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Exploring Myostatin
(Under the Direction of CLIFTON A. BAILE)

Myostatin, a negative regulator of muscle cell growth, is highly conserved across species. The loss of functional Myostatin is known to cause the "double-muscled" phenotype in several cattle breeds, and similar phenotypes in other species. For nearly 200 years, double-muscled animals have captured the attention of livestock breeders and researchers, boasting enlarged musculature but beset by production difficulties. With the advent of transgenic technology, researchers have created a "knockout" mouse model with which to efficiently explore the biochemical pathways and influences of Myostatin. Research involving this model has both agricultural and biomedical applications, and involves several cell growth and regulation mechanisms. Analysis of growth and development patterns in Myostatin-null mice is necessary to link these findings with past research. Developmental patterns for adult Myostatin knockout mice are well-defined, however, early patterns of growth were not previously delineated. We found variations between GDF-$8^{+/+}$ and GDF-$8^{-/-}$ mice 4-12 weeks of age.

INDEX WORDS: Myostatin, GDF-8, Mouse, Double-Muscled, Knockout, Growth and development, Cattle, Model organism

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by

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DEDICATION

For Susan and Kenneth Arnold. Mom and Dad, without your support and strength, I could never have come so far. And for my beloved Michael, who made it all worthwhile.

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CHAPTER 1

INTRODUCTION

For nearly two hundred years, the phenomenon of the "double-muscled" animal, a spectacular model of muscular hypertrophy, has represented a never-ending source of inspiration and argument for both livestock producers and animal scientists. Though livestock other than cattle produce double-muscled individuals of more or less desirability, recent characterization of the protein Myostatin, a specific inhibitor of muscle cell growth, has rekindled interest in the condition (McPherron et al., 1997). Myostatin "knockouts" are the genetic and biochemical basis for one form of muscular hypertrophy in the bovine (McPherron and Lee, 1997), and advances in the field of biotechnology have shown new means to exploit the gene involved. Far from burying the old controversy, contemporary understanding of double-muscling in cattle has reawakened and added new issues to the old debate.

On one hand, the stunning muscularity of such animals embodies the extreme in meat-producing efficiency, each animal yielding a large proportion of retail cuts that are low in fat and characteristically tender (Westhusin, 1997). At a glance, double-muscled breeds of cattle such as the Belgian Blue and Piedmontese would seem to represent a model of the modern agricultural philosophy - lean, commercially desirable meat produced in comparable quantities by fewer animals. They are economically important breeds, both at home and abroad. The Belgian Blue (*Blanc Bleu Belge* or BBB) is currently the premier cattle breed in Belgium, comprising 45% of the total population of

cattle in that country, as of a 1995 study (Fiems et al, 1995; Webb et al., 1998). Piedmontese and Belgian Blue bulls have both been researched as possible terminal sires, in the hopes of conferring some of their production advantage to heterozygous offspring (Kieffer and Cartwright, 1980; Baker and Lunt, 1990).

On the other hand, producers that raise these animals today encounter many of the same undesirable traits that farmers lamented two centuries ago. Issues of dystocia, or difficult calving, that are directly related to the coveted hypermuscularity have not been completely remedied, although current veterinary technology has helped to reduce the mortality rate. These and other undesirable physiological characteristics of double-muscled cattle have traditionally curbed widespread production, fueling the ongoing debate over whether continued propagation of such animals can ever be a part of ethical and responsible animal agriculture.

It seems logical that the same technological revolution that uncovered the basis for double-muscling in cattle should have a hand in alleviating the associated concerns and providing new ways to exploit the trait. New understanding of the role of Myostatin gene expression in growth and development, along with research into the structural and functional characteristics of the Myostatin protein has offered researchers several potential methods to manipulate the pathway. Altering the time of expression, for instance, may provide a way to circumvent some of the problems at parturition and in newborn calves. With the advent of transgenic technology, it may also be possible to induce the mutation in livestock animals other than the bovine species. The Myostatin gene is highly conserved across species, and there is evidence to suggest that its function in muscle growth regulation is similarly conserved. Changes in Myostatin function

induced either by targeted mutation of the gene or pharmacological or immunological targeting of the Myostatin pathway have already produced comparable growth and body composition alterations in mice, chickens, and sheep. The high conservation of myostatin function across such livestock species, along with the inability to easily differentiate hemizygous and normal animals, may also mean that endogenous Myostatin mutations already exist, undetected, within breeding herds today. Plans already underway to screen large numbers of various livestock breeds and species will help producers to identify the mutation within their own herds and develop a breeding strategy to maximize its potential.

Along with the direct applications of such functional Myostatin knockouts among livestock animals, the creation of Myostatin knockout mice by homologous recombination has provided an invaluable model animal for the further study of the biological pathway Myostatin follows. Corresponding function of the human Myostatin protein suggests that murine models with altered Myostatin expression may be of value in biomedical research. Identification of the human Myostatin gene and analysis of its expression patterns has indicated that Myostatin may play a role in certain diseases characterized by cachexia or muscle wasting, including AIDS (Gonzalez-Cadavid et al., 1998).

It is in this area that the novel research of this thesis concentrates. Establishing basic comparative growth and development information for normal and knockout mice age 1-4 months provides a platform for further studies into the characteristics of the mutant phenotype and possible control points in the Myostatin pathway, and further supports the use of Myostatin knockout mice as viable research animal models.

CHAPTER 2

HISTORY AND ORIGINS OF DOUBLE-MUSCLED BREEDS

The phenomenon of the double muscled animal presents an interesting puzzle for the dedicated genetic explorer. The history of the mutation can be traced back to the middle of the eighteenth century, and was first documented in the livestock almanac of a British farmer named George Culley. However, considering the diverse breeds in which the mutation has been observed, a true chronicle of the condition would likely span several million years of history, perhaps even beyond the Pleistocene period that saw the fragmentation of the *bos* genus into the earliest progenitors of our modern breeds.

Over two million years ago, an ancient ancestor of modern cattle gave rise to two distinct species, the aurochs, or *Bos primigenius*, progenitor of modern Asian and European cattle, and *Bos namadicus*, a forebear of *Bos indicus*, the humped zebu cattle of India (Friend, 1978). Aurochs skulls found in the swamps of Scotland and elsewhere in western Europe provide evidence of the immense size of the prehistoric animal, and although there are considerable structural differences between aurochs fossils and the bones of modern cattle, the aurochs is generally accepted as an ancestor of modern European cattle breeds (Wilson, 1909a). Double-muscled breeds are found among descendants of both these groups. It is not known at what point the various mutations that result in the double-muscled condition occurred. However, because several of the knockout mutations are breed-specific, it is worthwhile to discuss the origins of breeds

now characterized by their double-muscled trait, specifically the Belgian Blue breed of Belgium and the Piedmontese of Italy.

It should be noted that tracing the lineage of cattle breeds in Europe is much like tracing the origins of a bowl of stew. Most historic accounts of the evolution of breeds in Europe and western Asia world admit that thorough crossbreeding and blending of types occurred at many points in the pursuit of breed improvement. Therefore, any speculation about the origin of the Myostatin mutation among such diverse groups as Belgian Blue and Piedmontese cattle must take into account the polymorphic nature of the Myostatin "knockout" observed between breeds (Grobet et al, 1997; Grobet et al, 1998) and the most likely path by which the breeds developed. As will be explored in chapter 4, the double-muscled condition in the Belgian Blue is due to a deletion in the GDF-8 coding region that truncates the protein (Grobet et al, 1997; Grobet et al, 1998; Vaiman, 1997). Double-muscled character in the Piedmontese breed is an entirely different mutation (McPherron and Lee, 1997; Kambadur et al., 1997; Grobet et al., 1997).

Culley and other historians cited the appearance of the double-muscled character among shorthorn herds sometime in the late 1700's. This was consistent with the period of the so-called "Dutch Invasion", when political and economic incentives led farmers to import foreign cattle to cross with their domestic herds (Culley, 1804; Wilson, 1909b). Culley, in particular, stressed a difference between early imports and crosses, which "were of much service in improving the breed" (Culley, 1804), and the later appearance of the "double-lyery" cattle, which he considered a separate and inferior type.

The rise of several cattle breeds in which the double-muscled condition is considered breed standard begs the question of what selective advantage both

heterozygous and homozygous Myostatin knockouts held in the beef industry through their early years. This point is particularly interesting, considering the varying and volatile reaction of producers, consumers, and agricultural policymakers throughout the last two centuries. The extremely high dressing percentage and meat quality offered by these animals has traditionally been the obvious advantage for producers, but as mentioned above, these advantages are severely extenuated by the production difficulties of double-muscled animals. In many cases, double-muscling is considered a hereditary disease condition because of these reproductive, cardiopulmonary, and conformational sequelae (Hanset, 1991), and it remains a condition that can negatively impact the sale price of affected animals (Ohio State University, 2000)

Still, they have survived, and according to their respective breed associations at least, they have survived in grand style. In a 1982 paper, Thiessen and Rollins (1982) stated, "the high ranking of m+ [hemizygous knockout] males in live animal conformation and in the expected superiority of the carcase [sic] traits of their progeny would be expected to favor m+ genotypes in selection programmes [sic] and thereby maintain the gene in the population."

Although this particular study concerned muscular hypertrophy in the Angus breed, the same logic could be applied to the early development of breeds now distinguished by high incidence of the double-muscled phenotype. This was supported by the research of Hanset (1986), who stated that, as the double-muscled mode of inheritance in the Belgian Blue seems to favor a "partial recessive" nature, heterozygous animals tend to have a selective advantage in heavier muscling, without the problems of dystocia. The major double-muscled breeds all have unique variations of interest to the

livestock industry, and producers of all three breeds have faced and either overcome or taken steps to alleviate some of their endogenous production problems.

The Belgian Blue breed has existed in one form or another since the mid-1800's, and the breed has long been scrutinized by researchers as a model for the double-muscled condition. The breed originally resulted from crosses of Dutch Friesians and English shorthorns with native cattle circa 1850 (Friend, 1978). The herd book of the Belgian blue was begun in 1919. Belgian breeders, driven by consumer demand for lean, tender meat, likely selected individuals with the characteristic conformation of hypermuscularity, purposefully or accidentally fostering the persistence of *culard* (double-muscled) individuals in Belgian Blue herds (Hanset, 1991). Economic conditions have also supported the development and maintenance of the Belgian Blue breed. Michaux et al. reported in 1983 that the premium paid for '*viandeux*' or 'meaty' animals of the Belgian Blue type is substantially more than would be expected, simply based on the yield of lean meat. This premium, along with the large proportion of the carcass sold as higher-priced roasts and steaks rather than hamburger, contributes to the high market value of Belgian Blue animals (DARD, 2000).

Figure 1: Belgian blue (OSU,

Double-muscled Piedmontese cattle (referred to as the "Albese" variety) are believed to have resulted from the fusion of Zebu and Aurochs cattle followed by 25,000 years of evolution (OSU, 1998). Their herd book began in 1887, and the breed society was established in 1934. (Friend, 1978) Like the Belgian Blue, the Piedmontese has also been considered as a possible sire for terminal crosses, and has been used successfully in such programs for over 60 years (Swatland and Kieffer, 1974). Because the degree of muscling is not so extreme in heterozygotes and the problems of large birth weight are not compounded by some of the physical limitations in the double-muscled dam, dystocia is often less frequent and less severe (Baker and Lunt, 1990; Casas et al, 1999). In one crossbreeding study (Baker and Lunt, 1990), Piedmontese sires were not found to significantly impact incidence of dystocia in crossbred heifers when compared to Angus and Charolais sires. The offspring of Piedmontese sires failed to pick up any additional feed conversion efficiency from their double-muscled dads that would set them apart from conventional crossbred calves. However, carcass traits such as dressing percentage and ribeye area were considerably higher, suggesting that at least some of the Piedmontese hypermuscularity was transmissible to heterozygous progeny.

