University of Alberta

CONSEQUENCES OF UTERINE CROWDING DURING EARLY GESTATION ON MYOGENESIS IN THE PIG

by

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ABSTRACT

In a litter-bearing species such as the pig, 'crowding' *in utero* due to high numbers of conceptuses has been shown to adversely affect muscle fiber numbers of the resulting piglets. Unilateral oviduct-ligation surgery was used in the current study to generate conceptuses arising from relatively crowded (CTR) or uncrowded (LIG) uterine environments for comparison of myogenic events. Analysis of the myogenic regulatory factors (MRFs) *MyoD*, and *myogenin*, was performed on day 30 conceptuses from each group. *Myogenin* expression was significantly lower in conceptuses from CTR sows, while *MyoD* expression was unaffected. These differences in *myogenin* expression were only seen to be significant in male conceptuses. *In* situ hybridization techniques were developed to examine spatiotemporal differences in expression of MRFs in the two groups of conceptuses. Although MRF expression could be detected, this technique was not sufficiently sensitive to reveal differences in MRF expression between the two groups. Combined, these studies indicate that uterine crowding affects early myogenesis (day 30), and may have the most pronounced effects on male conceptuses.

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ABBREVIATIONS

ATP	adenosine tri-phosphate
BCIP	5-bromo,4-chloro,3-indolylphosphate
bHLH	beta helix-loop-helix
BMP	bone morphogenic protein
c-Met	c-Met gene or protein
C_T	cycle threshold
CTR	control
DEPC	diethylpyrocarbonate
DML	dorsal-medial lip
Dsh	dishevelled gene or protein
EGTA	Ethylenebis(oxyethylenenitrilo)tetraacetic acid
GH	growth hormone
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLH	helix-loop-helix
ICM	inner cell mass
Id	Id gene or protein
IGF	insulin-like growth factor
IUGR	intrauterine growth-retardation
Lbx1	Lbx1 gene or protein
LIG	ligated
Mef2c	Mef2c gene
MGB	major groove binding
MRF	myogenic regulatory factor
MRF4	MRF4 gene or protein
MRF4	MRF4 mRNA transcript
mRNA	messenger ribonucleic acid
MHC	myosin heavy chain
Myf-5	Myf-5 gene or protein
Myf-5	Myf-5 mRNA transcript
MyoD	MyoD gene or protein
MyoD	MyoD mRNA transcript
NBT	nitroblue tetrazolium
Pax3	Pax3 gene or protein
PBS	phosphate buffered saline
p.c.	*post coitum*
PCR	polymerase chainreaction
PGF2α	prostaglandin factor 2α
pGH	porcine growth hormone
PIPES	piperazine-1,4-bis(2-ethanesulfonic acid)
PSM	presomitic mesoderm
pST	porcine somatotropin
Ptc	patched recpetor gene or protein
rRNA	ribosomal ribonucleic acid

RT-PCR	real-time polymerase chain reaction
Tris-HCl	Tris hydrochloric acid
SDS	sodium dodecyl sulphate
Shh	sonic hedgehog gene or protein
Smo	smoothened gene or protein
SRY	Y chromosome specific region
SSC	sodium chloride-sodium citrate
Tcf	T-cell factor
VLL	ventro-lateral lip
Wnt-1	Wnt-1 gene or protein
Wnt-3	Wnt3 gene or protein
Wnt-7a	Wnt-7a gene or protein
ZFX	X chromosome specific region

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CHAPTER ONE

1.1. INTRODUCTION

The potential for muscle mass is primarily determined by the size and number of muscle fibers formed. In the pig, postnatal growth of muscle occurs through the increase in size of muscle fibers rather than number (hypertrophy versus hyperplasia). The formation of muscle fibers is complete prior to birth and, once established, cannot be altered during postnatal development. Therefore, factors impacting the determination of muscle fibers numbers during gestation are of critical importance.

Myogenesis in the pig follows a biphasic pattern of development. An initial wave of primary muscle fibers is formed, and is followed by a second wave of secondary fiber formation that uses primary fibers as a framework (Wigmore and Stickland, 1983 and references therein). A group of transcription factors, called the myogenic regulatory factors (MRFs), play an integral role during myogenesis (reviewed by Arnold and Braun 2000). The MRFs; Myf-5, MyoD, myogenin, and MRF4 act together, along with several other genes, in a tightly coordinated pattern of development to ensure correct spatiotemporal development of muscle.

Secondary muscle fibers make up the bulk of the total number of muscle fibers formed, and it is generally accepted that by day 90 of gestation in pigs, the formation of secondary muscle fibers is complete. Studies into the effects of maternal nutrition

(Dwyer et al., 1994) and the administration of porcine somatotropin (Rehfeldt et al. 1993) suggest that the determination of secondary muscle fiber numbers occurs during a critical time period prior to day 50, well before the onset of actual secondary fiber formation.

In addition to maternal nutrition and levels of porcine somatotropin, several other factors, including litter size, have been implicated in the determination of muscle fiber numbers. Using unilateral oviduct-ligation to limit the number of embryos *in utero*, an examination of muscle cellularity in fetuses at day 90 of gestation indicated that relatively higher states of uterine crowding resulted in a reduction in the total number of secondary muscle fibers formed (Town et al., 2004).

Additional observations by Town et al. (2004) at day 30 of gestation revealed that average placental mass was also reduced in sows whose uteri were relatively more crowded with embryos. Studies into the effect of maternal undernutrition of guinea pigs showed a decrease in the number of muscle fibers formed (Dwyer et al., 1995), and this has been suggested to be due to a decrease in placental size. Thus, the effect of uterine crowding on placental weight observed by Town et al. (2004) during the critical period of determination of muscle fiber numbers may influence, in a manner similar to that of undernutrition, the number of secondary muscle fibers formed.

Town et al. (2004) also examined embryo weight at both day 30 and day 90 of gestation. Although no difference was seen in embryo weights at day 30 of gestation,

day 90 fetuses from relatively crowded uterine environments were lighter than fetuses from a relatively non-crowded uterus. Based on an examination of intrauterine growth-retarded (IUGR) piglets, as compared with the largest normal littermates, Wigmore and Stickland (1983) reported that IUGR piglets exhibited lower numbers of total fiber numbers than large littermates. This was manifested through a reduction in the formation of secondary muscle fibers. These authors also noted that IUGR piglets exhibited signs of prenatal malnutrition and, therefore, a natural state of undernutrition *in utero* may have been responsible for the observed effects on muscle fibers. Handel and Stickland (1987) further observed that low birth weight was associated with lower total fiber numbers but low fiber numbers were not necessarily associated with low birth weight. Based on these studies combined, it appeared therefore that the weight of a embryo may be an indicator of potential muscle mass.

Whether or not gender plays a role in the effects of uterine crowding on muscle fiber formation has not been studied, but it is also possible that embryo sex has an effect. A study at day 70 of gestation indicated that the weight of male fetuses and placentae were heavier than their female counterparts (Wise and Christenson, 1992). In the same study, it was observed that female fetuses that were located in the uterus at sites between two members of the opposite sex were lighter than other fetuses, suggesting possible influences of gender-specific effects of one embryo on the development of another. The authors further suggested that endocrine differences during day 20 to 40 of gestation might result in the effects seen during the later stages of gestation.

In light of the evidence provided by the work of Town et al. (2004) on the effects of uterine crowding on secondary muscle fiber formation, a major objective of the research reported in this thesis was to determine whether evidence for a mechanism existed which might already determine the potential for muscle fiber development at day 30 of gestation. A quantitative examination of the expression of selected MRFs at day 30 of gestation was, therefore, carried out. Chapter two presents a literature review of myogenesis, and possible factors that influence the determination of muscle fiber numbers.

Chapter three addresses the possible influence of uterine crowding on myogenesis at day 30 of gestation. The expression levels of the MRFs MyoD, a gene involved in the determination of pluripotent precursor cells into myoblasts committed to the myogenic lineage, and myogenin, a gene responsible for the initiation of terminal differentiation of myoblasts into muscle cells, were quantified using real-time RT-PCR.

Chapter four addresses the possible role of gender in the development of muscle against a background of varying states of uterine crowding. PCR was used to amplify sex-specific genes to identify the gender of individual day 30 embryos. After the identification of sex, data gathered in chapter three was reanalyzed with respect to the sex of the embryos.

Any deficits in the expression levels of MRFs may have been due to active down-regulation, or to a delay in the onset and subsequent rise in MRF expression. Chapter 5 attempted to address this issue using *in situ* hybridization. Since myogenesis occurs in a tightly coordinated pattern of expression, it was hoped that *in situ* analysis might reveal differences in the timing of the onset of expression.

A summary of the results, and potential implications of the evidence provided by chapters three through five are discussed in the final chapter.

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CHAPTER TWO

2. LITERATURE REVIEW

In the commercial production of a meat-producing animal such as the pig, increasing or decreasing the potential for the development of muscle mass will have important economic consequences. Litter size is also an important economic component of pig production, as increasing the size of a litter can be beneficial in terms of increased reproductive efficiency. However, in a study by Town et al. (2004), increases in the numbers of embryos *in utero* were found to be detrimental to muscle development. Therefore, breeding strategies designed to increase the size of a litter may have undesirable consequences for the potential development of muscle mass in individual offspring.

The development of management strategies to minimize the effects of uterine crowding on muscle development would be valuable. A first step towards this goal would be to increase our knowledge of the mechanisms by which uterine crowding may be compromising muscle development. The purpose of this chapter is to review relevant information that may be useful in identifying such mechanisms.

2.1 EMBRYO DEVELOPMENT

Stroband and Van der Lende (1990) provide an excellent review of preimplantation development of the porcine embryo and this will be briefly summarized here (Figure

2.1.). Approximately 2-3 after fertilization, embryos reach the 2-4 cell stage and begin their entry into the uterus. Around day 4 of gestation embryos reach the 8-16 cell morula stage. It is around the 12-16 cell stage that the differentiation of inner and outer cells occur (blastulation). Within the morula, a fluid filled cavity called the blastocoele forms, which rapidly enlarges until the embryo becomes a hollow sphere termed the blastocyst. The blastocyst is composed of two cellular structures: the inner cell mass (ICM) and the trophoblast. The ICM gives rise to the embryo, while the trophoblast forms the placenta. At day 6-7 hatching from the zona pelucida occurs and the blastocyst remains free in the uterine lumen until day 13 (Stroband and Van der Lende, 1990).

Between hatching and day 11, a rapid expansion of the blastocyst occurs (Stroband and Van der Lende, 1990). It is also around this time that the embryo begins its utilization of uterine substances for growth. Polar trophoblast, or Rauber cells, cover the embryoblast until day 10 of gestation when these cells are shed. Afterwards, from day 11 to 12 onwards, the blastocysts begin elongating and reducing their diameter, resulting in a filamentous structure up to 100 cm in length. At around day 13-14, attachment of the embryos to the uterus begins. By day 18 this process is complete, resulting in an intermingling of uterine and trophoblastic microvilli (Stroband and Van der Lende, 1990).

The development *in utero* of embryos continues until birth at approximately day 114 of pregnancy. Gestation involves the overall development of all organ systems within

the embryo. However, in the following sections, only an in-depth review of skeletal muscle development will be made.

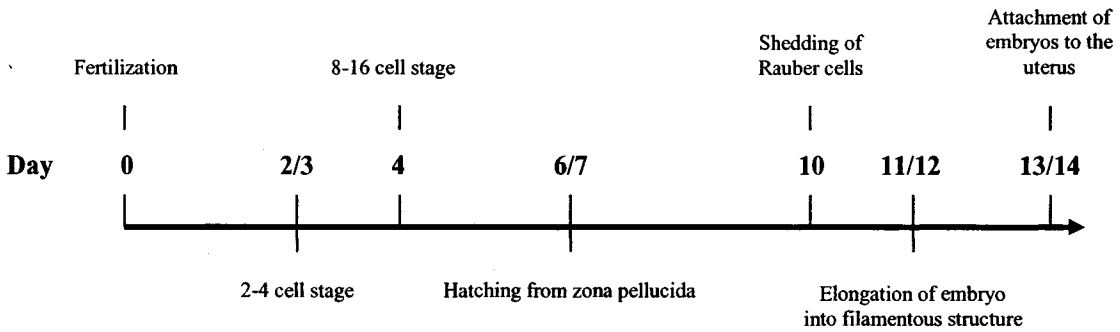


Figure 2.1. Timeline summary of the early gestational events of the porcine embryo.

2.2 EMBRYONIC MUSCLE DEVELOPMENT

Muscle fiber formation in the pig follows a biphasic pattern of development during gestation (Wigmore and Stickland, 1983 and references therein). A population of primary muscle fibers is formed first, and is followed by a wave of secondary muscle fiber formation. The initial population of primary fibers provides a 'scaffold' that is used by secondary muscle fibers as a framework for their subsequent establishment, growth, and development. In the pig, primary fiber formation occurs between day 35 and 60 of gestation. Secondary muscle fibers formation begins at approximately day 55 of gestation, and is complete by day 90 to 95 (summarized in Figure 2.2.).

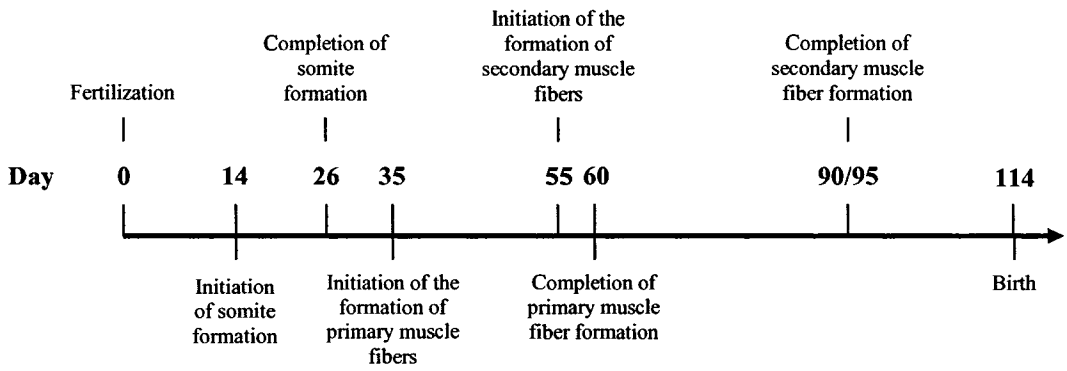


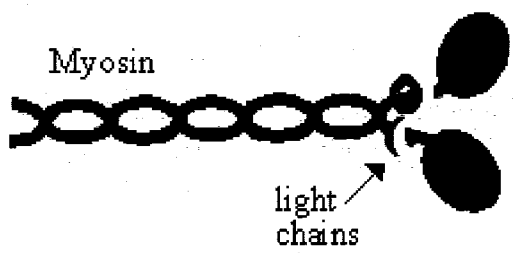
Figure 2.2. Timeline summary muscle development of embryos during gestation.

Muscle fibers are the primary determinant of muscle mass (discussed in section 2.4). The formation of muscle fibers is complete by around day 90 of gestation (Wigmore and Stickland, 1983), and once completed, the number of muscle fibers formed cannot be changed during postnatal development. Since muscle fiber formation is completed prior to birth, gestation is a critical time period in the determination of potential muscle mass of offspring. Although the development of muscle fibers continues until day 90 of gestation, there is evidence to suggest that the total number of muscle fibers that an offspring will attain is established prior to day 50 of gestation (discussed in section 2.5). An evaluation of the potential factors influencing muscle fiber formation may be useful in understanding the development of muscle in the pig.

2.2.1 Muscle Composition

The primary component of muscle is myosin (reviewed in Wigmore and Evans, 2002). Myosin is an asymmetric hexameric protein consisting of two myosin heavy chains (MHC), with two myosin light chains associated with each heavy chain

(Figure 2.3.). ATP and actin binding domains reside within a globular head region of MHCs, and the interaction of the MHCs and actin act in the hydrolysis of ATP. Different MHCs have differing rates of ATP hydrolysis and thus, determine the speed of contraction of a muscle fiber. There is a good correlation between muscle fiber characteristics and the isoform of MHC expressed and therefore, MHC isoforms are most commonly used in classifying muscle fiber types. In mammals there are four predominant isoforms (one slow and three fast) of MHC. Muscle fibers can be formed by either a single type of MHC, or by hybrids of two or more MHCs. In very broad terms primary fibers are classified by being a slow type fiber, while secondary fibers can be classified during the gestational period (Wigmore and Evans, 2002).



Adapted from Alberts et. al., 1994

Figure 2.3. Myosin protein. Two myosin heavy chains (red and blue). Two myosin light chains (yellow and green) are associated with each heavy chain. The isoform of the heavy chains determines the contractile properties of the myosin protein.

2.2.2 Somite Development

Most skeletal muscles in vertebrate muscle arise from structures known as somites (reviewed by Christ and Ordahl, 1995). In the pig, somite development begins at the rostral end of the embryo at around day 14 of gestation, and the last somite is formed

at the caudal end at approximately day 26 of gestation (summarized in Figure 2.4.). The presomitic mesoderm (PSM) is a subpopulation of mesoderm that is formed on either side of the neural tube, and somites are transient segments of the PSM that form sequentially in a rostral-to-caudal direction (Figure 2.4a.). Somites then differentiate into two compartments along a dorsal-ventral axis (Figure 2.4b.). The dorsal compartment forms the epithelial dermomyotome and will ultimately be responsible for the formation of the dermis, and the skeletal muscles of the trunk and limbs. The ventral compartment forms the sclerotome and will develop into the bones and ribs. Because differentiation of somites occurs along the dorsal axis of the embryo in a rostral-to-caudal direction, this results in somites at different stages of development present at the same time, with somites located towards the anterior end being more developmentally advanced than somites located towards the posterior end (Christ and Ordahl, 1995).

Cells in the dermomyotome remain in an undifferentiated and proliferative state. As somitogenesis progresses the dermomyotome begins both a dorsomedial and ventrolateral invagination, forming both a dorsomedial lip (DML) and a ventrolateral lip (VLL) respectively (Figure 2.4c and 2.4d). These lips form a third layer in the somite, called the myotome. The DML provides cells to form the epaxial myotome, which will form the muscles of the back. The VLL forms the hypaxial myotome that will provide muscles of the body wall. The VLL of somites at the level of limb buds also provides cells that delaminate and migrate to form the musculature of the limbs (Figure 2.4e).

