Identification of QTL that Interact with Myostatin Genotype in C57BL/6 x M16i Mice

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Identification of QTL that interact with myostatin genotype in C57BL/6 x M16i mice

by

Ye Cheng

A dissertation submitted to the graduate faculty in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Genetics

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Ames, Iowa

2011

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DEDICATION

I would like to dedicate this thesis to my husband Su Chang and to my parents Huaqing Cheng and Peihua Dong, without whose support I would not have been able to complete this work.
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ABSTRACT

Myostatin, or GDF8, regulates skeletal muscle growth and adipose development in mice, cattle and humans. Individuals with non-functional myostatin mutation exhibit excessive muscle growth, decrease fat mass and huge body size. Previous studies indicated that epistatic interaction might play an important role in the myostatin pathway. However, only several loci that have a significant interaction with myostatin genotype have been identified in mice and cattle.

In this dissertation work, we performed a QTL mapping study in F2 mice derived from a C57BL/6 myostatin-null mouse line and the M16i obese mouse line. A large number of traits were collected, which included 14 body weight traits, 10 obesity-related traits, 11 body composition traits, three bone strength traits and eight eQTL traits from 1000 F2. A total of 242 SNPs across the genome were genotyped for each mouse by Sequenom. A linkage map was constructed using the marker and pedigree information. Interval mapping was applied to identify main effect QTL for these traits. In addition, additive, dominance and imprinting effects were evaluated for each QTL position. The corresponding variation accounted for by the QTL was computed as well. Comparing different QTL models with QTL effect, myostatin genotype effect and the interaction between QTL and myostatin genotype, comparison-wise \( P \)-values were obtained to identify possible epistatic loci that interact with myostatin genotype. A similar approach was utilized to search for loci that have significant interactions with sex or reciprocal cross.
We identified a total of 115 main effect QTL. Among them, 10 QTL exhibited a significant imprinting pattern. In addition, 38 QTL were detected for their interaction with myostatin genotype with comparison-wise $P$-values less than 0.05. Most of them were associated with body weight and obesity-related traits. A total of 44 sex-specific and 41 cross-specific loci were discovered as well. Some of these QTL regions overlapped and this indicates possible pleiotropic effects. These QTL explained a large amount of phenotypic variation.

To the best of our knowledge, a large proportion of these QTL have not been identified in previous studies. This work is the first research to investigate epistatic interaction between genetic variation and myostatin genotype for bone strength and organ weight traits. The outcome of this work elucidates that epistatic interacting patterns widely exist between genetic loci that regulate muscle mass and fat accumulation. Moreover, the results from this dissertation work provide a foundation for future fine mapping work.
CHAPTER 1. GENERAL INTRODUCTION

Introduction

Double muscling phenotype was first documented in cattle (CULLEY 1807). Common double muscled cattle breeds include Belgian blue and Piedmontese (MASERO and POUJARDIEU 1982). The most significant phenotype of these cattle is a huge muscle mass and body size. While this increased muscle mass results in increased meat yield, which is a benefit to the producer, it also causes some problems, such as increased incidence of dystocia. People were curious about the genetic mechanisms underlying the double muscling phenotype. At the beginning of 90’s, the only genetic knowledge about double muscled cattle was a possible mutation on chromosome 2 (CHARLIER et al. 1995). With the discovery of a similar phenotype in myostatin-null mice, researchers quickly identified the double muscle phenotype in cattle is also caused by a mutation in myostatin gene. Myostatin was a new member of the transforming growth factor beta superfamily (MCPhERRON et al. 1997). Myostatin-null mice exhibited a heavily muscling phenotype that was similar to double muscled cattle. Following that observation, mutations in myostatin gene were successfully found in several cattle breeds (MCPhERRON and Lee 1997).

Myostatin is not the only gene that regulates skeletal muscle growth. Skeltal muscle mass, which is a complex trait, is controlled by a number of quantitative trait loci (QTL) that have small individual effects. Interestingly, the double muscling phenotype is influenced by both breed and sex of the animal. These results indicate the possibility that other genes or factors may interact with myostatin genotype to impact skeletal muscle mass. However, no genes have been shown to epistatically interact with myostatin and only several genome regions have been identified for their ability to interact with myostatin to affect carcass traits.
in cattle (Casas et al. 2000; Casas et al. 2001). A potential way to increase the QTL mapping power is to conduct a large-sample size study, but this is a costly and time consuming effort in cattle. In contrast, a mouse based study is another option that is less costive and feasible considering the presence of the myostatin-null mouse line.

In addition to its role in skeletal muscle growth, myostatin also affects adipose growth. For example, myostatin-null mice have less fat accumulation than wild-type mice (McPherron and Lee 2002). Considering that fat content is a key point in meat production, the myostatin-null mice also provide an opportunity to investigate the molecular details of adipose regulation. The approach we propose in this dissertation project is to utilize the myostatin-null C57BL/6 mouse line and the polygeneic obese mouse line, M16i, to identify QTL that interact with myostatin in an epistatic manner. The M16i line was developed from the outbred ICR line after continuous selection for high weight gain (Hanrahan et al. 1973). In contrast to myostatin-null mice, M16i mice exhibit extensive adipose accumulation and a few obesity-related phenotypes. We hypothesized that loci that interact with myostatin to impact skeletal muscle and fat growth would segregate in the F2 mice derived from the crossing of these two lines. These loci could be identified with current QTL mapping methodology.

Myostatin has also been shown to affect organ weights, such as heart and liver weights (Bunger et al. 2004), although myostatin gene expression is only detected in muscle and adipose tissue. Since there is a close correlation between body weights and individual organ weights, we performed a QTL mapping of body composition traits in our study to identify main effect QTL that impacted these traits. In addition, we analyzed the interaction effect between QTL and the myostatin genotype to detect potential epistatic loci that interact
with myostatin gene. Moreover, myostatin might regulate bone strength either directly or indirectly due to an increased skeletal muscle mass. Therefore, the bone breaking strength and bone stiffness were measured for femurs and used as traits for QTL mapping.

We recently completed a microarray experiment in myostatin-null and myostatin wild-type mice. The gene expression profiles were obtained from the pectoralis muscle tissue of mouse embryo. More than 2100 genes were differentially expressed in the muscle tissue while only 73 genes were differentially expressed in the embryos. In this dissertation work, we performed a eQTL mapping for nine genes selected from these 2100 genes. These nine genes were selected for their important effects on cellular signaling, muscle and adipose development, and fiber type alternation. The gene expression levels were obtained by a multiplex real time PCR experiment and then used for eQTL identification.

In summary, the goal of this dissertation work was to identify QTL that interacted with myostatin genotype to affect body weight, muscle and fat weight, body composition, and bone strength traits. eQTL mapping of important genes was performed as well. More importantly, the interaction effects between QTL and myostatin gene, QTL and sex, QTL and reciprocal cross were evaluated. It was expected that the results from this study would provide numerous QTL regions that interacted with myostatin genotype. It would also give a general idea how much of the phenotypic variation seen in these traits were contributed from QTL by myostatin interaction effects.

**Thesis organization**

This dissertation presents the author’s research in a journal format. The first chapter contains a general introduction and a literature review as background information for the dissertation work.
Chapter 2 through Chapter 5 are papers in preparation for four journals. Chapter 2 is a manuscript submitted to the Heredity Journal. It describes the QTL mapping work of body weights in F2 mice derived from myostatin-null C57BL/6 mouse line and M16i obese mouse line. The statistical methods for QTL mapping and interaction analysis of this project were described in most detail in Chapter 2. Chapter 3 continues Chapter 2’s QTL mapping work for muscle weight, fat weight and eQTL traits with an additional statistical analysis of imprinting effect and will be submitted to PLoS Genetics. Chapter 4 and Chapter 5 consist of QTL mapping of body composition and bone strength traits and they will be submitted to Mammalian Genome and Genetics Notes respectively. Chapter 6 is a general conclusion of the research presented in this dissertation. In Chapter 2, 3, 4 and 5, the contributions of the authors were as follows: Ye Cheng was the primary researcher and author of all chapters, under the direction of James Reecy. The work described in Chapter 2 to 5 was assisted by collaborative work from Satyanarayana Rachigani (mouse raising, DNA isolation and phenotype collection), Jack C M Dekkers (direction of statistical analysis), Mary Sue Mayes (coordination of phenotype collection) and Richard Tait (bone strength data collection). RNA isolation and real time PCR work described in Chapter 3 was assisted by Angela Cánovas Tienda.

**Literature review**

**Myostatin**

1) Introduction

It has always been fascinating how tissue growth is regulated. Animals stop their growth when they reach their normal body mass. Cells maintain a perfect balance between amplification and apoptosis when they are healthy. Liver has the ability to regenerate even
after severe trauma. One of the most studied tissues in this area is skeletal muscle. Research in skeletal muscle is mostly focused on the development, maintenance, and regeneration of muscle cells (Bryson-Richardson and Currie 2008; Parker et al. 2003; Peault et al. 2007). Many internal signal pathways have been identified for their regulation on muscle growth (Clemmons 2009; Zhao and Hoffman 2004). Both promotion and inhibition of growth have been observed in these signal pathways (Pampusch et al. 2003; Sacheck et al. 2004; Xi et al. 2004). Over 40 years ago, Bullough came up with the chalone hypothesis (Bullough 1962; Bullough 1965). He proposed that there might be some molecules secreted by certain tissues to circulate in the body and inhibit tissue growth. He named these molecules chalones. There may be some chalones that specially regulate muscle tissue. Therefore, this hypothesis provides a way to explain why muscle stops growing when it reaches the size limitation. The first known evidence to support the chalone hypothesis is the discovery of the myostatin gene.

2) Discovery of myostatin gene

Myostatin, or GDF-8, was first discovered during a screen for new a TGF-β superfamily member using degenerative PCR primers (McPherron et al. 1997). Following this, the entire myostatin cDNA sequence was obtained from the cDNA library. They discovered that the myostatin gene carried all of the hallmarks of members of the TGF-β superfamily. For example, it has a secretion signal sequence, a proteolytic processing site and conserved cysteine residues in its carboxy-terminal region. Myostatin was given its name based on its function.

In mouse, myostatin mutant mice that lost normal myostatin gene function, by gene targeting approach, exhibited a significant increase in both body weight and muscle weight
(McPherron et al. 1997). Even heterozygous myostatin mice showed some muscle gain and this indicated that the myostatin gene might be dose dependent.

In agriculture, modification of the myostatin gene might help improve meat production yield. Therefore, the myostatin gene has been widely analyzed in many species in addition to the mouse, e.g cattle (Grobet et al. 1997; McPherron and Lee 1997), pigs (Sonstegard et al. 1998), chicken (McFarland et al. 2007), turkeys (McFarland et al. 2006), sheep (Clop et al. 2006) and humans (Williams 2004).

The most interesting example here is cattle. Double-muscling, which is a phenotype of growing extremely large skeletal muscle, was documented in cattle quite early (Culley 1807). Afterwards, this phenotype was mapped to a chromosomal region on bovine chromosome 2 (Charlier et al. 1995). After the discovery of the myostatin gene in mouse, the double muscling cattle were re-analyzed to search for possible mutations in the myostatin gene. Not surprisingly, double muscle cattle breeds, e.g Belgian blue and Piedmontese, carry a mutation in their myostatin gene, which resulted in a truncated non-functional myostatin protein (McPherron and Lee 1997). These cattle breeds have been selected for significant muscle growth historically (Masoero and Poujardieu 1982).

On the other hand, the identification of myostatin mutations in the human genome may provide some ideas to develop new therapies for muscle diseases. In humans, this mutation was firstly identified in a German boy whose mother was an athlete (Williams 2004). The mother’s genome carried a mutated copy of the myostatin gene which contributed to this double muscled phenotype in the boy. This super boy was able to carry two 3-kg dumbbells in his arms when he was about half years old. This is the first report of human muscle hypertrophy caused by myostatin gene mutation. In addition, alterations of the
myostatin expression level were also found to be associated with some muscle diseases in clinic researches, e.g. muscle atrophy (MA et al. 2003) and muscle hypertrophy (ZHU et al. 2000).

Despite the relative conservation of the myostatin gene in the species mentioned above, fish appear to be an exception. Studies have found that the predicted myostatin protein sequence in fish is more closely related to GDF-11 (OSTBYE et al. 2001; ROBERTS and GOETZ 2001). This phenomenon suggests that more complicated events might have happened during the evolution of the fish genome and the myostatin gene.

3) Expression pattern of myostatin gene

Myostatin expression is initiated as early as embryonic day 9.5 (MCPhERRON et al. 1997). This expression continues in the myotome afterwards. In adult mouse, myostatin gene expression is mainly detected in skeletal muscle with a wide range of different expression levels. The knock-out experiment of myostatin gene supports the fact that the major function of myostatin is to control muscle mass.

In addition, a detectable myostatin RNA level is present in adult fat tissue (MCPhERRON et al. 1997), which suggests some possible function of myostatin gene in adipose accumulation. Further study showed that myostatin mutation could cause significant lose in fat accumulation and this evidence makes myostatin one of the possible genes related to obesity (MCPhERRON and LEE 2002).

4) Biosynthesis and activity regulation of myostatin

Myostatin pathway research began with looking into its biosynthesis and activity regulation. Myostatin is synthesized in a precursor form (MCPhERRON et al. 1997). Two proteolytic processing events occur after that (LEE and MCPhERRON 2001).
removes 24 amino acids from the N-terminal. The second event removes another 240-243 amino acids from the first cutting site. This results in a latent complex of myostatin protein. However, the full activity of myostatin can only be gained after removal of the propeptide which is noncovalently linked to the C-terminal (Lee and McPherron 2001; Thies et al. 2001; Wolfman et al. 2003). The propeptide has two important functions. First, it ensures the proper folding of the precursor protein. Evidence supports this function in that only a small proportion of myostatin product was correctly folded to gain their biological activity from bacteria without the presence of the propeptide (Taylor et al. 2001; Thomas et al. 2000). Secondly, the propeptide might also participate in the regulation of myostatin activity, since experimental results indicated that over-expression of the propeptide leaded to a similar phenotype as seen in a myostatin-null mouse (Lee and McPherron 2001; Yang et al. 2001). Additional evidence clearly shows that the propeptide can block the binding of myostatin to the receptor (Lee and McPherron 2001; Thies et al. 2001).

5) Myostatin pathway

TGF-beta family members bind to two types of serine/threonine kinase receptor (type I receptor and type II receptor) to active the downstream signals. Myostatin is known to bind the activin type II receptor (ActRIIB and ActRII) first. These activated receptors then phosphorylate SMAD proteins. After phosphorylation, the SMAD protein functions as intracellular mediators that enter the nucleus and activate downstream target genes. The downstream genes that are regulated by the myostatin gene include some myogenic regulators, such as MyoD, myogenin, or Myf5 (Joula et al. 2003; Langley et al. 2002; Rios et al. 2002). The target genes impact myogenic differentiation.

6) Control the specificity of myostatin
As long as myostatin is activated, it follows the usual TGF-beta signaling pathway. The most important question is how myostatin to reaches its specificity using this common pathway at specific times and in different tissues.

The first level of specificity can be controlled at the protein level, which is the transition from a latent complex to an active myostatin protein. Researchers found that this level can only be reached by the specific expression of metalloproteinase, e.g. BMP-1/ tollloid (Wolfman et al. 2003). The second level of specificity comes from the existence of a special co-receptor. Although no evidence have shown that a co-receptor is needed to myostatin to bind the ActRIIA and ActRIIB, other studies found that a co-receptor or protein was required for other TGF-beta members to bind to the receptor (Cheng et al. 2003; Lewis et al. 2000; Schier and Shen 2000). In addition to a co-receptor, a type I receptor might also be required to combine with a type II receptor to gain specificity, e.g. ALK4 and ALK5 are two candidates for this because of their ability to bind myostatin (Rebbapragada et al. 2003). Further evidence is needed to prove that these two type I receptors can promote the binding of myostatin to type II receptors.

Certain important signaling pathway, e.g. SMAD protein, also participate in the specificity of myostatin signaling. For example, myostatin may promote the phosphorylation of SMAD2 and SMAD3 to active the downstream genes (Langley et al. 2002; Rebbapragada et al. 2003; Thies et al. 2001). The result is that nuclear protein c-ski was shown to block the activity of SMAD makes c-ski a candidate for controlling muscle growth (Akiyoshi et al. 1999; Sun et al. 1999a; Sun et al. 1999b). In fact, experiments showing that over-expression of c-ski in mouse have lead to mice with muscle hypertrophy gave further support for this conclusion (Sutraive et al. 1990).
7) Proteins that interact with myostatin

Except for the propeptide, other proteins are able to interact with myostatin. The most important one is follistatin. Follistatin expression happens at approximately the same development stage as myostatin gene expression (AMTHOR et al. 2002a; AMTHOR et al. 1996; AMTHOR et al. 2002b). Experiments using receptor binding assays show that follistatin can inhibit myostatin function (ZIMMERS et al. 2002). In addition, mice that over-express follistatin have the same amount of muscle mass increase as myostatin knockout mice (LEE and MCPHERRON 2001). Besides follistatin, FLRG and GASP-1 are two other important proteins that interact with myostatin (HILL et al. 2002; HILL et al. 2003). They both bind to the c-terminal of myostatin. This blocks the active site of myostatin protein. All these proteins join together and regulate myostatin activity in order for it to remain at a normal level. More details need to be clarified for this complicated regulation mechanism to be understood.

8) Myostatin and satellite cell

Satellite cells are a mononucleated myogenic cells which stops in the middle of the cell cycle (CAMPION 1984; SCHULTZ and MCCORMICK 1994). After birth, they can re-enter the mitotic cycle and differentiate into new muscle cells while stimulated by other signals (BISCHOFF 1994). Several evidences supported that myostatin could inhibit the proliferation of satellite cells. For example, myostatin-null mice have more satellite cells than wild-type mice (MCCROSKERY et al. 2003). p21 gene is one of the downstream genes that interact with myostatin gene to impact satellite cell activity. It has been shown that myostatin raises p21 expression, and this lowers cdk2 expression than in turn inhibits cell proliferation and differentiation (MCCROSKERY et al. 2003; RIOS et al. 2001). Therefore in myostatin-null
individuals, this inhibition affect is removed, and the satellite cell becomes active again. Rb might also be a mediator for myostatin might affect cell proliferation through p21. Researches found that increasing the expression level of the p21 gene resulted in a suppression of cdk2 expression, which further caused less phosphorylation of Rb2 protein (Rios et al. 2001). The final consequence of this regulation is lower myogenic regulator expression, which in turn leads to the inhibition of differentiation in the myoblast.

9) Myostatin and muscle diseases

Some severe muscle atrophy diseases are caused by different reasons. For example, muscular dystrophy in mdx mice is caused by a mutation in dystrophin gene (Sicinski et al. 1989). Muscle degeneration has also been reported in some HIV patients (Gonzalez-Cadavid et al. 1998). In these cases, patients have significant muscle mass loss although this is via different mechanisms. Therefore, modifying myostatin gene expression levels has been suggested to be a potential direction for developing new therapies. Experiments in cachexia mouse showed that the suppression myostatin by RNA in vivo lead to a significant increases in skeletal muscle and improvement of muscle fiber (Liu et al. 2008). This might be the consequence of satellite cell reactivation. This raises concerns that this kind of treatment might cause this tissue to run out of the storage of satellite cells and put patients in an even more dangerous situation.

QTL mapping

1) Introduction

A large number of traits show a continuous distribution, e.g. body weight, height and disease susceptibility. These traits are often defined as quantitative traits or complex traits (Falconer and Mackay 1996). Some other traits, which fall into discrete phenotype
variation, can also be treated as quantitative traits, e.g. number of tumors in the mouse intestine that carries cancer. Quantitative traits have a close relationship with agriculture, medicine, evolutionary theory etc. Quantitative traits are controlled by numerous genes simultaneously and are often influenced by both genetic and environmental factors. The term Quantitative Trait Locus (QTL) was suggested in quantitative marker analysis (GELDERMANN 1975). It is used to refer to the multiple genetic loci (more than one) that cause the phenotypic variation in quantitative traits. In some articles, QTL can also be used to stand for a single quantitative trait locus. The first definition is used throughout this thesis. Modifiers are described as QTL as well (GLAZIER et al. 2002; KORSTANJE and PAIGEN 2002). Modifiers are genes that alter the phenotypic effect of other genes.

Usually, QTLs possess several or all of the following characteristics (TANKSLEY 1993). Quantitative traits are controlled by a large number of genes with a small individual gene effect. Each QTL follows Mendelian rules and their effect can be dominant or co-dominant. Gene by gene interaction, or epistasis, and gene by environment interaction often exist.

It is known that each of the QTL might only contribute to a very small proportion of the variation observed. Mapping QTL that lie underneath complex traits provides an efficient way to search for candidate regions that can be used in the next step for candidate gene identification. There is growing evidence which suggests that many phenotypic traits are controlled by QTL (Complex Trait Consortium, 2003). Studies also demonstrate that obesity and other human diseases are associated with QTL (BOUCHARD 1997; RANKINEN et al. 2006). Mapping QTL related to human disease is helpful in order to discover the genetic components and the molecular mechanism underlying the disease. Moreover, considering the
other issues involved in QTL mapping, such as pleiotropy, pair-wise and high-order gene interactions and gene by environmental interaction, it helps to explore the genetic mechanisms behind quantitative traits. This perspective of integration is very important in this post-genome era.

With the knowledge of statistics, many methods have been developed to identify QTL (DA 2003; KENDZIORSKI and WANG 2006). In addition, total phenotypic variance can be partitioned into polygenic (fixed effect), additive, dominance and epistatic genetic variation. Putting all these pieces of information together, help establish a better model of how each quantitative trait is controlled by genetic and environmental factors.

2) QTL mapping stages and methods used in each stage

The principle of QTL mapping is quite straightforward. It is a statistical test of association between a particular phenotype and polymorphic marker genotype (FLINT and MOTT 2001). If a significant association is there, this implies a trait QTL is linked to the marker. The first markers that were used in a QTL mapping study are allelic forms of enzymes. Different forms have different amino acid structures that can be separated on electrophoretic gels. Therefore, genes coding for these enzymes can be used as polymorphic markers for a mapping study. As time passed, a larger number of DNA makers have been discovered and widely used. The most commonly used molecular markers include SNP (single nucleotide polymorphism), microsatellites and RFLP. The phenotype to be tested can be any quantitative phenotypic or gene expression profile (see eQTL section).