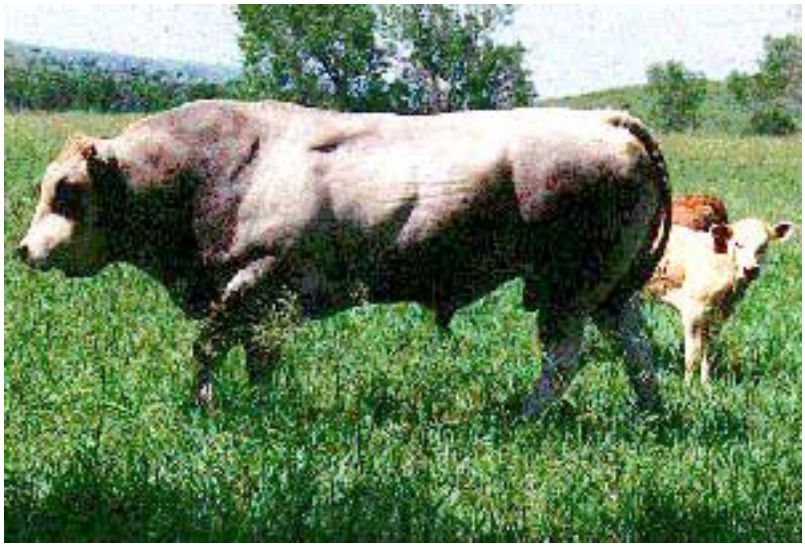

Figure 2: Piedmontese (OSU, 1998)

CHAPTER 3

PHYSIOLOGICAL CHARACTERISTICS OF GDF-8 KNOCKOUT ANIMALS

Interest in the double-muscled or "mh" (muscle hypertrophy) mutation can be traced back to the middle of the eighteenth century (Culley, 1804). While categorizing the various breeds of cattle occupying the western part of Europe at that time, Culley noted the appearance of "double-lyery" or black-fleshed cattle among the shorthorn herds on one side of the river Tees. From this initial observation, he went on to give both a description of their conformation and performance, as well as speculation on their origins.

> "...[S]ome other people of less knowledge going over [to Holland], brought home some bulls that in all probability introduced into that coast the disagreeable kind of cattle, well known to the breeders upon the river Tees, and called lyery, or double-lyered, that is, black-fleshed, for one of these creatures, notwithstanding it will feed to a vast weight, and though you feed it ever so long, yet will not have one pound of fat about it, neither within nor without, and the flesh (for it does not deserve to be called beef) is as black and coarse grained, as we generally suppose horse-flesh to be. However, by the pains and attention of the breeders, this useless disagreeable breed is now pretty well out of the country. No man will buy one of this kind, if he knows any thing of the matter; and if he should be once taken in he will remember it well for the future; for people conversant with cattle very readily find them out, from their round form all over, particularly their buttocks, which are turned like a black coach-horse, and the smallness of the tail: but they are best known to the graziers and dealers in cattle, by feel or touch of the fingers."

Since Culley's day, considerable time and effort has been directed toward understanding the nature of double-muscled cattle, and in the intervening two centuries,

both useful and misleading observations have been made about such outstanding animals. When considering various studies of physiology in hypertrophied and normal beef cattle, it is important to note that prior to localization of the "mh" locus (Charlier et al., 1995), it was not always possible to accurately differentiate muscular hypertrophy due to myostatin dysfunction and muscular hypertrophy of other origins. As mentioned before, there are several physiological conditions besides a myostatin knockout that can induce muscular hypertrophy among livestock species. Among these are the hypermuscularity associated with porcine stress syndrome and malignant hyperthermia (mh) in swine (Archibald, 1991) and the callipyge condition in sheep (Pringle, 1995). No gene-specific relationship has been shown between these conditions and myostatin-related double-muscling in cattle (Archibald, 1991; Pringle, 1995). In other cattle breeds known for hypermuscularity, including the Limousin and Blonde d'Aquitaine, double-muscled individuals appear to owe a generous portion of their bulk to sources other than a faulty myostatin gene (Grobet, 1998). An accurate analysis of research, therefore, requires one to take findings of the 70's and 80's with a grain of salt.

Gross physical characteristics of the GDF-8 knockout

One of the properties that sustained interest in the "mh" mutation and ultimately led researchers to link dysfunctional Myostatin to the double-muscled condition is the aforementioned easily recognizable extreme phenotype of the Myostatin-null individual. Culley's (1804) description of cattle like "a black cart-horse" is not far from accurate, and though Culley never saw a double-muscled mouse, he might have described them in much the same way.

The mice are characterized by bulging muscular development visible all over their bodies, with the most extreme hypertrophy apparent in the shoulders and hindquarters. This impression of extreme muscularity is enhanced by the lack of visible fat anywhere on the body. Myostatin-null mice have a rigid tension in their bodies that I have not observed in normal mice, and instead of becoming sleek and fat in retirement, they maintain a hard-lined, sparse-furred, "coke-bottle" shape throughout their lives. The muscles of their jaws and throats are heavy, their stance is wide and stiff, their respiration fast and their movement somewhat restrained. Nevertheless, from a colony-wide view, they give every indication of normal health, often remaining fertile past the average age of retirement for the background strain, and living at least as long as their normal contemporaries.

Double-muscled cattle are even more easily discernable than their murine counterparts. On Belgian Blue bulls, every intramuscular groove is readily visible, due to an almost complete lack of subcutaneous fat. Instead of the "boxy" build of typical cattle, double-muscled animals have tight, "greyhound" bellies and a muscular roundness to their quarters that probably prompted the original "carthorse" description (Culley, 1804; Menissier, 1982; Amory, 1993). Indeed, they have a muscular conformation most often reserved for draft horses and bodybuilders - and such a blatant advertisement of retail product that the industry interest is obvious.

Beyond a superficial appraisal, double-muscled animals exhibit a number of physiological variations and diatheses that distinguish them from other phenotypes. Differences between the types include variations in overall body composition, weights of bones and individual muscles, and in the histology of muscular and fatty tissue, as well as

differences in growth and development patterns, and hormonal abnormalities. Double-muscled individuals have been reported to have noticeably smaller weights of internal organs, particularly the thymus, liver, lungs, and spleen (Hanset, 1991). Pathological conditions that can develop in double-muscled cattle include subfertility and dystocia, stress intolerance, cardiomyopathies, and respiratory difficulties. All of these factors can influence the final production of saleable meat for better or worse, altering both the yield and quality of retail product.

Variations in body composition

Overall body composition of double-muscled cattle varies by individual, sex, and breed. In general, however, the double-muscled phenotype is characterized by lower proportions of bone, much higher proportions of muscle, and much lower proportions of fat than conventional cattle of comparable background (Dumont, 1982; Hanset, 1986; Hanset, 1991). Although the exact values of these differences vary by age and breed, independent researchers have consistently supported the general trends. Research supports these general trends not only across breeds but across species lines as well (McPherron et al, 1997; Arnold et al., unpublished data).

Variations in tissue development

The bones of double-muscled cattle, while significantly hypotrophied, are not affected as drastically as other tissues in the body. Hanset (1991) reported percent losses in bone mass of double-muscled bulls compared to conventional bulls that ranged from -4.8% (tibia) to -9.1% (femur). These losses were greater in a comparison of double-muscled and normal females. Hamrick et al. (2000) found that in mice, the size and

shape of the femoral bone were not altered in any way by a myostatin knockout. This is likely due to the fact that stress on the bones of myostatin-knockout mice is virtually identical to that of control mice in similar environmental situations, as their exercise level within the cage is nearly the same (Turner, 2000).

In cattle, the double-muscled condition causes a 20-25% increase in overall muscle mass (McPherron and Lee, 1997), and, as mentioned earlier, the degree of muscle tissue hypertrophy varies by anatomical region. In a 1980 symposium addressing muscular hypertrophy and possible methods to exploit the condition, attendees cited an overall anteroposterior gradient to the muscle increase, compared to conventional cattle; the greatest difference observed in the upper region of the hindquarters (the rump and round) and the least difference in the neck and fore ribs. The sex of the animal was also found to play a role in the degree of this gradient (Menissier, 1982). The double muscled condition in a variety of cattle breeds was found to have a greater hypertrophic effect in the forequarter muscles of bulls, while in cows, the muscles of the back were most affected (Shahin et al, 1986; Shahin et al, 1995). The "hypertrophy ratio", (or percent variation) between the individual muscles of double-muscled and normal calves was recorded by and Boccard and Dumont (1974) and Hanset and Ansay (1972). Results from the Hanset and Ansay (1972) study are shown in Table 1.

Individual muscle weights were also found to vary in normal versus Myostatin knockout mice, although the increase was much more drastic, ranging from 200-300% larger in knockout animals (McPherron et al., 1997). We also found a significant increase in the weight of the gluteal of knockout mice versus control mice (Arnold et al.,

unpublished data). Details of the original knockout mouse study (McPherron et al., 1997) are shown in Table 2.

Muscle	Percent increase
Triceps brachii caput laterale	50
Triceps brachii caput longum	31
Supraspinatus	29
Teres major	27
Subscapularis	23
Brachialis	20
Deltoideus	15
Biceps brachii	8
Lastissimus dorsi	30
Longissimus dorsi	19
Serratus ventralis	17
Pectoralis superficialis	16
Rhomboideus	13
Splenius	10
Semispinalis capitis	6
Spinalis dorsi	5
Biceps femoris	35
Gluteus medius	25
Semitendinosus	25
Quadriceps femoris	25
Sartorius	21
Tensor fasciae latae	19
Gracilis	18
Pectineus	14

Table 1: Percent increase in muscle weights of double-muscled cattle versus conventional cattle. (Hanset and

Muscle	Percent increase
Digastric	205
Pectoralis	262
Triceps brachii	235
Quadriceps	203
Gastrocnemius/plantaris	219
Tibialis cranialis	202
Soleus	200

Table 2: Percent increase in muscle weights of Myostatin knockout versus control mice (McPherron et al., 1997).

In spite of the name, a double-muscled animal has no more muscles than a conventional animal. Rather, hypertrophy of the muscular tissue and the extreme scarcity of fat cover sharply define every muscle in the animal's body. There are, however, some striking differences in muscle structure between the types. Double-muscled cattle develop more muscle fibers than do cattle of normal conformation, and it is this hyperplastic growth of cells which leads to the gross muscular hypertrophy. This hyperplastic growth is pronounced in the fetal stage of growth in double-muscled cattle, with cell increase proceeding at a rate nearly three times that of normal cattle (Swatland and Kieffer, 1974). This hyperplastic growth is not normally accompanied by cellular hypertrophy (Swatland and Kieffer, 1974; Gerrard and Judge, 1993). Because analysis of development at the fetal stage gives information of muscle growth independent of bone growth, double muscled cattle fetuses have been examined to characterize muscle histological development and structure in double-muscled cattle. Much of the hyperplasia observed was found to occur between 85 and 210 days after conception (Gerrard and Judge, 1993). However, because muscle fiber density is determined in the embryonic stages of development, it is not surprising that the hyperplastic condition persists in adult cattle.

There are also variations in the ratios of different muscle cell types. Microscopic analysis of the semitendinosus muscle of Belgian Blues has shown that double-muscled cattle develop approximately twice as many cells as normal cattle, and that this aggregate cell count contains far more of the smaller type IIB cells than found in normal counterparts (Wegner et al, 2000). The hypertrophy is specific to muscle, and is observed in skeletal muscles throughout the entire body (Lee and McPherron, 2000;

Swatland and Kieffer, 1974). The muscle of double-muscled cattle also contains less connective tissue, which also contributes to its tenderness (Bailey et al, 1982; Dumont and Schmitt, 1973; Hanset, 1986).

As mentioned before, the amount of fat in the carcasses of double-muscled cattle is significantly less than that observed in the carcasses of conventional cattle. Amounts of intramuscular fat, or marbling, are particularly affected by the double-muscled condition (Webb et al, 1998; De Smet et al, 2000; Thiessen and Rollins, 1982; Hocquette et al., 1999; Hanset, 1982; Hanset, 1991). This lack of marbling contributes to the lower quality grade that often classifies double-muscled carcasses of typically conventional breeds as inferior (Thiessen and Rollins, 1982; USDA, 1997). Adipocytes comprising the subcutaneous and internal fatty tissues of double-muscled cattle seem to be smaller than in conventional cattle, although adipocyte size within the intramuscular fat appeared to be similar between both types (Hocquette et al., 1999)

Variations also exist in the composition of fat depots in the Belgian Blue, compared to values for cattle of normal conformation. In particular, a lower total lipid content and a much higher percentage of polyunsaturated fats (11% of fatty acids, compared to 2.7-6.1% reported for other breeds) has been observed in the intramuscular fat of double-muscled animals (Webb et al, 1998). Significantly higher proportions of polar lipid fatty acids and linoleic acid have been observed in the intramuscular fat of Belgian Blue cattle (Webb et al, 1998; De Smet et al, 2000). The overall composition of fatty acids in the intramuscular fat of the Belgian Blue animals was considered to be closer to reported observations for pork (Webb et al, 1998).