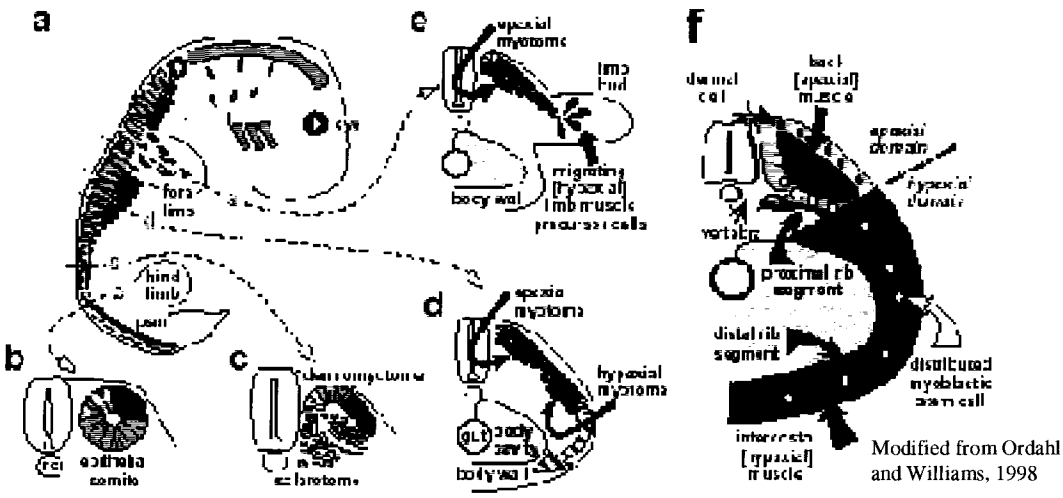


Figure 2.4. Summary of muscle development in the embryo. (a) Sagittal view of embryo showing the elongation of the presomitic mesoderm, and the formation of somites in rostral-caudal direction. (b-d) Cross sections of somites of differing levels of maturity with (b) being the least developed and (d) being the most developed. (c) cells from the dermomyotome delaminate to form the myotome. (d) Cells of the epaxial and hypaxial myotomes form the muscle of the back and of the body respectively (shown in f). (e) The development a somite at the level of the limb. Cells from the hypaxial dermomyotome delaminate to form the muscle of the limbs (adapted from Ordahl and Williams, 1998).

2.3 REGULATION OF MUSCLE DEVELOPMENT

2.3.1. Myogenic Regulatory factors (MRFSs)

During the development of somites, a highly coordinated pattern of gene expression occurs to initiate myogenesis. Although several genes are involved in the development of muscle, the myogenic regulatory factors (MRFs) have been the most thoroughly described. A great deal of literature on the functions of MRFs exists and some excellent reviews include those by Arnold and Braun (2000), Tajbakhsh and Buckingham (2000), and Parker et al. (2003). The MRFs consist of four genes, Myf-5 (myogenic factor 5), MyoD, myogenin, and MRF4 (myogenic regulatory factor 4) that have been identified in vertebrates by their ability to convert various non-muscle

cell types into muscle. MRFs are part of a superfamily of basic helix-loop-helix (HLH) transcription factors. The HLH is a structural domain of a given protein that mediates binding with other HLH-containing proteins. Each MRF protein contains a conserved DNA-binding domain that binds to a DNA binding motif known as the E-box, containing the sequence CANNTG. These E-boxes are found in the promoters of several muscle-specific genes, and are regulated by binding of MRFs. MRFs are also able to dimerize with the E-protein family of transcription factors. These heterodimers also bind to E-box motifs to regulate the transcription of genes involved in terminal differentiation.

2.3.2. Spatial and temporal expression of MRFs

In vivo studies using *in situ* hybridization in the mouse have shown distinct temporal patterns of expression of each MRF gene (genes in italics refer to mRNA transcripts; non-italicized genes refer to either the gene or the protein). Expression of each gene is first seen in the dermomyotomes of the rostral somites and progresses successively in somites in a rostral-caudal direction. Myf-5 is the first MRF to be expressed in the rostral somites at 8.5 days *post coitum* (*p.c.*), before formation of the dermomyotome and the sclerotome. There is a gradual down-regulation of expression of *Myf-5* mRNA in the rostral somites at 11.5 days *p.c.* and transcripts are last seen in the caudal somites at 13.5 days *p.c.* (Ott et al., 1991). *Myogenin* transcripts are also first detected in the rostral somites at 8.5 days *p.c.* while *MyoD* is first detected at 10.5 days *p.c.* After initial expression, both *myogenin* and *MyoD* are expressed throughout

prenatal development (Sassoon et al., 1989). The MRF4 gene shows a biphasic pattern of expression. In the most rostral somites, MRF4 is first expressed at 9 days *p.c.* and expression in the somites disappears by 12 days *p.c.* It then is expressed again at 16 days *p.c.* (Bober et al., 1991) and maintains a high level of expression thereafter, becoming the predominantly expressed gene in all pre- and postnatal muscle (Hinterberger et al., 1991).

The timing of MRF expression in the limb differs from the timing of expression in the somites. Cells that delaminate from the dermomyotome do not express MRFs but remain in an undifferentiated state until their arrival at proper locations in the limb. In this location, expression of *Myf-5* begins at 10.5 days *p.c.* (Ott et al., 1991) and is followed by expression of *MyoD* and *myogenin* at 11 days *p.c.* (Sassoon et al., 1989). Unlike expression in the somites, *MRF4* does not show a biphasic pattern of expression in the limbs, and is not expressed until after the onset of expression of the other MRFs (Bober et al., 1991; Hinterberger et al., 1991).

2.3.3. MRF function

The classification of MRFs by their ability to convert non-muscle cells towards the myogenic lineage, suggests a functional redundancy of each gene (reviewed by Chanoine et al., 2004). Studies of the binding site specificity of MyoD and myogenin have not identified any unique features of either MRF that might determine specific binding to a given E-box, supporting the idea of functional redundancy. However,

observations in the mouse, of distinctive windows of MRF expression, suggested a more unique function for each MRF. Studies that have used targeted inactivation of each MRF have helped elucidate their individual roles.

Knock-outs of the Myf-5 gene are lethal in the perinatal period (Braun et al., 1992). The development of the ribs is severely truncated, and death results from the inability of mutant mice to breathe due to the lack of a functional ribcage. However, skeletal muscles are morphologically normal, and have normal expression of muscle-specific gene markers, including the other MRFs.

In MyoD knock-outs there is even less evidence of effects due to the lack of *MyoD* expression, as mice are born alive and no alterations in the development of muscle is apparent (Rudnicki et al., 1992). Expression of *myogenin* and *MRF4* is also is normal. However, the expression of *Myf-5* is drastically increased, suggesting that the elevated levels of *Myf-5* may compensate for the lack of *MyoD*. Furthermore, although no visible phenotype is apparent at birth, MyoD mutant mice are deficient in muscle regeneration as adults (Megeney et al., 1996).

When double knock-out mice were generated that lacked both Myf-5 and MyoD, there was a complete absence of any muscle development (Rudnicki et al. 1993). In addition, no expression of muscle-specific mRNAs could be observed, indicating that there was a complete lack of myoblast formation. Thus, the expression of *Myf-5* or *MyoD* appears to be essential to the establishment of cells towards a myogenic

lineage. Furthermore, this study showed that the presence of at least one of these genes, MyoD or Myf-5, was essential for proper muscle development.

Inactivation of the myogenin locus in mice resulted in death shortly after birth. Unlike mice lacking in *Myf-5*, or mice that were deficient in both *Myf-5* and *MyoD*, myogenin-null mice showed a severe deficiency in the development of differentiated muscle fibers (Hasty et al., 1993; Nabeshima et al., 1993). In myogenin-null mice however, there was development of normal numbers of undifferentiated myoblasts that were capable of differentiation *in vitro*, and which expressed normal amounts of MyoD (Nabeshima et al., 1993). Due to the formation of viable myoblasts in myogenin-null mice, but lack thereof in MyoD/Myf-5 double knockout mice, it was concluded that myogenin plays a role in myogenesis downstream of Myf-5 and MyoD. Double homozygous null mutants lacking Myf-5 and myogenin, MyoD and myogenin (Rawls et al., 1995), and myogenin and MRF4 (Rawls et al., 1998) are all phenotypically similar to myogenin-null mice. This further supports the conclusion that myogenin acts downstream of both Myf-5 and MyoD.

Knock-in mice that express myogenin under the control of the Myf-5 locus were shown to be able to rescue the rib defects exhibited by Myf-5 null mutants (Wang et al., 1996). This study showed that there is an overlap in the functional capabilities of myogenin and Myf-5. When these mice were crossed into a MyoD-null or a myogenin-null mutant however, there was no rescue of muscle formation (Wang and Jaenisch, 1997). Therefore, *myogenin* expression from the Myf-5 locus cannot

completely replace the function of Myf-5. Wang and Jaenisch (1997) also proposed that the total level of MRFs rather than specific roles of MRFs may be critical in myogenesis, and the role of Myf-5 and MyoD are to promote the elevation of *myogenin*, thereby promoting terminal differentiation.

The initiation of differentiation by myogenin is a highly ordered sequence of separable events (Andrès and Walsh, 1996). Cells that begin to express *myogenin* initially maintain their ability to replicate DNA. This initial period ends with the expression of p21 that signifies withdrawal from the cell cycle, and ultimately the phenotypic differentiation and cell fusion.

Vivian et al. (1999) investigated the quantitative requirements of myogenin in skeletal muscle development. Using a hypomorphic allele of myogenin that expressed approximately one-fourth the level of a normal allele, the authors generated a series of mutant mice expressing varying levels of *myogenin*. At *myogenin* expression levels less than one-half of normal, neonatal viability was compromised, developmental defects in the ribs increased, and myofibers decreased. At one-half expression or above, neonates appeared to be normal. Although no specific muscle fiber count was performed, myofiber numbers did not appear to be affected. From these results the authors concluded that muscle fiber development was sensitive to absolute levels of myogenin.

Interestingly, upon further investigation of myogenin-deficient mice, it was concluded that myogenin played a more important role in the second wave of myogenesis, rather than in the first (Venuti et al., 1995). Development of myogenin-deficient mice exhibited similar myogenic developmental patterns with wild-type mice from between 9.5 days *p.c.* and 15.5 days *p.c.*, during the initial wave of myogenesis (occurring between 10.5 days *p.c.* and 14.5 days *p.c.*), and any differences occured from 15.5 days until birth. The authors further suggested that lack of myogenin had a greater impact on the development of secondary fibers than primary fibers. However, in myogenin-mutant mice, expression levels of muscle-specific gene products showed a significant reduction as compared to wild-type mice. This suggested that although differentiation does occur in the absence of myogenin, the differentiation of myoblasts into pre-muscle masses was delayed in mutant embryos. This is consistent with an earlier study that showed myogenin-independent differentiation of primordial myoblasts (Cusella-De Angelis et al., 1992). In this study it was observed that myogenin protein expression did not coincide with the expression of the transcript, yet differentiated myoblasts could still be observed. Cells that were cultured from the first myogenic cells in the myotome expressed muscle structural proteins without expressing any detectable levels of myogenin protein (even though the transcript was expressed), suggesting occurrence of post-transcriptional regulation of myogenin.

Studies addressing the inactivation of the MRF4 gene have shown variable consequences for postnatal viability. However, in all cases mutant mice showed

apparently normal muscle formation (Braun and Arnold 1995; Patapoutian et al., 1995; Zhang et al., 1995). In addition, the absence of normal *MRF4* expression appears to lead to an upregulation of *myogenin* expression (Zhang et al., 1995). These observations tentatively led to the hypothesis that MRF4 shares some functional redundancy with myogenin, and that it acts downstream of Myf-5 and MyoD, and works with myogenin to initiate terminal differentiation. However, a very recent study suggests that MRF4 is in fact a determination gene that acts with Myf-5 upstream of MyoD to initiate myogenic determination (Kassar-Duchossoy et al., 2004).

Although Myf-5 and MyoD appear to share some functional redundancy, they are responsible for distinct regions of muscle development. A study involving a lacZ-transgene under a MyoD promoter suggested that epaxial muscle development was dependent on *Myf-5* expression, while hypaxial muscle development depended on *MyoD* expression (Braun and Arnold, 1996; Kablar et al., 1997; 1998). Further evidence suggests that two distinct pathways independently give rise to either a *Myf-5*- or *MyoD*- expressing cell line, rather than a single cell line capable expressing both genes (Kablar et al., 2003).

There is also evidence to suggest that lineages of myoblasts that form primary and secondary fibers are predetermined early in gestation. Studies of myoblasts from limbs in avian models (see Stockdale, 1992) showed that early embryonic myoblasts in culture could form cells that expressed either fast or slow MHC, whereas cells

cultured from later stages of development expressed predominantly fast MHC. The broad classifications of primary and secondary fibers as slow and fast fibers respectively, suggest that myoblasts that form secondary fibers are present at early stages of development. A study of muscle cells from the human limb also indicated the presence of cells early on in development that express differentiation markers, including myogenin, that may be involved in the formation of secondary fibers later in gestation (Edom-Vovard et al., 1999).

2.3.4. Regulation of MRFs

In addition to the direct role of MRFs, the overall regulation of myogenesis has been studied extensively, and involves a complex network of genes and signaling pathways to control differentiation into myofibers. In order to begin differentiation a myoblast must exit the cell cycle, which has consequences of in itself (discussed by Füchtbauer, 2002). Adequate numbers of undifferentiated cells must be maintained prior to migration to limbs, for use in the subsequent waves of muscle fiber formation, and to provide a pool of cells for growth and repair of injuries to muscle later in life. Because the pool of undifferentiated cells is determined early on, tight control of gene expression is necessary to maintain an intricate balance between proliferation and differentiation in order to ensure proper muscle development. Several recent reviews (including Füchtbauer, 2002; Parker et al., 2003) describe the role and mechanism of action of many genes and a few of these will be briefly summarized here.

Three major signaling pathways have been described that regulate the expression of *Myf-5* (Figure 2.5a). The first pathway involves the gene sonic hedgehog (Shh), which binds to the receptor Patched (Ptc). This de-represses Smoothened (Smo), which activates the Gli family of transcription factors and induces *Myf-5* expression (Parker et al., 2003). A second pathway also induces *Myf-5* expression but, in this case, through the binding of Wnt-1 to its receptor Frizzled (Fzd). Binding activates the gene Dishevelled (Dsh), which in turn inhibits the gene GSK-3β (glycogen synthase kinase 3 beta). This allows the protein β-catenin to be translocated to the nucleus, in association with T-cell factor (Tcf), to activate Myf-5 (Parker et al., 2003). A third pathway involves the Bone Morphogenic Proteins (BMPs). BMPs bind to surface receptors that phosphorylate proteins called R-SMADs. This event allows R-SMAD, in association with Smad 4, to translocate to the nucleus. Unlike Shh and Wnt-1, BMP binding acts to inhibit *Myf-5* expression, and has been suggested to function in the establishment of adequate numbers of myogenic progenitor cells. The gene noggin is also an inducer of *Myf-5* expression, by acting through the antagonism of BMPs (Parker et al., 2003).

Three major pathways have also been described for the regulation of MyoD (Figure 2.5b). Shh does not appear to influence *MyoD* expression, but β-catenin/Tcf and the R-SMAD pathways described above act in a similar fashion on the expression of *MyoD*. In the case of the β-catenin/Tcf pathway, Wnt-3a rather than Wnt-1 binds to the Fzd receptor. The third pathway described involves Wnt-7a binding to a Fzd

receptor. However, the mechanims by which Wnt-7a binding acts have not been fully elucidated (Parker et al., 2003).

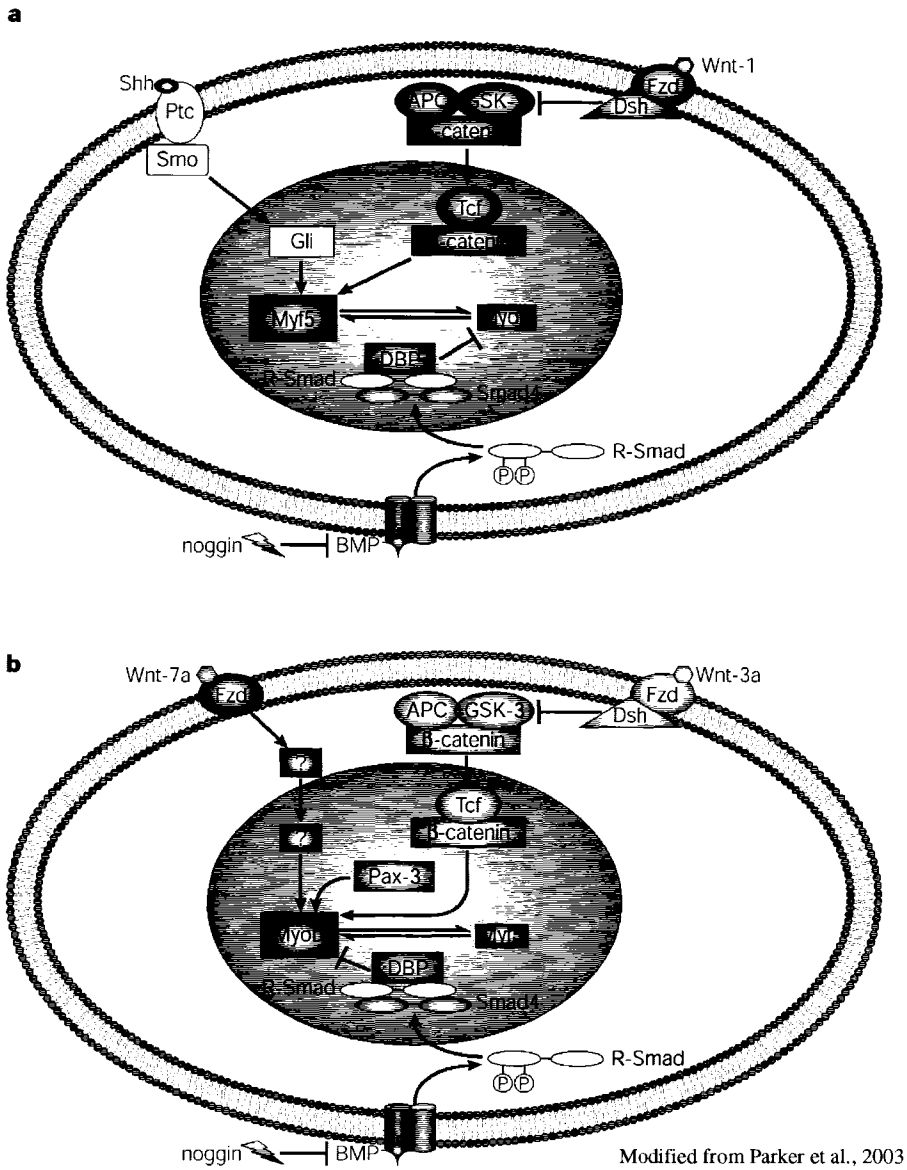


Figure 2.5. Overview of signaling pathways involved in the regulation of the MRFs Myf-5 (a) and MyoD (b). Myf-5 (a) is activated by binding of sonic hedgehog (Shh) to the receptor Patched (Ptc), and inducing Myf-5 through the Gli family of transcription factors. Myf-5 is also induced through a β-catenin/Tcf pathway that is activated by the binding of Wnt-1 to the receptor Frizzled (Fzd). Bone morphogenic proteins (BMP) act through a R-SMAD/Smad 4 pathway that binds to DNA-binding proteins (DBP) to inhibit Myf-5 expression. Noggin acts to induce Myf-5 expression by inhibiting the action of BMP. MyoD (b) is activated through a β-catenin/Tcf pathway from the binding of Wnt-3a to the Fzd receptor. Wnt-7a also binds to Fzd to induce MyoD expression. However, the precise mechanism is unknown. Noggin also acts to inhibit the action of BMP to induce expression of MyoD. MyoD expression is also induced by expression of Pax-3.