(1) Stages of QTL mapping

According to a review given by members of the Complex Trait Consortium (ABIOLA et al. 2003), there are generally three stages of any QTL mapping project, which includes
coarse mapping, fine mapping and identification of candidate genes. The difference between these three mapping stages is the resolution that can be reached in position of the QTL on the genome. Many factors affect the ability to detect a QTL and the resolution that can be reached. For example, how tightly the QTL is linked to the flanking markers. In addition, QTL having larger effect or higher heritability is easier to be identified. The size of the mapping population is also closely related to the mapping result. The most important factor that affects mapping resolution is the number of recombination events that occurred in the mapping population. A successful mapping strategy increases the recombination frequency in the mapping population, and consequently achieves a better mapping resolution.

Usually, coarse mapping gives the QTL location within a range of 10-30 cM. The second fine mapping step further shrinks this range to about 1-5 cM. Some important factors for fine mapping QTLs include: marker density, crossover density and molecular architecture of the QTL. Increasing marker density and crossover density solve the resolution problem (DARVASI 1998). In addition, some specially constructed mice populations have been utilized for QTL fine mapping, e.g. recombinant inbred lines (BAILEY 1971; SILVER 1995), recombinant congenic lines (STASSEN et al. 1996), recombinant intercross lines (VADASZ et al. 2000) and chromosomal substitution lines (HUNTER and WILLIAMS 2002).

After the fine mapping stage, the region that might contain the QTL is significantly smaller and therefore only a few genes fall into this QTL range. These genes become the candidate genes that can be examined for polymorphism in the third mapping stage. Many methods are used in this stage to identify the causal mutation and clone the gene (GLAZIER et al. 2002; KORSTANJE and PAIGEN 2002). The first criterion is to select candidates that have a strong effect on the given phenotype. The hypothetical function of these candidate genes can
be verified by molecular biology methods. For example, knockout and transgenic animals are often used to confirm the true function of the gene. Then, protein analysis can be used to give some proof of this gene function on a protein level.

(2) Statistical methods for QTL mapping

Many statistical approaches have been developed for QTL mapping. The three most important mapping methods are single marker analysis, interval mapping, and composite interval mapping.

i. Single marker analysis

In the single marker analysis, a statistical test is used to determine if there is a significant association between genotype at a single marker and phenotype. For example, in the backcross mapping population, there are two different genotypes, marker Aa and AA. If AA individuals have a phenotypic value significantly different from Aa individuals, this suggests a QTL might link to this AA marker, which in turn affects the phenotype. A simple t-test (SOKAL and ROHLF 1981) can be used here if there are only two different genotypes at the marker position. In addition, linear regression (LONG et al. 1995; LYNCH and WALSH 1998) and ANOVA (LYNCH and WALSH 1998) methods can also be applied here if there are more than two genotypes at the marker position. Linear regression provides a method to evaluate not only the QTL effect (additive and dominance effect) but also gene by gene and gene by environment interactions. The likelihood approach is also used for single marker analysis (LUCIEN 1990). In practice, the maximum likelihood estimates of the QTL genotypic values are obtained by an iterative approach.

ii. Interval mapping method
In the interval mapping method, a chromosomal region between two flanking genetic markers is analyzed for the existence of QTL. Maximum likelihood (Landerc and Botstein 1989; Lynch and Walsh 1998) and Haley Knott regression (Haley and Knott 1992; Martinez and Curnow 1992) are two common techniques used here. In maximum likelihood, the observed phenotype data is used to obtain the likelihood of a QTL within flanking markers. The likelihood of odds (LOD) score is calculated by taking the minus log of the likelihood-ratio test statistics (maximum likelihood of no segregating QTL versus maximum likelihood of segregating QTL). For each trait, the LOD score is mapped along each chromosome. Positions where the LOD score is above a significance threshold might harbor potential QTL. One important issue for this technique is to set the proper threshold to maintain a relatively low false positive rate. Another problem for the maximum likelihood method is the high computational demand. One new improvement of maximum likelihood is called order-restricted interval mapping (Nettleton and Praestgaard 1998). This method orders the genotype effects and then use it to calculate LOD scores based on restricted likelihood ratio test statistics. They suggest that this change in testing hypothesis can improve the QTL detection capability. Haley Knott regression method uses an approximate likelihood ratio test to test a full QTL and a reduced QTL model. This QTL model is a regression model that includes both additive and dominance QTL effect. It also includes the probabilities of QTL genotypes conditioning on the corresponding flanking markers.

iii. Composite interval mapping and multiple interval mapping method

Composite interval mapping method (Zeng 1994) is a multipoint QTL mapping method based on the interval mapping method. Composite interval method makes improvements on the efficiency and precision of QTL mapping by incorporating other
marker information into the analysis. In this analysis, additional markers outside the mapping region are used as cofactors in the regression, which will partly explain the phenotypic variation. In addition, multiple interval mapping (KAO et al. 1999) can also be applied to map multiple QTL simultaneously. Different from composite interval mapping method, multiple interval mapping methods simultaneously consider multiple interval regions instead of additional markers. The basic method of multiple interval mapping is still maximum likelihood, which takes into account a mix of several normal distributions. A potential problem of this method is an increase in parameters to estimate with the increase of QTL numbers.

iv. Bayesian QTL mapping method

Besides the traditional methods discussed above, Bayesian methods have been applied to QTL mapping (SATAGOPAN et al. 1996; SILLANPAA and ARJAS 1999; UIMARI et al. 1996). In the Bayesian analysis, QTL number, QTL genotypes and QTL effects are treated as unknown. The observed data, including phenotypic values, marker genotypes and linkage map are treated as known. The Bayesian approach accounts for all the uncertainties in the system conditional on the observed data. From the observed data, Bayesian theory is used to sample from the posterior and update the prior statistical model. Most of the sampling procedure is done with Metropolis-Hastings or Gibbs sampling. Some software has been developed to implement the Bayesian approach in QTL mapping (MARTINEZ et al. 2005; SILLANPAA and ARJAS 1998; YANDELL et al. 2007).

3) Experimental cross and population used in mouse QTL mapping

QTL mapping in mouse models is favored over human or other animal populations. Besides the lower cost and less time consumption, the primary reason to use mouse models is
to control environment exposure (Hunter and Crawford 2008). The ability to account for
environment variation can increase the power of QTL analysis. The second reason to use
mouse models is to tap into the power of using a known population structure and the
corresponding breeding scheme. Adding more complete pedigree information to the analysis
can reduce false negative and false positive rates (Serre et al. 2008). In mouse QTL
mapping, backcross (BC) and F2 populations are commonly used. In addition, other existing
mapping populations are also good candidates for QTL mapping.

(1) Recombinant inbred lines

Recombinant inbred strains were originally proposed by Bailey (Bailey 1971) and
developed by Taylor (Taylor 1978). Recombinant inbred (RI) lines are constructed by
inbreeding an F2 generation from two genetically distinct strains. A large number of RI lines
have been developed, genotyped, and are commercially available, refer to www.jax.org.

(2) Advanced intercross lines

Advanced intercross lines (AI) are produced by semi-random intercrossing within
each generation after a standard F2 cross. It has been proven that AI lines can systematically
increase the accumulation of recombination events (Darvasi and Soller 1995). Therefore,
AI lines are useful for the fine mapping stage.

(3) Chromosome substitution strains (CSS)

Chromosome substitution strains (CSS) are mice strains with one single chromosome
replaced by the corresponding chromosome from a donor strain (Hill et al. 2006). CCS are
developed by recurrent backcrossing progeny to one parent strain. They can provide QTL
location to a chromosome in the first coarse mapping stage.

(4) Congenic strains
Congenic strains are another way to construct QTL mapping populations using repeated backcrossing (Hill 1998). Different from CCS, conegenic strains keep one chromosome segment from the donor strain instead of a whole chromosome. Many conegenic strains have been developed and genotyped. They are an ideal source for QTL fine mapping.

(5) Collaborative Cross

The concept of the Collaborative Cross was first brought up by members of the Complex Trait Consortium at the Edinburgh meeting of the International Mouse Genome Conference in October of 2001 (Threadgill et al. 2002). The Collaborative Cross was started with eight commercial inbred mouse lines (A/J, C57BL/6J, 129S1/SvImJ, NOD/LtJ, NZO, CAST/Ei, PWK/Ph and WSB/Ei) (Chesler et al. 2008). They were mated pair wise to get the first generation (G1). From all 56 possible combinations of G0, G1 lines were crossed to obtain two four-way G2 progeny. Afterwards, G2 lines were crossed to yield 8-way hybrids (G2:F1). Lines were propagated by sib mating until they were inbred (Threadgill et al. 2002). It is estimated that the collaborative cross can shrink the QTL interval to only 1 to 5 candidate genes (William et al. 2002). SNP information, population structure recombination rate and other genome information will be available for each line (Chesler et al. 2008). The current status of this project is continually updated online (http://mouse.ornl.gov/projects/cc_breeding_progress.html).

eQTL (expression QTL) Mapping

1) Introduction

Microarray techniques for analysis of gene expression were first brought up and applied to molecular genetics in 2000. These experiments have been widely used to discover gene expression differences between two different biological conditions, e.g. cross
differences, healthy versus diseased individuals (PRIMIG et al. 2000; SANDBERG et al. 2000). Compared to traditional methods, microarrays provide an efficient way to obtain a large number of gene expressions simultaneously. With this advanced high-throughput technique, it has become possible to study the global gene expression profiles in parallel. In the meantime, statistical and bioinformatic tools make it possible to begin to look at and analyze this high dimensional data.

Combining traditional quantitative genetic approaches, Jansen and Nap proposed genetic mapping of genome-wide gene expression data, which treats gene expression level as a quantitative trait and follows this with quantitative genetic analysis (JANSEN and NAP 2001). They termed this strategy ‘genetical genomics’. The first study that successfully realized this was a global gene expression mapping study performed in yeast (BREM et al. 2002). Following this study, the QTL that are associated with expression variation of genes are named expression QTL (eQTL). Many eQTL have been mapped in more than a dozen species, e.g. mice, maize, human, rats, eucalyptus and Arabidopsis thaliana (BREM et al. 2002; BYSTRYKH et al. 2005; CHESLER et al. 2005; CHEUNG et al. 2005; DECOOK et al. 2006; HUBNER et al. 2005; KIRST et al. 2004; MEHRABIAN et al. 2005; MONKS et al. 2004; MORLEY et al. 2004; SCHADT et al. 2003; STRANGER et al. 2005; YVERT et al. 2003).

One reason to study gene expression as quantitative traits is that many gene expressions have a high heritability. Obtaining more details about these genetic factors is needed to get a full picture of how gene expression is regulated. For example, in a lymphoblastic cell line study of nearly 15,000 traits, the average heritability was larger than 0.3 (DIXON et al. 2007). eQTL with heritability>0.8 have mapped to more than one chromosome position. This again indicates that genetic background impactes a large number
of gene expression traits. Moreover, these genetic factors, including *cis* and *trans* regulatory elements, were widespread across the whole genome.

Another reason to study eQTL is the special intermediate role that gene expression serves between genotypic and phenotypic variation. Phenotypic QTL makes a connection between DNA sequence and regular trait phenotypes. eQTL further provides complementary information on an mRNA level about how the relevant gene expression is regulated by the genome. The collocation of eQTL and phenotypic QTL provides much stronger evidence of causal mutation.

2) Important approaches used in eQTL study

(1) eQTL mapping method and FDR control

The standard QTL mapping methods discussed above are used for eQTL mapping as well. Therefore, these approaches will not be discussed in this section. An important question that is addressed by eQTL mapping, and also regular QTL mapping, is the multiple-comparison problem. This is not surprising when we consider the statistical approaches and problems in microarray experiments. Historically, some methods have been developed to control FDR (false discovery rate) for multiple testing problems. For example, q-value measures the proportion of false positives incurred when the result of the test is significant (GRUNENFELDER and WINZELER 2002; HUBNER et al. 2005; STOREY and TIBSHIRANI 2003). In other words, q-value can be viewed as a transformed form of p-value. According to the q-value, significance cut-offs can be made similar to a p-value. This method has been used to solve many genomic problems. Another option to solve multiple-comparison problems is the permutation method (BREM and KRUGLYAK 2005). This method can be used to control FDR for a single location. Moreover, an empirical Bayesian approach discussed by Kendzierski
(Kendziorski et al. 2006) is also applicable to control FDR for multiple locations. The Bayesian approach adjusts for multiple tests across both traits (mRNA transcripts) and markers used in the analysis. This Bayesian approach is more accurate when multiple eQTL are detected for one transcript.

(2) Cis- vs. trans- eQTL

From the eQTL mapping result, cis-acting modulators or local modulators and trans-acting modulators or distant modulators can be distinguished by comparing the transcript location and the eQTL location (Rockman and Kruglyak 2006).

i. Cis- eQTL

Usually, SNPs within 100 kb upstream and downstream of the target gene can be viewed as cis-acting eQTL (Cookson et al. 2009). It is suggested that cis- regulation is more often caused by genetic variation in the gene itself. This is supported by evidence that eQTL are more densely dispersed within the 250 bp around the transcription initiation sites (Veyrieras et al. 2008).

ii. Trans- eQTL

Trans- regulation is caused by polymorphism of other regions across the genome. It has been found in eQTL studies that the frequency of a long-distance regulatory element is much less than that of a short-distance regulatory element (Dixon et al. 2007; Stranger et al. 2007), especially in rats (Morley et al. 2004; Schadt et al. 2003) and humans (Hubner et al. 2005). The trans-eQTL might be candidate genes for transcription factors. Therefore, it is expected that some trans-eQTL might have multiple effects on a number of transcripts at the same time. The trans-eQTL are sometime also defined as ‘master regulators’ and have been identified in many different species, e.g Drosophila (Dixon et al. 2007; Emilsson et al.
(3) eQTL hot spots

Master regulator is a special case of eQTL hot spots. eQTL hot spots are genomic regions where multiple transcripts have been mapped (Kendziorski and Wang 2006). Kendziorski and Wang (2006) provided a criterion to identify eQTL hot spots by. For example, the number of mapping transcripts and the sum of LOD scores need to be considered. In addition, statistical tests have been considered to determine which spots are truly hot. A Poisson-based test was proposed by Brem et al. (2002) to test if particular chromosomal regions are “ghost” hot spots. This test was also been discussed by another researcher (Perez-Enciso 2004). Other statistical approaches for detection of eQTL hot spots have been developed (BARRY et al. 2005; Subramanian et al. 2005).

(4) eQTL and GWA study

Another important application of eQTL study is to combine it with a genome-wide association (GWA) study to identify candidate genes for disease study. A GWA study builds a connection between genetic variation through the whole genome and disease. With the aid of eQTL, GWA can be used to identify genetic markers that affect both the disease and the gene expression profiles at the same time (Cookson et al. 2009). This approach is especially useful when a GWA study alone is not powerful enough to target a candidate gene. There are two common examples. First, the genome regions identified by a GWA study covers a couple of genes and no gene is an obvious candidate gene for the disease. An eQTL study can be used to find transcripts that co-localize in this region. If the transcripts and a certain gene

2008), rat (Hubner et al. 2005) and humans (Morley et al. 2004). But trans-regulation can also be caused by other indirect regulations, e.g. non-cell-autonomous effects and (Rockman and Kruglyak 2006), interchromosomal interactions (Spilianakis et al. 2005).
from the GWA study list overlap, it is very possible that this gene is highly associated with the disease. Therefore, this gene can be chosen as a candidate gene. Success stories using this application comes from a GWA study of asthma (DIXON et al. 2007; MOFFATT et al. 2007).

Another situation where these two tools can be applied is when the genome regions suggested by the GWA study does not provide any candidate gene list. Sometimes, the gene that causes the disease phenotype is not located in the GWA polymorphism region. It is indirectly influenced by another long distance cis- or trans- regulator factor, and this regulator is located in the GWA polymorphism region. An eQTL study provides additional evidence for the association between GWA polymorphism and the candidate gene expression. One example is the PTGER4 gene that impacts Crohn’s disease (CD) in a mouse study (KABASHIMA et al. 2002; LIBIOULLE et al. 2007). The direct region identified by GWA in this study was in a gene desert. However, through another eQTL study, a connection was built between GWA polymorphism and the PTGER4 transcripts.

(5) Pathway and integration of other -omics

eQTL hot spots that influence many transcripts at the same time are likely to be the hub of a regulatory pathway. The mapping transcripts from an eQTL study give a list of candidates that connect to the hub (KENDZIORSKI and WANG 2006). The first procedures to fit the pieces from this biological jigsaw together were proposed in 1998 (EISEN et al. 1998). In Jansen and Nap (2001), they expressed the similar ideas more formally. After summarizing the information gained from eQTL study, a putative pathway could be reconstituted (JANSEN and NAP 2001; SERVIN and STEPHENS 2007).

One method used in a neural synapse function study (CHESLER et al. 2005) was to calculate pair-wise correlations among the mapping transcripts. The extent of correlation
could be used to identify cliques. The relationship between any two transcripts among cliques could be studied by their correlation. It has also suggested to narrow down the transcripts lists in the network by including only highly correlated eQTL candidates and transcripts (BING and HOESCHELE 2005). One potential problem is a risk to falsely place closely linked loci from different networks together.

It has been proposed that information from an eQTL mapping study can be used to find the best network model (ZHU et al. 2004). The number of hubs in the network and the complexity of the network could be reduced. Moreover, after calculating the correlation between transcript abundance and phenotype values, an indirect association is made between eQTL and phenotypes.

However, pathway assembly is a demanding job because a regulatory factor is plastic and susceptible to special and temporal change (LI et al. 2006; PETRETTO et al. 2006; SERVIN and STEPHENS 2007; STERN et al. 2007; VAN SWINDEREN and GREENSPAN 2005). Nevertheless, a combination of genomics, transcriptomics, proteomics and metabolomics is expected to elucidate the genetic architecture and molecular mechanism of complex traits in the future (DITTRICH et al. 2008; MELZER et al. 2008).

Epistasis

1) Introduction

Genetic modifiers are genes that alter the expression of another gene (BRIDGES 1919; GRUNEBERG 1950). Epistasis is one special form of genetic modification (PHILLIPS 1998). Approximately 100 years ago, the word ‘epistasis’ was invented by William Bateson to explain the difference between the predicated Mendelian segregation ratio and the actual outcome of the flower color in sweet pea (BATESON 1909). In his paper, this kind of epistatic
interaction was caused by one gene masking the effect of other gene. An additional statistical meaning of this term was defined by R. A. Fisher, as any statistical deviation from the additive combination of two loci in their effects on a phenotype. This is defined as ‘epistacy’ by him (Fisher 1918). This statistical definition of epistasis is widely used in population and quantitative genetics. The original definition from Bateson is more often used by geneticist for segregation in a specific cross (Phillips 2008). These two definitions are not equal. A lack of statistical evidence for epistasis does not mean a lack of genetic interaction (Cordell 2002; Moore and Williams 2005).

There are currently three main categories of epistasis (Phillips 2008). The first category of epistasis is ‘functional epistasis’. This kind of interaction can also be viewed as protein-protein interaction. Proteins in the same pathway or same complex interact with each other (Boone et al. 2007). The evidence of functional epistasis comes from biochemistry experiments. The second category of epistasis is ‘compositional epistasis’. This kind of epistasis is the same as the one defined by Bateson (Bateson 1909). To study compositional epistasis, only the loci of interest are altered while the other genetic loci throughout the genome are kept the same. Both quantitative and qualitative phenotypes can be measured for compositional epistasis. The third category of epistasis is ‘statistical epistasis’, which is the same as the one proposed by Fisher. Statistical epistasis measures the phenotypes of samples from a population while compositional epistasis ‘intentionally constructs’ the phenotype (Phillips 2008). A very detailed example of mouse coat color was given by Philips (2008) to illustrate the difference between these three categories of epistasis. The interaction between the two loci that were involved in this coat color study could be viewed as an example of compositional epistasis. A further dissection of proteins that participated in this pathway was
an example of functional epistasis. A quantitative genetics way to look at the coat color was
used to combine marker genotypes, phenotypic values and give an estimate of genetic
estimate of these loci. This is a statistical epistasis way to look at coat color.

The concept of epistasis is also emphasized by systems biology (MOORE and
WILLIAMS 2005). In systems biology, results from genomics, transcriptomics, proteomics and
metabolomics are integrated to explore molecular mechanisms behind variation. This kind of
multifactorial analysis increases the power to find gene interactions.

2) How to measure epistasis

Epistasis is the deviation of phenotype from what is expected. It is very important to
find the proper way to measure this “expected” phenotypic value. Different mathematical
models have been used to measure the phenotype. The most common model for epistasis is
derived from Fisher’s definition of epistasis (FISHER 1918). This model includes the additive
and dominance effects of two loci and four epistasis terms (additive by additive, additive by
dominance, dominance by additive, dominance by dominance). If epistasis does not exist,
then the coefficients for the four epistasis terms are defined to be zero. Both a haploid and
diploid model can be derived from it. This linear model is easy to analyze with statistical
methods. The problem with Fisher’s definition is that the scale of measurement can be
affected if the phenotype is additive and epistatic. After some transformation, an additive
phenotype may show epistasis (FRANKEL and SCHORK 1996; GREENLAND and ROTHMAN
1998). In addition, population geneticists use multiplicative (HODGE 1981; RISCH 1990) and
heterogeneity (NEUMAN and RICE 1992; RISCH 1990) models to define epistasis. These two
models will not be discussed in detail here.

3) Epistasis in QTL mapping
Epistasis also plays an important role in complex traits. Historically, it has not been elucidated or even neglected in complex trait studies (Barton and Keightley 2002; Carlborg and Haley 2004; Doerge 2002; Flint and Mott 2001; Hoh and Ott 2003; Jansen 2003). This might be one the reasons why it is difficult to repeat human association studies (Hirschhorn et al. 2002). In addition, it might also lead to the disappearance of an identified QTL effect after these QTL are isolated (Carlborg and Haley 2004).