The relative ratios of muscle, fat, and bone all contribute to a final estimate of beef carcass yield and dressing percentage, while intramuscular fat, tissue structure, and specific tissue composition affect the quality of the meat. Belgian Blue cattle have a high dressing percentage - up to 70% - according to a 1995 study comparing Belgian Blues and conventional cattle (Fiems et al, 1995). In another study, the carcasses of Belgian Blues, measured at the 8th rib of 3-4 year old cull cows were estimated to contain 75.7±1.6% muscle, 11.8±0.7% bone, and 12.6±1.5% fat (Webb et al, 1998).

Double-muscled animals within other breeds also show a higher dressing percentage and higher cutability than normal animals (Thiessen and Rollins, 1982; Hocquette et al, 1999). The ribeye (longissimus dorsi) area for double-muscled animals is generally much greater than that of normal animals, even within the same breed. In a 1982 study of double-muscled and normal Angus cattle, the ribeye areas were 139, 97 and 90 cm^2 for homozygous double-muscled, heterozygous, and normal bulls, respectively (Thiessen and Rollins, 1982)

The significance for producers and consumers is that double-muscled animals produce a higher proportion of lean meat than conventional cattle types. The meat of double-muscled animals is generally much lower in fat, and what fat does remain is higher in the polyunsaturated varieties, both of which more closely conform to current nutritional guidelines. Unfortunately for the consumer, meat that most closely conforms to the standards of better nutrition generally has all the appeal of shoe leather. Fortunately, in many cases, beef from double muscled cattle is reported to be a welcome exception to the rule. The tenderness or shear force value for the meat of Belgian Blue animals is comparable to that of Angus, Galloway, and the ubiquitous Holstein-Friesian

breed (Wegner et al, 2000). This characteristic of the meat is usually attributed to the higher number of small muscle cells comprising the skeletal muscle of double-muscled animals (Wegner et al, 2000), and offers an interesting deviation from the traditional standard, which equates tenderness with a high degree of intramuscular fat, or marbling.

Meat quality problems associated with the double-muscled phenotype are also related to the structure of muscle tissues in such animals. Paler-colored meat is a condition related to higher numbers of small type IIB muscle fibers in the meat of Belgian Blues (Wegner et al, 2000), and depending on the market, the very low levels of intramuscular fat can be considered an undesirable characteristic (Wegner et al, 2000). Incidence of "dark cutter" beef in double-muscled animal is related both to its chemical nature and the tendency of such animals to be stress-prone.

Physiological abnormalities and pathologies

Physiological problems associated with double muscling are varied, and have historically dominated the controversy over the desirability of breeding for the double-muscled phenotype. These undesirable companion traits generally fall into several major categories: reproductive difficulties, conformational abnormalities, and respiratory and cardiovascular disadvantages. In addition, the tendency toward "dark-cutting" meat in double-muscled cattle has historically soured the demand for such animals, and can be readily linked to the abnormal muscle physiology of such animals.

The well-documented reproductive difficulties are perhaps the most serious set of faults associated with the double-muscled cattle, although cases of reproductive dysfunction have not been reported to the same degree in other species. Myostatin-null breeder mice in the colony, for instance, showed no signs of reproductive difficulty at all,

compared to control counterparts, and were often fertile well past the usual age of breeder retirement observed in other colonies (Arnold et al., unpublished observation). In cattle, however, the coincidental inhibition of reproductive development, coupled with increased incidence of dystocia, presents a major challenge to breeders. Studies from the late 1800's to the latter part of the 20th century documented case after case of slow development and genital infantilism in females, late-onset puberty, reduced scrotal circumference, and a lowered libido in males (Menissier, 1982). Although semen volume is lower in double-muscled bulls, the concentration is not significantly different from that of normal animals (Menissier, 1982; Vissac et al, 1974; Hanset, 1991).

The gestation length for double-muscled calves is significantly longer, and the resulting offspring usually have a higher birth weight than conventional calves (Hanset, 1986; Hanset, 1991; Vissac, 1973; Vissac, 1974). Due to the radically increased size of calves, cases of dystocia in double-muscled cows are the greatest concern. Studies in the mid-1970's, (Vissac et al., 1973; Vissac et al., 1974) indicated that the percent dystocia was 85% for double-muscled dams, compared to 43% in normal dams, and 62% of parturitions in the double-muscled dams required caesarian delivery, as opposed to 20% of parturitions among normal dams. Aside from the 5.8 kg average increase in the birth weight of calves from double-muscled dams however, Vissac documented an unusual reduction in the pelvic area of double-muscled dams. Unlike normal cows that showed an average pelvic area of 342 cm^2, double-muscled dams only averaged 307 cm^2, a loss of 36 square centimeters. This discrepancy between calf size and the dam's calving weight and pelvic area is a likely cause of dystocia, particularly in double-muscled heifers bearing double-muscled calves (Menissier, 1982). In other areas of reproductive

performance, including onset of sexual maturity, fertility, and milking ability, double-muscled cows are consistently characterized as inferior to normal cows (Menissier, 1982; Hanset, 1991; Vissac et al., 1974).

Undesirable conformational traits associated with the double-muscled condition in cattle include rachitism, and developmental abnormalities of the mouth such as macroglossy and brachygnathism (Hanset, 1991; Menissier, 1982). Many of these may be indirectly related to other abnormal traits of double-muscled animals. Rachitism (or rickets), for example, is a tendency to develop elongated, weak bones, and is a deficiency disease most often caused by lack of vitamin D or phosphorus (Miller and West, 1970). Macroglossy, or swollen tongue, and brachygnathism, or undershot lower jaw, are developmental abnormalities that can inhibit suckling and lead to illness or poor growth in neonatal or young double-muscled calves. It is important to keep in mind that although such conformational abnormalities have been reported at a higher frequency in double-muscled cattle, they are by no means universal. Stringent selection pressure against such conditions has traditionally limited their occurrence in double-muscled beef breeds (Hanset, 1982).

Aside from conformational disadvantages directly caused by the increased weight and growth patterns of muscles in double-muscled cattle, such individuals are considered more prone to coincidental developmental defects, particularly of the heart and lungs. Septal defects resulting from the persistence of embryonic cardiac structures in the adult animal and abnormally thickened ventricular walls have been shown to occur in double-muscled cattle (Halnan et al., 1970; Oliver and Cartwright, 1968).

Researchers considering the environmental sensitivity and stress-prone nature of double-muscled cattle examined, in particular, cardiac and pulmonary performance. A tendency toward increased incidence and severity of laryngitis and pulmonary disease, particularly in double-muscled calves, prompted studies into respiratory abnormalities, while a long history of cardiac defects and environmental sensitivity led researchers to consider abnormalities in the structure and function of the heart in double-muscled breeds. Many of the findings provided consistent evidence of a general decrease in the capacity of organs and internal structures in double-muscled cattle breeds.

One investigation indicated that increased upper-airway resistance in double-muscled calves could lead to an increased velocity of airflow and irritation of mucous membranes in the upper airway, causing laryngitis and other respiratory problems (Gustin et al., 1987). Another related study (Lekeux, et al. 1994), linked hypersensitivity to heat, exercise, and respiratory infection in double-muscled cattle to variations in oxygen transport between double-muscled and conventional (Friesian) cattle. The differences found between the types, that is, lower volume at inspiration and increased respiratory rate, increased pulmonary resistance and sensitivity to hypoxia could all be explained by the 15% decrease in the lung size of double-muscled cattle (Lekeux, et al., 1994; Ansay and Hanset, 1979), and all contributed to the lack of hardiness in some double-muscled cattle. Lekeux et al. (1994) specifically cited a reduction in pulmonary and cardiac reserve capacities in double-muscled animals, which left them especially sensitive to environmental stressors.

Cardiac performance and efficiency was also found to be inferior among double-muscled cattle, compared to Friesians (Amory, 1992; Amory, 1993; Amory, 1994;

Amory, 1995). Maximal cardiac performance, determined by pharmacological stress-test, was shown to be lower in double-muscled cattle than in conventional cattle (Amory, 1993). This condition was later attributed both to reduced heart size relative to body weight, as well as indications of reduced myocardial contractile properties (strength of contraction) in double muscled cattle (Amory, 1995).

Prior to identification of the Myostatin gene and subsequent characterization of the protein, one favorite hypothesis for the double-muscled condition was that of an endocrine hormone imbalance (Pomeroy and Williams, 1962; Arthur et al, 1990). Studies throughout the late 1980's indicated that, unlike other hypermuscular individuals, the muscular hypertrophy in double-muscled animals was not related to higher growth hormone levels. In fact, the blood plasma of double-muscled animals was found to have consistently lower levels of both growth hormone (Arthur et al, 1990) and insulin (Arthur et al. 1990; Hocquette et al., 1999) than normal calves. The latter suggests that insulin-regulated glucose metabolism might be affected, perhaps enhanced, in the double-muscled animal (Hocquette et al., 1999). Levels of both insulin and growth hormone could be modulated through feed restriction and the subsequent compensatory growth following refeeding (Hornick et al, 1998).

Variations in meat quality related to the physiological state of double-muscled animals have been observed as long as the phenotype itself. The "black-fleshed" meat described by Culley (1804) is thought to be a reference to the so-called "dark-cutter" condition, which is often observed in double muscled beef cattle. His analysis of the beef of double-muscled cattle was, "[T]he flesh (for it does not deserve to be called beef) is as black and coarse grained, as we generally suppose horse-flesh to be."

"Dark-cutting" beef describes meat with a dark red appearance instead of the desirable cherry-red color of normal beef. The phenomenon of dark-cutting beef is related to a high muscle pH and the corresponding increase in water-holding capacity and decrease in oxygen uptake. The predominance of myoglobin over oxymyoglobin in the muscle causes the dark color. The condition is related to stress and stress-prone breeds of pigs and cattle (Preston and Willis, 1974), including the double-muscled varieties.

Obviously, the double-muscled phenotype is well researched, particularly in cattle breeds that commonly display the trait. For the first century of investigation, research was basically limited to such analysis, with occasional forays into the inheritance patterns through simple breeding studies. Since the development of techniques that can directly identify the presence or absence of a particular allele, analysis of expression patterns in the double-muscled animal have diverged into new areas, offering information about the genetic and biochemical mechanisms of Myostatin transmission and function.

CHAPTER 4

GENETIC BASIS OF THE DOUBLE-MUSCLED PHENOTYPE

The genetic origin of double-muscling in cattle was never truly in doubt. In fact, the original reference (Culley, 1804) contains an observation that double-muscled animals resulted from breeding programs that used imported, double-muscled Teeswater cattle - along with the injunction not to breed such animals! In the following 200 years, a precise understanding of the inheritance pattern of muscular hypertrophy paralleled the technology of the time. Several patterns of inheritance were hypothesized, although by the end of the 1980's, the most favored theory was a single, autosomal recessive pattern. This idea was supported by long-standing observations of heterozygous "carrier" animals which often displayed growth characteristics between those of the normal and knockout homozygotes (Kieffer and Cartwright, 1980; Baker and Lunt, 1990).

Early crossbreeding experiments generally consisted of selecting a phenotypically double-muscled animal from a line known to consistently produce such individuals and crossing with a dairy type, usually Friesian, to produce a supposed heterozygous animal. Subsequent crosses and back-crosses generated population data that could be compared to known ratios for other expression patterns (Hanset, 1986). Dairy types such as Friesian and Holstein/Friesian typically made up the control group because there has been virtually no incidence of double-muscling in these breeds, and they are not under selection pressure for high muscling as are beef breeds.