In addition to the pathways described above, several other individual genes that have been implicated in myogenesis. The paired-domain transcription factor Pax3 also plays a role in the regulation on *MyoD* expression, and is, in fact necessary for myogenesis (Parker et al., 2003). However, the mode of action of Pax3 is not entirely clear, although it is believed to act upstream of MyoD. On its own, Pax3 is a poor transcriptional activator and may even act to repress transcription. It has been suggested that Pax3 may need transcriptional co-activators for proper function. Furthermore Pax3, along with other genes, such as c-Met and Lbx1, have also been shown to be responsible for delamination and subsequent proper migration of muscle precursor cells to the limbs (Parker et al., 2003).

Id genes encode helix-loop-helix proteins without a basic DNA-binding domain. These proteins can bind with bHLH factors to inhibit DNA binding as shown through studies in C2C12 cells (Füchtbauer, 2002). The likely binding targets of Id proteins are the E-proteins, thereby reducing the promoting activity of the dimerization of E-proteins with MRFs to bind E-box motifs. In C2C12 cells the overexpression of Id results in inhibition of differentiation, and Id and E-proteins cofractionate and co-immunoprecipitate in whole cell extracts of myoblasts. Id and bHLH factors are expressed in a mutually exclusive manner, and it is not clear whether expression levels are high enough for Id to consume all dimerization partners of MRFs. However, the evidence still suggests a role for Id in the regulation of myogenesis (Füchtbauer, 2002).

The gene myostatin has generated a great deal of interest in the study of myogenesis, and is thought to inhibit proliferation of myoblasts through down regulation of MyoD (Langley et al., 2002). Mutations in the myostatin gene have resulted in the double-muscling phenotype in cattle (Grobet et al., 1997; Kambadur et al., 1997; McPherron et al., 1997), and in one very recent case report in an extraordinarily muscled newborn human (Schuelke et al., 2004). In the pig, greater *myostatin* expression levels have been linked to lower birth weight (Ji et al., 1998).

2.4. MUSCLE FIBER DEVELOPMENT

2.4.1. Role of muscle fiber numbers

Potential muscle mass is determined by the overall number of muscle fibers formed and the size that these fibers attain. Comparisons of muscle fibers between different animal species suggests that although muscle fiber size makes a contribution, it is the number of muscle fibers rather than the size of individual fibers that is the primary determinant of muscle mass (reviewed briefly in Rehfeldt et al., 1999).

A comparative study of two pig breeds that were genetically different in size (Large White and Göttingen miniature) determined the mechanism by which smaller species developed fewer muscle fibers. It was observed that Large White pigs had a significantly greater number of primary muscle fibers than the miniature breed. The authors also observed that Large White pigs had a significantly higher secondary-to-

primary fiber ratio than miniature swine. However, it was concluded that primary fiber numbers played a much more significant role than fiber ratios in determining myofiber numbers when comparing two different sized species (Stickland and Handel, 1986).

Dwyer et al. (1993) also observed that muscle fiber number was an important characteristic in postnatal development. They determined that after 10 weeks of age, pigs with higher muscle fiber numbers tended to grow faster and more efficiently than littermates with a lower fiber number. Interestingly, prior to 10 weeks of age, growth was associated with birth weight and not total muscle fiber number, whereas after 10 weeks of age the reverse was observed. Nissen et al. (2004) also observed that muscle fiber characteristics contributed to intra-litter variation in postnatal growth performance. Pigs with relatively lower growth performance formed lower numbers of muscle fibers and had lowered growth rate of those fibers.

2.4.2. Postnatal Catch-up Growth

Catch-up growth (reviewed by Boersma and Wit, 1997) is described as a growth phase that is faster than statistically normal for a defined time period following a transient period of growth inhibition. The authors also define a difference between catch-up growth (growth of the whole organism) and compensatory growth (both growth of the whole organism and the overgrowth of a single organ). A key distinction between the two terms is that catch-up growth anticipates a future lack of

tissue, while compensatory growth reacts to the present loss of tissue. In other words, catch-up growth is a response over time to reach an inherent potential for growth not yet realized, due to initial growth inhibition. In contrast, compensatory growth is an immediate response in an individual to a loss of tissue that has already been established.

In animals, the authors describe factors important for the extent of compensatory growth after undernutrition. These include the nature, severity, duration and stage of development at the onset of undernutrition. The rate that a species matures is also important, since slow-maturing animals recover more rapidly than fast maturing ones. Furthermore the level of realimentation is important, as higher planes of nutrition appear to result in greater recovery.

A study by Handel and Stickland (1988) showed the existence of a relationship between muscle fiber number and catch-up growth. Twenty three low birth weight pigs were compared with their largest littermates. The small littermates had both a lower live weight at slaughter and lower muscle fiber numbers than the larger littermates, and exhibited differing catch-up growth potentials. The relative proportion of live slaughter weight and muscle fiber number between the two sizes of pig was determined, and small littermates that reached >0.84 of the relative slaughter weight of their large littermates were considered to exhibit a high degree of catch-up growth. In small pigs that did exhibit good catch-up growth, the proportion of total muscle fiber numbers did not differ significantly from that of their large littermates.

Those that did not exhibit good catch-up growth showed a range of muscle fiber numbers and, in some cases, external factors prevented some individuals from reaching their potential. In any case, those that did exhibit good catch-up growth always had relatively higher numbers of total muscle fiber numbers than those that did not, and the authors suggested that muscle fiber number at birth was an indicator of growth potential.

2.4.3. Tertiary Muscle Fibers

Although it is a controversial aspect of muscle development, there is evidence to suggest that, in addition to primary and secondary muscle fibers, there may be a tertiary wave of hyperplasia in the pig (Mascarello et al. 1992; Lefaucheur 1995). These tertiary fibers are closely associated with secondary fibers, analogous to the association of secondary fibers with primary fibers, and have also been observed in sheep and humans. Additionally, in humans and sheep, tertiary fibers are seen during gestation (Wilson et al. 1992; Draeger et al. 1997), while the tertiary fibers in the pig are observed around birth (Lefaucheur 1995). This however, is in contrast to previous data that suggested that total muscle fiber number is determined prenatally. The presence of these tertiary fibers has been suggested to be part of a mechanism that leads to larger muscle mass in large animals (Lefaucheur 1995). If tertiary fibers do indeed exist, the precise role and factors affecting their development have yet to be elucidated, and further research is necessary.

2.5. FACTORS INFLUENCING THE DETERMINATION OF MUSCLE FIBER NUMBERS

2.5.1. Intrauterine growth retardation (IUGR)

Litters often contain at least one piglet that is significantly lighter than its littermates, and is regarded as representative of a form of natural undernutrition. Several terms such as intrauterine growth retardation, and runting have been used to describe these piglets. The classification of intrauterine growth retardation (IUGR) piglets is much more variable, as several definitions of IUGR have been presented, including fetuses weighing less than two standard deviations below the mean body weight, and fetuses that are in the lowest tenth percentile for body weight (reviewed briefly in Ashworth et al., 2001). In any case, all definitions refer to individuals who are significantly lighter than the rest of the litter in terms of weight at a given gestational age.

The time point at which IUGR is established has been examined by several studies (reviewed in Ashworth et al., 2001). Runts that were two thirds of the average weight for the uterine horn were found at day 31 to 49 of gestation. In an examination of piglets with body weights less than two standard deviations or more below the mean litter weight, runting was apparent by day 44 of gestation. By plotting the cumulative probability of fetal weights in a litter, it was reported that runt littermates could be identified as early as day 30 of gestation. These observations are consistent with the conclusion that the within-litter weight distribution is determined by day 35 of

gestation. The placenta is likely an important contributing factor to heterogeneity, as placental weight at day 29 of gestation is an accurate determinant of fetal weight later in gestation (Ashworth et al., 2001).

In addition to decreased growth *in utero*, there are several postnatal consequences associated with growth retardation (reviewed by Ashworth et al., 2001). Reduced birth weight piglets have increased risks of fetal and neonatal death and do not achieve the same body weight as normal littermates, and internal organs are disproportionately small.

In study by Hegarty and Allen (1978), it was observed that runt littermates grow slower and less efficiently postnatally than large littermates. In a study examining the effects of IUGR on muscle development, it was observed that runts produce carcasses with higher proportions of fat and less muscle than larger pigs when compared at a constant slaughter weight (Powell and Aberle, 1980). Indirect measurements of muscle fibers have suggested that runts had a reduced fiber number at birth (Hegarty and Allen, 1978; Powell and Aberle 1981).

Particular attention to total fiber numbers was paid in a comparison of muscle development in the pig between large and small littermates (Wigmore and Stickland, 1983). The timing of primary and secondary fiber formation, and the number of primary fibers formed, was not different between large and small littermates. There were however, a lower number of secondary fibers in small littermates, which

resulted in a 17% reduction in the total fiber number at birth. Furthermore, it was found that primary fibers were smaller in small littermates than in large littermates. The authors concluded, therefore, that the reduced number of secondary fibers was due to reduced area for the attachment and fusion of myoblasts in the formation of secondary fibers. An estimation was made which showed that large littermates had approximately 20 secondary fibers per primary fiber, while small littermates had 16. The authors also noted, from unpublished observations, that small littermates exhibit signs consistent with prenatal malnutrition, and, therefore, *in utero* nutrition status was a potential contributing factor in the determination of muscle fiber numbers.

In a comparison of muscle cellularity between large and runt littermates, low birth weight was associated with reduced total muscle fiber numbers. However, low fiber number was not always associated with low birth weight. When this was the case there was a reduction in secondary to primary fiber ratio. Primary fiber numbers were not significantly different except in cases of extreme runting (Handel and Stickland 1987). An expansion of this study by Dwyer et al. (1991) showed that primary fiber numbers differed between litters, and was responsible for the variation in total fiber numbers found. Taken together, it was suggested from these two studies that primary fiber number was a relatively fixed genetic component determined mainly by the genotype of the animal. Secondary fiber number, although also having a genetic component, were more susceptible to environmental factors *in utero*.

2.5.2. Nutrition

Dwyer et al. (1994) tested the hypothesis that increased feed intake in third parity sows would affect muscle development by increasing the number of secondary muscle fibers. The authors studied the effect of doubling a standard commercial feed intake (5% oil; 17% protein; 6.5% fiber; 12.5 MJ of energy/kg of feed) at various gestational ages on myogenesis. Three time periods were chosen, with consideration given to the time periods of primary and secondary formation (Wigmore and Stickland, 1983): day 25 to 50 (up to the point of secondary fiber formation); day 50 to 80 (during secondary fiber hyperplasia); and day 25 to 80 that covered both time periods. The authors concluded that doubling feed intake did indeed improve the mean number of total fibers formed. However, the observed increases in total muscle fibers were observed only when feed intake was increased between the time periods of day 25 to 50, and day 25 to 80. During the period from day 50 to 80, increased feed intake had no effect on the number of total muscle fibers formed. Based on these observations, as well as calculations of the ratio of secondary to primary fibers, the authors concluded that the period prior to the onset of secondary fiber hyperplasia was a critical time period for increased feed intake to increase the number of secondary fiber numbers. It was also observed that the pigs derived from sows with increased maternal feeding from day 25 to 80 had increased growth rates from day 70 to the 80 kg slaughter weight (Dwyer et al., 1994).

A study addressing the effects of maternal undernutrion on the development of muscle fibers in the guinea-pig has also been performed (Dwyer et al., 1995). In this study it was observed that a 40% reduction in maternal nutrition resulted in a 20-25% decrease in the total number muscle fibers in the offspring. A previous study by Dwyer et al. (1992) also showed that maternal undernutrition resulted in decreased placental size, and it was suggested that the effects of maternal nutrition were mediated through effects on the development of the placenta. It has been suggested that the reason for the lack of effect of undernutrition on the number of primary fibers formed may be due to the fact that the critical period for primary fiber formation occurs when nutritional demands by the embryos are relatively low (Ward et al., 1992).

However, the effects of maternal nutrition are still somewhat controversial. In contrast to Dwyer et al. (1994), Nissen et al. (2004) observed that there was no effect of increased maternal nutrition from day 25 to 50 or day 25 to 70 of gestation on the number of muscle fibers formed. It appears however, that the feed composition between the two studies was not similar, and this was not discussed. In particular, protein content (17% protein in Dwyer et al., 1994; 13% protein in Nissen et al., 2004) and net energy (12.5 MJ/kg in Dwyer et al., 1994; 7.5 MJ/kg in Nissen et al., 2004) differed between the two studies. This is of note, as studies in the guinea pig have shown that supplementation of either protein or carbohydrate alone prevents a decrease in the muscle fiber number of offspring caused by a feed-restricted maternal diet (Dwyer and Stickland, 1994).

2.5.3. Insulin-like growth factors (IGFs)

Both the IGF proteins, and their receptors and binding proteins, play vital roles in the growth of placenta and embryo (reviewed in van Kleffens et al., 1998; Fowden, 2003). Knock-outs of either IGF-I or IGF-II resulted in similar reductions in birth weight in mice. In the case of IGF-I, neonatal death also occurred. When a knock-out of the IGF-1 receptor occurred, birth weight was even more reduced and also resulted in neonatal death. However, IGF-II knock-outs resulted in an increase in birth weight and higher levels of circulating IGF-II, but are also usually lethal due to growth abnormalities. Combined, these studies suggested that the IGF-I receptor serves as a receptor for both IGF-I and IGF-II in fetal development, and the IGF-II receptor serves as a receptor for only IGF-II in order to remove it from circulation.

Studies of the ontogeny of IGF levels in the pig have shown that levels of IGF-II were greater than IGF-I at all periods of development (Lee et al., 1991). From these results it was suggested that IGF-II acts as both a growth factor in fetal and postnatal growth, while IGF-I acts as a growth mediator.

In the case of placental development, gene ablation studies in mice have implicated IGF-II in the growth of the placenta (reviewed in Fowden, 2003). Mutants that were deficient in all placental cell types, or in the labyrinth trophoblast cells only (P0), were examined. In IGF-II nullizygous mice, placental growth was retarded and the

mice had structural abnormalities, while P0 mutants were smaller but morphologically normal. Both mutants begin retardation of placental growth at around the same period during midgestation, but fetal growth retardation is slowed until later in gestation in P0 mutants. Thus, it has been suggested that growth of the fetus is inhibited through effects of IGF-II on the functional capacity of the placenta to transfer nutrients.

An excellent review by Oksbjerg et al. (2004) thoroughly discusses the role of the insulin-like growth factor IGF system on muscle development and growth. The effects of IGFs on myogenesis have mainly been characterized from mutant mice and different cell culture models. The development of muscle fibers is linked to IGFs, and is the only known growth factor that stimulates both proliferation and differentiation of muscle cells. In a study of LA61 cells (L6 derived muscle cells), addition of IGF-I and/or IGF-II initially stimulates proliferation in a dose-dependent manner. Subsequent addition of low levels of IGFs stimulates differentiation, while high levels decrease differentiation. Myoblasts treated in culture with IGF-I, showed an initial decrease in MRFs. After withdrawal of myoblasts from the cell cycle, IGF-I promotes differentiation through induction of myogenin and MRF4 expression and activation of the Mef2c (myocyte enhacer factor 2c) gene. The use of an antisense oligonucleotide to myogenin blocks the stimulation of muscle differentiation by IGF-I, thus implicating the importance of up-regulation of myogenin (Florini et al., 1990).

2.5.4. Growth Hormone

There have been several studies examining the effects of maternally-administered porcine growth hormone (pGH) on the development of offspring (reviewed extensively by Rehfeldt et al., 2004). A study on the administration of human growth hormone in pregnant rats suggest that GH does not cross the placenta (Fhölenhag et al., 1994), and therefore acts to influence growth of the fetus indirectly through effects on the placenta.

GH treatment of pregnant sows led to increases in the concentrations of maternal IGF-I, and increases in IGF-I and IGF-II concentrations in fetal blood. However, in contrast to effects on birth weight, the effect of GH hormone on IGF concentrations seems to be limited to early gestation, as serum levels of IGF-I from neonatal pigs showed an increase when treatment occurred in early gestation, but not when it was applied in mid or late gestation (Rehfeldt et al., 2004).

The effect of porcine somatotropin (pST) on muscle fiber development has also been investigated. The administration of pST to sows from day 10 to 24 of gestation resulted in significantly higher numbers of muscle fibers in the *semitendinosus* muscle of the fetus (Rehfeldt et al., 1993). Furthermore, in agreement with nutritional manipulations that determined that a critical period exists in the

determination of muscle fiber numbers, no effect of exogenous pST was seen when treatment occurred after day 50 of gestation.