In some QTL mapping studies, epistasis is detected between loci, in which one or both have significant effects on the phenotype (Fijneeman et al. 1996; Li et al. 1997; Long et al. 1996). Recent studies also identified epistasis between loci that had no individual effects (Carlborg and Andersson 2002; Sen and Churchill 2001). Empirical and theoretical results support the conclusion that inclusion of epistasis into QTL mapping analyses could improve the statistical power to identify QTL (Gauderman and Thomas 2001; Kao and Zeng 2002).

4) Methods used to identify epistatic QTL

In the previous QTL mapping section, several common QTL mapping methods were discussed. The principle of these methods can also be used to map epistatic QTL. Epistatic QTL mapping requires use of a multi-QTL model instead of a single QTL model. Marginal QTL effects and the epistatic effects associated with pairs of loci are considered simultaneously. This is the main difference from traditional QTL mapping. Several methods have been used to search for epistatic QTL. One method is to first conduct a single QTL scanning. Following the scan, epistatic interaction is tested between QTL with significant marginal effects. This method is discussed in detail in (Culverhouse et al. 2002). The second method is to perform a two dimensional QTL scan across the genome (Haley and
KNOTT 1992; WANG 1999). This method does not require significant main effects of QTL for the epistatic interaction to be detected. In addition, stepwise selection has been applied to find the best genetic model by fitting additive, dominance and epistatic effects into the model. This approach was applied in the multiple interval mapping (MIM) (KAO et al. 1999; ZENG et al. 1999) in backcross experiments to identify epistatic QTL. Similar to QTL mapping, Bayesian methods have been studied and applied in epistatic QTL mapping (SEN and CHURCHILL 2001). After the reversible jump Markov Chain Monte Carlo (MCMC) algorithm was proposed (GREEN 1995), it was applied to QTL mapping to estimate epistatic interactions (YI and XU 2002; YI et al. 2003).

5) Epistasis studies in livestock animals

Many important traits in livestock animals are complex traits. Elucidating more detail about the role of epistasis in livestock animals will help to better understand the molecular mechanism that controls these traits. However, very few epistatic QTL have been identified in livestock animals. The reason is that only interactions with an effect greater than 0.4 standard deviations can be detected. The same power problem also happens in swine studies (VARONA et al. 2002). Nevertheless, several epistatic QTL were successfully identified in livestock animals. For example, two QTL were identified in beef cattle for significant interaction on backfat thickness and meat tenderness (CASAS et al. 2000). Another cattle study of the calpain gene family identified epistatic interactions between a QTL at calpain 1 (CAPN1), located on chromosome 29, with a QTL at calpastatin (CAST), located on chromosome 7 (BARENDSE et al. 2007). A more successful story of epistatic QTL mapping was performed in chicken (CARLBOG et al. 2003). With a large population size, epistatic
interactions were observed to contribute a large amount of variation in early growth traits, while later growth was mostly regulated by additive effects.

6) Epistasis studies in mice and humans

In mammals, gene interaction impacts coat color (Silvers 1979). In this book, he discussed the loci and the interactions between these loci that control coat color in mice in detail. It has been estimated that epistasis could explain about 30% of phenotypic variation in body weight and fat accumulation (Brockmann et al. 2000). They also estimated that about 20-33% of phenotypic variation in muscle weight and hormone concentration in serum was from epistatic interactions. Therefore, epistasis is an important factor of phenotypic variation in mice. Other studies in mice reached the same conclusion (Kim et al. 2001; Shimomura et al. 2001; Sugiyama et al. 2001). Carlborg and Haley pointed out that the estimation of epistasis in mice might be overestimated because of small population sizes (Carlborg and Haley 2004). Relatively speaking, studies with a larger sample size provide more accurate estimations.

As expected, epistasis also plays an important role in human health. This is not surprising because most of human diseases are complex traits (Moore 2003; Sugiyama et al. 2001). For example, coronary heart disease (CHD) is a complex disease affected by many traditional risk factors such as age, smoking, and body mass index (Kardia et al. 2003). Studies found that epistasis and gene-environment interactions play an important role in disease susceptibility (Rea et al. 2006; Sing et al. 2003). Moreover, gene interactions were identified in other human diseases, e.g. coronary artery disease (Kardia et al. 2006; Mendonca et al. 2009), diabetes (Phillips et al. 2008; Wu et al.; Zuniga et al. 2006),
bipolar effective disorder and autism (Abou Jamra et al. 2007; Tsai et al. 2007; Wiltshire et al. 2006).

7) Challenges faced in epistasis studies

Despite increasing recognition in genetic studies, epistasis analysis faces some practical challenges as well. One potential problem that an epistasis study encounters is the huge dimension of data that needs to be manipulated. With the increasing number of genes considered, the number of pair-wise interactions is boosted rapidly. If three-way or even higher interactions need to be explored, some special approaches must be applied. Logistic regression (Millstein et al. 2005), machine learning and data mining methods (McKinney et al. 2006) and multifactor dimensionality reduction (MDR) (Hahn and Moore 2004; Moore 2004; Moore et al. 2006; Moore and Williams 2002; Ritchie et al. 2003; Ritchie et al. 2001) were developed for this purpose.

The second problem facing an epistasis analysis is to set a reasonable significance threshold. In multiple testing, the threshold can be obtained by randomization tests (Churchill and Doerge 1994; Kao et al. 1999; Sen and Churchill 2001). However, the randomization test sets a very stringent threshold when a large number of gene interactions are tested. As a result, only large epistatic effects will be identified. New approaches are needed to solve this problem.

The sample size for epistasis study is another challenge. When the number of interacting loci increases, the number of individuals in each genotype combination decreases. Consequently, this requires a large sample size so that a moderate epistatic effect can be detected (Demuth and Wade 2006).
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CHAPTER 2. MAPPING GENETIC LOCI THAT INTERACT WITH MYOSTATIN TO AFFECT GROWTH TRAITS

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Abstract

Myostatin, or GDF8, is an inhibitor of muscle growth. A non-functional myostatin mutation leads to a double muscling phenotype in some animals. Previous studies have indicated that there are loci in the genome that interact with myostatin to control backfat depth and other complex traits. We now report a QTL (quantitative trait loci) mapping study designed to identify loci that interact with myostatin to impact growth traits in mice. Body weight and average daily gain traits were collected on F2 progeny derived from a myostatin-null C57BL/6 strain by M16i cross. Forty-four main effect QTL were detected above a 5% genome-wide significance threshold when an interval mapping method was used. In addition, we identified 37 QTL that significantly interacted with myostatin, sex, or reciprocal cross. Twelve of these QTL interacted with myostatin genotype. These results provide a foundation for the further fine mapping of genome regions that harbor loci that interact with myostatin.

Conflict of interest

The authors declare no conflict of interest.

Introduction

Growth traits are complex traits of economic importance to animal agriculture. A good understanding of mechanisms that control growth will benefit both meat production and human health. Therefore, growth traits have been widely studied in animal models (Corva
and Medrano, 2001). Heritability studies of growth-related traits (Eisen, 1974; McCarthy, 1982) indicate that there are genetic mechanisms that control these traits. Previous publications have estimated the amount of phenotypic variation in growth traits that can be explained by different types of genetic effects (Brockmann et al., 2000; Carlborg et al., 2003). It has been demonstrated that, among these genetic effects, epistasis explains a large amount of phenotypic variation, as compared to additive and dominance effects. For example, Brockmann (2000) estimated that about 33% of the phenotypic variation of body weight in mice could be attributed to epistasis. Another study indicated that there might be a time dependent pattern for these genetic effects and epistasis might be more important for early growth traits (Carlborg et al., 2003).

Identification of key genes that control growth traits has shed light on the molecular mechanisms that regulate growth. For example, myostatin has been shown to inhibit muscle growth in cattle, dogs, mice, and humans (Grobet et al., 1997; McPherron et al., 1997; McPherron and Lee, 1997; Schuelke et al., 2004; Mosher et al., 2007). As a member of the transforming growth factor beta family, myostatin was first identified in mice for its significant effect on skeletal muscle growth (McPherron et al., 1997). Interestingly, not all myostatin-null animals show an obvious difference in their muscle mass. For example, some South Devon cattle that are homozygous for myostatin-null mutations do not exhibit the double-muscling phenotype (Smith et al., 2000). In addition, Grobet (1998) showed that Limousin and Blonde dAquitaine, which both possess wild-type myostatin alleles, exhibited a double muscling phenotype.

Other loci might epistatically interact with myostatin to control skeletal muscle growth. To date, only loci that interact with myostatin to control backfat depth and meat tenderness
have been identified and mapped in cattle (Casas et al., 2000; Casas et al., 2001). Since the main function of myostatin is to control skeletal muscle growth, it will be critical to map these loci. Furthermore, the knowledge gained will help elucidate the molecular details behind the genetic architecture of growth traits.

Here, we developed an F2 population from myostatin-null C57BL/6 mice by M16i obese mice. A whole genome scan was performed to identify main effect QTL. Subsequently, a model comparison approach was applied to search for potential epistatic QTL. We further investigated the phenotypic and genetic variation that could be accounted for by these QTL.

**Methods**

**Mapping population**

An F2 cross design was used for this study. We first derived the mapping population from reciprocal crosses between the two founder strains, myostatin-null C57BL/6 (McPherron et al., 1997) and M16i high body weight mice (Hanrahan et al., 1973). Four myostatin-null male mice were mated with eight M16i females to produce 35 male and 37 female F1 offspring. In addition, two M16i males and seven myostatin-null females were crossed to generate 31 male and 55 female F1 offspring. Within each of these two crosses, F1 mice were intercrossed to produce the F2 mapping population.

**Phenotyping and genotyping procedure**

Each F2 litter was standardized to 9 pups one day after birth. At 1 week of age, mice were individually identified. Mice were weaned when they were three weeks old. Isolated DNA was used to determine the myostatin genotype by a standard PCR reaction. A total of 1000 F2 generation progeny that were either homozygous myostatin wild-type or homozygous myostatin-null were collected for genotype and phenotype information. Among
these 1000 F2 mice, 552 mice were from the myostatin-null male by M16i female cross while 448 mice were from the reciprocal cross. Each of the F2 individuals had its body weight recorded at 7, 14, 21, 28, 35 and 42 days of age. Based on the measurements of body weights, growth rate was calculated for different growth periods. For the pre-weaning period, average daily gain was calculated for the 2nd week and 3rd week, along with the 1st to 3rd week interval. During the post-weaning period, average daily gain was calculated for the 4th week, 5th week, 6th week, and the 4th to 6th week interval. Furthermore, average daily gain for the 1st to 6th week interval was calculated and included as a measurement of overall growth rate. Genomic DNA from each of the F0, F1 and F2 generation mice was purified by a phenol chloroform method. All animal procedures were approved by the Iowa State University Animal Care and Use Committee prior to this study.

**Linkage map**

We genotyped 242 SNPs on the Sequenom® platform at GeneSeek® (Lincoln, Nebraska). These SNPs were evenly distributed on 19 autosomes and the X chromosome. First, genotyping data were checked for genotyping errors on the basis of Mendelian inheritance and pedigree information. SNPs with low call rates (less than 80%) and genotyping errors were discarded. After this first selection, 152 SNPs were left and they were distributed on 17 autosomes (no informative SNPs were present on chromosome 15 and chromosome 16) and the X chromosome. Second, a Chi-square test was used to evaluate the extent of segregation distortion in the F2 population. Most markers did not significantly deviate from the expected Mendelian segregation ratios, except for SNPs close to the myostatin locus, which was expected because only homozygotes at the myostatin locus were included. Therefore, the remaining 152 SNPs were used for our statistical analysis. Finally, a
linkage map was constructed in Kosambi centimorgans with Cri-map (Green, et al., 1990). Marker order and position in this map (Supplemental Table 1) was in reasonable agreement with those from the Wellcome-CTC Mouse Strain SNP Genotype Set (http://www.well.ox.ac.uk/mouse/INBREDS). Thus, we performed the following QTL mapping analysis based on our linkage map.

**General statistical analysis**

The SAS 9.2 (SAS Institute, Inc., Cary, NC) was used to explore the data structure of the F2 phenotypic data. First, simple statistics were calculated on the fourteen growth traits. Second, Q-Q plot and normality tests were used to verify that each trait was normally distributed. Third, the Proc GLM procedure was applied to each trait to identify significant factors that need to be accounted for in the QTL mapping model. Specifically, effects of myostatin genotype, coat color, sex, reciprocal cross and interactions among these factors were evaluated. All these factors have been previously shown to impact growth traits. Finally, after removing fixed effects that were identified in the previous step, residual correlations were calculated and tested between each pair of traits by the Proc CORR procedure.

**Main effect QTL analysis**

GridQTL (Seaton, et al., 2006), a web-based QTL analysis program, was used to identify QTL associated with the 14 growth traits by interval mapping. The QTL model included the main effects of myostatin genotype, coat color, sex, and reciprocal cross, and the interaction effects of sex × myostatin genotype, sex × reciprocal cross, myostatin genotype × reciprocal cross, sex × coat color and sex × myostatin genotype × reciprocal cross along with the additive and dominance effect at the single QTL position. For each trait, both F-values and LOD-values were provided by GridQTL. Genome-wide significance levels (1% and 5%)
were determined by the genome-wide permutation procedure (Churchill and Doerge, 1994) implemented in GridQTL, using 1000 permutations. Additive and dominance effects, along with the corresponding standard errors, were estimated for each significant QTL peaks. The percentage of phenotypic variation accounted for by a QTL position was computed as the percentage of residual sum of squares explained by the additive and dominance effects at the QTL. For each trait, the total phenotypic variation of all QTL was calculated by summing the percentage of phenotypic variation together.

**QTL interaction with myostatin genotype, sex and reciprocal cross effect**

To test the potential interaction between a QTL and myostatin genotype, we first applied a forward QTL selection strategy. In this step, the dataset was split into two subsets by myostatin genotype. Interval mapping analysis was completed using the main effect QTL model without myostatin genotype effect within each subset. All positions that reached a 5% genome-wide significance level were identified. In the second step, the interaction between each of these positions and myostatin genotype was tested by comparing four models in the full data. Fixed effects in the following effect were same as before.

**Model 1:**

\[
\text{Phenotypic value} = \text{fixed effects} + \text{additive effect (QTL position)} + \text{dominance effect (QTL position)} + \text{Myostatin} \times \text{additive effect (QTL position)} + \text{Myostatin} \times \text{dominance effect (QTL position)} + \epsilon
\]

**Model 2:**

\[
\text{Phenotypic value} = \text{fixed effects} + \text{additive effect (QTL position)} + \text{dominance effect (QTL position)} + \text{Myostatin} \times \text{additive effect (QTL position)} + \epsilon
\]

**Model 3:**

\[
\text{Phenotypic value} = \text{fixed effects} + \text{additive effect (QTL position)} + \text{dominance effect (QTL position)} + \text{Myostatin} \times \text{dominance effect (QTL position)} + \epsilon
\]
Model 4:

Phenotypic value = fixed effects + additive effect (QTL position) + dominance effect (QTL position) + ε

The F-value was computed with the following formula:

To test overall interaction:

\[ F = \frac{\Delta \text{SSE} / 2}{SSE(\text{model 1}) / \text{d.f. (model 1)}} \]
\[ \Delta \text{SSE} = SSE(\text{model 4}) - SSE(\text{model 1}) \]
\[ \text{d.f.: degree of freedom of error term} \]

To test additive interaction:

\[ F = \frac{\Delta \text{SSE}}{SSE(\text{model 2}) / \text{d.f. (model 2)}} \]
\[ \Delta \text{SSE} = SSE(\text{model 4}) - SSE(\text{model 2}) \]
\[ \text{d.f.: degree of freedom of error term} \]

To test dominance interaction:

\[ F = \frac{\Delta \text{SSE}}{SSE(\text{model 3}) / \text{d.f. (model 3)}} \]
\[ \Delta \text{SSE} = SSE(\text{model 4}) - SSE(\text{model 3}) \]
\[ \text{d.f.: degree of freedom for error term} \]

The corresponding \( p \)-value was calculated from the F-distribution. A comparison-wise \( p \)-value less than 0.05 was considered significant.

To analyze the interaction effect between QTL and sex, the full dataset was split into two subsets according to gender: female F2 mice and male F2 mice data. A similar approach to the one described above was used by substituting the myostatin ×additive effect with the Sex ×additive effect and the myostatin ×dominance effect with the Sex ×dominance effect.

To analyze the interaction effect between QTL and reciprocal cross, the full mouse dataset was again split into two subsets according to reciprocal cross information: the M16i
female × myostatin male cross and the M16i male × myostatin female cross data. Again, a similar approach as described above was used by substituting the myostatin × additive effect with the Cross × additive effect and the myostatin × dominance effect with the Cross × dominance effect.

For each of the detected QTL, the additive and dominance effects were estimated. The amount of phenotypic variation accounted for by each was calculated using the percentage of sum of squares explained as described above for main effect QTL. For each trait, the total variation explained by QTL was calculated as the sum of phenotypic variation from all main effect QTL and interaction QTL that were identified for the trait.

Results

General statistics

Our initial statistical analyses indicated that all 14 phenotype traits were normally distributed (supplemental Figure 1). The histograms in supplemental Figure 1 illustrate that the effect of myostatin-null genotype and male sex increased with age from 1st to 6th week of age. The general statistics of these 14 traits are presented in Table 1. Most of the body weight and average daily gain traits had significant correlations with one another (Table 2). Early body weights (1st to 3rd week) were negatively correlated with body weights during the post-weaning growth period (4th to 6th week). In addition, the 14 body weight traits were identified for significant main effects of myostatin genotype, sex, and reciprocal cross. Interactions between main effects were significant for some traits (unpublished results).

Main effect QTL

Based on the genome wide scan in the whole F2 population with the additive and dominance QTL model specified in the method section, we observed 26 and 44 QTL that
were associated with these 14 growth traits at a 1% and 5% genome-wide significance level, respectively (Table 3). These 44 QTL were distributed on 10 autosomes (chromosome 1, 2, 3, 4, 5, 6, 10, 11, 12 and 18; Figure 1). Chromosome 2 harbored the greatest number of QTL, while chromosome 12 contained the least number of QTL. We identified four pre-weaning body weight QTL, 14 post-weaning body weight QTL, eight pre-weaning growth rate QTL, 12 post-weaning growth rate QTL, and six overall growth rate QTL (Figure 1). The total phenotypic variation explained by these QTL was summed for each trait in Table 4, along with the number of QTL. The average phenotypic variation accounted for by each of the 44 QTL was about 2.5%. Average daily gain for the 1st to 6th week period (Figure 2) had the most QTL identified and these QTL also explained the largest amount, about 15.6%, of the phenotypic variance (Table 4).

**Epistasis and interaction effect**

We identified 12 epistatic QTL that significantly interacted \( (p\text{-value}<0.05) \) with myostatin genotype (Table 5). Similarly, 11 and 14 QTL were detected with a significant interaction with reciprocal cross and sex, respectively (Table 6 and 7). These 37 QTL were distributed on chromosomes 1, 3, 4, 6, 7, 8, 10, 11, 12, 14 and X chromosome (Figure 3). Twenty-one of these QTL were also detected for their significant additive and dominance QTL effect when the whole genome scan was performed on the full data. However, the other 16 QTL did not have a significant QTL effect when the whole F2 population was analyzed. The phenotypic variation accounted for by these additive and dominance interaction effects are summarized in Table 5, 6 and 7, along with the corresponding F and p-values. The amount of total phenotypic variation of each trait that was explained by these interaction effects ranged from 1.18% to 6.36% (Table 8). Epistatic interaction effects accounted for the
greatest amount of phenotypic variation for 3rd week average daily gain. Sex × QTL interactions were detected during both the pre-weaning and post-weaning periods. However, no myostatin × QTL or cross × QTL interaction effects were detected for pre-weaning body growth traits (Table 9).

**Partitioning the total genetic variation**

The total genetic variation accounted for by QTL was partitioned into four genetic components, e.g. additive, dominance, additive interaction, and dominance interaction. The phenotypic variation explained by these four types of effects was summarized in Figure 4. About 30% of the genetic variation could be attributed to an interaction effect for the 1st week body weight. Additive and dominance effects accounted for more genetic variation in body weights as age increased. No interaction effects were detected for 2nd week body weight and average daily gain traits. For most of the growth traits, dominance interactions explained more of the variation than did additive interactions.

**Discussion**

**Comparison with other growth trait QTL studies**

In this study, we analyzed 14 highly correlated growth traits in an F2 population derived from a myostatin-null C57BL/6 by M16i high body weight mouse line cross. Compared with previous QTL mapping studies in the mouse, some of the 44 main effect QTL were coincident to QTL positions that had been previously discovered (supplemental Table 2). In particular, the QTL with the largest F value detected in our study, which was associated with ADG3 on chromosome 2, was very close to the largest QTL identified by Rocha et al. (2004). Although it was shown to be associated with week 10 body weight, it was very possible that these two QTL came from one common QTL with pleitropic effect. In
addition, no body weight QTL have been reported on chromosomes 1, 14 and X. For example, we detected main effect QTL associated with ADG5, ADG6 and ADG64 on chromosome 1 in our F2 population. In addition to these four chromosomes, we also detected QTL in several regions on chromosomes 3, 6, 10 and 18, which had never been shown to be associated with body weight traits. Interestingly, these regions were not detected in the Rocha et al. growth study either (Rocha et al., 2004). Similar to our study, their study also used M16i as one of the founders for its mapping population. This indicated that the unique QTL we identified was most likely from the C57BL/6 genetic background and these loci might not be polymorphic in other mouse strains, which made the F2 progenies derived from C57BL/6 and M16i a valuable mapping population. Moreover, some of the QTL that had been identified in previous studies were associated with different traits in our studies. Since the traits names and their definition are not very consistent between studies, this makes it difficult to conclude whether or not these QTL really control the same traits. Even for our own study, many of the QTL that we detected for different traits were localized to the same chromosomal region. One reason might be that some traits were highly correlated. Moreover, this might indicate the presence of pleitropic QTL. Without testing for pleiotropy, we cannot tell whether this is caused by pleitropic QTL or multiple QTL that are tightly linked. Further investigation, e.g. fine mapping, can help to increase the mapping resolution and provide more information.