In 1995, the research of Charlier et al. localized the mh locus to bovine chromosome 2, yielding strong evidence to support the idea that the mh locus described the single, autosomal, major gene underlying the double-muscled phenotype. This claim of simple, monofactorial Mendellian segregation was verified by an F1 backcross of presumed hemizygous cattle to known homozygous knockouts (mh+/- X mh-/-), which yielded a 1:1 ratio of double-muscled to normal offspring. Although upon first evaluation the researchers found no evidence to support linking the mh locus to any particular autosome, they constructed a marker map of bovine chromosome 2, and analyzed the relative rates of recombination between markers. The results of this experiment indicated that the marker that had the lowest incidence of recombination with mh - and therefore the shortest physical distance - was indeed on chromosome 2, at the centromeric end of the BTA2 linkage group.

The year 1997 marked the world's first formal introduction to the debutante growth and development factor named "Myostatin", by Johns Hopkins researchers Se Jin Lee and Alexandra McPherron. While looking for possible cousins of the well-known TGF-β superfamily of growth factors, the team discovered a novel gene that closely resembled previously investigated members of the family and crossed species barriers with a high degree of fidelity (McPherron et al., 1997; McPherron and Lee, 1997). Subsequent targeted mutation of the gene in mice resulted in an animal that showed runaway muscle development: dramatic, muscle-specific, and altogether like the condition observed for nearly two centuries in double-muscled cattle.

Soon after, independent researchers established that the novel protein did indeed map to the mh locus (Smith, 1997), and showed via the first successful bovine positional

cloning experiment that defects in Myostatin were responsible for the double-muscled phenotype in both Belgian Blue and Asturiana de los Valles cattle (Grobet et al, 1997; Vaiman, 1999). A flurry of other research followed, yielding more evidence to support the link between Myostatin and muscle hypertrophy. Casas et al. (1998) documented the "partial recessive character" of Myostatin in cattle, submitting that hemizygous ($mh^{+/-}$) animals in that study had a muscle mass 1.6 standard deviations higher than homozygous normal ($mh^{+/+}$) animals.

DNA sequencing of the Myostatin gene in other breeds known to produce a large number of double-muscled animals showed that several mutations were capable of inducing the double-muscled phenotype. In addition to the 11 base pair deletion at bovine Myostatin nucleotide 821 (nt821(del11)) found in double muscled Belgian Blue and Asturiana de los Valles (McPherron and Lee, 1997; Grobet et al., 1997) and the G to A transition at nucleotide 938, which causes a cysteine to tyrosine shift in the mature Piedmontese Myostatin protein (McPherron and Lee, 1997; Kambadur et al., 1997; Grobet et al., 1997), other polymorphisms can disrupt Myostatin function. Grobet et al. (1998) pointed out seven different possible mutations in the coding region of the Myostatin gene, five of which were likely to have severe effects on Myostatin function. These results (shown in Table 3) also suggested a hypothetical model for the evolution of Myostatin haplotypes among beef cattle.

Isolating the Myostatin gene and protein and definitively linking mutant Myostatin to the double-muscled phenotype had a broader effect than simply characterizing a cause of muscle cell hyperplasia. It allowed researchers to put away the nearly two-century long subjective selection of subjects and the corresponding mixture of

Breed	Mutation
Belgian Blue	nt821(del11)
Asturiana de los Valles	nt821(del11)
Rubia Gallega	nt821(del11)
Parthenaise	nt821(del11)
Piedmontese	C313Y
Gasconne	C313Y
Maine-Anjou	nt419(del7-ins10)
Charolais	Q204X

Table 3: Mutations in the Myostatin gene, by breed (McPherron and Lee, 1997; Grobet et al., 1997; Kambadur et al., 1997; Grobet et al., 1998)

genotypes that previously made up studies of double-muscled beef cattle. Instead of the physical observation once used, it became possible to directly genotype individuals, differentiating both those individuals who carry one copy of dysfunctional Myostatin, and those "homozygous" individuals that receive two different varieties of dysfunctional Myostatin (Casas et al, 1998; Casas et al, 1999).

This knowledge is of particular importance as researchers move from breeding programs that employ simple selection of the double muscled phenotype to inducing functional changes in the Myostatin genes of other species. Biochemically speaking, structure *is* function, so identifying these differences between species, breeds, and types allows us to better understand how small changes in the molecular structure of a protein can radically alter its effects.

CHAPTER 5

GDF-8 STRUCTURE

Members of the TGF-β superfamily of signaling cytokines have characteristic sequence and structural patterns which dictate their function (Piek, 1999). Their physiological duties run the gamut of cell growth regulation from directing the differentiation of neural tissue to inducing growth of mesenchymal cells (Piek, 1999), but most relevant to this discussion are those family members that act as highly specific, highly potent inhibitors of cell proliferation. Myostatin is dissimilar enough, especially in the C-terminal region, to defy classification into any of the major TGF-β subfamilies, such as inhibins, TGF-βs, and bone morphogenic proteins (McPherron et al., 1997), however, it shares several characteristics in common with other members of the superfamily (See Figure 3). TGF-β superfamily sequences encode a secretion signal sequence, proteolytic processing site, and a conserved pattern of cysteine residues in the C-terminal end, and are highly conserved across species (McPherron et al., 1997).

Figure 3: Myostatin gene - conserved regions (cysteine position are shown in yellow)

After translation, the large TGF-β superfamily precursor molecules are delivered to the Golgi apparatus, where they are proteolytically cleaved by the endoprotease furin. The amino-terminal remnant of the original precursor molecule (or LAP) and TGF-β

binding proteins form latent TGF-β complexes targeted for the cell surface, where they are activated by proteolytic cleavage of LAP from the mature, homodimeric complex (Piek, 1999) (See Figure 4).

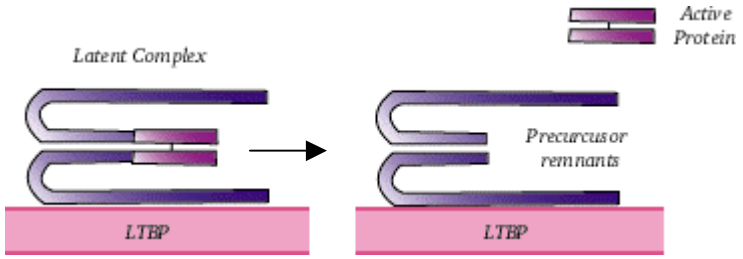

Figure 4: TGF-β formation

As mentioned before, TGF-β superfamily peptides are characterized by a conserved pattern of nine cysteine residues in the carboxy-terminal end (McPherron and Lee, 1997). In other TGF-β family members, similar cysteine patterns in the mature proteins form intramolecular and intermolecular disulfide bridges within the biologically active pocket of the homodimeric complex, the so-called "cysteine knot" structure (Thomas et al., 2000). This highly conserved pattern is one of the factors that originally led the Johns Hopkins researchers to Myostatin (McPherron and Lee, 1997). One of the nullifying mutations, C313Y, which changes the fifth of the nine cysteine residues in mature Myostatin to tyrosine, causes the functional loss seen in the Piedmontese breed (Grobet et al., 1998). The striking effects of this one-residue alteration gives some indication of the structural importance of this pattern in the mature protein function.

The amino acid sequence which targets the mature protein for secretion consists of a core of hydrophobic amino acids close to the N-terminal end, and the Arg-Ser-Arg-Arg (RSRR) proteolytic processing signal is located close to the protein's C-terminal end (Figure 3) (Thomas et al., 2000).

The active TGF-β family member Myostatin is a 26 kDa homodimeric protein expressed specifically in the myotome layer of developing somites during embryogenesis, and later in all skeletal muscles (McPherron and Lee, 1997; Lee and McPherron, 1999). Although it is not known whether Myostatin exists as a large, latent complex prior to activation like other TGF-β members, a similar activation procedure could explain the stringent specificity of its action (Lee and McPherron, 1999).

The actual amino acid sequence of Myostatin has a strikingly high degree of conservation across species boundaries, considering that the protein is patently not necessary for viability. Analyzing a sequence alignment of the published Myostatin amino acid sequences for 10 species, with Jellyfish™, I found that 85-90% or greater sequence identity between species were common (Figure 5). According to McPherron and Lee (1997) and Grobet et al (1997), in the 1,128 base pair overlapping region of the murine and bovine coding sequence, there is an 89.1% incidence of matching, while the predicted protein shows a 92.5% identity between the two species. As mentioned before, mutations that disrupt the bioactive carboxy-terminal region of the Myostatin gene or the structure of the cysteine knot can lead to functional knockouts, and the accumulation of two copies of dysfunctional Myostatin leads to the extreme double-muscled phenotype. A survey of Myostatin polymorphisms carried out by Grobet et al (1998) revealed a number of different ways to abrogate Myostatin's effect in cattle by structurally reorganizing the gene.

The double muscled phenotype in both the Belgian Blue and Asturiana de los Valles breeds is caused by an 11-bp deletion of nucleotides 821 to 831, which results in a frame shift and premature termination during translation (Grobet et al., 1997; Kambadur

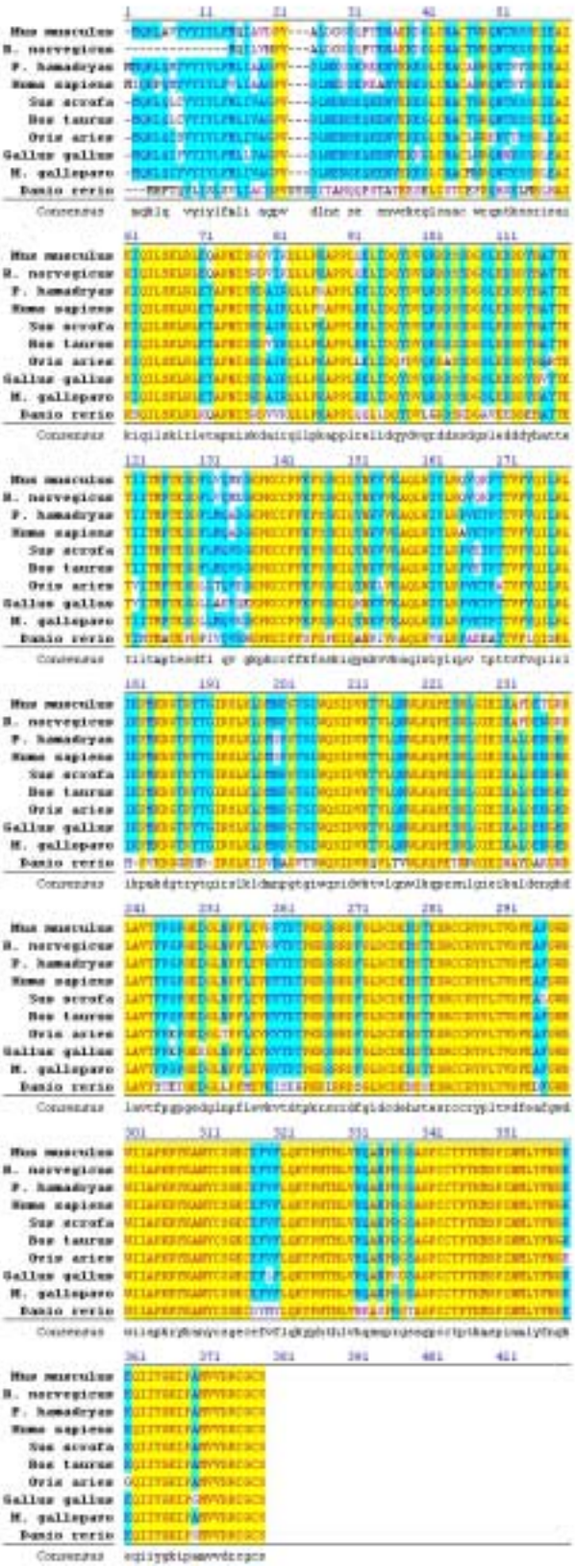

Figure 5: Sequence alignment

et al., 1997; McPherron and Lee, 1997). This protein is obviously truncated, lacking an important region of function, and it has been shown that inducing a similar abbreviation in the murine protein has very comparable effects (McPherron et al., 1997; McPherron and Lee, 1997) (Figure 6). The double-muscled phenotype in the Piedmontese breed is caused by a G-A transition in the same region, that replaces a highly reactive cysteine with a tyrosine (Kambadur et al., 1997; McPherron and Lee, 1997) (Figure 7). Additional mutations that appear to mangle the protein in other cattle breeds include the 7