Further evaluation of the effect of pST treatment from day 10 to 27, revealed that the increase in muscle fibers was due to both increase in primary and secondary fibers (Rehfeldt et al., 2001). This is in contrast to the previous suggestion that primary fiber numbers are genetically fixed (Handel and Stickland 1987; Dwyer et al., 1991), and suggests total primary fiber formation may also be affected by environmental influences. In addition it was also shown that treatment with pST resulted in higher expression levels of Myf-5 and MyoD, indicating a prolonged period of muscle cell proliferation. Interestingly, the effects of pST administration more strongly affected lower birth weight littermates than higher weight littermates. From these results, Rehfeldt et al. (2001) hypothesized that it is the early administration of pST in this study that influences primary fiber formation, which thus triggers a later increase of secondary fiber formation. The authors suggested that pST treatment might act by increasing nutrient availability to the embryo, which in turn acts directly on cell proliferation, or indirectly through hormones and growth factors influenced by nutrient availability.

2.5.6. Estrogen

Estrogen plays a critical role in the recognition, establishment, and maintenance of pregnancy in pigs (reviewed by Spencer et al., 2004). Prostaglandin F2α (PGF) is a

hormone that acts as to induce luteolysis in domestic farm species including swine. The secretion of estrogen by the embryo is antiluteolytic, and is thought act by to sequestering PGF2α in the uterine lumen by altering its pattern of secretion from an endocrine to exocrine direction. Initially, estrogens produced by embryos between day 11 and 12 of gestation initiate the recognition of pregnancy. This is followed by a second period of estrogen secretion between day 15 and 25-30.

There has been little research into the effects of estrogen on skeletal muscle. However, estrogen has been suggested to play a role, as the presence of estrogen receptor α has been observed in human skeletal muscle (Lemoine et al., 2003). An analysis of the effects of estrogen on the female reproductive tract has shown *Wnt7a* expression to be sensitive to levels of sex steroids (Miller et al., 1998), and that increases in the expression of *estrogen receptor α* in human uterine leiomyoma were associated with decreases in Wnt7a (Li et al., 2001). As mentioned above in section 2.3.3 Wnt7a acts to positively induce *MyoD* expression (Parker et al., 2003). Thus, if early myoblasts are sensitive to estrogen, a link between the production of estrogen by the embryo during early gestation and myogenesis may exist.

2.5.7. Gender

Gender of pig embryos during gestation has been shown to have an effect on the development of both the placenta and the fetus (Wise and Christenson 1992). From at least day 70 of gestation onwards, males fetuses were heavier than female fetuses.

The placentas of male fetuses were also observed to be heavier than those of females. By day 104 of gestation fetal weight was affected by the sex of neighbouring fetuses within the uterus. Fetuses that were surrounded by neighbours of the opposite sex were lighter than those that were surrounded by the same sex, and such fetuses may represent runts that will have compromised postnatal development. Analysis of hormonal effects showed no relationship of testosterone, or estrogens from surrounding fetuses on growth as measured by fetal weight. Levels of thymosin ß4, a peptide that stimulates immunological functions, was significantly lower in females surrounded by males, but not in males surrounded by females. From these results the authors suggested that the observed fetal weight differences may be due to immunological differences between fetuses, or endocrine influences may have occurred during early development but did not appear until later gestation (Wise and Christenson, 1992).

The effect of sex on the determination of muscle fibers in different species has shown contradictory results. Species, including human and rat, have shown males to exhibit higher numbers of muscle fibers than females. In an analysis of pig *Longissimus* muscle, there was no observable difference between males and females (reviewed briefly in Rehfeldt et al., 1999).

2.5.8. Litter Size and Uterine Crowding

Using unilateral oviduct-ligation to limit the number of embryos *in utero*, an examination was done on the difference in muscle cellularity of day 90 fetuses between ligated (LIG) and control (CTR) sows (Town et al., 2004). It was observed that fetuses from the relatively less crowded uterine environment of the LIG sows had higher numbers of secondary muscle fibers than fetuses from CTR sows. Nissen et al. (2004) also found that there was a significant negative correlation between the number of muscle fibers formed and the pigs born per litter.

Town et al. (2004) also examined the effects of uterine crowding on development at day 30 of gestation. It was observed in embryos at this time stage of gestation, that average placental weight in relatively crowded sows was significantly lighter than average placental weights of relatively non-crowded sows. This effect was observed during the critical time period for the determination of secondary muscle fiber numbers (Dwyer et al., 1994). As discussed in section 2.5.2., the determination of muscle fiber numbers in this experimental paradigm may be mediated through effects on placental size. Therefore, there is evidence to suggest that uterine crowding affects early events of myogenesis at day 30 of gestation.

2.6. CONCLUSION

Based on the observations of Town et al. (2004; discussed in section 2.5.8.) uterine crowding may affect basic events of myogenesis as early as day 30 of gestation. Since myogenesis requires a tightly regulated and coordinated series of events, it was

hypothesized in this thesis that uterine crowding will affect the expression of MRF's during this period. To help elucidate whether effects of uterine crowding at day 30 of gestation were responsible for reduced secondary muscle fiber numbers, real-time RT-PCR will be used to quantify gene expression of MRFs, as uterine crowding may result in a reduction in the expression of genes critical to the formation of muscle fibers. *In situ* hybridization was used to determine qualitatively whether there were any differences between embryos from crowded and non-crowded uterine environments. The possibility existed that relative uterine crowding might cause delays in the timing of expression of MRF's, and that such differences might be visualized by a reduced progression of MRF expression along the rostral-caudal axis.

The second goal of this thesis was to determine whether the gender of the embryo might play any role in this crowding-induced effect. Sex-typing PCR was used to determine the sex of each embryo, and the results of the real-time RT-PCR were interpreted in the context of this information.

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CHAPTER THREE

EXPRESSION OF MYOGENIC REGULATORY FACTORS IN DAY 30 PIG EMBRYOS IS AFFECTED BY UTERINE CROWDING

3.1. INTRODUCTION

The total number of muscle fibers formed is a critical determinant of total muscle mass. In the pig, myogenesis follows a biphasic pattern of development (Wigmore and Stickland, 1983 and references therein), whereby an initial population of primary fibers are formed that are used as a site for the subsequent attachment and fusion of myoblasts to form secondary fibers. By approximately day 90 of gestation, fiber hyperplasia is complete and total fiber numbers have been determined (Wigmore and Stickland, 1983). Since muscle fiber numbers are determined prior to birth, and cannot be altered during postnatal development, gestation is a critical period in the determination of the ultimate number of muscle fibers formed.

In a study of nutrition in pregnant sows, it was found that a doubling of a standard commercial diet during gestation resulted in a higher number of secondary fibers formed in offspring (Dwyer et al., 1994). The authors also observed that the effects of increased maternal nutrition only occurred when feed intake was increased between day 25 and 50 of gestation, but not afterwards. This time period, which coincides with the period prior to the onset of secondary fiber formation, was

therefore suggested to be a critical period in the determination of secondary muscle fibers. In the guinea pig, the effect of maternal undernutrition has been shown to result in a reduction of the total muscle fiber number of offspring (Dwyer et al., 1995). Furthermore, undernutrition has been shown to result in a reduction in placental size, and has been suggested as the mechanism by which maternal undernutrition is mediated (Dwyer et al., 1992)

Litter size is an important component of swine production, and increases to reproductive efficiency through increased litter size can be economically beneficial. However, uterine crowding during gestation may negatively impact the number of muscle fibers formed. Previously, unilateral oviduct-ligation surgery was used to study the effects of litter size on muscle cellularity by limiting the number of embryos in the uterus. Analysis of muscle fibers in day 90 fetuses from ligated (LIG) and control (CTR) showed that fetuses from the relatively less crowded uterine environment of the LIG sows had higher numbers of secondary muscle fibers than fetuses from CTR sows (Town et al., 2004). In the same study, an analysis at day 30 of gestation also revealed that LIG sows had higher average placental weights than CTR sows. These results suggest that the effects of uterine crowding in the determination of secondary muscle fibers may be due to effects on myogenic development during early gestation, analogous to those of undernutrition.

The myogenic regulatory factors (MRFs) Myf-5, MyoD, myogenin, and MRF4 play are critical transcription factors involved in the development of muscle (for review

see Arnold and Braun, 2000). Although initially identified by their common ability to convert non-muscle cells into muscle cells *in vitro*, knock-out studies in mice have identified distinct roles for each MRF in myogenesis. Myf-5 and MyoD act to determine pluripotent cells into myoblast precursor cells, while myogenin initiates terminal differentiation of myoblasts into muscle cells. MRF4 has long been thought also to be a factor involved in terminal differentiation; however, recent evidence suggests that *MRF4* acts to regulate *MyoD* expression (Kassar-Duchossoy, 2004).

The purpose of this study was to investigate whether the effects of uterine crowding on the determination of secondary muscle fiber numbers occurs through changes in expression of MRFs at day 30 of gestation. Because average placental weight was observed to be lower in day 30 CTR sows than in LIG sows, the relative state of uterine crowding may have an effect on development analogous to the effect of undernutrition. Since early gestation between day 25 and day 50 is a critical time period in the determination of secondary fiber numbers, we hypothesized that a decrease in MRF expression would be observed that reflected the reduced secondary fiber numbers observed in day 90 fetuses. The MRFs MyoD and myogenin, which play roles in myoblast determination and terminal differentiation, respectively, were selected for analysis. Real-time RT-PCR was used to quantify expression of these genes in day 30 embryos representing both LIG group and CTR group sows.

3.2. MATERIALS AND METHODS

3.2.1. Animals

Day 30 embryos for real-time RT-PCR were collected from animals used by Town et al. (2004). Thirty Hybrid F1 third parity sows (Genex Swine Group, Regina, Saskatchewan, Canada) were raised in a controlled environment barn at the Swine Research and Technology Centre at the University of Alberta during July to October 2001. Animals were managed and fed as per standard protocols during the previous gestation and lactation, and were weaned at 23 days after farrowing. Sows were randomly assigned to one of two groups. Oviduct ligation surgery (LIG) was performed on fifteen sows approximately 3 days after the end of their first post-weaning oestrus. This surgery was used to prevent oocytes ovulated from the ovary ipsilateral to the ligated oviduct from being fertilized and subsequently entering the uterus, thereby reducing the number of embryos *in utero*. The remaining fifteen animals did not undergo surgery and formed the control (CTR) group. All embryos slaughtered at day 30 of gestation analyzed for MRF expression were obtained from sows.

3.2.2. Collection and Paraformaldehyde fixation of Embryos

The reproductive tract were recovered and dissected from all sows within one hour after slaughter. Collected embryos were treated with paraformaldehyde in order to

preserve the cellular morphology for possible qualitative analysis. Whole day 30 embryos were pierced at the base of the skull with a 27-gauge needle to allow easier perfusion of paraformaldehyde. Embryos were then immersed in a 4% (w/v) buffered paraformaldehyde (85 mM PIPES, 25 mM HEPES, 5 mM EGTA, pH 7.0) and incubated at 4°C overnight. The embryos were then dehydrated with increasing concentrations of methanol with the final step in 100% (v/v) methanol and stored at -30°C. The total experimental group consisted of twenty sows (N=20), with all embryos from ten sows selected at random from the LIG group (N=10), and all embryos from ten sows selected at random from the CTR group (N=10), used for analysis of MyoD and myogenin expression. 93 embryos were collected from sows from LIG group, and 158 embryos were collected from the CTR group.

3.2.3. RNA Extraction

Extraction of paraformaldehyde-fixed tissues was performed using a modified proteinase K protocol described by Masuda et al. (1999). 1.0 ml homogenization buffer (200 mM Tris-HCl, 200 mM NaCl, 1.5 mM $MgCl_2$, 2% (w/v) SDS, pH 7.5) was added for every 100 mg of whole embryo tissue, and homogenized. 500 μg proteinase K (Sigma Chemical Co.) was added to 1.0 ml aliquots of the resulting tissue lysate and then incubated at 45°C for 1 h. 1.0 ml of buffer-saturated phenol (pH 4.3)/chloroform/isoamyl alcohol (25:24:1), followed by 1.0 ml chloroform, was then used for total RNA extraction. RNA was precipitated using 2.0 ml isopropanol, and the RNA pellet was then dissolved in DEPC-treated RNase-free water (Qbiogene)

and diluted to a concentration of approximately 1 μg/μl. Total RNA was run on a 1% (w/v) agarose gel to verify RNA integrity. DNase I treatment using DNA-*free* kit (Ambion Inc.) was performed after RNA extraction to ensure removal of residual genomic DNA.

3.2.4. Quantitative real-time RT-PCR

Quantitative real-time two-step RT-PCR was used to measure expression levels of the MRF genes MyoD and myogenin. Approximately 1 μg of total RNA was reverse-transcribed to cDNA for real-time analysis using MMLV RT (Invitrogen) and a poly-dT primer. Real-time PCR reactions for each gene were run in duplicate in 96-well optical plates in an ABI Prism 7700 Sequence Detection System (Applied Biosystems). Primers and TaqMan MGB probes sets for porcine cyclophilin, MyoD, and myogenin were designed using Primer Express software (Applied Biosystems) (Table 3.1.). Primer and probe sets for MyoD and myogenin were designed to avoid homologous regions between the two genes. Each primer and probe set was also designed to cross an exon-exon junction, and to generate amplicons of 110 bp or less, in order to reduce the possibility of genomic DNA amplification. Primer specificity was determined by agarose gel electrophoresis. RT-PCR products produced under real-time amplification conditions were run on a 2% (w/v) agarose gel and stained with ethidium bromide. Gels were placed over a UV lamp and examined for the presence of amplification products from target mRNAs, as well as the possible presence of amplification products from DNA contamination.

3.2.5. Analysis of real-time RT-PCR data

Analysis of expression levels was done using the $2^{-\Delta\Delta C_T}$ comparative C_T method (Livak and Schmittgen 2001; see Appendix A.1.). The threshold was set above the measured baseline and in the logarithmic phase of amplification. The threshold cycle value (C_T), defined as the cycle number at which detected fluorescence passes the fixed threshold value, was determined for each a gene. Amplification efficiencies for each gene were determined in order to establish the suitability of the comparative C_T method for analysis of gene expression (see Appendix A.1.).

3.2.6. Statistical Analysis

Data were analysed using the mixed classification model of SAS (SAS Inst., Cary, N.C., USA) to determine the effect of treatment on MyoD and myogenin expression. Treatment was used as a fixed factor, and the sow was included as a random factor. Significance was considered at $P < 0.05$. The results are presented as means of sows ± SEM.

Average gene expression levels of MyoD and myogenin, average embryo number and placental weight, as well as the number of viable embryos at day 30 of gestation were determined for all sows. Correlations were performed across all sows to determine if

any possible relationships existed between expression level and embryo weight, placental weight, or viable embryo number.

3.3. RESULTS

3.3.1. RNA integrity from paraformaldehyde fixed embryos

All extracted RNA samples were run on 1% (w/v) agarose gels (a representative gel is shown in Figure 3.1.). RNA samples showed the strong presence of both intact 18S and 23S rRNA, indicating that RNA extraction from paraformaldehyde fixed tissues was successful.

3.3.2. Real-time PCR amplification specificities and efficiencies

RT-PCR was performed to verify if RNA extracted from the paraformaldehyde fixed tissues was suitable for real-time RT-PCR analysis. 2.25% (w/v) agarose gels shown in Figure 3.2 indicate the expected specific amplicons in sample wells but not in negative control lanes. In addition, products for primer sets for each gene showed only the expected amplicon size from mRNA transcripts, and not the amplicon size expected if contaminating genomic DNA had been amplified. This shows that the primer sets were specific for amplification from target mRNA. A sample amplification plot is shown in Figure 3.3. The higher the Ct value for a given ΔRn, the lower the initial template concentration for amplification. Comparisons of the

amplification efficiencies of MyoD, myogenin, and cyclophilin were highly similar, thus making each gene suitable for the comparative Ct method (Figure 3.4).

3.3.3. Analysis of MyoD and myogenin expression in embryos from CTR and LIG sows.

The relative expression levels (ΔC_T) of MyoD were determined to be 9.10 ± 0.53 and 8.92 ± 0.79 for embryos from CTR and LIG sows, respectively, and were not different ($P = 0.57$). For myogenin ΔC_T of 5.59 ± 0.25 and 4.89 ± 0.29 for embryos from CTR sows and LIG respectively, were different ($P < 0.05$; summarized in Table 3.2). Analysis using the $2^{-\Delta\Delta C_T}$ method indicated embryos from LIG had an approximately 1.7-fold higher expression of myogenin than in embryos from CTR sows (Table 3.3).

A comparison of the average ΔC_T of all embryos within a sow showed no correlation between either MyoD or myogenin (Figure 3.5). Similarly, no correlation was observed between the average ΔC_T of MyoD, or myogenin and placental weight (Figure 3.6). When compared against the total number of viable embryos at day 30 in each sow there was also no correlation between the ΔC_T of MyoD and embryo number (Figure 3.7). However, a significant positive correlation was observed when the ΔC_T of myogenin was compared with viable embryo number at day 30 ($R^2 = 0.40$; $P < 0.003$; Figure 3.8). As embryo number increased, the ΔC_T also increased,

signifying a decrease in expression as the total number of viable embryos at day 30 increased.

3.4. DISCUSSION

In this study, a relatively more crowded uterine environment already resulted in effects on MRF expression in day 30 embryos. Although no change was observed in the expression of MyoD, a significant reduction in the expression of myogenin was observed in embryos from CTR sows compared to LIG sows. However, MyoD is a lowly expressed gene in relatively few cells, and the real-time RT-PCR technique used here may not be sensitive enough to detect these small changes in the expression of MyoD. Therefore, small changes in the number of proliferating myoblasts may not be detectable at this stage of development. In any case, the previously observed effect of uterine crowding on the number of secondary muscle fibers formed in day 90 fetuses (Town et al., 2004) may be due to effects on basic events of myogenesis during this critical period in the determination of secondary fiber numbers later in gestationas established earlier by Dwyer et al. (1994).

Previous studies into the roles of MRFs indicate that MyoD is responsible for the determination of myoblasts, while the role of myogenin is to initiate terminal differentiation (reviewed by Arnold and Braun, 2000). The observations from this study suggest that an up-regulation in the expression of myogenin resulted from a reduction in the relative degree of uterine crowding. If MyoD expression is indeed

unaffected, the effects of uterine crowding may act through differentiation of a pre-existing population of myoblasts rather than proliferation of myoblasts.

Another potential explanation for the observed differences is a delay in the onset of expression of MRFs, rather than a change in total expression. The lower myogenin levels in embryos from CTR sows might, therefore, indicate a less advanced state of development of the myogenic lineage. The lack of a difference between embryos of the two treatments in MyoD expression may be due to a threshold level of myoblast formation reached during an earlier stage of development that may mask any previous asynchrony in expression.