Epistatic QTL interactions with myostatin gene

Myostatin protein binds the activin type II receptor (ActRIIB) (Lee and McPherron, 2001). Previous research found that transgenic mice that over express follistatin, a myostatin antagonist, exhibit more muscle mass than myostatin knockout mice (McPherron et al.,
This indicates that other molecular signals might be involved in this growth-related pathway through multi-level interactions. This information was consistent with the fact that significant myostatin × QTL interactions were detected in our study. A study of mapping myostatin modifiers that impact muscle growth in the Compact mouse line was previously reported (Varga et al., 2003). However, only a Chi-square test was used to identify chromosomal regions linked to the putative myostatin modifiers. Compared to their study, our results were based on a more quantitative analysis. Therefore, our study was the first to successfully identify QTL that interacted with myostatin to control growth traits. Most of the QTL that were detected in this study were associated with post-weaning (3-6 week after birth) traits, which corresponds with the onset of puberty. This indicates that these QTL might interact with sex hormones. Moreover, it has been suggested that myostatin effects might be time-dependent (Lee, 2004). Therefore, these QTL regions could provide more information about how this kind of time-dependent mechanism is regulated. On the other hand, body weight is composed of multiple organ weights. Therefore, it will be of further interest to dissect whole body weight gain into single organ weights, e.g. adipose, muscle and etc. It has been shown that myostatin is also expressed in adipose tissue of the adult mouse (McPherron et al., 1997). Other studies have indicated that QTL by myostatin interactions can influence some adipose traits in cattle (Casas et al., 2000).

**Gene interaction with sex effect and cross effect**

Sexual dimorphism in QTL mapping has been studied previously (Kenney-Hunt et al., 2006; Fawcett et al., 2008). These QTL that significantly interact with sex might truly function differently between male and female. However, they can be a false positive result in QTL analysis. In our study, we identified 14 QTL that had a significant sex × QTL
interaction. Compared with QTL that exhibited a myostatin × QTL interaction, we did not observe a clear time-dependent trend in sex × QTL interactions (Table 9). We found that among these 14 QTL, four were located on chromosome X. These QTL associations can be a result of X chromosome dosage compensation or sex-specific genes. In mammals, X chromosome dosage compensation results in equal gene expression between different sexes. Further investigation could be performed to narrow these regions and identify causal mutations. The most significant sex × QTL interaction controlled growth traits during the pre-weaning period. Functional studies of genes that underlie these QTL could be important to elucidate the role that sex plays in growth.

Among the 11 QTL that had a significant reciprocal cross × QTL interaction, six were located on chromosome 4. This type of interaction indicated a strain-dependent gene expression pattern. There are several possible ways to explain this kind of reciprocal cross interaction. First, there might be an interaction between the mitochondrial and nuclear genome, as F2 individuals only inherited mitochondrial DNA from one of the F0 founder lines. Different alleles of these QTL might interact directly with mitochondrial DNA or its protein product to affect body growth. Given the importance of mitochondria in energy and metabolism processes, it was not surprising that such interactions were observed. Similarly, differential reciprocal cross effects have been documented in other QTL mapping studies (Smith Richards et al., 2002; Lagerholm et al., 2009). Another explanation of this reciprocal cross effect could be a result of some effects from the Y chromosome. Since F2 mice within each cross only inherited their Y chromosome from either M16i or C57BL/6 founders. These QTL loci might interact with genes on the Y chromosome to affect growth. Statistically speaking, any effect resulting from the Y chromosome was confounded with the effect of
mitochondrial DNA. Therefore, further investigation is needed to identify the real reason behind this cross × QTL interaction.

**Variation explained by epistatic interactions**

In spite of the fact that we identified 37 QTL with significant interactions with myostatin, sex or reciprocal cross effect, the total phenotypic variation accounted for by these QTL was not very large (Table 8). The QTL with the largest effect accounted for only about 6.36% of the phenotypic variance of ADG3 trait. This is much smaller than that estimated by Brockmann et al. and Carlborg et al. (Brockmann *et al.* 2000; Carlborg *et al.*, 2003). They reported that epistasis could account for approximately 33-36% of the phenotypic variance observed in body weight and fat accumulation in mice. However, both these studies and our study show that there might be a time-dependent trend for epistatic or interaction effects. Gene interactions seem to be more important for early growth (Table 9). In addition, dominance QTL interactions with myostatin genotype explained more phenotypic variation than did additive QTL interactions. Dominance interaction effects might be a preferred mechanism for the myostatin associated growth pathway. There are several possible explanations for the difference between Carlborg’s (Carlborg *et al.*, 2003) and our estimates of genetic variation. One obvious reason could be that we only considered the interactions between the myostatin locus and QTL. This is a small proportion of overall pair-wise gene interactions that might be involved in the control of body weight. A further analysis of the interaction between these QTL and interaction between non-significant genome locations could possibly explain more phenotypic variance. In addition, Carlborg et al. used a 20% genome wide significant threshold to identify epistatic QTL while we used a 5% level. The difference of significance threshold might lead to a difference in the total number of QTL
detected. Furthermore, the allele segregation status in M16i and C57BL/6 genome can also be a reason for this. Some QTL that associate with body weight traits might not be segregating in the F2 population derived from these two strains and this limits the number of QTL that can be detected. One possible way to solve this problem is to use a mapping population that has more genetic variation. For example, the collaborative cross is a good candidate (Threadgill et al., 2002). By an 8-way intercross, a collaborative cross represents more abundant genetic variation than any single mouse strains, because a large number of recombination events occurred in the genome during the development of the collaborative cross. QTL mapping studies in these mice will provide a higher statistical power and better resolution.

Conclusions

We identified a substantial number of QTL that control body weight and growth rate traits. In addition, we also detected QTL that significantly interact with myostatin genotype, sex and reciprocal cross. Further investigation of individual tissues will help to elucidate more details on how myostatin regulates growth. In future studies, these QTL regions could be used to search for candidate genes that affect the myostatin signaling pathway.

Acknowledgement

We would like to thank Dr. Daniel Pomp for providing the M16i mice used in our study and technical insights. This research was supported by grants from the USDA CSREES 2006-35205-16696.
Tables and Figures

Tables

Table 1. Summary statistics for growth traits in this study

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Table 2. Phenotypic correlations among growth traits

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Table 3. (continued)

Table 4. Total percentage of phenotypic variance accounted for by QTL detected

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Table 5. Statistics of QTL with significant interactions with myostatin genotype

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Table 6. Statistics of QTL that interacted with reciprocal cross

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<th>Position (cM)b</th>
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<th>AI %var</th>
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Table 7. Statistics of QTL interacted with sex

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Table 8. Total percentage of phenotypic variance accounted for by QTL interactions

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### Table 9. Total percentage of phenotypic variance accounted for by QTL interactions

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<td>Overall GR</td>
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### Figures

**Figure 1**

![Graph showing distribution across chromosomes](image)
Figure 2

![Graph of F-value against Chromosomes with thresholds indicated.]

Figure 3

![Bar chart showing number of QTL on different chromosomes. Legends: Cross, Sex, Myostatin.]
Figure 4

Legends to Tables

Table 1. Summary statistics for growth traits in this study

Abbreviation: BW1, BW2, BW3, BW4, BW5 and BW6 are body weights (gram) measured 7, 14, 21, 28, 35 and 42 days after mice were born. ADG2, ADG3, ADG4, ADG5, ADG6, ADG31, ADG64 and ADG61 are average daily gain calculated for the 1\textsuperscript{st} - 2\textsuperscript{nd}, 2\textsuperscript{nd} - 3\textsuperscript{rd}, 3\textsuperscript{rd} - 4\textsuperscript{th}, 4\textsuperscript{th} - 5\textsuperscript{th}, 5\textsuperscript{th} - 6\textsuperscript{th}, 1\textsuperscript{st} - 3\textsuperscript{rd}, 4\textsuperscript{th} - 6\textsuperscript{th} and 1\textsuperscript{st} – 6\textsuperscript{th} week growth periods. S.D: standard deviation.

Table 2. Phenotypic correlation among growth traits

Phenotypic correlation coefficients have p-value less between 0.01 and 0.05 are in italic.

Phenotypic correlation coefficients have p-value larger than 0.05 are in bold.

All other phenotypic correlation coefficients have p-value less than 0.01.
Table 3. Statistics of growth trait QTL detected in genome wide scan

aQTL with an F value that exceeded the 1% genome-wide permutation threshold are denoted by **; other QTL exceeded the 5% threshold.
bPeak position of QTL detected in Kosambi centimorgans. cFlanking markers (left and right) of the QTL peak.

Abbreviation: a: additive effect; d: dominance effect; A positive estimate indicates that the effect of the recurrent QTL genotype is larger than the effect of the heterozygous genotype. s.e.a: standard error of additive effect; s.e.d: standard error of dominance effect. %var: percentage of phenotypic variance accounted for by QTL.

Table 4. Total percentage of phenotypic variance accounted for by QTL detected

aTotal number of QTL that were associated with each trait.
bTotal percentage of phenotypic variance accounted for by QTL for each trait.

Table 5. Statistics of QTL interacted with myostatin effect

aTrait name of QTL with significant interaction. Interactions with p-value less than 0.01 are denoted by **; others have p-value less than 0.05.
bPeak position of QTL with significant interaction in Kosambi centimorgans.

Abbreviation: AI: additive interaction; DI: dominance interaction. %var: percentage of phenotypic variance accounted for by QTL.

Table 6. Statistics of QTL interacted with reciprocal cross effect

aInteraction with p-value less than 0.01 is denoted by **; others have p-value less than 0.05.
bPeak position of QTL with significant interaction in Kosambi centimorgans.

Abbreviation: AI: additive interaction; DI: dominance interaction. %var: percentage of phenotypic variance accounted for by QTL.
Table 7. Statistics of QTL interacted with sex effect

aTrait name of QTL with significant interaction. Interaction with \( p \)-value less than 0.01 is denoted by **; others have \( p \)-value less than 0.05.

bPeak position of QTL with significant interaction in Kosambi centimorgans.

Abbreviation: AI: additive interaction; DI: dominance interaction. \%var: percentage of phenotypic variance accounted for by QTL.

Table 8. Total percentage of phenotypic variance accounted for by QTL interaction

aTotal number of QTL that significantly interact with myostatin, sex or cross.

bTotal percentage of phenotypic variance accounted for by QTL interaction.

Table 9. Total percentage of phenotypic variance accounted for by QTL interaction

aMain effect that QTL interact with. Cross: reciprocal cross; Myostatin: myostatin genotype; Sex: sex.

bTotal number of QTL that significantly interact with cross or myostatin or sex.

Abbreviation: Pre-weaning: 1st to 3rd week; Post-weaning: 4th to 6th week; BW: body weight; GR: growth rate, average daily gain. \%var: Total percentage of phenotypic variance accounted for by QTL interaction.

Legends to Figures

Figure 1. Chromosomal distribution of QTL for different growth periods.

Pre-weaning BW: pre-weaning body weight trait, include BW1, BW2 and BW3; Late BW: late body weight traits, include BW4, BW5 and BW6; Pre-weaning GR: pre-weaning growth rate traits, include ADG2, ADG3 and ADG31; Late GR: late growth rate traits, include ADG4, ADG5, ADG6 and ADG64; Overall GR: overall growth rate trait, includes ADG61.

Figure 2. QTL scan for average daily gain of 1st week and 6th week by interval mapping.
Figure 3. Chromosomal distribution of QTL that interact with different main effect.

Figure 4. Partitioning of the genetic variation explained by QTL mapped at a 5% genome-wide significance level.

Abbreviation: A: additive effect; D: dominance effect; AI: additive interaction effect; DI: dominance interaction effect.

References:


Corva P M and Medrano J F. (2001). Quantitative trait loci (QTLs) mapping for growth traits
in the mouse: a review. *Genet Sel Evol* **33**: 105-32.


### Supplementary Table 1. List of 152 SNPs used in the final QTL mapping

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ᵃPosition of markers in Kosambi centimorgans. This column of linkage map is build using Cri-map (Green and Crooks, 1990).

ᵇPosition of markers in Kosambi centimorgans. This column of linkage map is from the Wellcome-CTC Mouse Strain SNP Genotype Set (http://www.well.ox.au.uk/mouse/INBREDS).

ᶜPositions of the first marker on each chromosome is from the Wellcome-CTC Mouse Strain SNP Genotype Set.
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<sup>a</sup>Flanking marker physical position of QTL identified in our study.

<sup>b</sup>QTL names in Roach et al. growth traits study (Rocha et al., 2004).

<sup>c</sup>QTL names and associated traits in other studies.
References cited in Supplemental Table 2


Supplemental Figure 1.

Distribution of growth traits measured in this study. For each trait, the histogram is shown individually for all 4 subpopulations of different myostatin genotype and sex combination.

Abbreviation: BW1, BW2, BW3, BW4, BW5 and BW6 are body weights (gram) measured on 7, 14, 21, 28, 35 and 42 days after mice were born. ADG2, ADG3, ADG4, ADG5, ADG6, ADG31, ADG64 and ADG61 are average daily gain calculated for the 1st - 2nd, 2nd - 3rd, 3rd - 4th, 4th - 5th, 5th - 6th, 1st - 3rd, 4th - 6th and 1st - 6th week growth periods.
Supplemental Figure 1. (continued)
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Supplemental Figure 1. (continued)
Supplemental Figure 1. (continued)
Supplemental Figure 1. (continued)
Supplemental Figure 1. (continued)
CHAPTER 3. IDENTIFICATION OF IMPRINTING AND MYOSTATIN
INTERACTION EFFECTS ON MUSCLE AND ADIPOSE MASS, OBESITY AND
MORPHOMETRIC TRAITS IN MICE

A paper to be summited to PloS Genetics

Ye Cheng, Satyanarayana Rachigani, Jack C Dekkers,
Mary Sue Mayes, Richard Tait, Daniel Nettleton, James M Reecy

Abstract

Obesity has a close relationship to many known chronic human diseases. Efforts have
been made to investigate the genetic mechanisms behind obesity. Genome-wide association
studies are widely performed to discover quantitative trait loci (QTL) and causal mutations
that are associated with obesity-related traits. A number of obese mouse lines have been
developed for this purpose. Although some obesity QTL regions have been identified in mice,
limited research has been focused on the imprinting and interaction effects involved in
obesity traits in mice. In preliminary studies, we found that myostatin genotype, reciprocal
cross and sex effects interacted with numerous chromosomal regions to affect growth traits.
Here, we used obese mouse line M16i and muscular mouse line C57BL/6 to identify muscle,
adipose and morphometric phenotypic QTL (pQTL), translation and transcription QTL
(tQTL) and expression QTL (eQTL) by applying a QTL model with additive, dominance,
imprinting and interaction effects. Using an F2 population of 1000 mice, we identified a
number of pQTL and eQTL that significantly impacted obesity traits. In addition, six
imprinted pQTL were discovered on chromosomes 6, 9, 10, 11 and 18 that were associated
with fat-related and body size traits. pQTL and eQTL that interacted with myostatin,
reciprocal cross and sex effect were detected on some chromosomes. These interaction
effects accounted for a large amount of phenotypic variation in this study. Therefore, imprinting and interaction effects could be key players in the cause of obesity phenotypes.

**Author Summary**

Obesity genes have been widely identified in previous literature, however, the underlying genetic mechanism is still too complicated to be elucidated completely by candidate gene approach. Here, we applied QTL mapping methods to both phenotypic obesity-related traits and gene expression traits. Using F2 mice derived from a myostatin-null line and an obese line, we show that numerous genomic regions are associated with these traits. Some of them have significant interaction effects with myostatin genotype. Imprinting was also identified for several loci. Further study of these regions may provide novel genes that directly or indirectly regulate obesity. Our results also illustrate that gene expression profiles may be considered as a necessary part of QTL mapping to provide more information about regulating pathways.

**Introduction**

Obesity is an increasing problem faced globally. The World Health Organization estimated that around one billion adults in the world are overweight and more than 300 million are obese. It has been pointed out that weight gain significantly increases the risk of diabetes [1]. In addition, excess weight has a close relationship with other severe chronic diseases, such as, cardiovascular disease, hypertension and stroke, certain forms of cancer, insulin resistance, and hyperinsulinemia [2].

Environmental factors, such as consumption of sugars and less physical activity might also cause obesity [3,4]. In addition to this, genetics is another important cause of obesity. In human studies, several chromosomal regions that were associated with obesity-related traits
have been identified [5-7]. Novel genes involved in adipose accumulation might be harbored in these regions. Obesity loci have also been identified in pigs [8,9]. Unlike humans, it is easier to control environmental variation in model organisms, such as mice. Therefore, many QTL mapping studies have been conducted in mice to unravel loci that might play a role in obesity-related traits, e.g. fat depots, leptin, cholesterol, fatty acid, insulin and glucose levels [10-15].

In this study, an M16i mouse line and C56BL/6 myostatin-null mouse line were crossed to obtain an F2 population for mapping obesity-related traits. The M16i is an obese mouse line derived from an ICR mouse line after selection for 3-6 weeks body weight gain [16]. M16i mice exhibit many typical obesity phenotypes [17-20]. On the other hand, the myostatin-null mouse displays a significant decrease in body fat accumulation with an increase in skeletal muscle mass [21]. Therefore, the F2 population developed from these two lines might contain segregating genetic loci that are involved in adipose and muscle development. These loci can be identified with the aid of QTL mapping.

Here, we studied ten muscle, adipose and morphometric phenotypes, six transcription and translation traits and nine gene expression traits in this F2 population. The nine genes were identified differentially expressed in myostatin-null and myostatin wild-type mice in a previous microarray experiment [22]. An imprinted QTL model was tested against an additive and dominance QTL model with the aim to identify potential imprinted QTL. Interaction effects between QTL and the myostatin genotype, reciprocal cross and sex were evaluated as well. Finally, the amount phenotypic variation accounted for by each QTL was computed.

**Results**
Data evaluation

Summary statistics for all phenotype measurements are presented in Table 1 and pairwise phenotypic correlations are in Table 2. Most traits were highly correlated with other traits (Table 2). In particular, the two adipose traits, BMI and fat pad weight had significant correlations (P<0.05) with all other traits.

The significant main effects and interaction effects identified in Proc GLM were included as fixed effects in the QTL model. Polymorphism of SNPs within lines were detected for some SNPs, therefore, imprinted effects were also included in the QTL model for further testing. Details of these models are discussed in the method section and are not repeated here.

Additive and dominance effect of pQTL

We observed 21 and 38 non-imprinted pQTL at 1% and 5% genome-wide significance levels respectively, using the additive and dominance pQTL model (Table 3). We detected pQTL for all 10 traits measured in our study, except for the soleus muscle weight percentage. In particular, we detected the most number of pQTL for the gastrocnemius weight percentage (Table 3). Only one pQTL was detected for BMI and for tail length (Table 3). These two QTL were both located on chromosome 11. In addition, the 38 non-imprinted pQTL were distributed on 13 chromosomes. No pQTL were identified on chromosomes 4, 12, 13, 19 and X. Chromosome 1 harbored the greatest number of pQTL. The phenotypic variation accounted for by these 38 pQTL ranged from 0.86% to 9.88%. Interestingly, the pQTL that were associated with pectoralis and gastrocnemius on chromosome 1 had the two largest F-values. These two pQTL also explained the largest amount of phenotypic variation (Table 3). The distribution of estimated additive, dominance effects are shown individually in Figures
1a and 1b. Most additive and dominance effects were small. Compared with the dominance effects, more pQTL had larger additive effects.

**Imprinting effect of pQTL**

We identified six imprinted pQTL with a comparison-wise $P$-value less than 0.05 (Table 4). These pQTL were located on chromosomes 6, 9, 10, 11 and 18. Of these pQTL, three were associated with nasal to anal length. The imprinted pQTL on chromosome 10 for nasal to anal length had the largest imprinting effect (Figure 2). The two imprinted pQTL on chromosome 18 shared the same peak position and both were associated with adipose traits. The variation accounted for by these pQTL was similar, about 2.2-2.4% of the total phenotypic variation. Although only six imprinted pQTL were detected, three had relatively large effects (Figure 1c). When compared to the $P$-values of additive, dominance and imprinting effects, the most significant effects came from the additive pQTL effects (Table 5). Summing additive, dominance and imprinting effects together, the total phenotypic variation accounted for by pQTL is presented in Figure 3.

**Interaction effect of pQTL with myostatin genotype, reciprocal cross and sex effect**

We identified 14 chromosomal positions that significantly interacted with myostatin genotype (comparison $p$-value<0.05) (Table 6). In addition, another nine and 18 positions were detected for their significant interaction with reciprocal cross and sex effect, respectively (Table 7, Table 8). Tables 6-8 include the $P$-values that were associated with three different tests for interaction. For example, in Table 6, the first test (am + dm + im) gives the $P$-value of the overall interaction, which includes interactions of additive, dominance, and imprinted pQTL effects by myostatin genotype interaction. The second test (am + dm) gave the $P$-value of the non-imprinted interaction, which includes interactions of
additive and dominance pQTL effect by myostatin genotype interaction. The third test (am) gave the $P$-value of the additive interaction, which includes interaction of additive by myostatin genotype interaction. More than half of the pQTL detected with myostatin genotype or sex interactions were associated with adipose traits (Table 6, Table 8). Most of the interaction effects were significant for either the non-imprinted interaction or the additive interaction. One exception is the BMI pQTL on chromosome 14 that interacted with sex. This pQTL had a significant overall pQTL by sex interaction but no significant non-imprinted or additive pQTL by sex interaction. A pQTL with a similar interaction pQTL pattern was found for tail length on chromosome 7 for the cross interaction.

**Main effect eQTL and interaction with eQTL**

We identified two main effect eQTL on chromosomes 1 and 4 (Table 9) by a 5% genome-wide significance level. The eQTL on chromosome 1 was associated with Tnni1 expression level. The eQTL on chromosome 4 impacted Igf1 expression. Both eQTL explained about 2% of the phenotypic variation. In addition, two, four and one eQTL were detected with a significant interaction with reciprocal cross, myostatin genotype and sex effect (Table 10). These eQTL were located on chromosomes 1, 3, 6, 7, 8 and X. Similar to pQTL, the $P$-values from three interaction tests are presented in Table 10, along with the phenotypic variation that could be explained by these interactions.