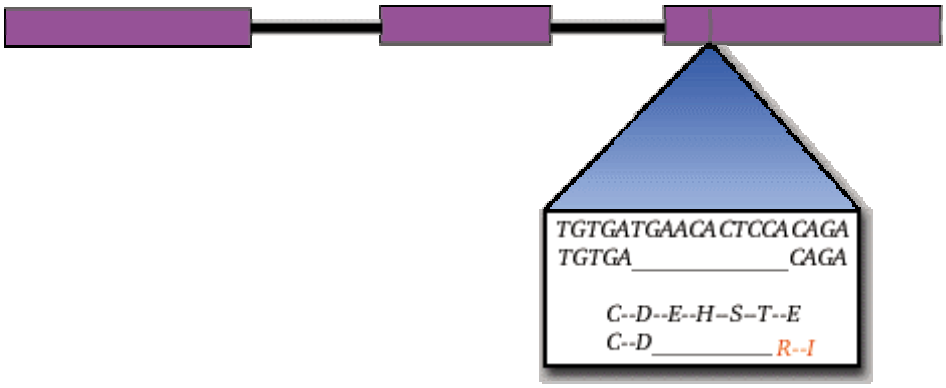

Figure 6: Normal Myostatin nucleotide and predicted amino acid sequence (top) vs. nt821(del11) mutant Myostatin (bottom)

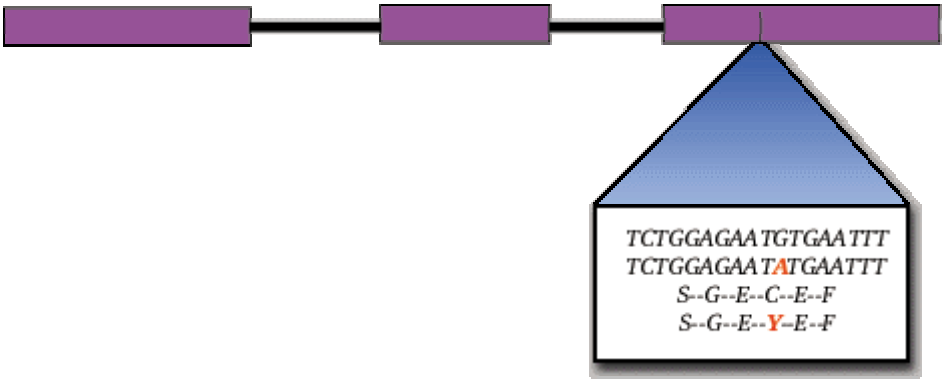

Figure 7: Normal Myostatin nucleotide and predicted amino acid sequence (top) vs. C313Y mutant Myostatin (bottom)

base pair deletion and 10 base pair insertion at nucleotide 419, found in the Maine-Anjou breed (Grobet et al., 1998), and the C to T point mutation at nucleotide 610, observed in Charolais animals, which results in the replacement of glutamine residue 204 with another amino acid (Grobet et al., 1998; Antoniou and Grosz, 1999). These polymorphisms are shown in Figures 8 and 9.

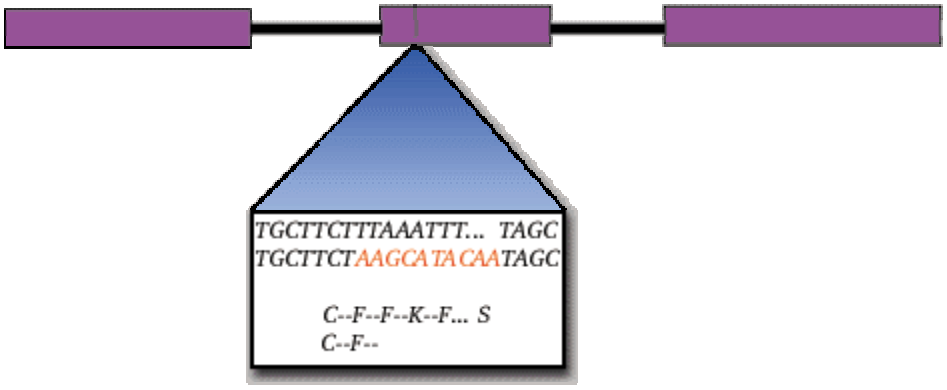

Figure 8: Normal Myostatin nucleotide and predicted amino acid sequence (top) vs. nt419(del7ins10) mutant Myostatin (bottom)

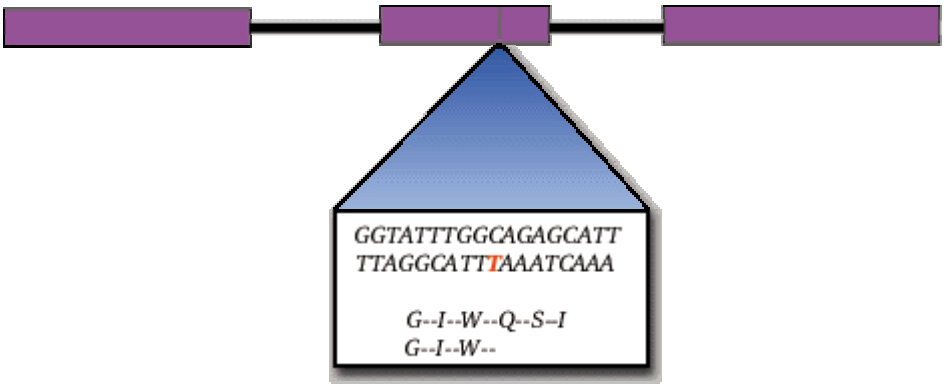

Figure 9: Normal Myostatin nucleotide and predicted amino acid sequence (top) vs. Q204X mutant Myostatin (bottom)

CHAPTER 6

GDF-8 MECHANISM

Myostatin has been identified as a circulating factor, secreted by muscle cells and acting upon those cells to inhibit growth (McPherron et al., 1997; Gonzales-Cadavid, 1998; McPherron and Lee, 2000). Previous work showed that serum from double muscled fetuses failed to inhibit the replication of myoblasts *in vitro*. This was first interpreted as a case of double-muscled fetal serum stimulating growth (Gerrard and Judge, 1993). Later supplemental research, however, identified the causal agent of double-muscling as a blood-borne factor that was capable of inhibiting muscle cell proliferation (Gerrard et al., 1995).

Given that overview of Myostatin function, it is possible to consider several different aspects of its *in vivo* role. First, it can be considered a limiting factor in normal muscle development. Given its pattern of expression: highly expressed in embryonic and fetal stages and expressed to a lesser degree in adult muscle tissue, it can be primarily viewed as a growth regulator in early development (McPherron and Lee, 2000). In spite of this variation of expression during phases of development, Myostatin is expressed in postnatal muscle tissue, and has been shown to affect adult tissue as well (Thomas et al., 2000). Overexpression of Myostatin has been linked to muscle wasting, such as that seen in individuals infected with HIV, limiting growth or regrowth in adult humans (Gonzales-Cadavid, 1998). Myostatin expression patterns have also been shown to change with

physiological state, becoming upregulated in cardiomyocytes after heart damage (Sharma, 1999), and downregulated in regenerating muscle (Sakuma et al, 2000).

In this way, Myostatin, like leptin, can be described as a route of continual communication between individual tissues and the organism as a whole, a "chalone" (McPherron and Lee, 2000; Slack, 1997), helping to report the status of tissue and maintain a global balance in tissue growth. Both of these approaches to understanding Myostatin's function are supported by an exploration of its mechanism of action in muscle and other tissues. The inhibitory effects of Myostatin have been shown to be reversible in vitro (Thomas et al., 2000), which tends to support the notion of Myostatin as a reporter and regulator whose effects can be modulated with changes in muscle tissue size and cell number.

Like all tissues of the body, the specialized cells that make up muscle begin as undifferentiated precursor cells or stem cells that commit to the mesoderm. Mesodermal progenitor cells then undergo modulation by growth factors at the determination and differentiation stages, which collectively assign the final identity of the cells (Kelvin et al; 1989). Members of the TGF-β superfamily all act as positive, and/or negative regulators of points in the pathway that sculpts a myocyte from an undifferentiated stem cell, and they operate by way of tissue specific cell-surface receptors (Kelvin et al, 1989). TGF-β regulates at the terminal differentiation level, blocking the differentiation of myoblasts (Kelvin et al.; 1989).

Myoblasts proliferate during myogenesis, then withdraw at G_1 of the cell cycle and commit to form myotubes. Progression through the cell cycle, and cell cycle arrest, are often controlled by cyclin-dependent kinase and cyclin-dependent kinase inhibitor

(CDK/CKI) complexes. Myostatin is thought to control the G_1 to S and G_2 to M transitions of the cell cycle for myoblasts through modulation of $p21^{cip1}$ and Cdk2 protein levels (Thomas et al., 2000). Myostatin upregulates expression of $p21^{cip1}$ (a CKI) (Ríos et al., 2001; Thomas et al., 2000), and downregulates expression of Cdk2, inactivating the Cyclin/CDK complex that allows progression from G_1 to S (Thomas et al., 2000). $p21^{cip1}$ is also a key factor in the survival of myocytes, strongly inhibiting myocyte apoptosis (Ríos et al., 2001). Overexpression of normal Myostatin, therefore, induces cell cycle arrest at the G_1 stage and termination of proliferation, and increases the survival of differentiating myocytes. A functional Myostatin knockout removes both the inhibition and the resistance to apoptosis, which may have some effect on the final structure of the muscle tissue: increased size due to hyperplastic growth of small myotubes (Kelvin et al., 1989) (Figure 10).

Myostatin has been shown repeatedly to specifically affect muscle cells. However, it is expressed in other tissues and there is some indication that it may carry out cell cycle control in these areas as well. Although Myostatin is expressed only at low levels in adipocytes (McPherron et al., 1997), it has been shown to inhibit the differentiation of preadipocytes into adipocytes, probably by inhibition of transcription factors (Kim et al., 2001). In this way, Myostatin can be said to have a direct effect on adipogenesis in addition to its well-described indirect effects that result from radically changing the ratio of muscle to adipose tissue.