There is evidence to suggest that the determination of secondary muscle fiber numbers occurs during the time-period coinciding with the formation of primary muscle fibers (Dwyer et al., 1994). Therefore, the effects of uterine crowding on secondary fiber numbers potentially act through an effect on the development of primary fibers. In the pig, primary fiber numbers have been suggested to be a relatively fixed genetic component, while secondary fiber numbers are sensitive to environmental influences (Handel and Stickland, 1987; Dwyer and Stickland 1991). In an analysis of the prenatal development of small and large littermates, primary fibers in small littermates, although equal in number to the large littermates, were smaller and may have restricted the surface area available for the establishment of secondary fibers (Wigmore and Stickland, 1983). Studies into myogenin knock-out mice have determined that myogenin is not completely necessary for the attachment

of primary fibers, although differentiation of these fibers is delayed (Venuti et al., 1995). Therefore, it is possible that the observed differences in secondary muscle fibers in day 90 fetuses from LIG and CTR sows (Town et al., 2004) may be due to the effect of myogenin expression at day 30 on the differentiation of primary fiber numbers.

Myogenin expression levels at early stages of development may also lead to direct effects on the development of secondary fibers. Studies in avian models have shown that myoblasts that have an ability to form both primary and secondary fibers are predetermined early on gestation (see Stockdale, 1992). A study of muscle cells in the human limb, further suggest that cells expressing myogenin are present prior to primary fiber formation and are involved directly in the formation of secondary fibers later in development (Edom-Vovard et al., 1999). It is, therefore, possible that lower myogenin expression at day 30 in the present study in CTR sows may represent a reduction in the number of cells available for the development of secondary fibers at later stages of gestation. Whether or not it is primary or secondary fiber development that is affected by altered myogenin expression levels is not clear. However, effects on both fiber types are not necessarily mutually exclusive.

Using all sows in this study, an analysis of the relationship between the average expression levels of MyoD and myogenin of all the embryos of each sow, with either average embryo weight or placental weight, showed no significant correlations. In addition, no significant correlation was found between the expression level of MyoD

and the total number of viable embryos present at day 30 of gestation. However, a significant correlation was observed between myogenin expression levels and the total number of viable embryos at day 30. The relative state of uterine crowding has been shown to negatively affect embryo weight at day 90 of gestation, as well as placental weight at both day 30 and day 90 of gestation (Town et al., 2004). Combined these results suggest that although uterine crowding may affect the growth and development of the embryo and the placenta, the establishment of myoblasts may not be affected by overall growth and development, or the state of crowding. Additionally, the level of myogenin expression also appears to be independent of either embryo or placental weight, but appears to be influenced by the presence of higher numbers of embryos. Thus, the effect of uterine crowding on myogenin expression may be somewhat independent of individual embryo growth *in utero*, or to access to nutrients through increased materno-fetal exchange provided by a greater placental mass.

In summary, the results from this study indicate that uterine crowding affects genes related to myogenesis, and is presumably a key factor in determining the reduction in total numbers of secondary muscle fibers reported at day 90 of gestation. Therefore, there may be critical considerations that need to be made when genetic selection is carried out to increase average litter size. Since fiber numbers may not be altered during postnatal development, selection criteria that favour large litters may, at the same time, be compromising the postnatal potential for muscle development. Strategies that effectively increase differentiation may be effective at alleviating the

negative effects of uterine crowding on the development of muscle fiber numbers. This may be an especially important consideration as the observations of this study may be a modest representation of the impact of uterine crowding on myogenesis with respect to the potential for embryo survival in modern commercial dam-lines. The ovulation rate of sows used in this study (19.90 ± 0.36; Town et al., 2004), is considerably lower than the ovulation rate of other modern dam-line populations, such as those observed by Vonnahme et al. (2002; 26.6 ± 0.40). Thus, an examination of these higher states of uterine crowding on myogenesis would be helpful in determining the potential of the effects of selection for increased litter size on muscle accretion. Further investigation into the mechanisms which reduce secondary fiber numbers, as well as greater knowledge of the specific timing of the effect, would also be beneficial in optimizing muscle development in this meat-producing species.

Gene	Primers	Sequence 5' --> 3'	Expected RNA amplification size	Expected DNA amplification size
Cyclophilin	Sense	AAT GCT GGC CCC AAC ACA		
	Antisense	TCA GTC TTG GCA GTG CAA ATG	55 bp	450 bp
	Probe	CAC AAA CGG TTC CCA GTT TTT		
MyoD	Sense	ACT CAG ACG CAT CCA GCC C		
	Antisense	GTA ATA GGT GCC GTC GTA GCA GT	107 bp	598 bp
	Probe	CGG CAT GAT GGA TTA T		
myogenin	Sense	GGC CCC AAC CCA GGG		
	Antisense	GGA GTG CAG ATT GTG GGC A	63 bp	682 bp
	Probe	ATC ATC TGC TCA CAG CTG		

Table 3.1. Oligonucleotides and TaqMan MGB probes.

Parameter	CTR (n=10) "Relatively Crowded" (ΔC_T)	LIG (n=10) "Non-Crowded" (ΔC_T)	P-value
MyoD ΔC_T	9.10 ± 0.53	8.92 ± 0.79	P = 0.5653
myogenin ΔC_T	5.59 ± 0.25	4.85 ± 0.29	< 0.05

Table 3.2. Expression levels normalized to cyclophilin(mean ± SEM) of MyoD and myogenin in control (CTR) and unilateral oviduct-ligated sows (LIG).

Treatment	ΔC_T	$\Delta\Delta C_T$ (ΔC_T - $\Delta C_{T,CTR}$)	Normalized myogenin amount relative to control $2^{-\Delta\Delta C_T}$
CTR	5.59 ± 0.25	0.00 ± 0.25	1.0 (0.8 – 1.2)
LIG	4.85 ± 0.29	-0.74 ± 0.29	1.7 (1.4 – 2.0)

Table 3.3. Relative fold difference of myogenin expression between control (CTR) sows and unilateral oviduct-ligated (LIG) sows.

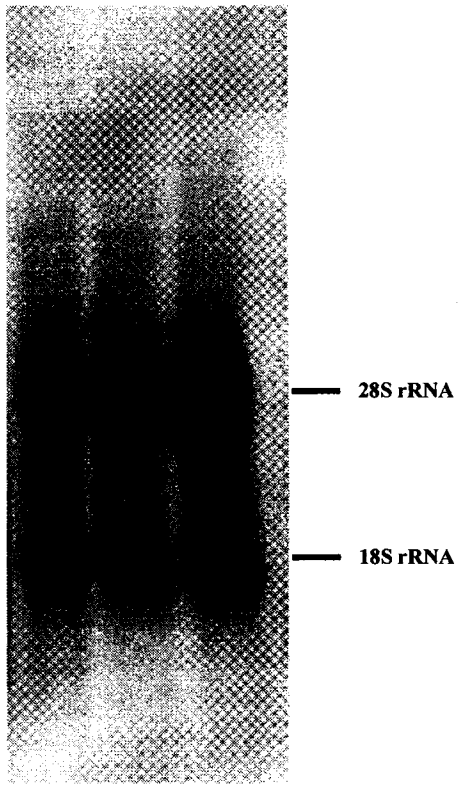


Figure 3.1. RNA extracted from paraformaldehyde-fixed embryos run on a 1% (w/v) agarose gel. The strong presence of bands representing 28S and 18S ribosomal RNA suggests the successful extraction of intact mRNA.

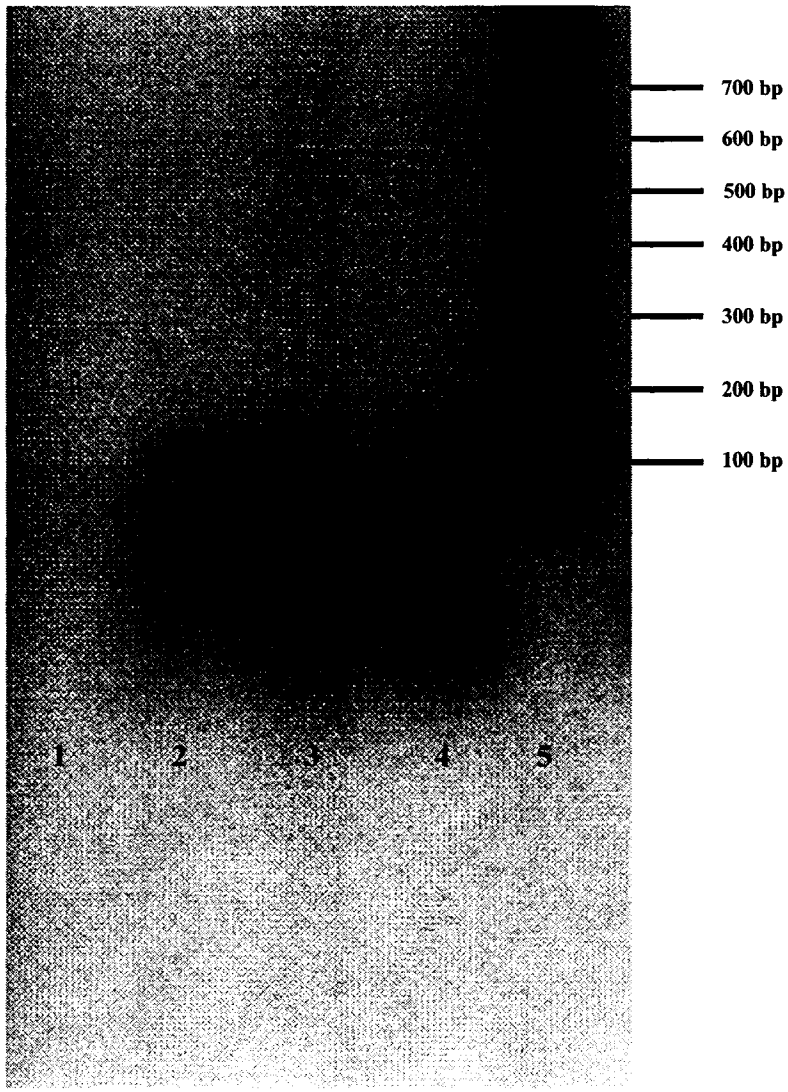


Figure 3.2. Products from real-time RT-PCR reaction run on a 2.25% agarose gel. Lane 1: no template control. Lane 2: MyoD. Lane 3: myogenin. Lane 4: cyclophilin. Lane 5: 100 bp marker. PCR showed products of expected size if amplification occurred from RNA but not from DNA (Table 1).

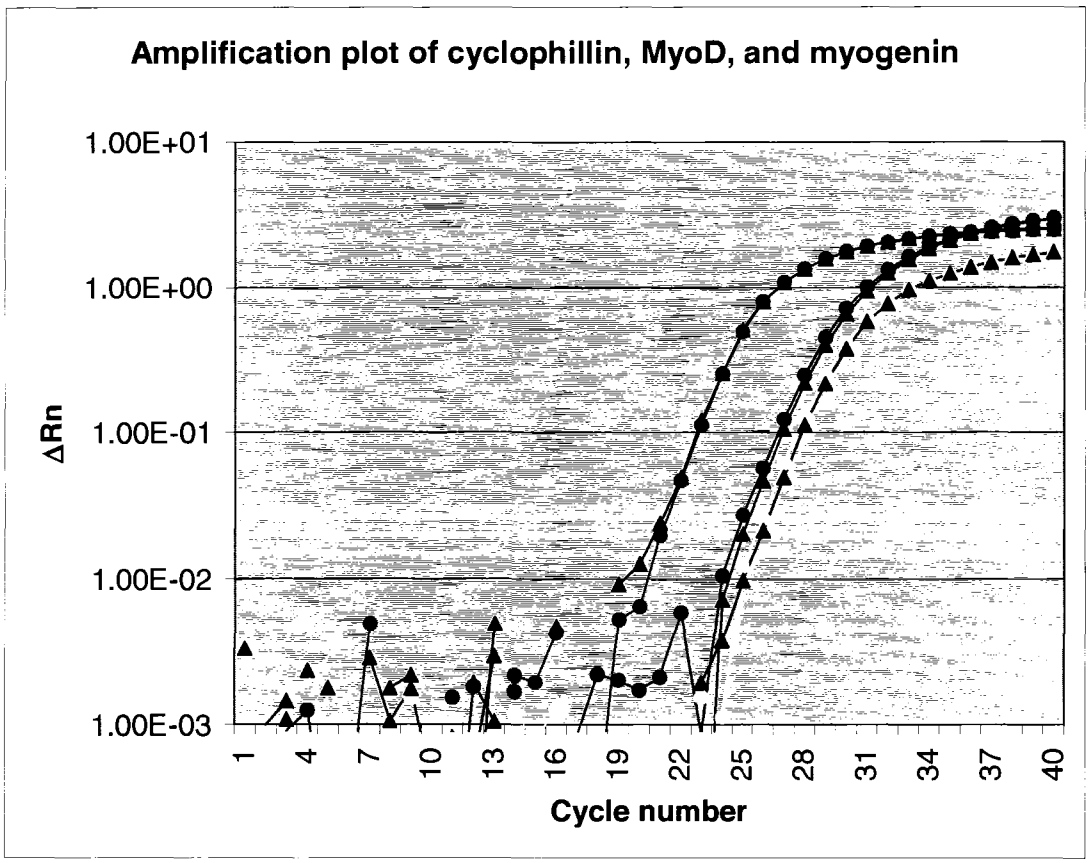


FIGURE 3.3. Amplification plots of real-time RT-PCR reactions. Amplification plots representing cyclophilin (blue), MyoD (red), and myogenin (green). All reactions were run in duplicate and averaged to compensate for inter-reaction variability (represented by corresponding lighter colour). The higher the cycle number for a given ΔRn for a given gene the lower the initial amount of transcript present (see appendix A.1.).

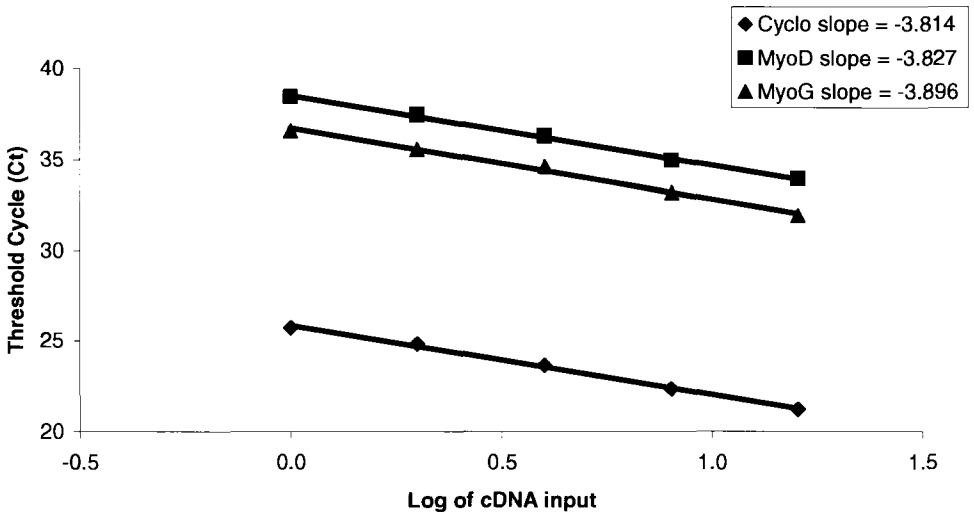
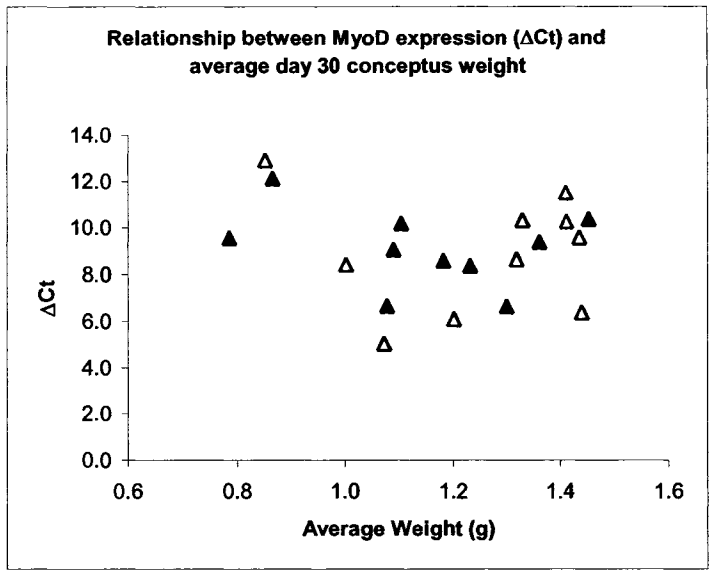


FIGURE 3.4. Real-time PCR efficiency determination of the reference gene cyclophilin (Cyclo), the target gene MyoD, and the target gene myogenin (MyoG). C_T at threshold cycle is plotted versus the log of relative input amounts of cDNA (see Appendix A.1.).



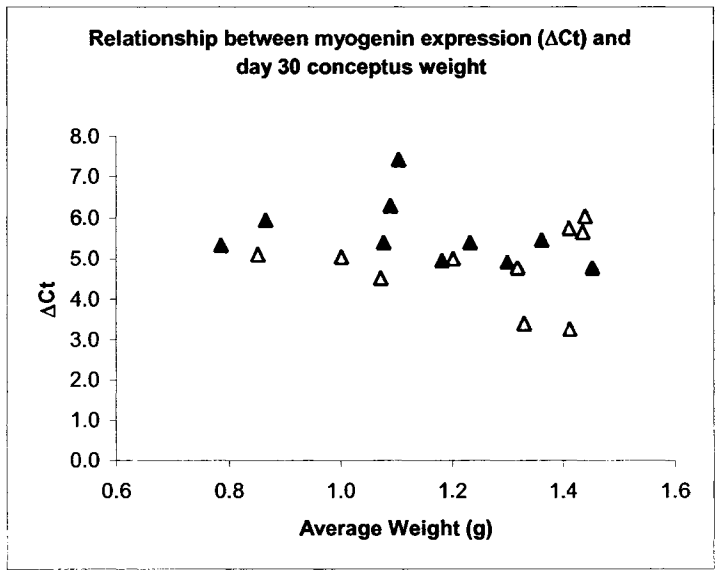
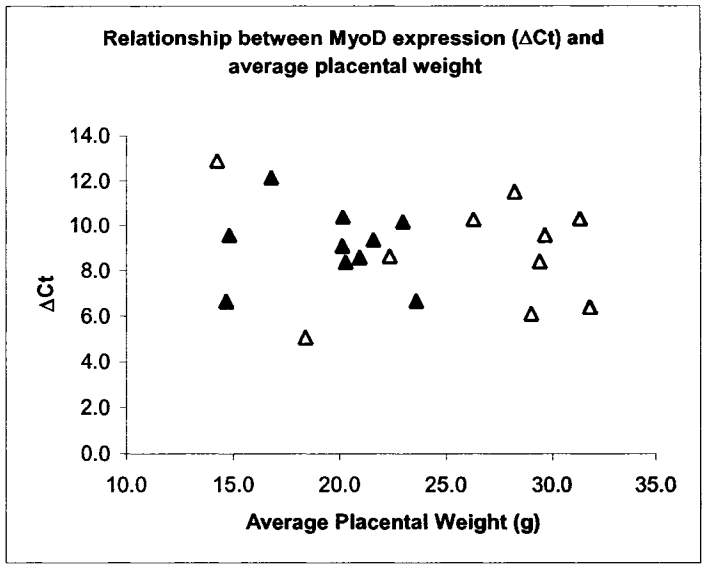


Figure 3.5. Lack of relationship between average expression levels of either MyoD (top) or myogenin (bottom) and average embryo weight within each sow from both CTR (▲) and LIG (Δ) treatment groups.