**Main effect tQTL and interaction with tQTL**

No significant main effect (additive, dominance or imprinted) QTL was identified for the six transcription and translation traits in our study by a 5% genome-wide significance level. Using a threshold of comparison-wise $P$-value less than 0.05, a total of seven tQTL were identified for their significant interaction with myostatin genotype, sex or reciprocal
cross effect (Table 11). Among these seven tQTL, five of them interacted with reciprocal cross, one of them interacted with myostatin genotype and one of them interacted with sex. After a testing of different interaction model, all of these seven QTL had significant overall interaction, additive and dominance interaction (Table 11). The average variation accounted for by these QTL is about 2.5%.

**Genetic variation components from pQTL**

For each trait, the total amount of phenotypic variation accounted for by additive, dominance and imprinted pQTL is presented in Figure 3. pQTL that were associated with gastrocnemius muscle weight explained the largest amount of phenotypic variation. The phenotypic variation accounted for by interaction effects is summarized in Figure 4. For fat related traits, most of the variation was caused by pQTL interaction effects with myostatin genotype and sex. Compared with adipose traits, cross by pQTL interaction effects explained more phenotypic variation for muscle weight traits. We did not identify any phenotypic variation resulting from sex by pQTL interactions for body length traits. Summing all additive, dominance, imprinting and interaction effects, the variation explained by different components of total variation is presented in Figure 5. The largest proportion of phenotypic variation came from the additive and dominance QTL effects for most traits. Additive, dominance, and interaction QTL effects explained almost equal amounts of genotypic variation for BMI. Most of the genotypic variation for soleus weight came from interaction effects. The variation from imprinting effects varied from trait to trait and was relatively small for most traits.

**Discussion**

**Imprinting effects**
We identified six imprinted QTL in this study. The reason that we were able to detect these imprinting effects was that the two mouse lines used were not fully inbred. Polymorphisms of QTL were detected within the parental lines. This is shown in information content graph (Supplemental Figure 1). We also found that three of these six QTL were body length traits.

In mice, imprinting effects have been studied by others. For example, a study by Leamy et al. [23] used the post hoc t-test from regression analyses and discovered several QTL that displayed an imprinting pattern for mandible size and shape in mice. These QTL were located on chromosomes 2, 3, 6 and 12. Imprinted QTL have also been identified on mouse chromosome 8 for a mature body mass trait [24]. In addition, there was evidence to support that some genomic regions on mouse chromosomes 3, 4, 5, 6, 7, 12 and 18 had imprinting effects on adult body composition [25,26]. Compared with these previous mapping results, the QTL regions we discovered here on chromosomes 9, 10 and 11 had not been previously identified to affect body length traits. These regions might harbor unknown genes that regulate body size.

The imprinted QTL we identified on chromosome 18 significantly impacted fat-related traits. This had not been described in other imprinted QTL studies. Limited research has been done in this area. In mice, imprinted obesity QTL were first identified in LGXSM recombinant inbred strains [27]. Other studies of imprinted fat-related QTL support an association between fat pad weight and chromosome 2 and 7 in mice [28]. Imprinted obesity QTL are more widely identified in other species such as humans and pigs [29,30]. For example, the most famous study of fat-related imprinted QTL in pigs was the mapping of IGF2 locus [31]. This locus had a huge effect on muscle mass and fat deposition.
Additive and dominance effect of pQTL

Using interval mapping and the genome-wide permutation method, we identified 38 pQTL with significant additive and dominance effects on the ten obesity traits. Most of the estimates of additive and dominance effect were very small. Six and one pQTL had relatively large additive and dominance effects, respectively (Figure 3). These results agree with the fact that quantitative traits are controlled by many genes of smaller effect and a few genes of larger effect. Furthermore, a large number of the identified additive and dominance pQTL in this study were associated with muscle weight or fat-related traits. This is understandable when considering the two mouse lines we used to set up the breeding. The biggest phenotypic difference between M16i and C57BL/6 myostatin-null lines are in skeletal muscle weight and fat accumulation. We expected that loci controlling these phenotypes were segregating in the F2 generation and could be identified through pQTL mapping.

The chromosomes we identified to be associated with significant pQTL effects contained some obvious candidate genes for muscle, adipose and body size development. For example, IGF-binding protein 2 on chromosome 1 modulated the activity of IGF1 to protect against obesity [32]. IGF-binding protein 5 on chromosome 1 was associated with whole-body growth and muscle development [33]. The insulin-like growth factor 1 receptor gene on chromosome 7 and the growth hormone gene and signal transduction factor on chromosome 11 were also located in our pQTL regions. Defects in these genes resulted in overgrowth [34], obesity [35] and insulin resistance [36]. However, QTL detected on chromosome 1 around the myostatin locus might be false positive due to segregation distortion.

Some of the pQTL that were associated with AI and fat weight overlapped. This agreed with the high positive correlation between these two traits. However, the pQTL identified on
chromosome 11 for BMI was not significant for either AI or fat weight. pQTL for fat-related traits, e.g. body fat mass and body mass, have been mapped to this region previously [37-39]. The fact that BMI, AI and fat weight pQTL were not totally identical supported the importance of using BMI as a measurement of obesity. BMI was first invented in the 19th century and after that it has been widely used in clinical obesity research. Studies found that the increase of body mass index in the United States during the 1980s coincided with an increase in the incidence of non-insulin-dependent diabetes [40]. Moreover, there has been tremendous interest in understanding the underlying genetic mechanism [41]. Genes have been identified for their significant impact on BMI at different growth periods [38,42,43]. BMI takes into account the body size information that might not be elucidated from AI and fat weight measurement alone.

**Interaction effect of pQTL**

We tested the pQTL by myostatin genotype, pQTL by reciprocal cross and pQTL by sex interaction in this study. We also calculated the different components of the pQTL interaction effects, including additive, dominance and imprinting effects. Correspondingly, the interaction tests were also itemized to include three different types of interaction. The first test was an overall interaction test that considered the additive pQTL effect by myostatin genotype, reciprocal cross or sex interaction (am), the dominance QTL effect by myostatin genotype, reciprocal cross or sex interaction (dm) and the imprinted pQTL effect by myostatin genotype, reciprocal cross or sex interaction (im). The second test was an additive and dominance interaction test that only considered the am and dm. The third test was an additive interaction test that only considerer the am. The results from these tests gave an idea of what might be the most important interaction effect. For example, from our results (Table
6-8), the combination of additive and dominance interaction effects were significant for most of the traits tested. We also noticed that some these pQTL did not have a significant combination effect (am + dm) but actually had a significant additive interaction effect (am), e.g. pectoralis weight pQTL on chromosome 1 interacting with reciprocal cross effect. Meanwhile, there were other pQTL that did not have a significant additive interaction effect (am) but actually had a significant combination interaction effect (am + dm), e.g. gastrocnemius weight pQTL on chromosome 1 interacting with myostatin genotype effect. This second example might be an indication of important dominance interaction effect. Due to the limited functions provided by GridQTL, we did not test the dominance interaction effect directly.

In addition, we estimated the phenotypic variation accounted for by the interaction effect in our study. Compared with the additive and dominance effect, the phenotypic variation explained by the interaction effect was much smaller (<2% of the total phenotypic variation). In our previous pQTL mapping study of growth traits (unpublished work), we discovered a large number of interaction effects that also explained a small amount of phenotypic variation. These results suggest that for quantitative traits, gene-gene interaction effects are usually smaller than additive and dominance effect. However, when we take into account the total number of possible gene-gene interaction in a certain pathway, the total amount phenotypic variation that accounted for for these interaction effects may be a fairly large.

**tQTL, eQTL and pQTL**

Two tQTL were identified on chromosomes 1 and 13. These two QTL significantly interact with cross to affect the total RNA amount and the RNA/DNA ratio in the F2 mice.
RNA/DNA ratio is a measurement of transcription rate. Therefore, these two QTL might be some cross-specific transcription factor that controls the RNA transcription rate from DNA. Another two tQTL that was associated with the total protein amount and the protein/DNA ratio were identified at close positions on chromosome 2. These two tQTL significantly interact with myostatin genotype and sex. Inactivation of myostatin post-natally can cause muscle hypertrophy [44] in mice. One important symptom of muscle hypertrophy is an increasing amount of protein or protein/DNA ratio in the cell. Therefore, these two QTL can be key mediators that are controlled by myostatin to affect protein synthesis and muscle hypertrophy.

It has been identified that Igf1 regulates skeletal muscle growth through promoting satellite cells and synthesizing muscle protein [45,46]. In our previous QTL study of growth traits in the same population, we discovered that the genomic region around 65 cM on chromosome 4 was significantly associated with average daily gain (from the 1st to 3rd week) and body weight (the 3rd, 4th and 5th week) traits. Here in this study, we identified an eQTL for Igf1 at 68cM on the same chromosome. Considering the fact that the Igf1 gene is on mouse chromosome 10, these results suggested that it is very possible that a trans-regulator element of Igf1 gene is on chromosome 4. This element might control the expression level of Igf1 gene to further regulate body growth. In addition, another Igf1 eQTL was identified on chromosome 7 for its significance interaction with reciprocal cross. We detected muscle pQTL with significant main and interaction effects with cross and sex near this position. Igf1 seems to be a very active mediator factor between regulating elements and growth, and its expression level appears regulated by sex and genetic backgrounds.
We identified two eQTL that interacted with myostatin genotype to affect Atp2a2 expression levels. These two eQTL did not locate on the same chromosome as Atp2a2 gene and might be long-distance regulating factors. Recently, it was shown that a change in Atp2a2 expression level represented a fiber-type transformation [47]. Another protein analysis of skeletal muscle in cattle demonstrated that myostatin impacted the number of different muscle fibers [48]. These pieces of information suggest that myostatin might interact with these two eQTL to regulate the Atp2a2 activity, and alter fiber type.

The two examples of eQTL we discussed above prove the important role of eQTL mapping in quantitative trait analysis. It provides complementary details about genetic mechanism on an mRNA level. This is a key link between DNA and phenotypes and makes it much easy to identify candidate genes for regulators in a pathway.

**Conclusions**

Imprinted pQTL were identified on chromosomes 6, 9, 10, 11 and 18 to impact muscle weight, fat-related and body length traits. Furthermore, pQTL, tQTL and eQTL with significant interaction effects with myostatin genotype, reciprocal cross and sex effect were identified across the genome. These results will help to search for genes involved in obesity formation. Particularly, these QTL were evaluated for their additive, dominance and imprinting interaction effects. This knowledge is important to understand how obesity genes function to regulate adipose accumulation.

**Materials and Methods**

**Mouse lines and breeding procedure**

Two founder mouse strains, myostatin-null C57BL/6 [49] and M16i high body weight [16], were reciprocally crossed to derive an F2 mapping population. The Myostatin-null
C57BL/6 line contained non-functional Myostatin gene on both chromosomes. The M16i obese mouse line was derived and selected from an outbred population (ICR) for high weight gain before 6 weeks of age. To generate the F2 progenies, four myostatin-null male mice were crossed with eight M16i females. This generated 35 male and 37 female F1 mice. Meanwhile, two M16i males and seven myostatin-null females were mated in the reciprocal cross to produce 31 male and 55 female F1 offspring. The resulting F1 mice were intercrossed within each reciprocal cross to obtain the final mapping population.

**Traits collection procedure**

One day after birth, each F2 litter was standardized to nine pups. Mice were identified by toe clipping seven days after birth. At three weeks of age, mice were weaned and placed in separate cages. We obtained 552 mice from the myostatin-null male by M16i female cross and 448 mice from the reciprocal cross. These 1000 F2 mice were either homozygous myostatin wild-type or homozygous myostatin-null genotype. At 42 days of age, the body weight was recorded for each F2 individual. Subsequently, mice were sacrificed. Skeletal muscles (soleus, gastrocnemius, EDL and pectoralis) from both left and right sides of body and gonadal fat pad (epididymal for males and perimetrial for females) were collected and weighed. Body size (nasal-tail length, nasal-anal length and tail length) was also measured. Based on these measurements, body mass index (BMI) and adiposity index (AI) were calculated and included as measurements of obesity.

A small cut of the right pectoralis muscle of each F2 mouse was first used for total RNA, DNA and protein isolation with standard protocol. The ratio of DNA/RNA, RNA/protein and DNA/protein were calculated based on these measurements. The rest of the muscle was then homogenized in liquid nitrogen. After homogenization, total RNA was
isolated using RNeasy (Qiagen). The resulting RNA was amplified and measured by a triplex qPCR using Quantitect (Qiagen). Three sets of triplex qPCR experiment were performed. The first set included beta-actin (Actb), adipophilin (Adfp) and ATPase2 (Atp2a2). The second set included epidermal growth factor (Egf), insulin-like growth factor 1 (Igf1) and insulin-like growth factor 2 (Igf2). The third set included myogenic factor 5 (Myf5), troponin I (Tnni1) and wingless-related MMTV integration site 4 (Wnt4). Each sample was measure twice and the averaged CT value was then normalized by the CT value of the Actb gene and by the plate efficiency. The adjusted CT values were then used as traits for eQTL mapping. All animal procedures were approved by the Iowa State University Animal Care and Use Committee prior to this study.

**Genotyping and linkage map**

Genomic DNA was isolated from toe clips and purified by a phenol chloroform method. A total of 242 SNPs evenly spanning 19 autosomes and X chromosome were genotyped, in addition to the myostatin locus on the 1000 F2 mice. The myostatin locus was genotyped by standard PCR and agarose gel electrophoresis protocols. The SNP genotyping procedure was performed on the Sequenom® platform at GeneSeek® (Lincoln, Nebraska). SNPs on chromosome 15 and chromosome 16 were discarded because no informative SNPs were present. Among the remaining SNPs, 152 SNPs with call rates greater than 80% and no observable genotyping errors were included in the analysis. These 152 SNPs were located on 17 autosomes and the X chromosome. Marker segregation distortion was evaluated in the F2 mice by a Chi-square test. Only SNPs close to the myostatin locus significantly deviated from the expected Mendelian segregation ratios. This was caused by the fact that only homozygotes at the myostatin locus were included. All 152 SNP markers were used to
generate a linkage map by Cri-map [50], with distances estimated in Kosambi centimorgans. The marker order and position in our map (Supplemental Table 1) were consistent with the map from the Wellcome-CTC Mouse Strain SNP Genotype Set. Therefore, we analyzed the data based on our linkage map.

Data analyses

Data exploration

Simple statistics (mean, standard deviation, minimum and maximum) were calculated for each trait. In addition, all main factors (myostatin genotype, sex, reciprocal cross, coat color) and interaction terms (interactions between main effects) were tested for each trait by fitting a generalized linear model. Effects with $P$-value less than 0.1 were included in the QTL model (Supplemental Table 2). Finally, the significant factors were removed from the linear model to evaluate the residual correlations between each pair of trait values. All general statistical analyses in the F2 data were carried out using a SAS® software package. The corresponding procedures used were Proc MEANS, Proc GLM and Proc CORR.

Additive, dominance and imprinting effects

First, we analyzed each trait to identify imprinted QTL. To achieve this, two different models were used to perform a whole genome scan. The imprinted QTL model included significant main effects, interaction effects, along with additive, dominance and parent of origin (imprinting) effects at a single QTL position. The non-imprinted QTL model included all the terms in the first model except for the imprinting effect. Each QTL model was individually analyzed in GridQTL [51], a web-based QTL analysis program to identify QTL by interval mapping. A genome-wide permutation procedure [52] with 1000 repetitions was applied to each model to obtain 1% and 5% genome-wide significance levels. All QTL
positions above the 5% significance level under the imprinted QTL model and the non-imprinted QTL model were used to evaluate the imprinting effect by calculating an F-value as follows:

\[
F = \frac{\Delta SSE}{SSE(\text{imprinted QTL model})/ \text{d.f. (imprinted QTL model)}}
\]

\[
\Delta SSE = SSE(\text{non-imprinted QTL model}) - SSE(\text{imprinted QTL model})
\]

\[
d.f. : \text{degree of freedom of error term (same after)}
\]

\[
SSE : \text{sum squares of error}
\]

The corresponding comparison-wise \( P \)-value was computed from a standard F-distribution with the corresponding degrees of freedom. If the \( P \)-value was less than 0.05, the imprinted QTL model was assumed to be more suitable for this QTL position. Otherwise, the non-imprinted model was chosen. After the best model for each trait was determined, it was applied to each QTL position again to obtain F-values, LOD-values and estimates for QTL effects, along with the corresponding standard errors for each QTL peak.

At each QTL position, a corresponding comparison-wise \( P \)-value was computed for the additive and dominance effect respectively as follows.

**Additive and dominance model (AD model):**

\[
\text{Phenotypic value} = \text{fixed effects} + \text{additive effect (QTL position)} + \text{dominance effect (QTL position)} + \varepsilon
\]

**Additive model (A model):**

\[
\text{Phenotypic value} = \text{fixed effects} + \text{additive effect (QTL position)} + \varepsilon
\]

**Full reduced model (R model):**

\[
\text{Phenotypic value} = \text{fixed effects} + \varepsilon
\]

For additive effect:
For dominance effect:

\[ F = \frac{SSE(A \text{ model}) - SSE(AD \text{ model})}{SSE(AD \text{ model}) / d.f.(AD \text{ model})} \]

The corresponding comparison-wise \( P \)-values were computed from an F-distribution with the corresponding degrees of freedom.

The phenotypic variance explained by the QTL was computed. For imprinted QTL, the percentage of phenotypic variation accounted for by a QTL position was computed as the percentage of residual sum of squares explained by the additive, dominance and imprinting effects at the QTL using the imprinted QTL model. For non-imprinted QTL, the percentage of phenotypic variation accounted for by a QTL position was computed as the percentage of residual sum of squares explained by the additive and dominance effect using the non-imprinted QTL model.

**QTL interaction analysis**

We first analyzed the interaction between a QTL position and myostatin genotype. To accomplish this, we split the data into two subsets based on myostatin genotype. Within each myostatin genotype subset, both the imprinted QTL model and the non-imprinted QTL model were evaluated by interval mapping. All QTL positions identified at 5% genome-wide significance levels in either model were considered for analysis in the next step, in which the following six QTL models were fitted to these identified QTL positions in the full whole F2 data.

**Model 1-1:**
Phenotypic value = fixed effects + additive effect (QTL position) + dominance effect (QTL position) + imprinting effect (QTL position) + Myostatin × additive effect (QTL position) + Myostatin × dominance effect (QTL position) + Myostatin × imprinting effect (QTL position) + ε

Model 1-2:

Phenotypic value = fixed effects + additive effect (QTL position) + dominance effect (QTL position) + imprinting effect (QTL position) + ε

Model 2-1:

Phenotypic value = fixed effects + additive effect (QTL position) + dominance effect (QTL position) + Myostatin × additive effect (QTL position) + Myostatin × dominance effect (QTL position) + ε

Model 2-2:

Phenotypic value = fixed effects + additive effect (QTL position) + dominance effect (QTL position) + ε

Model 3-1:

Phenotypic value = fixed effects + additive effect (QTL position) + Myostatin × additive effect (QTL position) + ε

Model 3.2:

Phenotypic value = fixed effects + additive effect (QTL position) + ε

By comparing the above models, we determined the F-values for different interaction effects as follows:

1) When considering the general interaction (additive, dominance and imprinting interaction):

\[
F = \frac{\Delta \text{SSE} / 3}{\text{SSE(model 1 - 1)} / \text{d.f. (model 1 - 1)}}
\]

\[
\Delta \text{SSE} = \text{SSE(model 1 - 2)} - \text{SSE(model 1 - 1)}
\]

2) When considering the non-imprinting interaction (additive and dominance interaction):
3) When considering the additive interaction:

\[
F = \frac{\Delta SSE / 2}{SSE(model 2 - 1)/ d.f. (model 2 - 1)}
\]

\[
\Delta SSE = SSE(model 2 - 2) - SSE(model 2 - 1)
\]

Correspondingly, each F-value gave a comparison-wise \( P \)-value. Interactions with a \( P \)-value less than 0.05 were considered significant.

Using a similar approach, reciprocal cross \( \times \) QTL interactions were analyzed. Initially, the data was split into two subsets by reciprocal crosses and potential QTL positions for testing reciprocal cross interactions were identified within each cross subset. Then, interactions at each position were tested in the full data set, similar to the procedure used for myostatin interactions but with the myostatin effect replaced by the reciprocal cross effect in models 1-1 to 3-2.

Finally, the same approach was applied to identify sex \( \times \) QTL interactions. The data was split into two subsets by sex and potential testing QTL positions were identified within each sex subset. Myostatin effect in the QTL models were all replaced by sex effect and the corresponding interaction effects were switched as well.

Estimates of additive and dominance effects and phenotypic variation accounted for by each identified QTL were estimated in the same way discussed above for main effect QTL. To calculate the total effect of pQTL, we summed the phenotypic variation explained by all main effect pQTL and interaction pQTL, for each trait respectively.

Acknowledgements
We would like to thank Dr. Daniel Pomp for providing the M16i mice used in our study and technical insights. This research was supported by grants from the USDA CSREES 2006-35205-16696.