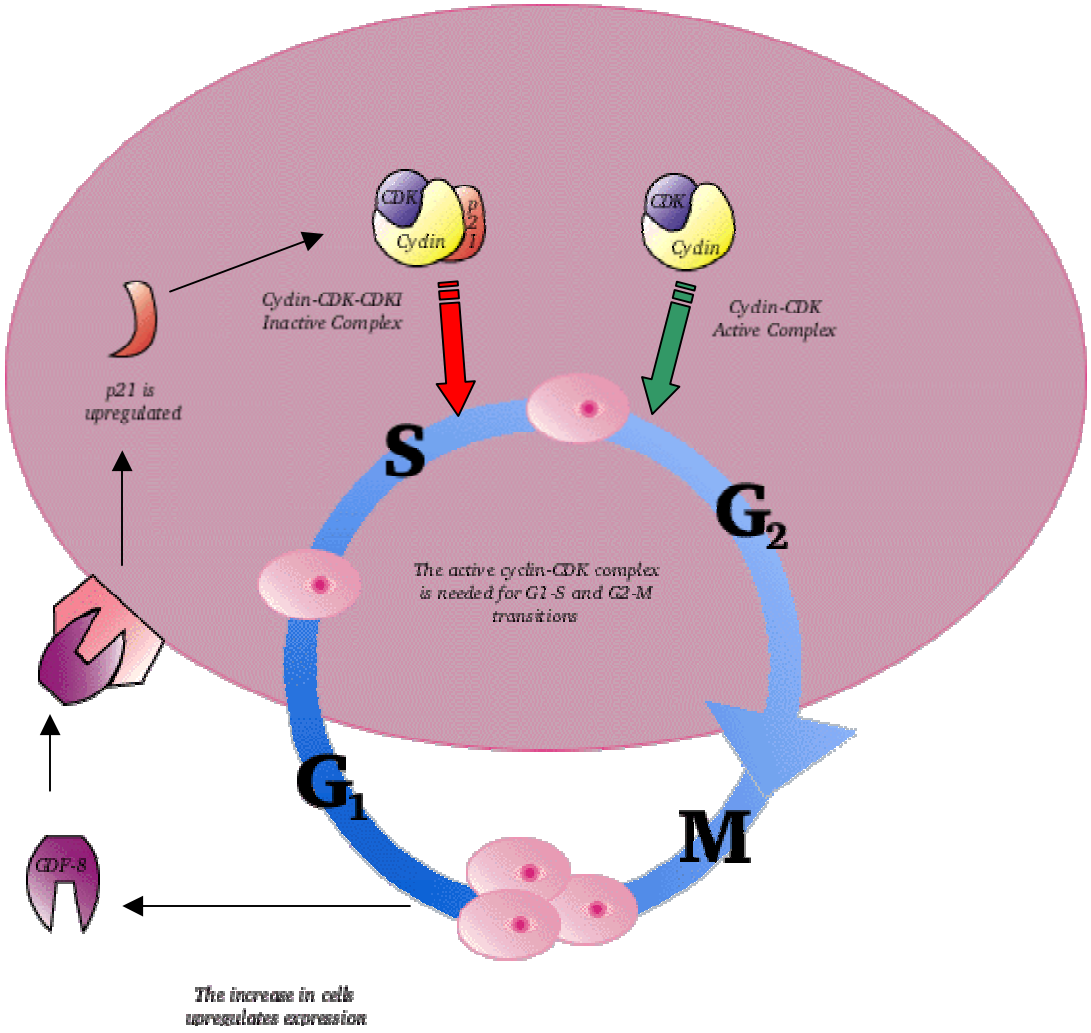

Figure 10: Myostatin mechanism

CHAPTER 7

REPORT: NONFUNCTIONAL GDF-8 CAUSES VARIATIONS IN THE GROWTH AND BODY COMPOSITION OF MICE 1 TO 4 MONTHS OF AGE

Abstract

GDF-8, a recently discovered member of the TGFβ superfamily of growth and differentiation factors, has been shown to act normally as an inhibitor of skeletal muscle growth. A natural loss of functional GDF-8 expression has been identified as the cause of "double-muscling" in the Belgian Blue and Piedmontese cattle breeds, and GDF-8 "knockout" mice display many of the same characteristics, including hypermuscularity and hypotrophy of adipose tissue. The body composition of these mice, determined by proximate analysis and nitrogen analysis, has not been determined for weanlings. We analyzed intake, body weight changes, tissue weights, and body composition variations between GDF-8 knockout and normal mice at 4, 8, and 12 weeks of age, and found significant, genotype-specific differences in the weights of adipose and muscle tissue weights,

Introduction

Since the development of genetic manipulation techniques, a major goal of the livestock biotechnology industry has been to increase the growth of agriculturally important livestock species. This goal involves not only the production of larger animals but the development of strains that produce larger proportions of saleable produce, and

those that follow a more efficient pattern of growth. The search for new methods to regulate growth patterns has targeted many scientific inquiries toward specific mutation or regulatory pharmaceutical control of growth mechanisms. Knockout models have shown promise for both the design of new breeds of animals with enhanced growth characteristics and the development of pharmaceuticals and therapies for the manipulation of growth in normal animals.

GDF-8, or Myostatin, a recently discovered member of the TGFβ superfamily of growth and differentiation factors, has been shown to act normally as an inhibitor of skeletal muscle growth (McPherron et al, 1997; McPherron and Lee, 1997; Grobet et al, 1997; Grobet et al., 1998; Kambadur et al, 1997; Slack, 1997). A natural loss of functional GDF-8 expression has been identified as the cause of muscle hypertrophy (mh) or double-muscling observed in cattle breeds such as the Belgian Blue and Piedmontese (McPherron et al, 1997b; Grobet et al, 1998; Westhusin, 1997). The creation of Myostatin "knockout" mice through homologous recombination has resulted in an experimental model organism with comparable phenotype (McPherron et al, 1997; McPherron and Lee, 1997). This allows some basic research to be conducted at the small-animal level, allowing efficient exploration of Myostatin knockout effects *in vivo* that may be related to agricultural and biomedical applications. Clearly, systematic analyses of Myostatin effects in the murine knockout model are required to demonstrate the applicability in agricultural and biomedical contexts.

While growth characteristics of adult Myostatin-null mice have been well-documented (McPherron et al., 1997; McPherron and Lee, 1997), little research has targeted the earlier phases of growth, including the periods between weaning and puberty.

Using Myostatin knockout mice and wild-type mice generously provided by MetaMorphix, Inc., we investigated the effects on the growth and development, intake, and body composition of mice 1-4 months of age.

Methods

Animal selection:

The 63 mice used in this experiment were selected from confirmed homozygous breeding lines of GDF-8+/+ (wild type) and GDF-8-/- (knockout) mice generously provided by MetaMorphix, Inc. Mice selected for the study included 15 female and 17 male GDF-8-/- mice and 17 female and 14 male GDF-8+/+ mice.

Growth, intake, and tissue weight measurements:

At weaning (21 days) the mice were randomly assigned to treatment groups balanced by sex and genotype and were isolated in wire-bottomed cages for a one-week acclimation period. The mice were provided with bedding cups, nestlets, toys, and were given access to 5020 rodent chow and tap water ad libitum for the duration of the study period. Feed intake and weight were measured weekly throughout the duration of the study. At 4, 8, and 12 weeks of age, balanced groups of individuals were sacrificed by CO_2 asphyxiation and decapitated. Tissues, including heart, lungs, liver, kidney, gluteal muscle, and retroperitoneal, inguinal, and epididymal or parametrial fat pads were removed, weighed, and flash-frozen in liquid nitrogen for later use. The eviscerated carcass was then frozen until proximate analysis of body composition.

Body composition (proximate) analysis:

Body composition was determined by proximate analysis, according to the methods of the M. Azain laboratory (Azain, unpublished communication), and are

summarized as follows. Frozen carcasses were weighed, then autoclaved for 1 hour at 120°C to soften tissues and allowed to cool to 4°C overnight. Distilled water, in an amount 3 times the previously recorded carcass weight, was added to the chilled carcasses. The mixture was then blended thoroughly and homogenized for 3 minutes. The resulting homogenate was then divided into triplicate samples of 3 ml for dry matter and ash determination, 3.5 ml for lipid extraction, and 2 ml for nitrogen analysis. Any remaining homogenate was stored at -20°C.

Crucibles containing the 3ml samples were allowed to dry in a 100°C oven for 48 hours, then weighed for a dry matter measurement. The dry homogenate was then baked at 600°C for 12 hours, and the resulting ash was weighed for an estimate of mineral content.

Tubes containing the 3ml ether extract were vortexed briefly, and 4 ml methanol and 2 ml chloroform were added to each. The tubes were then vortexed again and allowed to react at room temperature for 1 hr. Two ml chloroform and 2 ml 1M KCl were added to each tube. Tubes were vortexed and centrifuged at 2000 rpm for 20 minutes, at 4°C. The top layer and pellet were then removed from tubes, and the remaining ether extract was poured into pans and weighed. The chloroform was allowed to evaporate in the fume hood for 48 hrs, leaving the lipid content of the homogenate sample.

Nitrogen analysis:

Nitrogen analysis of lyophilized homogenate samples was carried out using the protocol for the LECO FP-528 Nitrogen analyzer (Leco Corporation, Warrendale, PA). The machine was calibrated for EDTA, and all samples were run in triplicate.

Results

Body weights were significantly higher in knockout mice only at 4, 5, and 6 weeks of age. Food intake was only significantly different at 4 and 7 weeks of age. Body weight changes are shown in Figure 11.

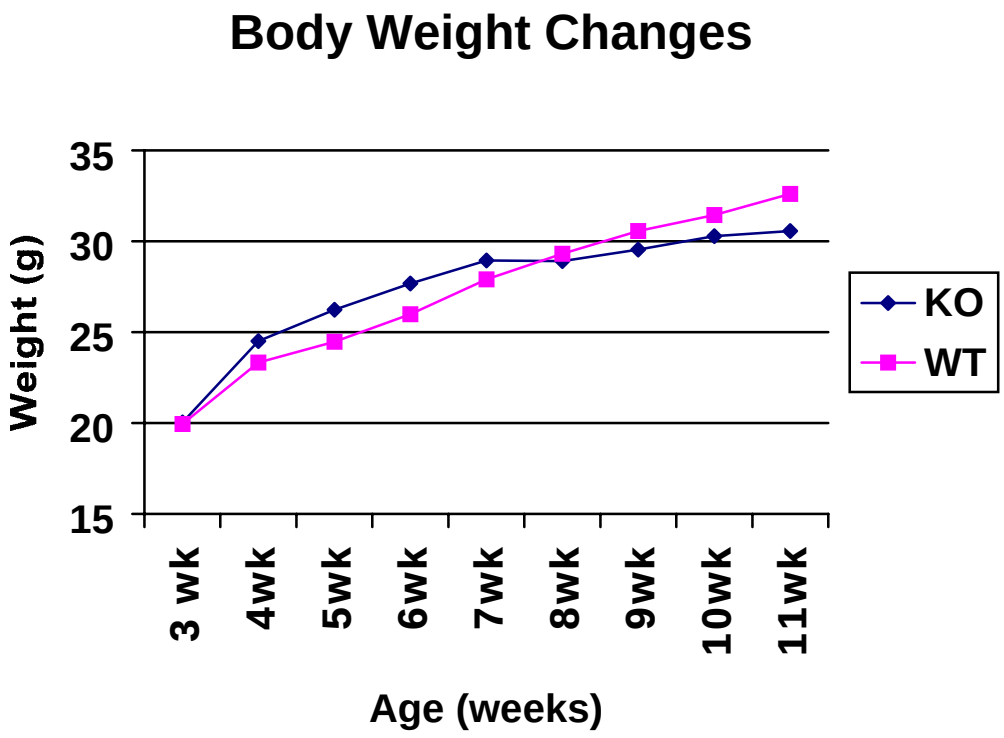

Figure 11: Body weight changes over time (asterisks denote significant differences)

All tissue weights were expressed both as a mean weight and as a proportion of total body weight. The mean weights for individual tissues at each age are shown in Table 4. Heart and lung weights showed no significant difference and are excluded from the table.

Tissue	Genotype	4 weeks	8 weeks	12 weeks
Rp	KO	0.01887±0.014	0.11799±0.094	0.10124±0.097 (p<.0001)
	WT	0.03317±0.011	0.14631±0.076	0.24691±0.139
Epi/Par	KO	0.12444±0.093	0.62509±0.308	0.42798±0.224 (p<.0001)
	WT	0.16264±0.074	0.73837±0.167	1.32444±0.571
Ing	KO	0.09707±0.059 (p<.05)	0.28143±0.163	0.19673±0.067 (p<.0001)
	WT	0.18658±0.061	0.32373±0.079	0.56292±0.355
GM	KO	0.09048±0.031	0.17338±0.048 (p<.0001)	0.20409±0.070 (p<.0001)
	WT	0.07936±0.015	0.12924±0.015	0.16012±0.017
Liver	KO	0.90994±0.324 (p<.05)	1.18590±0.287	1.19865±0.263 (p<.0003)
	WT	1.05193±0.177	1.23808±0.213	1.43741±0.267
Kidney	KO	0.24307±0.069 (p<.05)	0.35895±0.088	0.37436±0.093
	WT	0.28206±0.045	0.37738±0.068	0.40592±0.092

Table 4: mean tissue weights by age group and genotype ± SEM

The mean weights of all three fat pads as a proportion of body weight were significantly different (p<.0001) between genotypes, heavier in control mice and lighter in knockout mice. The fat pads of knockout mice tended to be represent a smaller portion of body weight than those of control mice at all age groups observed. Retroperitoneal fat pads were significantly lighter in knockout mice at 12 weeks of age (p<.0001). Parametrial and epididymal fat pads were significantly lighter in knockout mice at 8 (p<.05) and 12 (p<.0001) weeks of age. Inguinal fat pads were significantly lighter in knockout mice at 4 (p<.0001), 8 (p =.0535) and 12 (p<.0001) weeks of age.