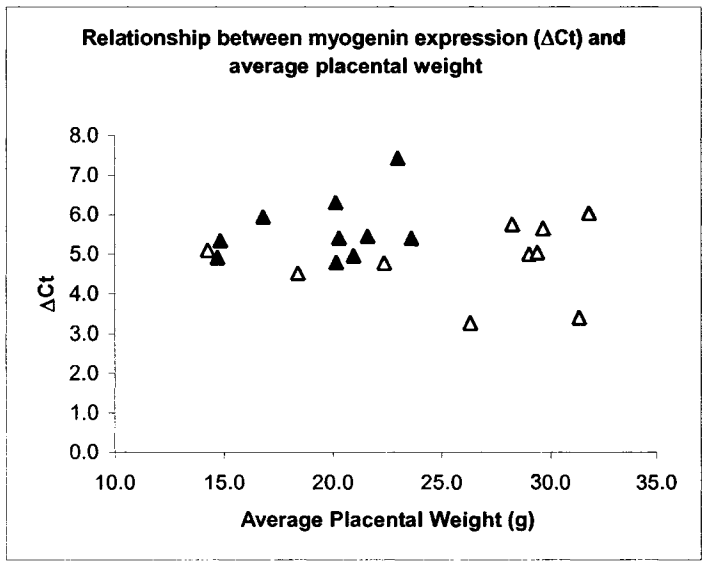


Figure 3.6. Lack of relationship between average expression levels of either MyoD (top) or myogenin (bottom) and average placental weight within each sow from both CTR (▲) and LIG (Δ) treatment groups.

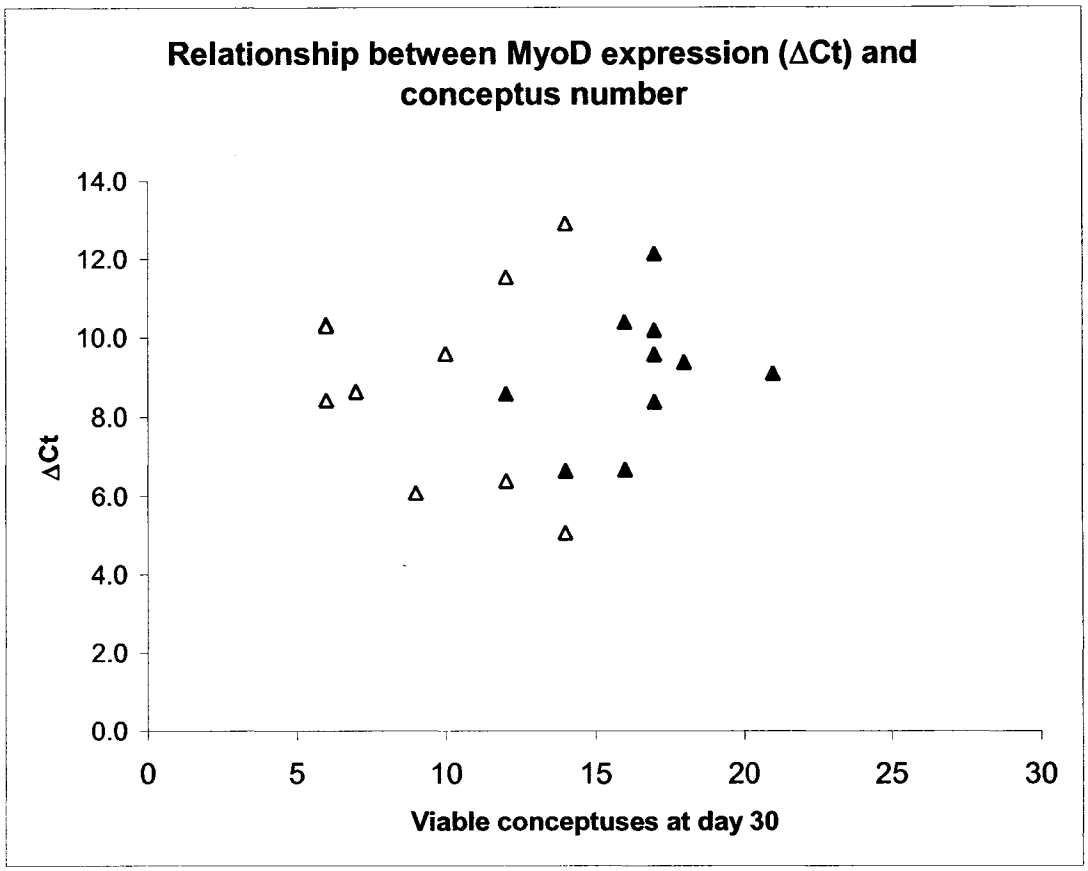


Figure 3.7. Lack of relationship between average expression levels of MyoD and viable embryo numbers at day 30 of gestation within each sow from both CTR (▲) and LIG (Δ) treatment groups.

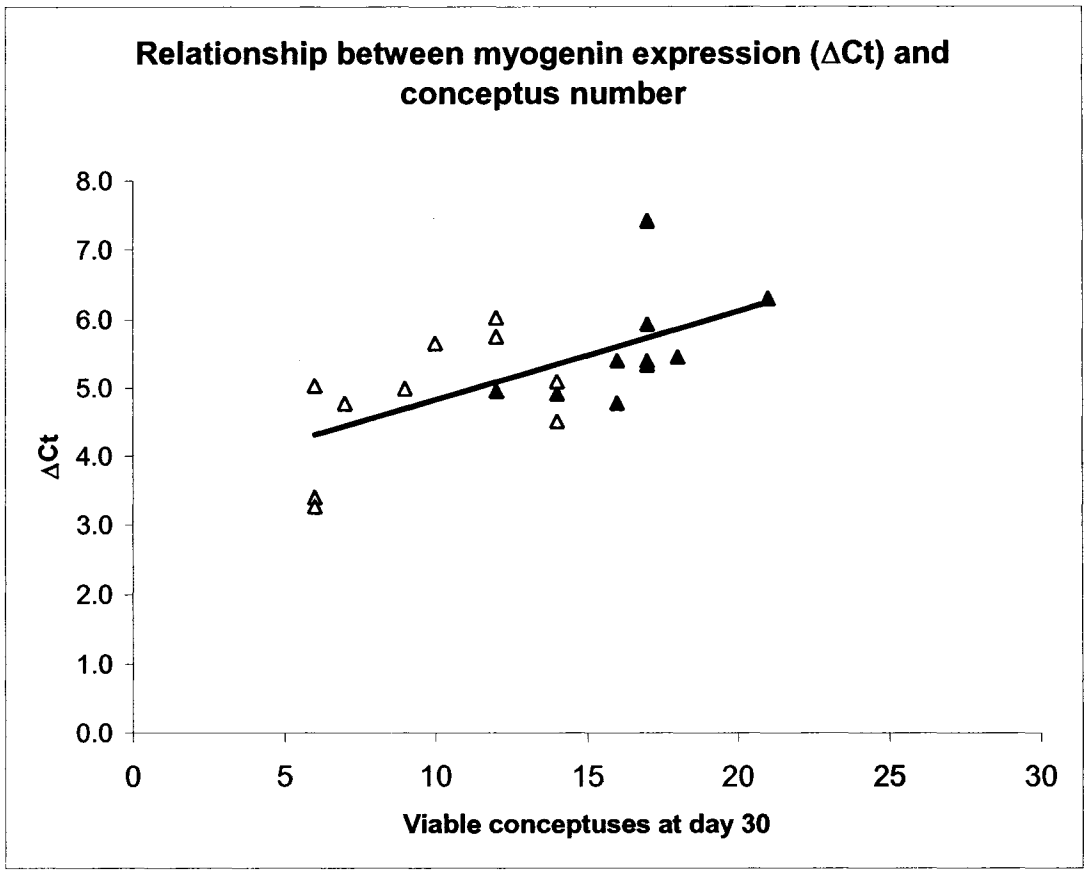


Figure 3.8. Positive correlation between expression levels (ΔCt) of myogenin and viable embryo numbers at day 30 of gestation within each sow from both CTR (▲) and LIG (Δ) treatment groups ($R^2 = 0.40$; $P < 0.0028$). This signifies that as embryo numbers increase the average expression levels of myogenin of all embryos within a sow decreases.

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CHAPTER FOUR

UTERINE CROWDING AFFECTS THE EXPRESSION OF MRFS IN MALES BUT NOT IN FEMALES

4.1. INTRODUCTION

In the pig, gender can play a significant role in the growth and development of a embryo during gestation. Wise and Christensen (1992) observed that male pig fetuses and placentas are heavier than their female counterparts by as early as day 70 of gestation. This same study also showed that the sex of surrounding fetuses appears to be important in determining fetal growth potential, as a male fetus surrounded by two females, or vice versa, was significantly lighter at day 104 of gestation. Thus, it appears that the gender of one embryo may exert an influence over the development of its neighbour. It was suggested that possible endocrine influences occurring between day 20 and 40 of gestation might not become apparent until later in gestation (Wise and Christenson, 1992).

Using reciprocal embryo transfer between animals of Yorkshire and Meishan breeds, a study on the effect of sex on embryo development at day 12 of gestation indicated that uterine environment rather than sex played a role in determining the growth rate of a embryo (Kaminski et al., 1996). Analysis of estrogen secretions also showed no significant differences between males and females. In addition, from unpublished

observations, the authors also found no apparent effects of sex on fetal or placental mass at day 30 of gestation.

Unilateral oviduct-ligation has been used previously as an experimental strategy to limit the number of embryos *in utero*, so that ligated (LIG) sows achieve a relatively lower state of crowding than control (CTR) animals. It was observed that higher numbers of secondary muscle fibers were present in day 90 fetuses from LIG sows than in fetuses from CTR sows (Town et al., 2004). It was also observed that placental mass at day 30 was adversely affected by increased uterine crowding. In chapter 3, analysis of expression of the myogenic regulatory factors (MRFs), MyoD and myogenin, showed that a higher expression level of myogenin was observed in day 30 embryos of LIG sows than in CTR sows. There was no significant correlation between expression levels and either embryo weight or placental weight. There was however, a significant inverse correlation between expression levels of myogenin and viable embryo number.

It therefore appears that muscle accretion of an individual embryo may not be apparent by measuring its embryo or placental weight alone at day 30 of gestation. Furthermore, the lack of differences due to gender in embryo growth and development may not indicate the lack of gender effect on myogenic development. Thus, the endocrine influences of gender on development that have been proposed to possibly act between day 20 and 40 of gestation (Wise and Christenson, 1992), may yet influence MRF expression.

Since gender appears to influence both the individual development of a embryo and the development of the litter as a whole, the purpose of this study was to reexamine the expression of the MRFs, MyoD and myogenin, reported in the previous chapter with respect to the gender of the embryo, and to determine if the relative state of uterine crowding influences any potential effects of gender. It is possible that the sex of the embryo influences the expression of MRFs, and that this effect is heightened due to increased numbers of viable embryos.

4.2. MATERIALS AND METHODS

4.2.1. Animals and Embryo collection

Embryos that were collected by Town et al. (2004), and used for real-time PCR analysis in chapter 3 three were used for analysis of the effects of gender in this study.

4.2.2. DNA Extraction

1.0 ml aliquots of tissue lysate (see chapter three) were supplemented with 500 μg proteinase K (Sigma Chemical Co.) and incubated at 45°C for 5 days. DNA extraction was performed using phenol (pH 7.9)/chloroform/isoamyl alcohol

(25:24:1), followed by further extraction with 1.0 ml chloroform. DNA was precipitated using 2ml of 100% ethanol and 100 µl of 6M ammonium acetate and incubated overnight at -20°C. This was followed by a wash of 75% ethanol. DNA pellets were then dissolved in DEPC treated H_2O to a final concentration of approximately 1µg/µl. Gel electrophoresis of extracted DNA samples was performed on a 1% (w/v) agarose gel to verify the integrity of the DNA sample.

4.2.3. Sex-Typing PCR

The sex of 260 day 30 embryos was determined using a previously developed PCR protocol (Pomp et al., 1995). One primer set was designed to amplify a 157 base pair segment of the Y chromosome specific-region (SRY) and another primer set was designed to amplify a 445 base pair segment of the X chromosome-specific region (ZFX). DNA samples were coamplified with both primer sets, and PCR products were resolved using a 2% (w/v) agarose gel. PCR of samples resulting in the amplification of both the 157 base pair fragment and the 445 base pair fragment were identified as males, while those showing only the 445 base pair fragments were identified as females. The PCR protocol was repeated with samples of DNA isolated from adult animals whose sex was verified visually, in order to verify the results of amplification of the samples.

4.2.4. Statistical Analysis

Data was analysed using the mixed classification model of SAS (SAS Inst., Cary, N.C., USA) to determine the effect of gender on MyoD and myogenin expression within each treatment group (LIG and CTR). Sex was used as a fixed factor, and the sow was included as a random factor. Embryos were also grouped by gender, and analysis was also performed to the determine the effect of the treatment within each gender, using treatment as a fixed factor, and the sow as a random factor. Significance was considered at $P < 0.05$. The results are presented as means of sows ± SEM.

4.3. RESULTS

PCR of DNA from individual day 30 embryo samples resulted in easily identifiable bands representing the SRY and ZFX genes, allowing the identification of the sex of the embryo. Banding patterns of embryos were compared to the banding patterns produced by PCR of porcine DNA from which the gender was known. An example of PCR reactions of embryo samples and the control samples are shown in Figure 4.1.

Table 4.1 shows a summary of embryo and placental weights of male and female embryos from CTR sows. Analysis of the weight of embryos from CTR sows showed no significant difference between male and female embryos. There was also no evidence of any significant difference in placental weights of male and female embryos.

An analysis of the weight of embryos from LIG sows also showed no significant difference between males and females. Likewise, placental weights of embryos from LIG sows also showed no significant differences between male and female embryos (Table 2).

MyoD and myogenin gene expression data was reanalyzed with respect to the sex of the embryo. When compared within treatment groups, male and female embryos from CTR sows did not significantly differ in the expression of MyoD. The expression of myogenin was also not significantly different between genders in CTR sows (Table 4.1.) Male and female embryos from LIG sows were also not significantly different in the expression either MyoD or myogenin (Table 4.2.).

Samples were then split male and female groups and analyzed between treatment groups (Table 4.3 and 4.4). Female embryos did not show a significant difference in embryo weight between the CTR and LIG. Analysis of male embryos also did not reveal a significant difference in embryo weight between CTR and LIG groups. However, there was a significant difference in placental weights between CTR and LIG males, as well as between CTR and LIG females.

MyoD expression also did not vary between CTR and LIG groups for either gender of embryo (Table 4.3 and 4.4). Analysis of myogenin expression comparing CTR and LIG treatments in females also showed no significant difference between female embryos (Table 4.3). However, in male embryos there was a significant difference

between the treatment groups ($P < 0.01$; Table 4.4). This represents an approximately 2.1 fold-increase in myogenin expression in male embryos from LIG sows than in male embryos from CTR sows (Table 4.5).

Further analysis of MyoD expression in females showed that no relationship existed with embryo weight, placental weight, or viable embryo numbers (Figures 4.2, and 4.3). This was likewise the case for male embryos, as no significant relationship was observed between MyoD expression and embryo weight, placental weight, or viable embryo numbers (Figures 4.4, and 4.5).

Myogenin expression in females also showed no significant relationship with embryo weight, placental weight, or viable embryo numbers (Figures 4.6, and 4.7). Male embryos showed no significant relationship between myogenin expression and embryo weight or placental weight (Figure 4.8). However, there was a significant positive correlation between ΔCt and viable embryo number for male embryos at day 30 ($R^2 = 0.5927$; $P < 0.0001$; Figure 4.9).

4.4. DISCUSSION

An initial analysis of embryo weights of male and female embryos showed no significant differences between the two sexes in either the CTR or the LIG groups. This is in agreement with a previous study that observed no effect of sex on these aspects of development during early gestation (Kaminski et al., 1996). It has been

previously shown that relatively crowded embryos have lower average placental weights at day 30 of gestation (Town et al., 2004). Results from this present study also suggest that the effects of uterine crowding on placental weight are independent of the gender of the embryo at day 30 of gestation.

No differences in the expression levels of MyoD or myogenin between male and female embryos were observed within either CTR or LIG treatment groups. These results suggest that MRF expression remained consistent regardless of sex. Comparisons of MyoD expression of a given gender between CTR and LIG treatment groups also showed no significant difference. Analysis into the relationship between MyoD expression and embryo weight, placental weight, or viable embryo number at day 30 indicated no significant correlations with either sex. All results were consistent with the results observed in chapter three.

Interestingly however, analysis of myogenin expression between CTR and LIG groups indicated a significant difference in males. This was not the case in females as no significant difference was observed between treatments. Additional analysis of the relationship of myogenin expression revealed no significant relationship between myogenin expression in either sex and embryo weight or placental weight. In Chapter 3 a, significant correlation was observed between average myogenin expression in all sows and the number of viable embryos. However, when each gender was examined individually, only males showed a significant correlation with

viable embryo number, while myogenin gene expression no longer showed any relationship.