Figure legends

Figure 1. Distribution of estimated additive effects (a), dominance effects (b) and imprinting effects (c)

Figure 2. Genome scan of imprinted QTL on chromosome 10 at 58 cM to control lengthNA

   IMP: imprinted QTL model
   AD: additive and dominance QTL model
   Vertical line indicates the position of the imprinted QTL

Figure 3. Phenotypic variation accounted for by QTL

   Trait abbreviations are the same as in Table 1.
   %var: phenotypic variation accounted for by QTL for each trait

Figure 4. Phenotypic variation accounted for by QTL that interacted with myostatin genotype, reciprocal cross and sex effect

   Trait abbreviations are the same as in Table 1.
   Mstn: Myostatin genotype by QTL interaction
   Cross: Reciprocal cross by QTL interaction
   Sex: Sex by QTL interaction

Figure 5. Partitioning of variation for different components

   interaction: the sum of myostatin genotype by QTL, reciprocal cross by QTL and sex by QTL effect
vi: imprinting QTL effect
vd: dominance QTL effect
va: additive QTL effect
Tables

Table 1. Basic statistics for obesity traits in the F2 mapping population

<table>
<thead>
<tr>
<th>Traits(^a)</th>
<th>Mean</th>
<th>Std. Dev(^b)</th>
<th>Range(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>lengthNA</td>
<td>9.926</td>
<td>0.728</td>
<td>7.000-16.700</td>
</tr>
<tr>
<td>lengthNT</td>
<td>17.691</td>
<td>1.203</td>
<td>12.000-20.500</td>
</tr>
<tr>
<td>AI</td>
<td>0.470</td>
<td>0.250</td>
<td>0.041-1.743</td>
</tr>
<tr>
<td>BMI</td>
<td>30.598</td>
<td>4.312</td>
<td>10.517-45.793</td>
</tr>
<tr>
<td>Tail</td>
<td>7.764</td>
<td>0.784</td>
<td>2.100-10.000</td>
</tr>
<tr>
<td>Soleus</td>
<td>0.032</td>
<td>0.015</td>
<td>0.010-0.381</td>
</tr>
<tr>
<td>Gastro</td>
<td>0.592</td>
<td>0.113</td>
<td>0.367-1.013</td>
</tr>
<tr>
<td>Edl</td>
<td>0.358</td>
<td>0.080</td>
<td>0.193-1.649</td>
</tr>
<tr>
<td>Pec</td>
<td>0.636</td>
<td>0.245</td>
<td>0.277-1.310</td>
</tr>
<tr>
<td>Fat</td>
<td>0.470</td>
<td>0.250</td>
<td>0.041-1.743</td>
</tr>
</tbody>
</table>

\(^a\) lengthNA: nasal to anal length (cm); lengthNT: nasal to tail length (cm); AI: adipose index; BMI: body mass index; Tail: tail length (cm); Soleus: soleus muscle weight percentage; Gastro: gastrocnemius muscle weight percentage; Edl: EDL muscle weight percentage; Pec: pectoralis muscle weight percentage; Fat: average gonadal fat pad weight percentage (epididymal for males and perimetrial for females).

\(^b\) standard deviation

\(^c\) minimum and maximum
Table 2. Phenotypic correlation among traits in this study\textsuperscript{a}

<table>
<thead>
<tr>
<th>Traits\textsuperscript{b}</th>
<th>lengthNA</th>
<th>lengthNT</th>
<th>AI</th>
<th>BMI</th>
<th>Tail</th>
<th>Soleus</th>
<th>Gastro</th>
<th>Edl</th>
<th>Pec</th>
<th>Fat</th>
</tr>
</thead>
<tbody>
<tr>
<td>lengthNA</td>
<td>1.000</td>
<td></td>
<td>0.778**</td>
<td>0.308**</td>
<td>0.013*</td>
<td>0.264**</td>
<td>-0.004*</td>
<td>0.112</td>
<td>-0.051*</td>
<td>0.097</td>
</tr>
<tr>
<td>lengthNT</td>
<td>1.000</td>
<td></td>
<td>0.377**</td>
<td>0.158**</td>
<td>0.812**</td>
<td>-0.043*</td>
<td>0.020*</td>
<td>-0.111</td>
<td>-0.014*</td>
<td>0.377**</td>
</tr>
<tr>
<td>AI</td>
<td>1.000</td>
<td></td>
<td>0.008*</td>
<td>0.291**</td>
<td>-0.016**</td>
<td>-0.454**</td>
<td>-0.439**</td>
<td>-0.382**</td>
<td>1.000**</td>
<td></td>
</tr>
<tr>
<td>BMI</td>
<td>1.000</td>
<td></td>
<td>0.231**</td>
<td>0.110</td>
<td>0.550**</td>
<td>0.347**</td>
<td>0.676**</td>
<td>0.008*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tail</td>
<td>1.000</td>
<td></td>
<td>-0.063</td>
<td>-0.073</td>
<td>-0.123**</td>
<td>-0.111</td>
<td>0.291**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soleus</td>
<td>1.000</td>
<td></td>
<td>0.351**</td>
<td>0.301**</td>
<td>0.290**</td>
<td>-0.164**</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gastro</td>
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<td></td>
<td>0.668**</td>
<td>0.886**</td>
<td>-0.454**</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Edl</td>
<td>1.000</td>
<td></td>
<td>0.606**</td>
<td>-0.439**</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Pec</td>
<td>1.000</td>
<td></td>
<td>-0.382**</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Fat</td>
<td></td>
<td></td>
<td>1.000</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

\textsuperscript{a} P-value for testing for significant correlation. **: \(P < 0.0001\); *: \(0.0001 < P < 0.05\); otherwise, \(P > 0.05\)

\textsuperscript{b} Same abbreviations for traits as in Table 1 are used here.
### Table 3. Statistics of non-imprinted QTL

<table>
<thead>
<tr>
<th>MMU</th>
<th>Traits&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Peak (cM)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Flanking Markers&lt;sup&gt;c&lt;/sup&gt;</th>
<th>F-value</th>
<th>LOD</th>
<th>Estimate&lt;sup&gt;d&lt;/sup&gt;</th>
<th>s.e&lt;sub&gt;a&lt;/sub&gt;</th>
<th>s.e&lt;sub&gt;d&lt;/sub&gt;</th>
<th>%var&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AI**</td>
<td>24</td>
<td>rs3696088 rs13472794</td>
<td>13.00</td>
<td>5.573</td>
<td>0.0813</td>
<td>0.0165</td>
<td>0.0332</td>
<td>0.0219 2.64</td>
</tr>
<tr>
<td>2</td>
<td>AI**</td>
<td>88</td>
<td>rs3144393 rs13476878</td>
<td>18.65</td>
<td>7.952</td>
<td>0.0519</td>
<td>0.0091</td>
<td>-0.0263</td>
<td>0.0138 3.65</td>
</tr>
<tr>
<td>6</td>
<td>AI**</td>
<td>27</td>
<td>rs13478727 rs13478839</td>
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<td>0.0393</td>
<td>0.0090</td>
<td>-0.0121</td>
<td>0.0136 1.99</td>
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<td>0.0030</td>
<td>0.0042 1.56</td>
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Table 3. (continued)

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<th>Peak (cM)^b</th>
<th>Flanking Markers^c</th>
<th>F-value</th>
<th>LOD</th>
<th>Estimate^d</th>
<th>%var^e</th>
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<td>Right</td>
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<td>s.e_a</td>
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</table>

^a Trait abbreviations are the same as in Table 1. QTL with an F-value that exceeded 1% genome-wide permutation threshold are denoted by **; QTL without ** exceeded 5% genome-wide permutation threshold.

^b Peak position of QTL detected in Kosambi centimorgans.

^c Flanking markers (left and right) of the QTL peak. A “-“ notation denotes the end of the chromosome. See Supplemental Table 1 for marker information.

^d a: additive effect; s.e_a: standard error of additive effect; d: dominance effect; s.e_d: standard error of dominance effect.

^e %var: percentage of phenotypic variance that a given QTL position could account for.
### Table 4. Statistics\(^a\) of imprinted QTL

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<th>MMU</th>
<th>Trait(^b)</th>
<th>Peak(^c) (cM)</th>
<th>Flanking Markers(^d)</th>
<th>Estimate(^e)</th>
<th>%var(^f)</th>
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\(^a\) Imprinted QTL with comparison-wise \(P\)-value <0.05.

\(^b\) Traits abbreviations are the same as in Table 1.

\(^c\) Peak position of QTL detected in Kosambi centimorgans.

\(^d\) Flanking markers (left and right) of the QTL peak. See Supplemental Table 1 for marker information.

\(^e\) a: additive effect; s.e\(_a\): standard error of additive effect; d: dominance effect; s.e\(_d\): standard error of dominance effect; i: imprinting effect; s.e\(_i\): standard error of imprinting effect.

\(^f\) %var: percentage of phenotypic variance that a given QTL position accounts for.
Table 5. Test statistics of additive, dominance and imprinting effects

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*a Traits abbreviations are the same as in Table 1.*
Peak position of QTL detected in Kosambi centimorgans.

Comparison $P$-value for testing QTL effect. $i$: imprinting effect; $a$: additive effect; $d$: dominance effect. Only imprinted QTL (Table 3) were estimated for the imprinting effect. A “-” notation indicates that the effect was not estimated. $P$-value $< 0.01$ is shown in bold type.
Table 6. Statistics of QTL interacting with myostatin genotype effect

<table>
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<th>Traits(^a)</th>
<th>Position (cM)(^b)</th>
<th>Flanking markers(^c)</th>
<th>(am + dm + im)(^d)</th>
<th>(am + dm)(^e)</th>
<th>(am)(^f)</th>
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<td>8.48E-03 0.69</td>
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<tr>
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<td>24</td>
<td>rs3696088 rs13472794</td>
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<td>1.77E-08 3.34</td>
<td>1.49E-01 0.20</td>
</tr>
<tr>
<td>1</td>
<td>Pec</td>
<td>25</td>
<td>rs3696088 rs13472794</td>
<td>3.95E-12 5.06</td>
<td>1.08E-11 4.56</td>
<td>3.52E-03 0.80</td>
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<tr>
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<td>Pec</td>
<td>43</td>
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<td>1.02E-02 0.91</td>
<td>8.95E-02 0.29</td>
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<tr>
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<td>Fat</td>
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<td>rs13478727 rs13478839</td>
<td>4.24E-03 1.31</td>
<td>1.36E-03 1.31</td>
<td>2.74E-04 1.31</td>
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<tr>
<td>6</td>
<td>lengthNT</td>
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<td>3.65E-02 0.43</td>
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<td>8</td>
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<tr>
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<td>Fat</td>
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<td>rs13479657 rs13479757</td>
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<td>4.50E-02 0.62</td>
<td>1.23E-01 0.24</td>
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<tr>
<td>17</td>
<td>Fat</td>
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<td>rs31482893 rs3719497</td>
<td>4.84E-02 0.74</td>
<td>3.38E-02 0.65</td>
<td>1.44E-02 0.64</td>
</tr>
<tr>
<td>17</td>
<td>AI</td>
<td>28</td>
<td>rs3023442 rs6395919</td>
<td>3.76E-02 0.83</td>
<td>2.68E-02 0.71</td>
<td>7.06E-03 0.71</td>
</tr>
<tr>
<td>17</td>
<td>Soleus</td>
<td>69</td>
<td>rs6257479 rs3663966</td>
<td>3.77E-02 0.86</td>
<td>1.82E-02 0.81</td>
<td>3.49E-02 0.45</td>
</tr>
<tr>
<td>18</td>
<td>AI</td>
<td>42</td>
<td>rs3718618 rs13483438</td>
<td>3.92E-02 0.82</td>
<td>2.97E-02 0.69</td>
<td>4.65E-02 0.39</td>
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<tr>
<td>18</td>
<td>Fat</td>
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<td>rs3718618 rs13483438</td>
<td>3.27E-02 0.87</td>
<td>2.78E-02 0.71</td>
<td>4.88E-02 0.39</td>
</tr>
</tbody>
</table>

\(^a\)Traits abbreviations are the same as in Table 1.

\(^b\)Peak position of QTL detected in Kosambi centimorgans.

\(^c\)Flanking markers (left and right) of the QTL peak. See Supplemental Table 1 for marker information.

\(^d\)\(am + dm + im\) tests the overall interaction, which includes interactions of additive, dominance, and imprinted pQTL effects by myostatin genotype interaction. \(P\)-value < 0.05 is shown in bold type. \%var: percentage of phenotypic variance accounted for at QTL position.
am + dm tests the non-imprinted interaction, which includes interactions of additive and dominance pQTL effect by myostatin genotype interaction. $P$-value< 0.05 is shown in bold type. %var: percentage of phenotypic variance accounted for at QTL position.

am tests the additive interaction, which includes interaction of additive by myostatin genotype interaction. $P$-value< 0.05 is shown in bold type. %var: percentage of phenotypic variance accounted for at QTL position.
Table 7. Statistics of QTL interacting with cross effect

<table>
<thead>
<tr>
<th>MMU</th>
<th>Traits&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Position (cM)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Flanking markers&lt;sup&gt;c&lt;/sup&gt;</th>
<th>am + dm + im&lt;sup&gt;d&lt;/sup&gt;</th>
<th>am + dm&lt;sup&gt;e&lt;/sup&gt;</th>
<th>am&lt;sup&gt;f&lt;/sup&gt;</th>
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<tbody>
<tr>
<td></td>
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<td>Left</td>
<td>Right</td>
<td>P-value</td>
<td>%var</td>
<td>P-value</td>
</tr>
<tr>
<td>1</td>
<td>Gastro</td>
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<td>rs3696088</td>
<td>rs13472794</td>
<td>2.85E-06</td>
<td>2.68</td>
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<tr>
<td>1</td>
<td>Pec</td>
<td>23</td>
<td>rs3696088</td>
<td>rs13472794</td>
<td>1.62E-02</td>
<td>0.94</td>
</tr>
<tr>
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<td>Edl</td>
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<td>rs3696088</td>
<td>rs13472794</td>
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<td>Pec</td>
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<td>rs3656205</td>
<td>3.82E-02</td>
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<tr>
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<td>rs13479471</td>
<td>rs6275579</td>
<td>3.45E-02</td>
<td>0.86</td>
</tr>
<tr>
<td>11</td>
<td>Fat</td>
<td>57</td>
<td>rs3701609</td>
<td>rs8270290</td>
<td>5.27E-02</td>
<td>0.77</td>
</tr>
<tr>
<td>11</td>
<td>AI</td>
<td>57</td>
<td>rs3701609</td>
<td>rs8270290</td>
<td>6.46E-02</td>
<td>0.72</td>
</tr>
<tr>
<td>17</td>
<td>Edl</td>
<td>31</td>
<td>rs3023442</td>
<td>rs6395919</td>
<td>2.00E-01</td>
<td>0.47</td>
</tr>
</tbody>
</table>

<sup>a</sup>Trait abbreviations are the same as in Table 1.

<sup>b</sup>Peak position of QTL detected in Kosambi centimorgans.

<sup>c</sup>Flanking markers (left and right) of the QTL peak. See Supplemental Table 1 for marker information.

<sup>d</sup>am + dm + im tests the overall interaction, which includes interactions of additive, dominance, and imprinted pQTL effects by myostatin genotype interaction. *P*-value< 0.05 is shown in bold type. %var: percentage of phenotypic variance accounted for at QTL position.

<sup>e</sup>am + dm tests the non-imprinted interaction, which includes interactions of additive and dominance pQTL effect by myostatin genotype interaction. *P*-value< 0.05 is shown in bold type. %var: percentage of phenotypic variance accounted for at QTL position.
fam tests the additive interaction, which includes interaction of additive by myostatin genotype interaction. $P$-value $< 0.05$ is shown in bold type. %var: percentage of phenotypic variance accounted for at QTL position.
Table 8. Statistics of QTL interacting with sex effect

<table>
<thead>
<tr>
<th>MMU</th>
<th>Traits&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Position (cM)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Flanking markers&lt;sup&gt;c&lt;/sup&gt;</th>
<th>am + dm + im&lt;sup&gt;d&lt;/sup&gt;</th>
<th>am + dm&lt;sup&gt;e&lt;/sup&gt;</th>
<th>am&lt;sup&gt;f&lt;/sup&gt;</th>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>Soleus</td>
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<td>rs3696088</td>
<td>rs13472794</td>
<td>1.57E-06</td>
<td>2.94</td>
</tr>
<tr>
<td>1</td>
<td>Pec</td>
<td>23</td>
<td>rs3696088</td>
<td>rs13472794</td>
<td>1.23E-03</td>
<td>1.45</td>
</tr>
<tr>
<td>1</td>
<td>Edl</td>
<td>89</td>
<td>rs3666905</td>
<td>rs13476312</td>
<td>1.65E-02</td>
<td>1.03</td>
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<tr>
<td>3</td>
<td>Pec</td>
<td>36</td>
<td>rs13477132</td>
<td>rs13477174</td>
<td>1.90E-02</td>
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<td>3</td>
<td>Edl</td>
<td>64</td>
<td>rs3663873</td>
<td>rs13477430</td>
<td>4.79E-02</td>
<td>0.80</td>
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<tr>
<td>6</td>
<td>Fat</td>
<td>10</td>
<td>petM-02094-1</td>
<td>rs3678887</td>
<td>3.61E-02</td>
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<tr>
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<td>petM-02094-1</td>
<td>rs3678887</td>
<td>6.05E-02</td>
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<tr>
<td>7</td>
<td>Pec</td>
<td>47</td>
<td>rs3676254</td>
<td>rs3656205</td>
<td>5.61E-03</td>
<td>1.25</td>
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<td>9</td>
<td>Fat</td>
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<td>rs3719607</td>
<td>rs8259427</td>
<td>2.31E-02</td>
<td>0.95</td>
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<tr>
<td>9</td>
<td>Al</td>
<td>15</td>
<td>rs3719607</td>
<td>rs8259427</td>
<td>3.50E-02</td>
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<td>rs6276300</td>
<td>rs6199956</td>
<td>3.75E-02</td>
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<td>rs13482893</td>
<td>rs3719498</td>
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<td>0.78</td>
</tr>
</tbody>
</table>

<sup>a</sup>Trait abbreviations are the same as in Table 1.

<sup>b</sup>Peak position of QTL detected in Kosambi centimorgans.

<sup>c</sup>Flanking markers (left and right) of the QTL peak. See Supplemental Table 1 for marker information.
\(d\) am + dm + im tests the overall interaction, which includes interactions of additive, dominance, and imprinted pQTL effects by myostatin genotype interaction. \(P\text{-value}< 0.05\) is shown in bold type. \%var: percentage of phenotypic variance accounted for at QTL position.

\(e\) am + dm tests the non-imprinted interaction, which includes interactions of additive and dominance pQTL effect by myostatin genotype interaction. \(P\text{-value}< 0.05\) is shown in bold type. \%var: percentage of phenotypic variance accounted for at QTL position.

\(f\) am tests the additive interaction, which includes interaction of additive by myostatin genotype interaction. \(P\text{-value}< 0.05\) is shown in bold type. \%var: percentage of phenotypic variance accounted for at QTL position.
Table 9. Results for genome-wide scan of eQTL

<table>
<thead>
<tr>
<th>MMU</th>
<th>Gene</th>
<th>Peak (cM)a</th>
<th>Flanking Markersb</th>
<th>F</th>
<th>LOD</th>
<th>Var%c</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Tnni1</td>
<td>23</td>
<td>rs3696088</td>
<td>rs13472794</td>
<td>8.73</td>
<td>3.747</td>
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<td>4</td>
<td>Igf1</td>
<td>68</td>
<td>rs6324470</td>
<td>rs3659226</td>
<td>8.46</td>
<td>3.633</td>
</tr>
</tbody>
</table>

a Peak position of eQTL detected in Kosambi centimorgans.
b Flanking markers (left and right) of the QTL peak.
c %var: percentage of phenotypic variance that a given QTL position could account for.
Table 10. Statistics of eQTL interacting with myostatin, cross and sex effect

<table>
<thead>
<tr>
<th>Factor</th>
<th>Gene</th>
<th>MMU</th>
<th>Position (cM)</th>
<th>Flanking markers&lt;sup&gt;a&lt;/sup&gt;</th>
<th>am + dm + im&lt;sup&gt;e&lt;/sup&gt;</th>
<th>am + dm&lt;sup&gt;d&lt;/sup&gt;</th>
<th>am&lt;sup&gt;e&lt;/sup&gt;</th>
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</thead>
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<td>Tnni1</td>
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<td>24</td>
<td>rs3696088</td>
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<td>4.84E-03</td>
</tr>
<tr>
<td>Cross</td>
<td>Igf1</td>
<td>7</td>
<td>53</td>
<td>rs13479422</td>
<td>1.62E-02</td>
<td>1.31</td>
<td>5.18E-03</td>
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<tr>
<td>Mstn</td>
<td>Atp2a2</td>
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<td>120</td>
<td>rs3724562</td>
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<td>3.72E-03</td>
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<td>Mstn</td>
<td>Igf2</td>
<td>8</td>
<td>33</td>
<td>rs13479657</td>
<td>6.63E-02</td>
<td>1.05</td>
<td>8.20E-01</td>
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<td>Mstn</td>
<td>Atp2a2</td>
<td>X</td>
<td>54</td>
<td>rs13484003</td>
<td>1.74E-04</td>
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<td>1.96E-03</td>
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<td>Mstn</td>
<td>EGF</td>
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<td>rs13478839</td>
<td>4.18E-03</td>
<td>1.79</td>
<td>4.85E-04</td>
</tr>
</tbody>
</table>

<sup>a</sup>Peak position of QTL detected in Kosambi centimorgans.

<sup>b</sup>Flanking markers (left and right) of the QTL peak. See Supplemental Table 1 for marker information.

<sup>c</sup>am + dm + im tests the overall interaction, which includes interactions of additive, dominance, and imprinted pQTL effects by myostatin genotype interaction. P-value< 0.05 is shown in bold type. %var: percentage of phenotypic variance accounted for at QTL position.

<sup>d</sup>am + dm tests the non-imprinted interaction, which includes interactions of additive and dominance pQTL effect by myostatin genotype interaction. P-value< 0.05 is shown in bold type. %var: percentage of phenotypic variance accounted for at QTL position.