The gluteal muscle represented a larger proportion of the total body weight in knockout animals at 4 (p<.005), 8 (p<.0001), and 12 (p<.0001) weeks of age. Heart muscle, on the other hand, tended to represent a smaller proportion of total body weight

in 4- and 8- week old knockout mice, but was significantly larger than that of control mice at 12 weeks ($p<.01$).

Adjusted kidney weights of knockout mice were significantly smaller than those of control mice at 4 ($p<.0001$) and 8 ($p<.0002$) weeks of age, and tended to remain fixed as a proportion of body weight in the knockout animals. Adjusted kidney weights of control animals tended to decrease over time, representing a smaller proportion of total body weight as the age of the mouse increased. Adjusted liver weights of knockout mice were significantly smaller 4, 8, and 12-week-old mice ($p<.0001$, $p<.0004$, and $p<.0005$, respectively). Lung weights were not significantly different between knockout and control mice of the same age group.

Body composition analysis showed differences between genotypes in the concentration of lipid, ash, and protein in the carcass. These changes are shown in Table 5.

Component	Genotype	4 weeks	8 weeks	12 weeks
Dry Matter	KO	36.790±8.051	41.374±6.158	36.988±5.652
	WT	37.469±6.177	43.285±2.791	46.799±5.183
Lipid	KO	9.898±4.009	13.753±5.999	10.196±2.950
	WT	12.557±2.813	15.658±2.179	21.029±5.468
Ash	KO	3.671±0.835	3.841±2.419	3.605±1.365
	WT	3.310±0.762	3.567±0.508	3.450±1.378
Protein	KO	23.220±4.731	23.576±2.865	23.097±3.252
	WT	21.602±4.011	23.908±1.622	22.385±1.914

Table 5: Body composition determined by proximate analysis, percent of carcass weight

Because the protein content of a sample is estimated indirectly with proximate analysis, percent nitrogen was also measured directly with the LECO nitrogen analyzer, and multiplied by 6.25 for a more direct estimate of protein composition. The mean percent protein for each age group determined by nitrogen analysis is shown in Table 6.

Body composition for all mice was significantly different by genotype, and is shown in Figure 12.

Genotype	4 weeks	8 weeks	12 weeks
KO	61.053±7.750	53.703±13.081	63.939±8.469
WT	55.600±5.081	51.717±9.856	47.468±11.039

Table 6: Percent protein estimated by nitrogen analysis.

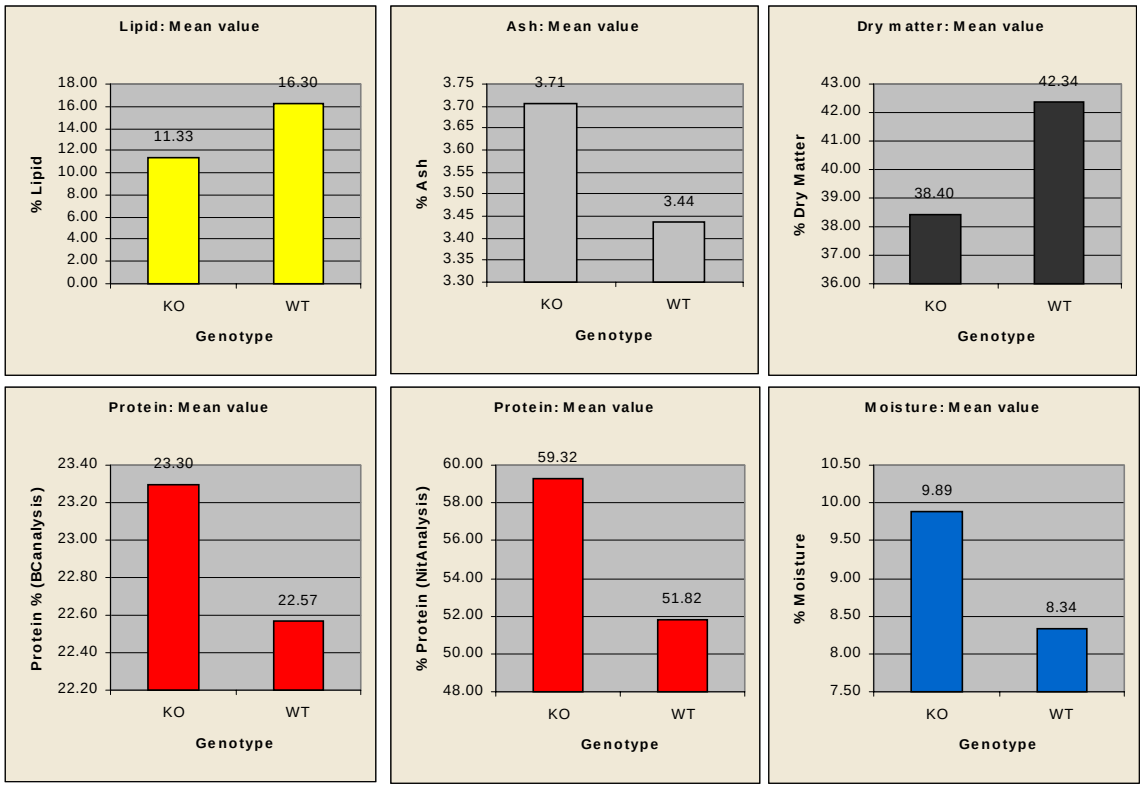

Figure 12: Body composition by genotype.

Discussion

The results of this study generally correspond to the trends shown in previous studies with older mice, although total weight was reduced in 8-12 week old knockout mice. Myostatin knockout mice tended to be leaner and heavier than control mice

between 4 and 8 weeks of age, with drastically reduced fat pad weights and lower carcass concentrations of fat and higher concentrations of protein in their carcasses. These trends varied by age, and the variation in percent fat and percent protein tended to increase in older mice. The actual fat pad weights only showed highly significant differences between genotypes at 12 weeks of age, however, when expressed as a percent of total body weight, significant differences began to appear at 8 weeks of age. This is consistent with the higher growth curve of Myostatin knockout mice reported previously (McPherron et al., 1997).

Several unusual interruptions in the development patterns occurred near the 8-week analysis mark, but it is possible that body changes associated with puberty (approximately 6 weeks for females and 7-9 weeks for males) may have influenced these values. It is also possible that the rodent diet fed did not contain a sufficient proportion of protein to support the accelerated growth of Myostatin knockout mice. Overall, feed intake was not significantly different between genotypes. Other research in mouse models of muscular hypertrophy has shown that a diet that does not meet the nutritional requirements of such animals can lead to inefficient growth (Lopez-Oliva et al., 2000). We found trends toward proportionately smaller organ weights in knockout mice, excluding the heart and lungs.

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CHAPTER 8

CONCLUSION

Month by month, new research has increased our understanding of the mechanism of Myostatin function and its specific roles in the development of diverse species. Two hundred years' worth of breeding for the double-muscled trait in cattle is some indication of our general willingness to exploit a condition without full understanding. However, with new information about the specific effects of a Myostatin knockout in model organisms such as the mouse, it becomes possible to explore this pathway in a economical and time-efficient way. We found that Myostatin knockout mice follow generally the same trends of growth and development in the first 1-4 months as observed in older mice, namely, lower proportions of fat and higher proportions of muscle than normal counterparts. This is particularly important because our research concerned growth trends in Myostatin-null mice that correspond to ages of importance to the livestock industry: weaning, puberty, and young adulthood. Although growth trends in the mouse model cannot be directly applied to other species, our findings can give further support to inter-species growth patterns related to the Myostatin gene.

The possible uses of such basic research are far-reaching, from the obvious agricultural applications to human medicine. An exploration of quantitative trait loci associated with the double-muscled phenotype and modes of inheritance of Myostatin in livestock animals may offer means to "tweak" the expression patterns of this protein and

manipulate other, related traits to compensate for the deleterious effects of a full knockout (Casas et al., 2000).

It is evident that certain economic atmospheres and production norms are more suited to the management of double-muscled cattle than others. In Belgium, for instance, a system that rewards beef leanness supports the production of Belgian Blue cattle, in spite of the cost of dystocia and lowered fertility (DARD, 2000). The selling price of a double-muscled calf and the cost of a caesarian section are in a ratio of 10:1 (Hanset, 1991). Production of heterozygous animals, using double-muscled bulls as terminal sires, may be a viable option when the value of increased retail product yield is greater than the increased cost associated with calving difficulty. Production systems that use mature cows instead of heifers in these crosses may also avoid some of the problems (Casas et al, 1999).

This sort of recourse does not escape debate either. Concern over the welfare of such extreme phenotypes has incited some animal rights groups to protest the continued breeding of Belgian Blue cattle and other double-muscled breeds, on the grounds that multiple caesarian deliveries amount to a form of torture and are not consistent with sound, compassionate animal husbandry. The same groups have vehemently protested the creation of Myostatin knockouts in other breeds for this and other reasons. Others argue that since routine caesarian in Belgian Blue herds eliminates the need for pulling calves, it may represent an improvement in the welfare of both cow and calf over that of normal cattle (Hanset, 1991).

Increased knowledge of expression patterns and Myostatin mechanisms of function have already hinted at some possible methods to circumvent the deleterious effects of a full Myostatin knockout. Pharmaceutical agents that block Myostatin at expression, transformation from latent complex to active complex, or receptor binding may offer a way to "turn off" Myostatin function in adult animals, bypassing the problems for gestating, fetal, and neonatal cattle. This method may have a place in human medicine as well, offering a way to fight AIDS-related or cancer-related forms of cachexia.

Transgenic technology may offer a way to induce Myostatin knockouts in other livestock species, such as pigs, which do not suffer the same difficulties with large birth weights. Finally, identification of Myostatin polymorphisms that can interrupt function opens the door for widespread screening of possible carrier animals and breeding strategies that can take advantage of the useful nature of a Myostatin knockout while selecting against undesirable companion traits. Taken together, rapidly increasing understanding of cell-cycle control mechanisms and these varied approaches to exploiting Myostatin mutations may represent a significant gain for several industries.

The two-century-long drive to explore Myostatin has been a study in tenacity for the livestock industry in particular, embodying the best outcome of perseverance and creative problem-solving. Now, new understanding and new technology have shown that this mechanism can be an extremely valuable in the pursuit of muscle-specific growth control.

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APPENDIX A

MYOSTATIN: NEW DEVELOPMENTS[1]

[1] Arnold, H.; D.L. Hartzell; H. Kim; K. Page; C.A. Baile. 2000. To be submitted to The Journal of Animal Science.

As biotechnology moves into the realm of animal agriculture, researchers find a host of new models and information at their disposal for building better livestock. The vagaries of markets, environment, and public opinion can upset the best plans, or bring to the forefront the most unexpected candidates for the latest explosion of interest. Nowhere is this more apparent than the long and often rocky relationship between cattle producers and the massively muscled beef breeds such as the Piedmontese and Belgian Blue. Call them muscular hypertrophy mutants, doppellender, or double-muscled. Call them Myostatin or GDF-8 knockouts - a centuries-old windfall of selective breeding, or a royal pain for producers. Any way you slice it, cattle with this mutation have offered producers and consumers the same dilemma from the start: big beef, for a price.

In the recorded history of this phenotype, it has had enough interest ups and downs to rival several other growth factors of genetic origin, and remains a condition with both obvious consumer appeal and dismal production difficulties. When George Culley described the “double-lyered” cattle in his 1804 almanac, he concluded that, though tasty and undeniably large, these animals so often had dark-colored, unacceptable meat that they were of dubious merit in production (1). This tendency, usually brought on by pre-slaughter stress in so-called “dark cutter” cattle, and the notorious reproductive difficulties of double-muscled breeds, led many countries to ban the production and import of such animals. In a world that valued hardy cattle for meat, milk, and labor, the easily-stressed double-muscled animal became a nuisance, and heated debate answered the suggestion that the bloodlines of a few outstanding shorthorn bulls were “tainted” with the trait (2). Only a few countries, including Belgium and Italy, saw a potential cash cow in the variation. Instead of prohibiting the breeding of double-muscled animals, they

selected for the trait, gradually shifting the emphasis of the dual-purpose Belgian Blue and Piedmontese cattle to that of massive meat production.