The fold difference of males between treatment groups represents an approximately 2.1-fold increase in myogenin expression in males from LIG sows over CTR sows. In the previous chapter a 1.6-fold increase of the treatment groups as a whole was also observed. Thus, the results from this study suggest that although differences in myogenin expression occur due to the relative state of uterine crowding, this difference may be largely attributed to an effect on male embryos.

The lack of difference in embryo and placental weights due to gender observed by Kaminsky et al. (1996) was also observed in this study. However, the lack of association between either embryo weight, or placental weight, and myogenin expression observed in the previous chapter suggest that myogenesis may be independent of these growth characteristics. The lack of correlation of either MyoD or myogenin between embryos of either gender and embryo weight or placental weight are consistent with the contention that MRF expression occurs independently of these two developmental characteristics.

Furthermore, although myogenin expression is affected by uterine crowding, this effect seems to be specific to male embryos, while female embryos appear to remain unaffected. Therefore, there may be other factors caused by uterine crowding to which male embryos are more sensitive. It may also be possible that a mechanism is

in place that protects female embryos from the effects of crowding, to help ensure the proper development of females.

Kochhar et al. (2001) proposed that the sex chromosomes provide such a protection mechanism. The Y-chromosome is believed to encode transcription factors that may accelerate growth, while the X-chromosome encodes genes that regulate growth. Therefore, before X-inactivation in females, higher levels of X-chromosome genes may help reduce the effects of environmental stress (Kockhar et al., 2001). If such a protection mechanism does in fact exist, it may also act to restrict any possible benefits of reduced crowding on myogenesis.

Further study into whether or not the effect of uterine crowding on secondary muscle fiber numbers remains consistent at day 90 of gestation, with respect to gender, would help support the idea of a gender-specific response. Based on the results of this study, strategies that target males may not only help to mitigate the effects of increased uterine crowding on the determination of secondary muscle fiber numbers, but may also be of interest in increasing the muscle fiber numbers in situations where uterine crowding is not an issue.

Parameter	CTR "Relatively Crowded" Males	CTR "Relatively Crowded" Females	P-Value
Embryo Weight	1.20 ± 0.05	1.16 ± 0.05	0.1172
Placental Weight	19.67 ± 1.03	19.39 ± 1.00	0.9742
MyoD ΔC_T	9.15 ± 0.45	8.96 ± 0.69	0.5959
myogenin ΔC_T	5.77 ± 0.23	5.35 ± 0.32	0.1606

Table 4.1. Influence of sex on embryo weight, placental weight, MyoD expression, and myogenin expression on embryos from a relatively crowded uterine environment.

Parameter	LIG "Non-Crowded" Males	LIG "Non-Crowded" Females	P-Value
Embryo Weight	1.27 ± 0.07	1.19 ± 0.06	0.3194
Placental Weight	27.08 ± 1.73	24.33 ± 1.98	0.1895
MyoD ΔC_T	8.84 ± 0.82	8.98 ± 0.77	0.3445
myogenin ΔC_T	4.71 ± 0.33	5.02 ± 0.30	0.2608

Table 4.2. Influence of sex on embryo weight, placental weight, MyoD expression, and myogenin expression on embryos from a relatively non-crowded uterine environment.

Parameter	CTR "Relatively Crowded" Females	LIG "Non-Crowded" Females	P-Value
Embryo Weight	1.16 ± 0.05	1.19 ± 0.06	0.4029
Placental Weight	19.39 ± 1.00	24.33 ± 1.98	< 0.001
MyoD ΔC_T	8.96 ± 0.69	8.98 ± 0.77	0.4136
myogenin ΔC_T	5.35 ± 0.32	5.02 ± 0.30	0.9336

Table 4.3. Influence of the relative state of crowding on embryo weight, placental weight, MyoD expression, and myogenin expression in female embryos.

Parameter	CTR "Relatively Crowded" Males	LIG "Non-Crowded" Males	P-Value
Embryo Weight	1.20 ± 0.05	1.27 ± 0.07	0.8270
Placental Weight	19.67 ± 1.03	27.08 ± 1.73	< 0.001
MyoD ΔC_T	9.15 ± 0.45	8.84 ± 0.82	0.7895
myogenin ΔC_T	5.77 ± 0.23	4.71 ± 0.33	0.0014

Table 4.4. Influence of the relative state of crowding on embryo weight, placental weight, MyoD expression, and myogenin expression in male embryos.

Treatment	ΔC_T	$\Delta\Delta C_T$ (ΔC_T - $\Delta C_{T,CTR}$)	Normalized myogenin amount relative to control $2^{-\Delta\Delta C_T}$
CTR	5.77 ± 0.23	0.00 ± 0.23	1.0 (0.8 – 1.2)
LIG	4.71 ± 0.33	-1.06 ± 0.33	2.1 (1.7 – 2.6)

Table 4.5. Relative fold difference of myogenin expression in male embryos between control (CTR) sows and unilateral oviduct-ligated (LIG) sows.

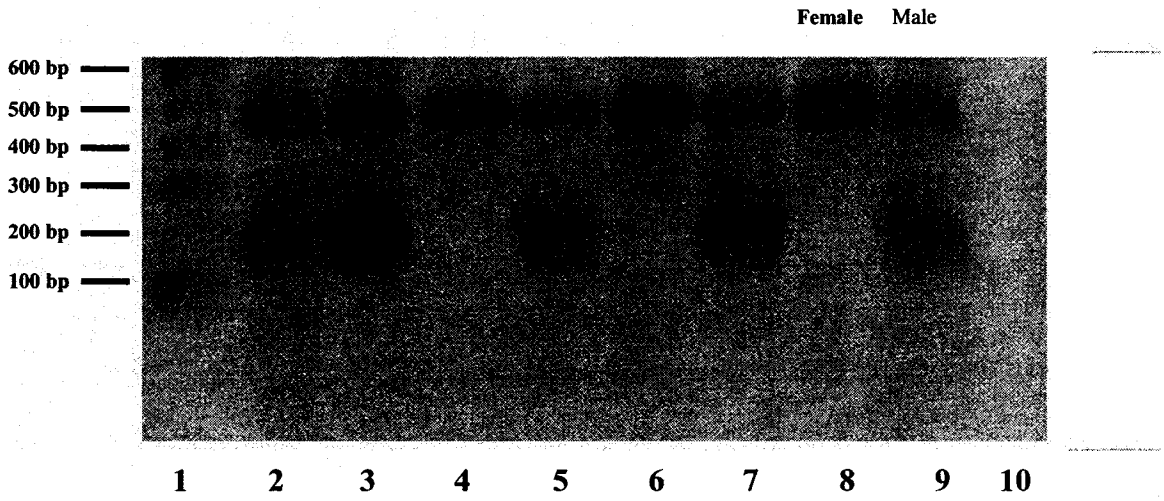


Figure 4.1. Determination of sex of embryos using PCR. Lane 1: 100kb marker. Lane 2-7: PCR products of embryos of which the sex was previously unknown. Lanes 8: PCR product from genomic DNA of a female embryo. Lane 9: PCR product from genomic DNA of a male embryo. Lane 10: No template control. The upper band represents the ZFY (445 bp) gene common to both male and females, while the lower band represents the male specific SRY gene (157 bp). Thus lanes 2, 3, 5, 7, and 9 represent males, while lanes 4, 6, and 8 represent females.

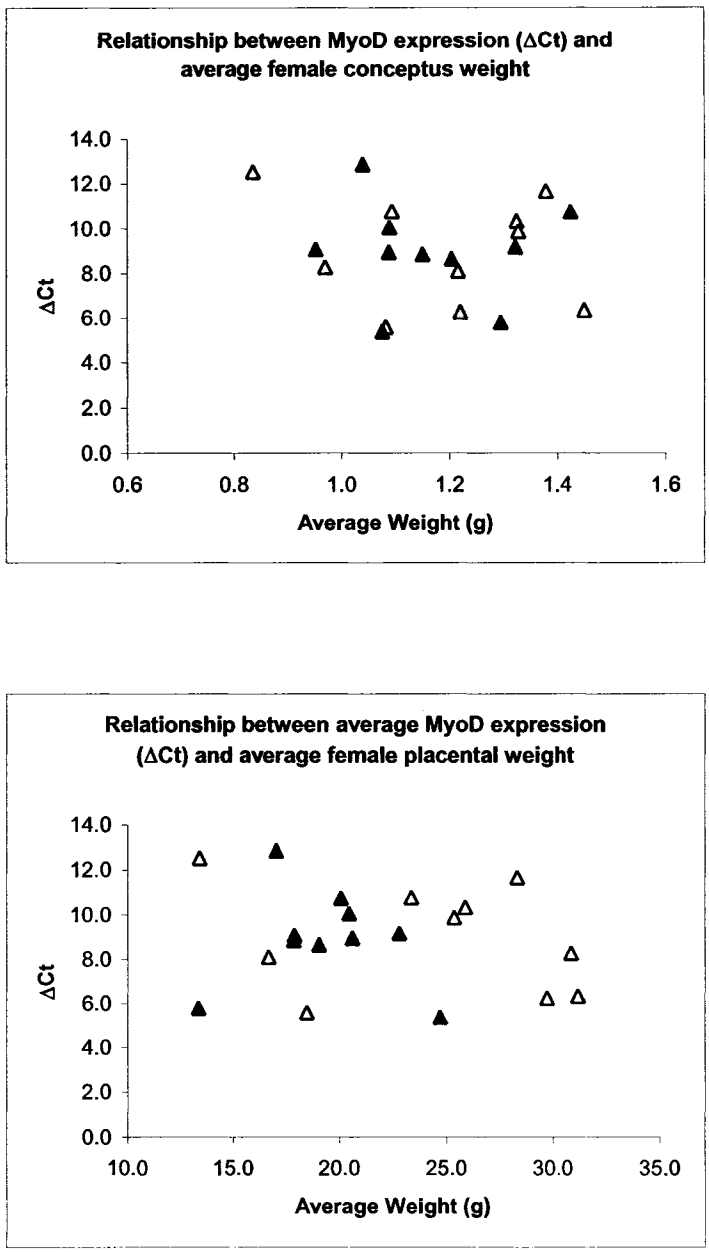


Figure 4.2. Lack of relationship between average MyoD expression (ΔCt) and either average female embryo weight (top) or average female placental weight (bottom) within sows from both CTR (▲) and LIG (Δ) treatment groups.

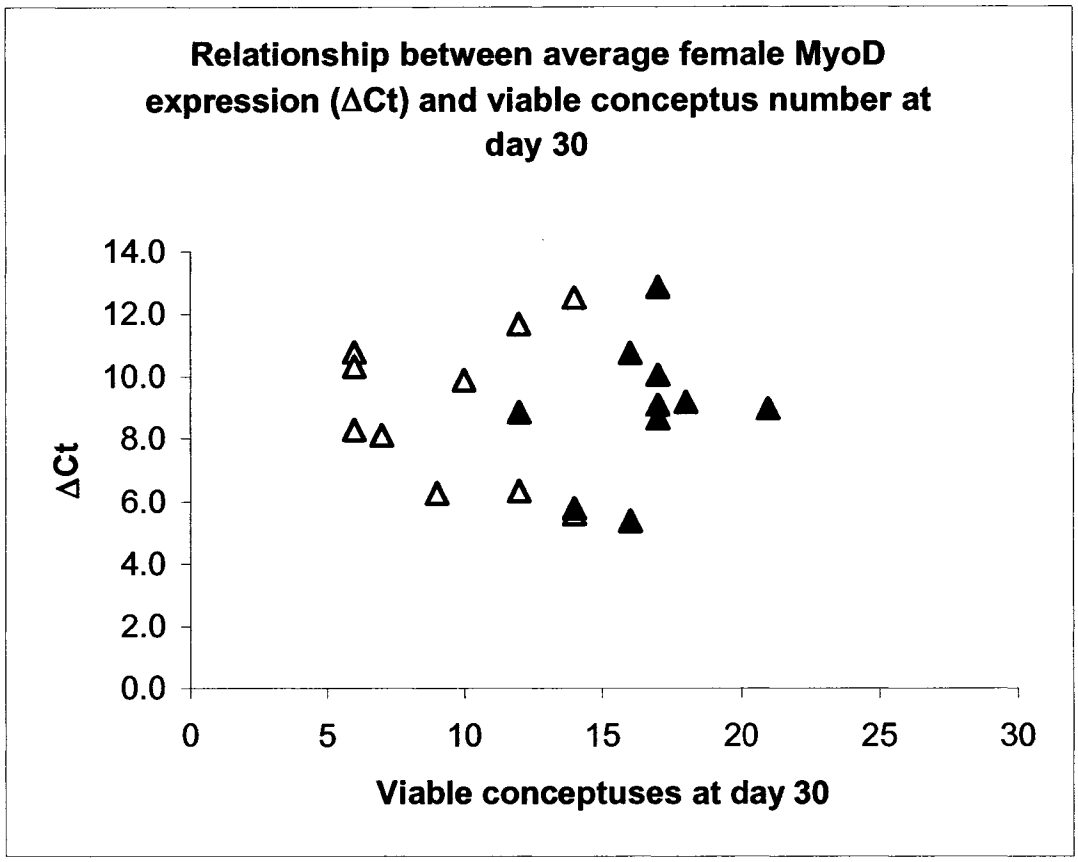
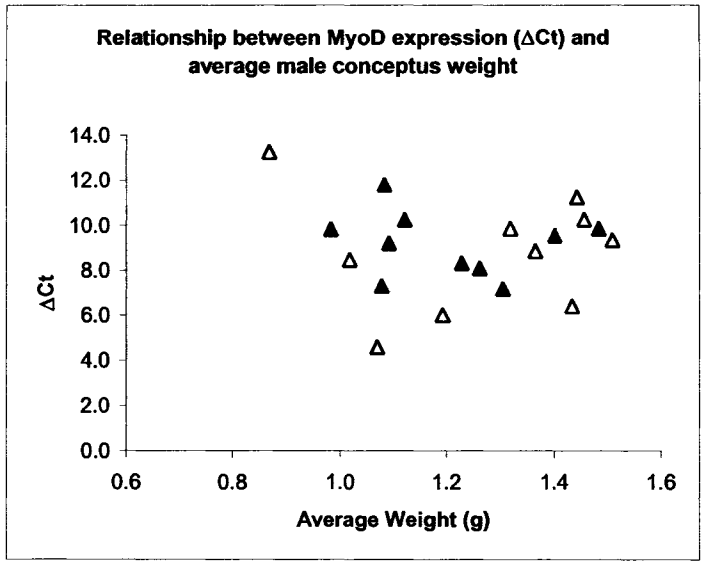


Figure 4.3. Lack of relationship between embryo number at day 30 and average MyoD (ΔCt) of female embryos of both CTR (▲) and LIG (Δ) sows.



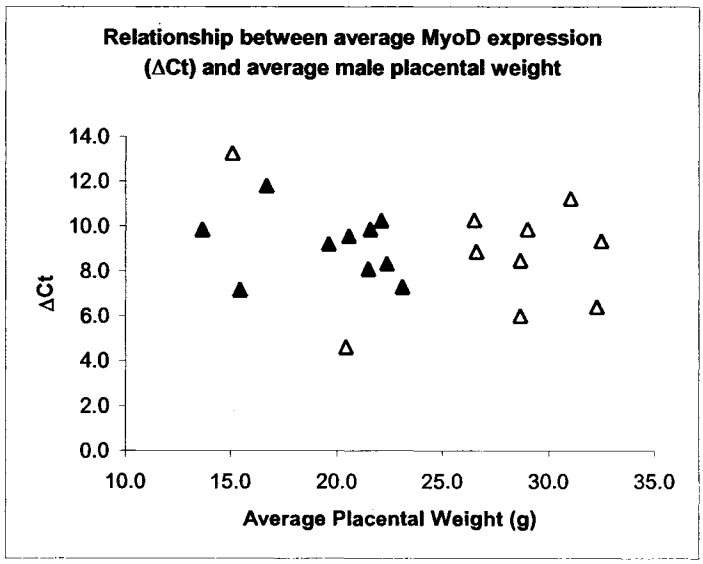


Figure 4.4. Lack of relationship between average MyoD expression (ΔCt) and either average male embryo weight (top) or average male placental weight (bottom) within sows from both CTR (▲) and LIG (Δ) treatment groups.