<sup>e</sup>am tests the additive interaction, which includes interaction of additive by myostatin genotype interaction. P-value< 0.05 is shown in bold type. %var: percentage of phenotypic variance accounted for at QTL position.
Table 11. Statistics of QTL that interact with myostatin, cross and sex effect to affect muscle hypertrophy

<table>
<thead>
<tr>
<th>Factor</th>
<th>Traits&lt;sup&gt;a&lt;/sup&gt;</th>
<th>MMU</th>
<th>Position (cM)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Flanking markers&lt;sup&gt;c&lt;/sup&gt;</th>
<th>am + dm + im&lt;sup&gt;d&lt;/sup&gt;</th>
<th>am + dm&lt;sup&gt;e&lt;/sup&gt;</th>
<th>am&lt;sup&gt;f&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td>Cross</td>
<td>R</td>
<td>1</td>
<td>29</td>
<td>rs13472794 rs13475931</td>
<td>1.09E-05 3.50</td>
<td>2.03E-02 1.09</td>
<td>1.10E-01 0.36</td>
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<tr>
<td>Cross</td>
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<td>1</td>
<td>29</td>
<td>rs13472794 rs13475931</td>
<td>1.39E-04 2.82</td>
<td>1.81E-02 1.12</td>
<td>1.65E-01 0.27</td>
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<tr>
<td>Cross</td>
<td>R</td>
<td>13</td>
<td>25</td>
<td>rs13481780 rs3678784</td>
<td>1.08E-03 2.21</td>
<td>2.08E-04 2.34</td>
<td>1.26E-03 1.44</td>
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<tr>
<td>Cross</td>
<td>R/D</td>
<td>13</td>
<td>25</td>
<td>rs13481780 rs3678784</td>
<td>2.05E-03 2.02</td>
<td>4.71E-04 2.12</td>
<td>4.36E-03 1.13</td>
</tr>
<tr>
<td>Cross</td>
<td>R/D</td>
<td>14</td>
<td>18</td>
<td>rs13482096 rs8251329</td>
<td>3.64E-04 2.56</td>
<td>6.77E-04 2.03</td>
<td>1.05E-03 1.50</td>
</tr>
<tr>
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<td>P/D</td>
<td>2</td>
<td>61</td>
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<td>2.66E-04 2.64</td>
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<tr>
<td>Sex</td>
<td>P</td>
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<td>34</td>
<td>rs6268714 rs13476554</td>
<td>1.18E-02 1.53</td>
<td>3.73E-03 1.55</td>
<td>2.04E-01 0.23</td>
</tr>
</tbody>
</table>

<sup>a</sup>R: total RNA (µg/mg sample); R/D: RNA/DNA ratio (µg/µg); P/D: protein/DNA ratio (µg/mg); P: Total protein (mg/mg sample).

<sup>b</sup>Peak position of QTL detected in Kosambi centimorgans.

<sup>c</sup>Flanking markers (left and right) of the QTL peak. See Supplemental Table 1 for marker information.

<sup>d</sup>am + dm + im tests the overall interaction, which includes interactions of additive, dominance, and imprinted pQTL effects by myostatin genotype interaction. P-value< 0.05 is shown in bold type. %var: percentage of phenotypic variance accounted for at QTL position.

<sup>e</sup>am + dm tests the non-imprinted interaction, which includes interactions of additive and dominance pQTL effect by myostatin genotype interaction. P-value< 0.05 is shown in bold type. %var: percentage of phenotypic variance accounted for at QTL position.

<sup>f</sup>am tests the additive interaction, which includes interaction of additive by myostatin genotype interaction. P-value< 0.05 is shown in bold type. %var: percentage of phenotypic variance accounted for at QTL position.
Figures

Figure 1. (a) Estimated additive effects

(b) Estimated dominance effect
Figure 1. (continued)

(c)

Estimated imprinting effects

QTL #

0.15 0.12 0.09 0.06 0.03 0 0.03 0.06 0.09 0.12 0.15 0.18 0.21

Figure 2.

Position (cM)

F-value

IMP

· AD

Position (cM)
Figure 5.
Supplementary information

**Supplemental Table 1. List of 152 SNPs used in the final QTL mapping**

<table>
<thead>
<tr>
<th>SNP name</th>
<th>MMU</th>
<th>cM (Crimap)$^a$</th>
<th>cM$^b$</th>
<th>Map Location$^c$</th>
</tr>
</thead>
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\(^a\)Position of markers in Kosambi centimorgans. This column of linkage map is build using Cri-map (Green and Crooks, 1990).

\(^b\)Position of markers in Kosambi centimorgans. This column of linkage map is from the Wellcome-CTC Mouse Strain SNP Genotype Set (http://www.well.ox.au.uk/mouse/INBREDS).

\(^c\)Positions of the first marker on each chromosome is from the Wellcome-CTC Mouse Strain SNP Genotype Set.
### Supplemental Table 2. Effects included in the QTL model for traits in this study

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*a* A notation of "*" indicates that the corresponding effect is included in the QTL model. Blank indicates that the corresponding effect is not included in the QTL model.

*b* Effects in the QTL model. M: myostatin genotype; S: sex; R: reciprocal cross; C: coat color; Group: group effect in DNA, RNA and protein isolation; Plate: plate effect in real time PCR experiment; Replicate: replicate effect in real time PCR experiment.
Supplemental Figure 1. Information content values of imprinted effects
References


CHAPTER 4. QTL ANALYSIS OF INTERNAL ORGAN WEIGHT IN MICE

REVEALS IMPRINTING AND INTERACTION EFFECTS

A paper to be submitted to Mammalian Genome

Ye Cheng, Satyanarayana Rachigani, Jack C Dekkers,
Mary Sue Mayes, Richard Tait, James M Reecy

Abstract

Myostatin is an important regulator of skeletal muscle mass. Our previous QTL mapping project utilized F2 mice derived from a myostatin-null C57BL/6 by M16i obese mice cross. The results from those studies indicated that a few regions in the mouse genome significantly interacted with myostatin genotype to affect growth, skeletal muscle and adipose traits. Here, we used the same F2 mapping population from our previous work to further explore the genome for loci that interacted with myostatin genotype to impact individual organ weights. We detected 18 main effect QTL on chromosomes 1, 2, 4, 5, 9, 10, 11, 13, 14, 17 and X. Among these QTL, three appear to be imprinted with comparison $P$-values less than 0.05. Moreover, six QTL on chromosomes 1, 4, 9 and 17 were identified for their significant interaction with myostatin genotype. Our analysis also indicated that seven and ten chromosomal positions interact with reciprocal cross and sex effect respectively. QTL interaction with myostatin genotype, sex and reciprocal cross could be an important contribution to the variation in body composition traits.

Introduction

Phenotypic variation in body weights is caused by both genetic and environmental factors. Many quantitative trait loci (QTL) that impact body weights have been successfully identified (Fisler and Warden 1997; Moody et al. 1999; Morris et al. 1999; Rocha et al.
However, it is still not clear how individual components of body weight are influenced by genetic factors due to the limited number of studies that have been conducted. The first published study of body composition discussed using genetic markers to predict adipose weight and bone size (Castle et al. 1936; Danforth and de Aberle 1927). Most research about body composition QTL mainly focused on fat, heart, kidney, liver and spleen (Brockmann et al. 1998; Brockmann et al. 2000; Kenney-Hunt et al. 2006; Leamy et al. 2002; Moody et al. 1999). It will also be interesting to identify QTL that impact other internal organ weights, e.g. lungs, intestines, stomach etc. In addition to the main effect QTL, epistatic QTL and sex specific QTL have been reported in mice, chicken and humans to affect organ weights (Carlborg et al. 2005; Eisen and Legates 1966; Segal et al. 2009; Wang et al. 2006; Weiss et al. 2006; Yi et al. 2006).

Myostatin, or GDF-8, was discovered as a new member of transforming growth factor-beta (TGF-beta) superfamily (McPherron et al. 1997). Myostatin is mainly expressed in skeletal muscle as a major regulator of muscle mass. Previous studies on myostatin knockout mice demonstrated that a non-functional myostatin gene caused an increase in muscle weight and a decrease in fat accumulation (McPherron et al. 1997; McPherron and Lee 2002). However, it is unknown how myostatin affects individual organ weights.

In our previous study, we developed a F2 mouse population to map QTL involved in a number of traits, e.g. body weight, fat pad weight and muscle weight etc. These F2 mice were derived from a myostatin null C57BL/6 line and M16i (Hanrahan et al. 1973), an obese mouse line that exhibits many obesity phenotypes. Our results identified many regions in the mouse genome that were associated with those growth and obesity related traits. In addition, a few QTL were detected for their significant interaction with myostatin genotype to affect
these traits. Since there is a close correlation between organ weights and those previously used traits in our study, we hypothesized that organ weight QTL might be also segregating in this F2 population.

Here, we described a QTL study of organ weight traits using this F2 data. In each QTL position, we studied the QTL effect by comparing different QTL models including additive, dominance or imprinting effects. In addition, the interaction between QTL and myostatin genotype, QTL and sex, QTL and reciprocal cross was evaluated.

**Materials and methods**

The mouse lines used in this study included myostatin-null C57BL/6 (McPherron et al. 1997) and M16i (Hanrahan et al. 1973) mice. Myostatin-null C57BL/6 mice carry non-functional myostatin genes and as a result are hypermuscled. Detailed information about the breeding design, QTL mapping and interaction analysis have been presented in Cheng et al., (Ph.D dissertation Chapter 2 and 3). F2 mice were sacrificed after having its body weight recorded at six weeks of age. Subsequently, internal organs (heart, kidney, large intestine, liver, pancreas, reproductive organ, small intestine, spleen, stomach, lungs and thymus) were collected and weighed. Based on this data, an internal organ weight percentage was calculated using the organ weight divided by the 6th week body weight. All animal procedures were approved by the Iowa State University Animal Care and Use Committee prior to this study.

**Results and discussion**

**Data summary**

Traits used in the study are summarized in Table 1. Both the normality test and Q-Q plots supported that the traits were normally distributed (unpublished results). The
phenotypic correlations between traits are present in Table 2. Most traits had significant correlations ($P$-value < 0.05) with one another. Significant fixed effects for each trait were identified by Proc GLM and were included in the QTL model (discussed in methods).

**Main effect QTL**

We identified three imprinted (comparison-wise $P$-value < 0.05, Table 3) and 15 non-imprinted QTL (5% genome-wide permutation level, Table 4).

The three imprinted QTL were located on chromosomes 1, 2 and X. The comparison-wise $P$-values for testing additive, dominance and imprinting effects are presented in Table 3. In particular, the imprinted heart QTL on chromosome 2 had significant additive, dominance and imprinting effects ($P$-value < 0.05).

The 15 non-imprinted QTL were detected on nine chromosomes (chromosomes 1, 4, 5, 9, 10, 11, 13, 14 and 17). Chromosome 1 and 17 harbored the greatest number of QTL. No genome positions were associated with either pancreas or lung weight. The largest number of QTL was associated with kidney weight. The largest two F-values were associated with the kidney QTL on chromosome 1 and the thymus QTL on chromosome 14. Except for the small intestine QTL on chromosome 9, all traits had comparison $P$-values less than 0.05 for additive QTL effects. The small intestine QTL on chromosome 9 exhibited only a significant dominance effect. In addition, the kidney QTL on chromosome 1 and chromosome 4, the liver QTL on chromosome 4 and the heart QTL on chromosome 5 had significant additive and dominance effects.

**QTL that interacted with myostatin genotype, reciprocal cross and sex**

We identified six, seven and ten chromosomal positions that significantly interacted with myostatin genotype, reciprocal cross and sex, respectively (comparison $p$-value < 0.05)
These 23 QTL were located on chromosomes 1, 4, 7, 9, 10, 11, 14, 17, 18 and X. In addition, chromosomes 1 and X had the largest number of QTL detected. The highest number of QTL was associated with stomach and large intestine weight traits.

The $P$-values for three different interaction tests are presented in Table 5. For example, we did three different tests to identify QTL by myostatin genotype interactions. The first test $(am + dm + im)$ tested the overall interaction, which was a combination of additive QTL by myostatin genotype, dominance QTL by myostatin genotype, and imprinted QTL by myostatin genotype. The second test $(am + dm)$ tested the non-imprinted interactions, which included additive QTL by myostatin genotype and dominance QTL by myostatin genotype. The third test $(am)$ tested for an additive QTL by myostatin genotype effect. Some of the identified QTL had $P$-values less than 0.05 for all three tests, e.g. the heart QTL on chromosome 9 that interacted with sex (Figure 1a). Other QTL had significant $P$-values for only one or two of these tests. For example, the stomach QTL on chromosome 4 was significant for the overall and non-imprinted interaction with myostatin genotype (Figure 1b). Whereas, the pancreas QTL on chromosome X was significant for the overall interaction with reciprocal cross effect only (Figure 1c).

**Phenotypic variation analyses**

The amount of phenotypic variation accounted for by the imprinted QTL is presented in Table 3. At each QTL position, the amount of variation associated with additive, dominance or imprinted QTL was also computed.

The amount of variation that could be explained by the additive and dominance effects of the non-imprinted QTL are presented in Table 4. Among all these QTL, the thymus QTL
on chromosome 14 accounted for the largest amount of phenotypic variation. Moreover, nearly all the explained variation at this position was attributed to the additive QTL effect.

In Table 5, we computed the phenotypic variation accounted for by QTL that interacted with myostatin genotype, reciprocal cross and sex. At each QTL position, this variation was computed as the variation caused by the overall interaction, the non-imprinting interaction and the additive interaction. The variation from overall interaction ranged from 0.45% to 2.61%. Specifically, the overall QTL by sex interaction effect from the large intestine QTL on chromosome X explained the largest amount of overall phenotypic variation.

A stacked histogram was graphed to summarize the variation accounted for by different variation components (Figure 2). The amount of phenotypic variation explained by all identified QTL is shown for each trait. QTL that were associated with stomach weight accounted for the most amount of variation. However, the QTL we identified for Liver and small intestine weights could only explain a small proportion of the total variation in these traits.

Discussion

We preformed a QTL scan to identify genomic regions that harbor genetic variants that influence internal organs weights in this study. Many of the traits we measured here had never been studied before. Previous studies of body composition traits mainly focused on the weight of heart, spleen, kidney, liver and fat pads due to their biological importance and relatively large weight proportion. Here we analyzed a large number of internal organs with the aim to identify genetic factors that controlled individual components of body weight.

Imprinted QTL
We identified three imprinted QTL that had a comparison $P$-value less than 0.05. One of these QTL, heart QTL on chromosome 2, was previously discovered as a main effect QTL in a large sample QTL study (Rocha et al. 2004a). This study also used M16i as one of the founder stains to construct the mapping population. However, the QTL was associated with the direct heart weight in their study while the weight was normalized by the 6th week body weight in our study. The reason for normalizing the direct weight was that a small number of QTL could be identified if we used the direct weight. Normalizing the data by the body weight also helped to eliminate some of the noise in the data and thus increase the mapping power.

In another study examining imprinting on body composition traits in mice (Cheverud et al. 2008), they discovered only one imprinted QTL over a genome-wide significance level. Another 13 imprinted QTL were significant at a chromosome-wide level. They suggested that the use of chromosome-wide significance level might help to avoid false positive according to the work done by (Chen and Storey 2006). It would be interesting to compare the results of mapping body composition traits by using different methods to set up the significance level. Unfortunately, Cheverud’s and our study performed the QTL mapping in different mapping populations. The traits we analyzed were different as well. We suggest that comparing the results in the same QTL mapping data by different significance levels might help to find a more appropriate significance level. Although we have not performed such a comparison, a comparison $P$-value might still be a good choice to set up the significance level according to the results in this study.

**Additive and dominance QTL**
We identified 15 non-imprinted QTL with 5% genome-wide significance level. Among these 15 QTL, several QTL were located in the same genomic regions as other body composition QTL that were previously discovered. For example, the positions of the kidney weight QTL on chromosome 4 and the spleen weight QTL on chromosome 9 were in agreement with QTL detected by (Rocha et al. 2004b). The kidney QTL on chromosome 1 had been mapped to the same position in previous QTL studies (Brockmann et al. 2004; Leamy et al. 2002).

We estimated the additive and dominance effects associated with each QTL position. In our study, more than half of the QTL had a more statistically significant additive effect than a dominance effect. This was also reflected in the fact that the additive estimates were often larger than the dominance estimates. In contrast, Rocha et al. (2003) reported very similar additive and dominance estimates. In addition, their QTL were able to explain more phenotypic variation than our QTL. However, we used 6 week old mice as compared to 10 week old mice in Rocha et al., (2003). The additive and dominance estimates of QTL identified in our study were almost the same order of magnitude as the body composition QTL estimates from (Rance et al. 2005). Moreover, the estimates of body composition QTL were much smaller in Leamy et al. (2002). Because each of the studies used different lines of mice, it was very possible that different QTL were segregating in the populations and each QTL had a different impact on the phenotypic values. Furthermore, as mentioned before, several traits used in this study, e.g. lungs, reproductive organs, have rarely been measured in previous QTL studies. Therefore, we could not compare the effect size of the QTL with other studies.
The most important point was that we did find a few regions within the genome that were associated with organ weights. This supported our hypothesis that there were indeed genetic factors that control these traits. Additional fine mapping in these regions will help to confirm these results and provide some knowledge to build a molecular connection between organ weights and body weights.

**Interactions with myostatin genotype, sex and reciprocal cross effect**

We analyzed the interactions between QTL and myostatin genotype, sex or reciprocal cross in this study. These interaction effects were significant for many organ weight traits measured here. To the best of our knowledge, testing the interaction between QTL and myostatin genotype had not been discussed in other body composition QTL studies. In addition to that, the way that interaction with myostatin genotype might affect organ weights has been seldom seen in the literature. Previous findings found that the myostatin gene was expressed in muscle and adipose tissue (Lee 2004). It is also known that liver is an important tissue for fatty acid synthesis and that the heart is mainly composed of muscle fiber. Therefore, it is expected that there might exist an interaction between the weights of these two organs and myostatin genotype. One finding that could support this hypothesis is from (Bunger et al. 2004). They noticed that myostatin null mice had reduced weights of liver, heart, kidney and digestive tract, but they did not look into the effect of myostatin gene on other internal organs.

In this study, seven QTL were shown to have a significant interaction with myostatin genotype. The phenotypic variation accounted for by these QTL was similar to that from the main effect QTL identified. This could be evidence to support the importance of QTL by myostatin interaction in controlling organ weights. In addition, among these six QTL, one
affected liver weight, two affected kidney weight, one affected reproductive organ weight and the other two affected stomach and small intestine weight. Therefore, myostatin might still have an impact on other internal organs although a significant expression of myostatin in these organs could not be detected. This interaction could be realized though other mediator factors or circulating myostatin in the blood. If the causal mutation underlying these QTL could be identified, this would provide more detailed information for us to gain a more complete picture of the myostatin gene pathway.

We identified ten and six QTL positions that had a significant interaction with sex and reciprocal cross, respectively. Sex and strain effects for spleen, liver, heart and kidney have been previously reported (Reed et al. 2008). Sex interaction might indicate sex-dependent gene expression. Reciprocal cross interactions could be caused by genetic variation in mitochondrial or Y chromosome DNA.

We further tested three different interaction models (overall interaction model, non-imprinted interaction model, and an additive interaction model) to each QTL position. Analysis of these three types of interactions gave more information about how these QTL interactions may function, e.g. additive vs. imprinted. Due to limited options provided in GridQTL, we did not directly test the interaction between imprinted QTL and fixed effects (myostatin, sex or reciprocal cross), or the interaction between dominance QTL effect and fixed effect. However, these results at least provide an estimate about genetic mechanism by which each interaction might operate.

In addition, we computed the phenotypic variation explained by QTL by myostatin genotype interaction, QTL by sex interaction and QTL by reciprocal cross interaction. The average amount of variation that these three different types of interactions could account for
were very similar, even though QTL by sex interactions accounted for a little bit more amount of variation. For some traits, e.g. large intestine, pancreas and reproductive organ weight, the sum of phenotypic variation from the different types of interactions was very close or even larger than the variation from the sum of additive, dominance and imprinting effects. This provided strong support for the importance of interaction between QTL and myostatin genotype, QTL and sex, QTL and reciprocal cross in body composition.

Acknowledgement

We gratefully acknowledge Dr. Daniel Pomp for providing the M16i mice used in our study and technical insights. This research was supported by funds from the USDA CSREES 2006-35205-16696.
### Tables

**Table 1. Summary statistics for phenotype traits in this study**

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<td>0.389-2.462</td>
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<tr>
<td>Thymus</td>
<td>0.326</td>
<td>0.119</td>
<td>0.074-0.800</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.664</td>
<td>0.113</td>
<td>0.331-1.084</td>
</tr>
<tr>
<td>Ovary</td>
<td>0.697</td>
<td>0.307</td>
<td>0.134-2.674</td>
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<tr>
<td>Testis</td>
<td>0.612</td>
<td>0.195</td>
<td>0.213-2.201</td>
</tr>
</tbody>
</table>

\(a\) Lintestine (large intestine); Sintestine (small intestine). Phenotypic values used here were computed as the corresponding organ weights (gram) divided by the 6\(^{th}\) week body weight (gram).

\(b\) S.D: standard deviation

\(c\) Range: minimum - maximum
Table 2. Pearson correlation coefficients among traits in this study\textsuperscript{a}

<table>
<thead>
<tr>
<th>Traits\textsuperscript{a}</th>
<th>Stomach</th>
<th>Lintestine</th>
<th>Sintestine</th>
<th>Spleen</th>
<th>Pancreas</th>
<th>Liver</th>
<th>Heart</th>
<th>Lungs</th>
<th>Thymus</th>
<th>Kidney</th>
<th>Ovary</th>
<th>Testis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stomach</td>
<td>1.000</td>
<td>0.309**</td>
<td>0.32**</td>
<td>0.268</td>
<td>0.163**</td>
<td>0.198**</td>
<td>0.264**</td>
<td>0.093*</td>
<td>0.164**</td>
<td>0.148**</td>
<td>0.014</td>
<td>0.167*</td>
</tr>
<tr>
<td>Lintestine</td>
<td>1.000</td>
<td>-0.031</td>
<td>-0.002</td>
<td>0.468**</td>
<td>0.061</td>
<td>0.334**</td>
<td>0.162**</td>
<td>0.269**</td>
<td>0.143**</td>
<td>0.054</td>
<td>0.258**</td>
<td></td>
</tr>
<tr>
<td>Sintestine</td>
<td>1.000</td>
<td>0.239**</td>
<td>-0.269**</td>
<td>0.350**</td>
<td>0.133**</td>
<td>0.317**</td>
<td>0.396**</td>
<td>0.222*</td>
<td>0.162*</td>
<td>0.350**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>1.000</td>
<td>-0.08*</td>
<td>0.159**</td>
<td>0.056</td>
<td>0.141**</td>
<td>0.201**</td>
<td>0.063*</td>
<td>0.107*</td>
<td>0.002</td>
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</tr>
<tr>
<td>Pancreas</td>
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<td>0.374**</td>
<td>0.081*</td>
<td>0.134**</td>
<td>0.178**</td>
<td>0.103*</td>
<td>0.216**</td>
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<td>Liver</td>
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<td>0.238**</td>
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<td>-0.042</td>
<td>0.673**</td>
<td>0.301**</td>
<td>0.504**</td>
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<tr>
<td>Heart</td>
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<td>0.236**</td>
<td>0.176**</td>
<td>0.350**</td>
<td>0.166*</td>
<td>0.381**</td>
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<tr>
<td>Lungs</td>
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<td>0.385**</td>
<td>0.076*</td>
<td>0.095*</td>
<td>0.263**</td>
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<tr>
<td>Thymus</td>
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<td>-0.128**</td>
<td>0.228**</td>
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<tr>
<td>Kidney</td>
<td>1.000</td>
<td>0.319**</td>
<td>0.632**</td>
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<tr>
<td>Ovary</td>
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<td>-</td>
<td></td>
<td></td>
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<td>1.000</td>
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\textsuperscript{a} P-value for testing for significant correlation. **: P < 0.0001; *: 0.0001 < P < 0.05; otherwise, P > 0.05. A “-“ notation denotes the correlation coefficient is not computed.