Later, as changing consumer demands led to a new evaluation of the double-muscled animal's merits in a leaner-meat market, a 1980 symposium of experts met to discuss the use of the double-muscled trait in beef cattle improvement (3). Their findings, although optimistic, were not particularly surprising. Double-muscled animals offered great potential from a standpoint of the quality and quantity of retail cuts, but only in a society that would allow caesarian-delivered calves in 5-70% of pregnancies, depending on breed, and only in a market willing to make the extra care and expense of breeding and raising the animals worthwhile for producers.

In 1997, Alexandra McPherron and Se-Jin Lee, researchers at Johns Hopkins University, described the single malfunctioning gene responsible for the condition in the Belgian Blue: Myostatin or GDF-8, a cell-cycle inhibitor specific to skeletal muscle (4, 5, 6). In the absence of functional GDF-8, muscle cells continue to multiply long after normal conditions would signal a stop, resulting in hyperplastic tissues - tissues made larger by the increased numbers of cells. In cattle, this hyperplasia accounts for the 20-30% increase in muscle mass. However, when Lee and McPherron designed mice with a nonfunctional GDF-8 gene, the Myostatin "knockout" mice grew muscles that were 200-300% larger than their normal littermates, and showed the bulging, muscular shoulders and hips so familiar to those studying double-muscled cattle. Subsequent analysis localized the bovine version of Myostatin at the muscular hypertrophy (*mh*) locus, suggesting that the gene driving the double-muscled phenotype had finally been found (7).

Identifying the GDF-8 knockout effects in model animals led to much the same conclusions that scientists and farmers have been drawing for over a century. The gene is highly specific to skeletal muscle. Loss of function results in hyperplasia only of muscle tissue, with a corresponding drastic decrease in fat and without added bone growth. In cattle, the quality of the meat is enhanced by the increased number of cells beefing up the tissue. While the giant muscle fibers that characterize some hypermuscular breeds tend to be tough, the many small muscle cells in the GDF-8 knockout reportedly result in steaks that are tender without added fat, a quality condition not well-supported by current meat-grading standards.

Researchers at the USDA's Meat Animal Research Center in Clay Center, Nebraska compared the production attributes of crossbred calves bred from Belgian Blue or Piedmontese sires to Angus or Hereford sired crosses. While the Belgian Blue and Piedmontese crossbred steers did show an increased ratio of muscle to fat and a better relative yield of lean meat, the Angus and Hereford showed higher quality grades. The most obvious reason for the lower quality grade was the reduction in intramuscular fat, or marbling, a traditional indicator of both tenderness and flavor. The Belgian Blue crossbred calves also showed a significant increase in calving difficulty when compared to groups with similarly large offspring (8).

This reproductive difficulty has been a long-standing problem for Belgian Blue producers. Females are generally slow coming into puberty, and the rate of reproductive failure is very high. Calves, which begin showing increased muscle growth at around day 16 of gestation, are generally large, leading to difficult births that often require intervention. When the calves are successfully delivered, producers still often have to

face the problems of poor mothering and poor milking in the cows (3). With calves that tend to be somewhat less vigorous at birth, this can add up to a higher death loss, or at the very least, a drastic increase in the cost of care and labor.

So the question remains, where do we go from here? It's hard to look at a double-muscled bull without seeing potential for the meat industry, but the undesirable companion traits continue to hinder large-scale development. Armed with better understanding of GDF-8 mechanisms, it may be possible to circumvent the reproductive drawbacks of GDF-8 mutation, either through intrabreed and interbreed screening and selection for GDF-8 deficient animals with superior reproductive traits, or through transgenesis.

A 1998 study identified seven unique polymorphisms of the GDF-8 coding sequence in European breeds of double-muscled beef cattle, five of which could partially or totally disrupt the function of GDF-8 (9). With so many variations of "knockouts" within one species, it seems very likely that some production problems in cattle could be reduced simply through traditional selection techniques, improving production without losing the valuable growth characteristics.

In a world where the debate over genetically modified food continues to intensify, such "naturally occurring" mutations may offer added confidence to those producers leery of future consumer response to transgenic livestock. The benefits of finding such variation are unlikely to end with cattle. Rapidly accumulating knowledge about the conservation of GDF-8 structure and function across species lines suggests that the applications in livestock animals could be far-reaching. The functional carboxy-terminal region of GDF-8 is identical in the pig, chicken, and turkey, and is highly conserved

across many other species (5). Plans already underway to screen exceptional hogs and chickens for endogenous forms of mutant GDF-8 will offer the producer a way to identify and select for the variations already present in a commercial herd, and will provide researchers with a new library of genotypes to search for other commercially valuable traits. This approach will give producers detailed information about the superior genetics within their herds, and will allow them to construct better breeding strategies to make use of those resources. Researchers, in turn, will have both genetic material and access to the performance information of individual superior animals from conception to slaughter, offering a means to search for patterns of exceptional traits.

Another recent study (10) supports the idea that GDF-8 functions as a circulating, secreted protein, produced by muscle cells and acting on muscle cells to limit proliferation. This understanding opens yet another route for developers to exploit the effects of GDF-8, by developing a Myostatin "vaccine" that would check the response of a normal animal to the endogenous GDF-8 already in the system. This vaccine could allow producers to shut off the inhibiting effects of GDF-8 in livestock at different points in production, possibly bypassing the high birth weight and related calving difficulties often seen in cattle that produce nonfunctional GDF-8 in embryonic tissue. Though levels of GDF-8 are variable and lower at maturity than fetal stages, (11) reduced response to GDF-8 in growing animals can allow increased growth throughout this important production period. Several groups have looked into the effects of such a vaccine in food animals of various sorts. A study by (12) showed that *in ovo* administration of a Myostatin antagonist significantly increased the weights of muscles, particularly the breast muscles of female birds, which were approximately 14% larger

than those of control females. This technique could most likely be applied to other species as well, giving producers control over the timing of GDF-8 presence.

Ongoing growth and development studies of GDF-8 knockout models will provide new and more effective strategies of feeding and management to support the increased growth. Inquiries into the phenomenon of pleurotrophic growth, which allow growth-enhancing mutations like the GDF-8 knockout to be combined with complementary transgene-stimulated overexpression of growth hormone, or single-gene knockout models of hyperphagia could eventually produce animals with larger cells in the hyperplastic tissue, or animals in which a higher nutrient availability could accelerate growth. Additive effects in these areas could mean production of an animal with even more exceptional growth characteristics.

These and many more challenges still lie ahead, but with the advent of greater understanding in the areas of tissue development, cell cycle control, and the importance of single-gene knockout models of growth, we can apply these new discoveries to animal agricultural production. The technology available may make it possible to choose the desirable characteristics of these animals without retaining the unwanted side-effects so often in these animals, putting a whole new spin on the nearly two-centuries-long quest to exploit the nature of the double-muscled animal. Tied to a process of great importance to the livestock industry, new understanding of this single-gene regulator of muscle development represents a milestone in modern livestock improvement.

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APPENDIX B

MYOSTATIN IN REVIEW[1]

[1] Arnold, H.A. 2000. Submitted to Feedstuffs 11/00.

When McPherron and Lee announced in 1997 that they had found the gene responsible for double-muscling in cattle, scientific communities and beef producers alike rushed to get news of the action. Hundreds of articles expanded or explained the discovery, and thousands of fevered imaginations went to work, once again, to find ways of exploiting this dramatically effective (and presumably lucrative) "new" gene.

This is not a new gene.

In his 1804 treatise, George Culley described a breed of cattle well known to pockets of Europe: a breed imported from Holland that one could "feed to a vast weight...yet will not have one pound of fat about it, neither within nor without." These cattle were called "double-lyered", or "black-fleshed", and were said to look more like draft horses than cattle in their extreme musculature.

Throughout the nineteenth century, instances of the condition in the shorthorn herds of Europe were met with either distinct interest or instant disgust, depending on the market climate and the favored production system of the region. In Belgium, market influences led producers within the country to gradually shift to selection that favored the round-rumped and lean double-muscled animals over the former milk-oriented makeup of the Belgian Blue (or Belgian Blue White) breed. Meanwhile, in England, a long and heated debate followed the suggestion that a few superior shorthorn bulls might be "tainted" with the double-muscled influence.

The variation has been around so long, one might wonder just what all the fuss is about, but new livestock applications for this well-traveled mutation are more than just a new label on an old product. Since the discovery and subsequent patenting of this gene, (called GDF-8 or Myostatin™) by Dr. Se-Jin Lee, founder of Metamorphix, Inc., the gene

has risen to the top of the pile in biotech research. It's a gene that controls growth. It's specific to muscle. Breeds that lack functional GDF-8 produce 20% more muscle - an increase that doesn't compromise the taste or quality of the meat.

This appeals to the steak lovers among us.

So if the meat is so desirable, if the cuts are so large, if the investment is so good, then why would anyone willingly throw the incendiary issue of bioengineering into the mix? The answer is twofold.

First of all, when Culley described the cattle as "black fleshed", he wasn't just being poetic. In cattle that are stressed before slaughter, individuals often become known as "dark cutters" when reduced muscle glycogen and high pH cause the meat to hold water, making it appear dark in color, instead of the desirable cherry-red. Double-muscled animals, which are more susceptible to stress even under today's production conditions, were sadly out of their element in a world that required hardy cattle for milk, meat, and labor. Even with more cautious handling, Double-muscled calves today can be more susceptible to disease and extreme conditions.

Double-muscled cattle are also notorious for reproductive problems. Females are generally slow coming into puberty, and the rate of reproductive failure is very high. To top it off, calves, which begin showing the increased muscle growth caused by a GDF-8 mutation at around day 16 of gestation, are generally large, which leads to difficult births. At a 1980 conference on the use of double-muscled animals in commercial beef systems, Dr. B. Vissac of France suggested that in some breeds, as many as 60 to -70% of double-muscled calves must be delivered by cesarean section. When the calves are successfully delivered, producers still often have to face the problems of poor mothering and poor

milking in the cows. With calves that tend to be somewhat less vigorous at birth, this can add up to a higher death loss, or at the very least, a drastic increase in the cost of care and labor.

With these challenges in mind and armed with over a century's worth of studies, including Alexandra McPherron and Se-Jin Lee's 1997 discovery and characterization of GDF-8, biotechnologists are already working to get around the drawbacks of the double-muscled condition -- and not just in beef production.

GDF-8 is highly conserved between species as diverse as baboons and zebrafish, and in several livestock species, including cattle, pigs, chickens, and turkeys, the functional end of the GDF-8 protein is identical. Moreover, GDF-8 seems to have similar biological effects, at least in cattle and mice. Loss of function results in the overgrown musculature so familiar to those studying double-muscling: bulging shoulders, hips, and jowls, and an overall weight increase.

This similarity could boost recent efforts to screen for mutations in GDF-8, in the hopes of identifying herculean specimens in the poultry, swine, or fish industries. Lines of double-muscled livestock could be identified and established in breeding programs. This opportunity to find useful variations already present in breeding animals could allow producers to take full advantage of the desirable mutations within their stock, while providing researchers with a vast library of material to screen for other interesting and potentially valuable genes.

Because GDF-8 is a secreted protein that acts on muscle cells, it may be possible to develop a GDF-8 "vaccine" that will immunize a normal animal against the protein already in its system, making it unresponsive to the normal slowing of muscle growth. In

effect, producers may be able to "turn off" the mechanism in newborn and growing calves, pigs, or other livestock, bypassing the reproductive difficulties historically associated with nonfunctional GDF-8 in cattle, while encouraging the extreme muscle development that develops in the absence of GDF-8.

Transgenic technology may also provide a new way to exploit GDF-8. By comparing the proteins in different species, or between breeds within a species, researchers may eventually be able to separate some of the valuable growth effects of GDF-8 mutations from the production difficulties that have limited its use. In beef cattle alone, researchers in Belgium described seven different versions of the GDF-8 gene, five of which can cripple GDF-8 function, resulting in a double-muscled animal. With the aid of transgenic and cloning technology, it may be possible to balance the exceptional fetal growth of GDF-8 mutants with better milking and mothering traits, improved calving or farrowing ease, better marbling, and improved neonatal vigor common to other breeds. New answers to old problems, as well as changing market conditions that put high value on lean, high-quality meat, may allow more producers to take advantage of the double-muscled animal's high yield. As biotechnology becomes a stronger presence in the production of food animals, more and more opportunities will arise for researchers and producers to work together to build better food.