\textsuperscript{b} Same abbreviations for traits as in Table 1 are used here.
<table>
<thead>
<tr>
<th>MMU</th>
<th>Traits^a</th>
<th>Peak (cM)^b</th>
<th>F-value</th>
<th>Flanking Markers^c</th>
<th>P-value^d</th>
<th>Estimates^e</th>
<th>%var^f</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>Sintestine</td>
<td>25</td>
<td>7.32</td>
<td>rs3696088, rs13472794</td>
<td>4.75E-03</td>
<td>3.62E-04</td>
<td>0.2008 0.1578 -0.5291 0.8151 0.0983 1.2772</td>
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<tr>
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<td>Heart</td>
<td>53</td>
<td>9.74</td>
<td>rs13476636, rs3144393</td>
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<td>3.90E-02</td>
<td>0.0778 0.3126 0.1703 0.0068 2.4966 0.7733</td>
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<tr>
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<td>12.61</td>
<td>rs13483724, rs13483748</td>
<td>7.96E-01</td>
<td>6.3E-07</td>
<td>5.36E-03</td>
</tr>
</tbody>
</table>

^a Trait abbreviations are the same as in Table 1.

^b Peak position of QTL detected in Kosambi centimorgans.

^c Flanking markers (left and right) of the QTL peak. See Supplemental Table 1 for marker information.

^d P-value for testing additive, dominance and imprinting effects. a: additive effect; d: dominance effect; i: imprinting effect.

^e Estimates for additive effect, dominance and imprinting effects. The corresponding standard errors are in parenthesis.

^f %var: percentage of phenotypic variation accounted for by the additive, dominance and imprinting effects.
<table>
<thead>
<tr>
<th>MMU</th>
<th>Traits^a</th>
<th>Peak (cM)^b</th>
<th>F-value Flanking Markers^c</th>
<th>%var^d</th>
<th>Estimates^e</th>
</tr>
</thead>
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</table>

^a Traits abbreviations are the same as in Table 1.

^b Peak position of QTL detected in Kosambi centimorgans.
c Flanking markers (left and right) of the QTL peak. See Supplemental Table 1 for marker information.

d $P$-value for testing additive and dominance effects. a: additive effect; d: dominance effect.

e Estimates for additive effect and dominance effects. The corresponding standard errors are in parenthesis.

f $\%$var: percentage of phenotypic variance accounted for by additive and dominance.
Table 5. Statistics of QTL that interacted with myostatin, reciprocal cross and sex

<table>
<thead>
<tr>
<th>MMU</th>
<th>Position</th>
<th>Traits</th>
<th>Factor</th>
<th>Flanking markers</th>
<th>AM+DM+IM</th>
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<th>AM</th>
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<tbody>
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<td></td>
<td>(cM)</td>
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<td></td>
<td></td>
<td>P</td>
<td>Var%</td>
<td>P</td>
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<tr>
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<td>20</td>
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<td>Sex</td>
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<tr>
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<td>22</td>
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<td>Mstn</td>
<td>rs3696088</td>
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</table>

a Traits abbreviations are the same as in Table 1.

b Peak position of QTL detected in Kosambi centimorgans.

c Interaction factors: Mstn (myostatin genotype); Cross (reciprocal cross); Sex (sex).
d Flanking markers (left and right) of the QTL peak. A "-" notation denotes the end of the chromosome. See Supplemental Table 1 for marker information.

c Comparison $P$-value for testing interaction effect. am: additive QTL interaction; dm: dominance QTL interaction; im: imprinting QTL interaction. $P$-value $< 0.05$ is shown in bold type. %var: percentage of phenotypic variance accounted for at QTL position.
Figure Captions

Figure 1. QTL that interacted with (a) sex, (b) myostatin genotype and (c) reciprocal cross

(a) QTL on chromosome 9 at 51cM that interacted with sex to control heart weight.

(b) QTL on chromosome 4 at 52cM that interacted myostatin genotype to control stomach weight.

(c) QTL on chromosome X at 46cM that interacted with reciprocal cross to control pancreas weight.

Figure 2. Phenotypic variation components
Figures

Figure 1. QTL that interacted with (a) sex, (b) myostatin genotype and (c) reciprocal cross
(a) QTL on chromosome 9 at 51cM that interacted with sex to control heart weight.
Figure 1. (continued)

(b) QTL on chromosome 4 at 52cM that interacted myostatin genotype to control stomach weight.
Figure 1. (continued)

(c) QTL on chromosome X at 46cM that interacted with reciprocal cross to control pancreas weight.

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- **IMP**: imprinting QTL model
- **AD**: additive and dominance QTL model
Figure 2. Phenotypic variation components

Traits abbreviations are the same as in Table 1.

%var: percentage of phenotypic variance accounted for by additive (A), dominance (D), imprinting (I), interaction with reciprocal cross (cross), sex (sex) and myostatin genotype (mstn) effects.
## Supplementary information

### Supplemental Table 1. List of 152 SNPs used in the final QTL mapping

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"aPosition of markers in Kosambi centimorgans. This column of linkage map is build using Cri-map (Green and Crooks, 1990)."

"bPosition of markers in Kosambi centimorgans. This column of linkage map is from the Wellcome-CTC Mouse Strain SNP Genotype Set (http://www.well.ox.au.uk/mouse/INBREDS)."

"cPositions of the first marker on each chromosome is from the Wellcome-CTC Mouse Strain SNP Genotype Set."
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CHAPTER 5. IDENTIFICATION OF INTERACTING QTL THAT CONTROL BONE STRENGTH TRAITS IN MICE

A paper to be submitted to Genetics Notes

Ye Cheng, Satyanarayana Rachigani, Jack C Dekkers,
Mary Sue Mayes, Richard Tait, James M Reecy

Abstract

In this study, quantitative trait loci (QTL) for bone strength were mapped in 1000 F2 mice, which were derived by crossing C57BL/6 myostatin-null by M16i polygenic obese mice. Six main effect QTL, including one imprinted QTL, were identified at a 5% genome-wise significance level. Numerous QTL that interacted with sex, cross or myostatin genotype were also detected. It appears that epistatic interaction controls bone strength traits in mice.

Introduction

Bone strength measures bone resistance to the exerted force. It has been estimated that bone mineral density (BMD) could explain about 60% of the variation in bone compression strength (Weinstein 2000). Other studies also indicate that BMD is associated with bone fracture risk (Cummings et al. 1985; Melton et al. 1989). Therefore, QTL research about bone strength has focused on BMD (Beamer et al. 1999; Benes et al. 2000; Klein et al. 1998; Koller et al. 2000; Shimizu et al. 1999).
In addition to BMD, another important measurement of bone strength is femur breaking strength (FBS). FBS measures the maximum force that a bone can bear before it breaks. Studies of mapping QTL on FBS in mice indicate that these QTL might account for part of the variation in bone strength (L1 et al. 2002; Yu et al. 2009). The remaining proportion of the phenotypic variation in bone strength might be explained by factors besides single gene effects or undetected QTL. For example, epistatic and sex-specific QTL have been identified for bone strength traits (L1 et al. 2002; Orwoll et al. 2001).

Previous literature demonstrates that muscle mass imposes a large amount of force on bones (Burr 1997). GDF-8, also known as myostatin, regulates the growth of skeletal muscle growth. To investigate the potential role of myostatin on bones, the bone mass was measured in myostatin null mice (Hamrick et al. 2003). They found that myostatin null animals had an increase in bone mass. Moreover, another study indicated that myostatin deficient mice had an increase in bone strength (Hamrick et al. 2006). However, it is still unknown whether there is any epistatic interaction with myostatin to regulate bone strength.

The objective of this study was to identify bone strength QTL in the F2 mice derived from myostatin null C57BL/6 and M16i polygenic obese mice. More importantly, we investigated the presence of interactions between bone strength QTL and myostatin genotype, with the aim to provide candidate regions for fine mapping these epistatic QTL. Interactions of QTL by sex and by reciprocal cross, and QTL imprinting effects were also analyzed.

Results and Discussion
After fitting a linear model to the full F2 phenotype data, we found the most appropriate statistical model, which included the fixed effects of myostatin genotype, sex, reciprocal cross, color, sex by myostatin genotype interaction, myostatin genotype by color interaction, myostatin by reciprocal cross interaction, sex by myostatin genotype by cross interaction, and myostatin by color by reciprocal cross interaction.

We identified six main effect QTL using a whole genome scan that utilized the full data set (Table 1). Among these six QTL, five had a significant impact on the maximum tolerated force (AvgForce2). Except for the two QTL on chromosome 11 and 18, all QTL were significant at the 1% genome-wise significance threshold. Compared with previous studies of bone strength QTL, the position of the QTL on chromosome 2 was close to the femur breaking strength QTL 1 (Fembrs1) that was reported by Li et al. 2002. Despite the QTL name difference, these QTL actually measured the same phenotype. The QTL on chromosome 11 overlapped with a previously discovered femur mechanical trait QTL 14 (Fmtq14) (WERGEDAL et al. 2006). The average phenotypic variation accounted for by these six QTL was about 2.28%, with a minimum of 1.75% and a maximum of 2.84%.

The QTL on chromosome 11 that affected AvgForce 2 exhibited an imprinting effect with a comparison-wise $P$-value of 0.022. Further testing of this QTL indicated that it also had significant additive and dominance effects, with comparison $P$-values of 0.022 and 0.012, respectively. This QTL had been previously identified (WERGEDAL et al. 2006) for its
significant additive and dominance effect. To the best of our knowledge, this might be the first imprinted QLT for bone strength to be documented in the mouse.

In this study, significant interactions of QTL with myostatin genotype, reciprocal cross, sex effects were detected (Table 2). For example, one QTL on chromosome 4 significantly interacted with myostatin genotype to control AvgForce1, a measure of bone elasticity. However, no QTL by myostatin genotype interaction was detected on this chromosome that affected body weight or muscle mass traits. Bone elasticity and muscle mass were two key factors that impacted bone strength. Therefore, the results here suggested that this QTL by myostatin interaction could affect bone strength only via its influence on bone elasticity. Some independent regulation pathway might exist for bone elasticity.

In addition, three QTL on chromosomes 4, 6 and 8 significantly interacted with reciprocal cross to control bone stiffness (AvgGrad) and AvgForce2. The phenotypic variation accounted for by these QTL ranged from 1% to 1.25%. The most significant \(P\)-value was from the QTL on chromosome 6 that was associated with sex interaction on AvgForce2. Sex-specific bone strength QTL have been reported in other studies in mice. For example, five female-specific and six male-specific BMD QTL were identified by Orwoll et al. 2001. Furthermore, other studies support the existence of sex-specific QTL on mouse chromosomes 1 and 11 (Turner et al. 2003). In humans, there is a known difference of osteoporosis rates between males and females, but the genetic mechanism underlying it is not clear. Therefore, the knowledge gained from the sex interaction in mice study will be useful
to elucidate this.

In conclusion, this study identified five additive and dominance QTL, and one imprinted QTL for bone strength traits. Significant interactions were detected between QTL and the myostatin genotype, which affect bone elasticity. In addition, interactions of QTL with sex and reciprocal cross explained some of the phenotypic variation in these traits. Some of these QTL were novel and the results indicated that some regions in the genome might interact with myostatin to affect bone strength.

We would like to thank Dr. Daniel Pomp for providing the M16i mice used in our study and technical insights. This research was supported by grants from the USDA CSREES 2006-35205-16696.
Table 1. Statistics of main effect bone strength QTL

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a AvgGrad: stiffness (Newton/second); AvgForce1: stress at maximum load (Newton); AvgForce2: maximum tolerated force (Newton). The imprinted QTL was in bold type.

b Chr: mouse chromosome.

c Peak position of QTL detected in Kosambi centimorgans. d Flanking markers (left and right) of the QTL peak.

e Flanking markers (left and right) of the QTL peak.

Materials and Methods: The mapping population consisted of F2 mice derived from the crossing of myostatin null C57BL/6 line (McPherron et al. 1997) and M16i obese mouse line (Hanrahan et al. 1973). Detailed information about the breeding design and the QTL mapping and interaction analysis are in Cheng et al., (Ph.D dissertation Chapters 2 and 3). F2 homozygotes were raised until six weeks of age. The mice were sacrificed and femurs were collected from both the left and right side. Bone strength of each femur was measured by three-point bending using a TA-XT2i Texture Analyzer system (Texture Technologies Corp.,...
Searsdale, New York) after it was chilled at 4°C overnight. The bone was placed horizontally on supports that were separated by a distance of 9 mm. The crosshead speed was 0.4 mm/min with a sampling speed of 100 samples per second. Stiffness, stress at maximum load and maximum tolerated force were determined by Stable Micro Systems Texture Expert Exceed version 2.6 (Stable Micro Systems). Stiffness measured the resistance of bone to the breaking force, and was calculated as the slope of the linear portion of the load displacement curve from 0.30 to 0.60 seconds (WERGEDAL et al. 2005). The stress at maximum load was measured at exactly 0.30 seconds. The maximum tolerated force was also called maximum force or femur breaking strength, and measured the maximum force that the femur could bear before it was broken. The average bone strength from both sides of each mouse was used as phenotypic values.
Table 2. Statistics of QTL interacting with myostatin, reciprocal cross and sex effect

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a Interaction factors: Mstn (myostatin genotype); Cross (reciprocal cross); Sex (sex).

b Traits abbreviations are the same as in Table 1.

c Chr: mouse chromosome.

d Peak position of QTL detected in Kosambi centimorgans.

e Flanking markers (left and right) of the QTL peak.

f Comparison P-value for testing interaction effect. P-value<0.01 is shown in bold type.

g %var: percentage of phenotypic variance accounted for by the interaction effect at QTL position.

References


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WERGEDAL, J. E., M. H. SHENG, C. L. ACKERT-BICKNELL, W. G. BEAMER and D. J.

BAYLINK, 2005 Genetic variation in femur extrinsic strength in 29 different inbred
strains of mice is dependent on variations in femur cross-sectional geometry and

YU, H., B. EDDERKAOUI, A. CORTEZ, H. M. DAVIDSON, J. E. WERGEDAL et al., 2009

Mapping of the chromosome 17 BMD QTL in the F(2) male mice of MRL/MpJ x
CHAPTER 6. GENERAL CONCLUSIONS

General discussions

The goal of this project was to identify loci that control muscle mass and fat accumulation in F2 mice generated from myostatin null C57BL/6 and M16i obese mouse lines. We measured 47 traits, including 14 body weight traits, ten obesity-related traits, 11 body composition traits, three bone strength traits and nine gene expression traits. The QTL mapping results for 46 traits are presented here because the gene expression level of beta-actin gene was used to normalize the other gene expression levels. Large phenotypic variation was observed in these 46 traits even before we began our statistical analysis. The linkage map that we constructed from our genotyping results was relatively consistent with the published mouse linkage map. In the previous chapters, we presented our work according to the different traits. Here we summarize the results of this dissertation work from a more general view. This is discussed from two main aspects, QTL that have additive, dominance and imprinting effects, and QTL that interact with myostatin genotype, sex or reciprocal cross effects.

QTL that have additive, dominance and imprinting effects

QTL with significant main effects (additive, dominance and imprinting effects) were identified by interval mapping. The significance levels were set at 1% and 5% genome-wise significance, which was determined with a permutation method. The additive, dominance and imprinting effects were individually evaluated and the corresponding comparison-wise $P$-values were provided. The variation explained by each QTL was computed as the percentage of the sum of squares that a given QTL could account for.
For the 46 traits we analyzed here, we identified a total of 115 main effect QTL at a 5% genome-wide significance level. This number might seem to be large, however, the actual number of causal mutations might be much smaller. Some of these QTL overlapped and it is possible that a given QTL may control multiple phenotypes. A test of pleiotropy using multivariate analysis will help to solve this issue. Among these 115 QTL, ten had comparison-wise $P$-values that indicate that they may be imprinted. Of these ten imprinted QTL, six were associated with obesity-related traits, three with body composition and one with bone strength. For the remaining 105 main effect QTL, 44 were associated with body weight, 38 with obesity-related traits, 15 with body composition, six with bone strength and two with gene expression levels. A few QTL with large effect were located close to the myostatin locus. These QTL need further study considering the fact that segregation distortion was detected for SNPs near the myostatin gene.

The largest number of QTL detected in this study was associated with body weight, muscle and fat accumulation. This was as expected considering the two founder mouse lines we used, one heavily muscled line and one obese line. In previous publications, QTL that controlled individual organ weights were not extensively studied except for those associated with heart, kidney and livers. We measured a large number of different internal organs, including heart, kidney, large intestine, liver, pancreas, reproductive organ, small intestine, spleen, stomach, lungs and thymus. Our study successfully identified QTL for most of the traits, which indicates that genetic factors contribute to the phenotypic variation observed in these traits. A few QTL identified overlapped with QTL that had been previously discovered in other studies. Other QTL discovered were novel. This suggests that our F2 mapping population contained numbers of segregating alleles that affected the traits we studied.
Therefore, these mice could be used for future fine mapping study to identify the causal mutation underlying the phenotypes.

We obtained some interesting results from the eQTL mapping portion of the study as well. For example, an eQTL for IGF1 was identified on chromosome 4 and several QTL for body weight traits were located very close to this eQTL. Combined with the fact that the IGF1 gene is on mouse chromosome 10, this might indicate that chromosome 4 harbors a trans-regulatory element for the IGF1 gene. eQTL mapping provides us an alternative way to link gene expression to a certain phenotype. In addition, it also provides some indication whether a QTL could be a cis- or trans- regulator. If we could perform a large-scale gene expression QTL analysis (microarray or RNA-seq) with the samples collected here, it would offer more clues to build genetic networks that control these traits. This may provide us with a better picture of the genetic mechanisms underlying a certain phenotype.

**QTL that interact with myostatin genotype, sex or reciprocal cross effects**

The interaction effects were statistically identified by comparing a full model with reduced models. Both models had the same fixed effects, but the full model included an extra interaction term. The interaction effects we analyzed here were interactions of QTL with myostatin genotype, sex, and reciprocal cross. The sex-specific and cross-specific interactions have been widely discussed in previous studies. To the best of our knowledge, QTL that interact with myostatin genotype have not been studied for most of the traits we analyzed.

We identified 38 QTL that interacted with myostatin genotype, 44 QTL that interacted with sex, and 31 QTL that interacted with reciprocal cross. Most of the 38 myostatin interacting QTL were associated with body weight or obesity-related traits. In
particular, the body weight traits that were impacted the most were post-weaning traits. This result indicates that the myostatin gene effect was time-dependent and it was more important for postnatal growth. Another interesting finding from this study was that myostatin interactions were associated with some internal organ weight traits, although myostatin expression was not detectable in these tissues. One possible explanation was that there might be other mediators between myostatin and genes that regulated these organs. Alternatively, circulating myostatin may bind to a receptor on the surface of these tissues. Through the analysis of myostatin effect on bone strength traits, we found that myostatin could influence bone elasticity and that this effect was caused by an alternation of muscle mass. Therefore, myostatin might be a key regulator of whole body growth, as it appears to affect muscle, adipose, internal organs and bone strength.

The phenotypic variation accounted for by the epistatic QTL effects that were identified in our study was relatively small compared to that reported by Brockmann et al. (BROCKMANN et al. 2000). One reason may be that we only investigated the interaction effects between QTL and myostatin, but they analyzed all genome-wide pairwise QTL interactions. This could also be caused by a difference in genetic background. Therefore, the difference in QTL identification leads to a difference in the variation explained by these QTL.

**Future Research**

The most obvious future work following this study is to fine map the QTL regions identified in this study. Many different approaches can be used to realize this. The key point here is to increase the number of recombination events and to shrink these regions. For example, an advanced intercross line (DARVASI and SOLLER 1995) provides an experimental
population for fine mapping. Advanced intercross lines may be used to fine map several QTL regions simultaneously. However, the cost for constructing advanced intercross lines might be high because it requires an effective number of 100 individuals for each of ten generations before it reaches the ideal mapping resolution. Recombinant inbred lines are another option for QTL fine mapping (TAYLOR 1978). These lines have very dense breaking points, which provide for a high mapping resolution. In addition, one strain just needs to be genotyped once and can be collected for multiple phenotypes to study environmental influence. Recently, a new project has been initiated to construct a large number of recombinant inbred lines from eight commercial mouse lines. This is named the Collaborative Cross (WILLIAM et al. 2002). With these eight carefully selected founder lines, the Collaborative Cross will provide better mapping resolution and greater statistical power to fine map any QTL. Moreover, speed congenic lines (HILL 1998) can be derived by intercrossing the myostatin null allele into the Collaborative Cross, which will only take five generations. Therefore, these mice can be used for fine mapping both the main effect QTL and epistatic QTL that interact with myostatin genotype. Although the construction of speed congenic lines itself does not require a huge number of mice, one congenic line needs to be developed for each QTL. Thus, the total number of mice is still large if we try to fine map several QTL.

Once a narrow QTL region is obtained from the fine mapping step, candidate genes can be selected according to the phenotype of the QTL and the known functions of the genes in this region. Causal mutations can be located by other molecular biology approaches. If eQTL are fine mapped in the fine mapping population as well, a pathway or gene network might be constructed from the myostatin gene.
Other future work that might be done includes testing the pleiotropic effects of these QTL; testing the effects of additive QTL by myostatin genotype interaction, dominance QTL by myostatin genotype interaction and imprinting QTL by myostatin genotype interaction individually.

References