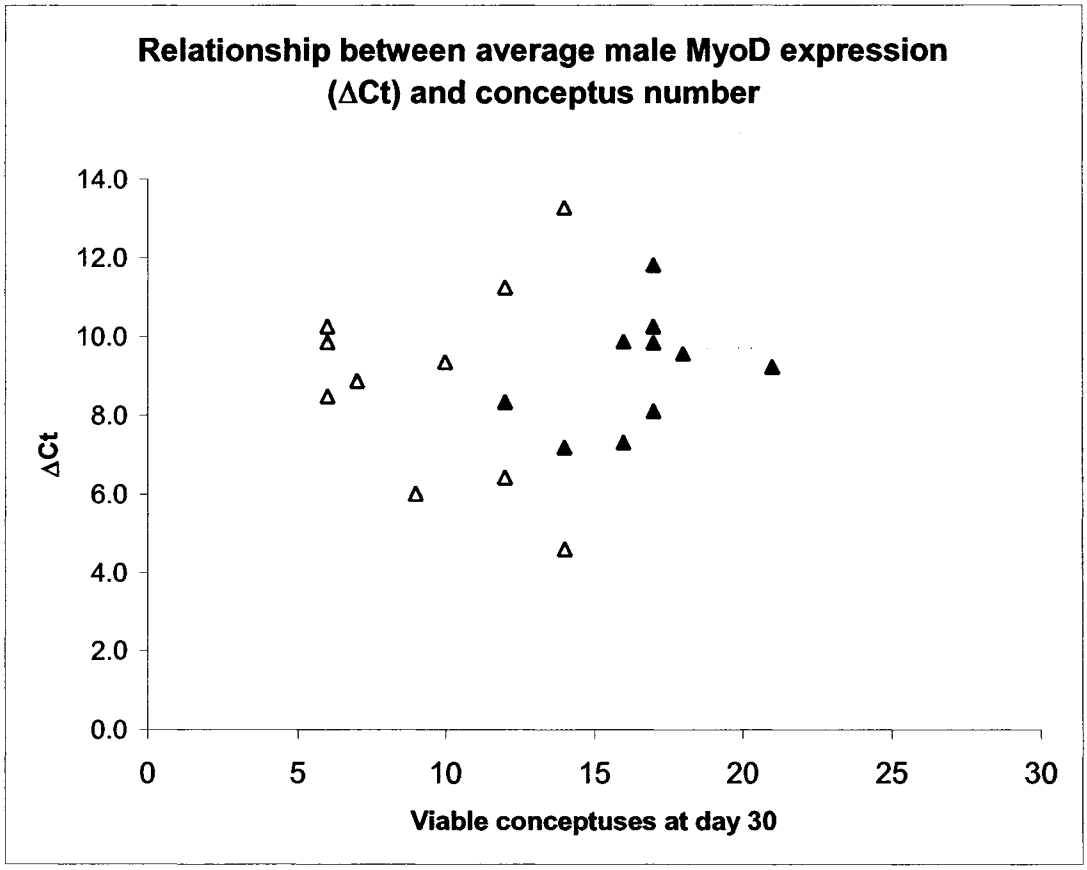
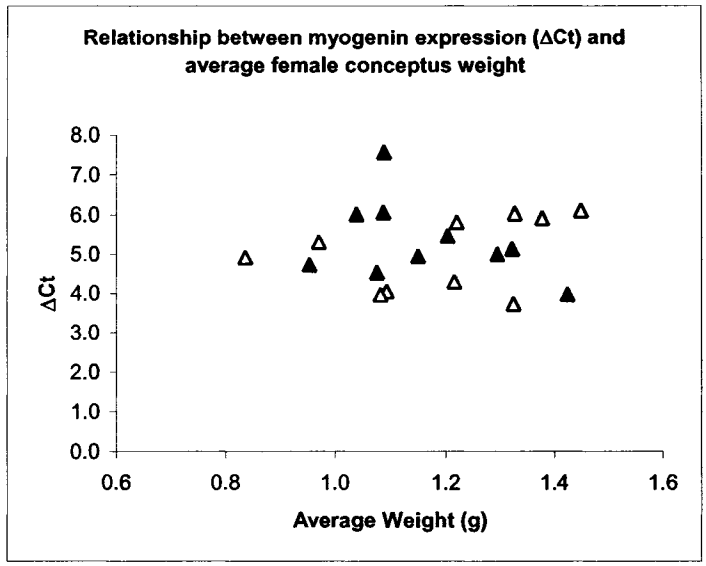


Figure 4.5. Lack of relationship between embryo number at day 30 and average MyoD (ΔCt) of male embryos of both CTR (▲) and LIG (Δ) sows.



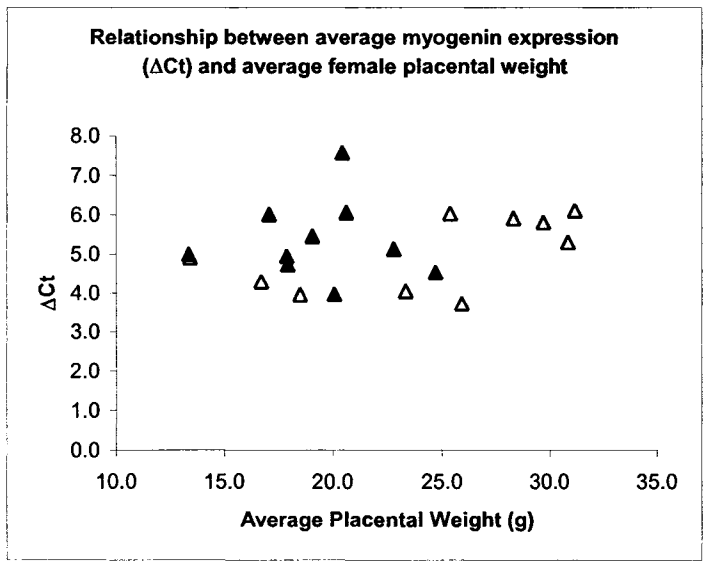


Figure 4.6. Lack of relationship between average myogenin expression (ΔCt) and either average female embryo weight (top) or average female placental weight (bottom) within sows from both CTR (▲) and LIG (Δ) treatment groups.

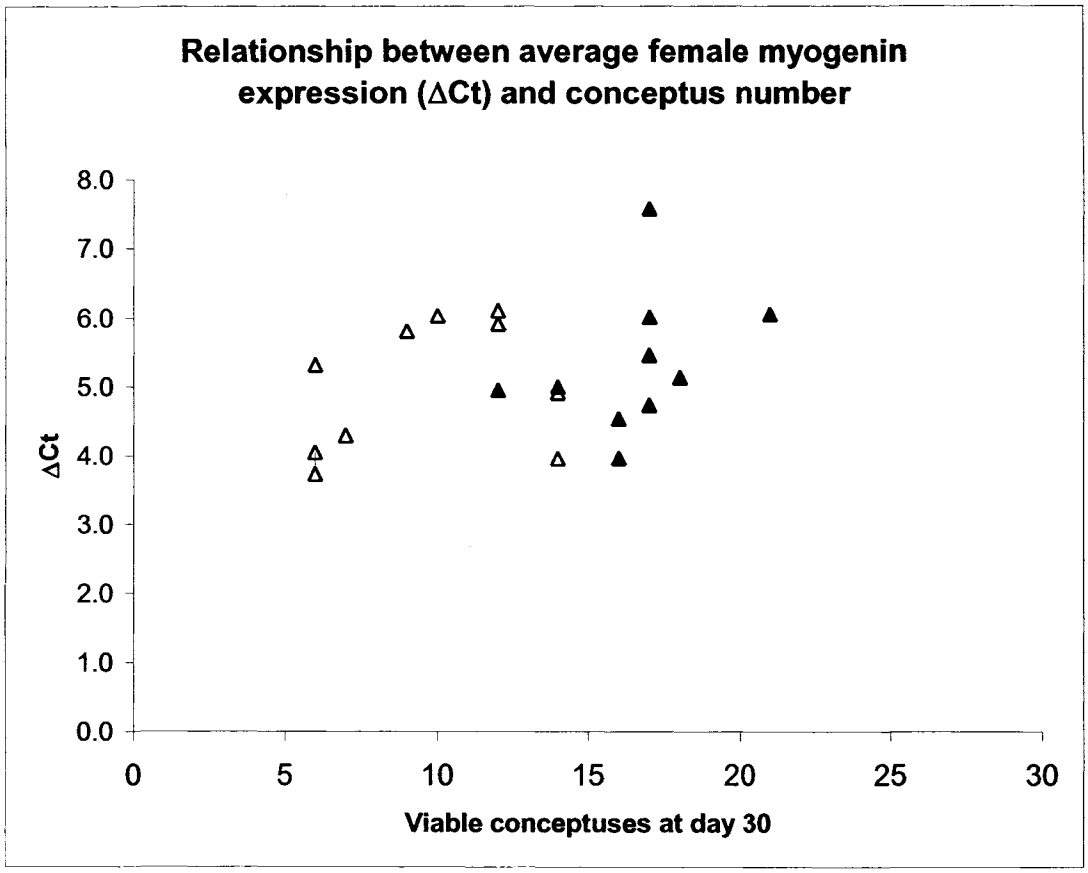
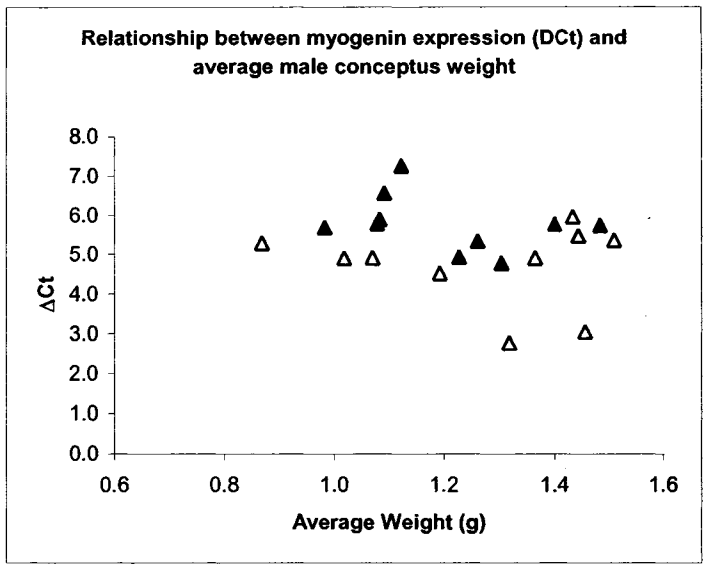


Figure 4.7. Lack of relationship between embryo number at day 30 and average myogenin (ΔCt) of female embryos of both CTR (▲) and LIG (Δ) sows.



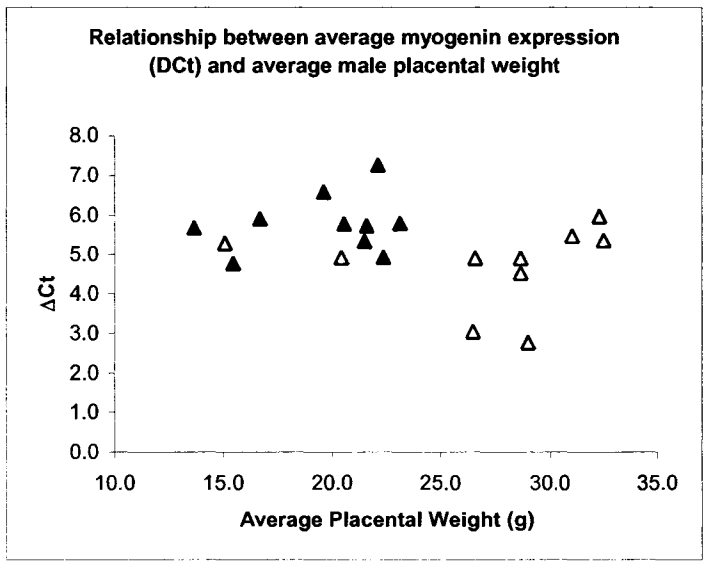


Figure 4.8. Lack of relationship between average myogenin expression (ΔCt) and either average male embryo weight (top) or average male placental weight (bottom) within sows from both CTR (▲) and LIG (Δ) treatment groups.

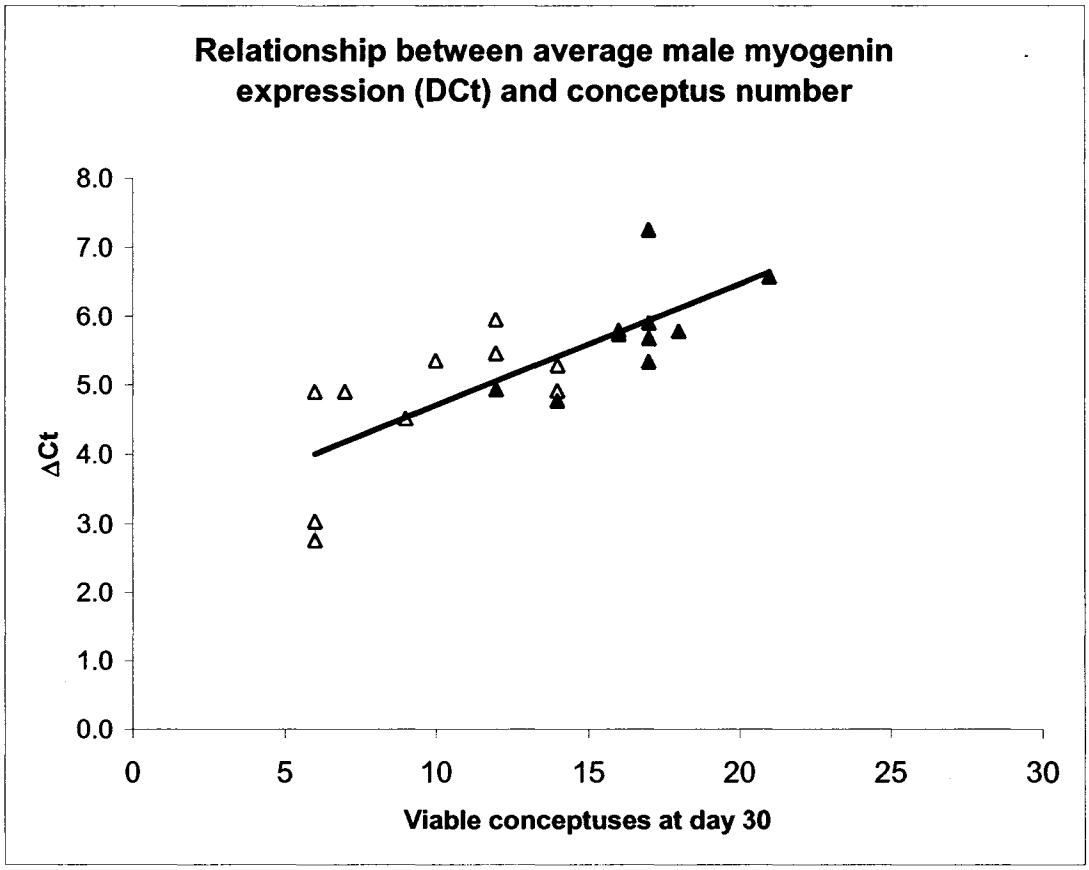


Figure 4.9. Positive relationship (R^2 = 0.5926; P < 0.0001) between average myogenin (ΔCt) of male embryos of both CTR (▲) and LIG (Δ) sows.

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CHAPTER FIVE

DETECTION OF THE EXPRESSION OF MYOGENIC REGULATORY FACTORS USING *IN SITU* HYBRIDIZATION

5.1. INTRODUCTION

The total number of muscle fibers is the primary determinant in the potential for muscle mass. The formation of muscle fibers is completed prior to birth, making gestation a critical period in the determination of muscle fiber numbers. Previously, a study was performed to analyze the effect of uterine crowding in the pig on development of muscle in offspring during gestation *in utero* (Town et al., 2004). In this study it was observed that a higher relative state of uterine crowding resulted in lower numbers of secondary muscle fibers in day 90 fetuses. The development of secondary muscle fibers begins at approximately day 50 and is completed by around day 90 of gestation (Wigmore and Stickland, 1983 and references therein). However, studies into the effects of maternal nutrition during pregnancy suggest that the determination of muscle fiber numbers occurs between day 25 and 50 (Dwyer et al., 1994). Thus, gestational conditions prior to the onset of secondary muscle fibers appear to be critical in the ultimate determination of muscle fiber numbers.

The effects of maternal undernutrition of guinea-pigs during pregnancy have established a negative effect on the number of muscle fibers formed in offspring

(Dwyer et al., 1995). Furthermore, maternal undernutrition in the guinea-pig results in a reduction in placental size, and it was suggested that the effects of undernutrition on embryo development are mediated by effects on the placenta (Dwyer et al. 1992). Additional analysis by Town et al. (2004) on the effects of uterine crowding at day 30 of gestation revealed a significant decrease in placental weight in relatively crowded sows than in relatively non-crowded sows. It is therefore possible that the effects of uterine crowding on secondary muscle fiber numbers are caused through changes at day 30 of gestation.

All skeletal muscle, with the exception of the muscles of the head, are derived from structures known as somites. The somites are structures formed from the presomitic mesoderm that ultimately give rise to most of the skeletal muscle in the vertebrate body (reviewed by Christ and Ordahl, 1995). The formation of somites begins near the head and progresses on either side of the neural tube in a rostral-caudal direction. As a somite matures, a structure termed the dermomyotome is formed, and it is this region of the somite that is responsible for providing cells for myogenesis.

The myogenic regulatory factors (MRFs) consist of four genes, Myf-5, MyoD, myogenin and MRF4 that play pivotal roles during myogenesis (reviewed by Arnold and Braun, 2000). From studies in the mouse, the expression patterns of the MRFs have been shown to follow distinct rostral-caudal spatiotemporal patterns of development (Sassoon et al., 1989; Bober et al., 1991; Hinterberger et al., 1991; Ott et al., 1991; see section 2.3.1.). *In situ* hybridization of MyoD in day 30 pig embryos

pig shows that the expression of MyoD is seen in the somites and developing muscles of the back (Chang et al., 1995)

Since it has previously been observed that relatively higher states of uterine crowding result in a reduction in the formation of secondary fiber numbers (Town et al., 2004), an elucidation of the mechanism by which MRF expression is altered would be useful in developing strategies to minimize any negative impacts of uterine crowding on the development of muscle. Because of the highly coordinated pattern of expression of the MRFs, a delay in the onset of expression of these genes may result in significant consequences for the formation of muscle fibers. The purpose of this chapter was to determine, using *in situ* hybridization, if any differences in the pattern of MRF expression could be observed at day 30 of gestation. It is hypothesized that an investigation of the expression of MRFs will reveal a significant delay in the normal spatiotemporal expression pattern of MRFs in relatively crowded embryos that will account for the eventual reduction of secondary fiber numbers observed at day 90 of gestation.

5.2. MATERIALS AND METHODS

5.2.1. Animals

290 embryos that were collected from animals from Town et al. (2004) were used for *in situ* hybridization analysis. Unilateral oviduct-ligation was used to limit the

number of embryos *in utero*. Day 30 embryos were collected from both ligated (LIG) and non-ligated control (CTR) sows for comparison.

5.2.2. Preparation of tissue

Reproductive tracts were recovered and dissected from all sows within one hour of slaughter as described by Town et al. (2004). Collected embryos were treated with paraformaldehyde in order to preserve the cellular morphology for possible qualitative analysis. Whole embryos were soaked in a 4% (w/v) buffered paraformaldehyde (85 mM PIPES, 25 mM HEPES, 5 mM EGTA, pH 7.0) and incubated at 4°C overnight. The embryos were then dehydrated with a series of increasing concentrations of methanol up to the final step in 100% methanol and stored at - 30°C.

Paraformaldehyde fixed embryos were then embedded in paraffin wax. Tissues were first incubated in a Histomatic Tissue Processor (Fisher, Model 166). The tissue processor performed two one-hour xylene incubations, followed by a series (100%, 70%, 50%, 30%) of half-hour methanol washes. The tissues were then left to incubate in melted paraffin at 60°C overnight. Embryos were then placed in a metal cup mold and melted paraffin was poured into the mold to create a block for thin sections to be cut from. After the paraffin had hardened, 7μm thin saggital sections of embryos were cut with a microtome and then mounted on poly-l-lysine slides.

5.2.3. Preparation of probes for *in situ* hybridization

RNA from frozen porcine muscle tissue was extracted using TRIZOL (Sigma Chemical Co.), for the preparation of the probes used for *in situ* hybridization by RT-PCR. Using previously reported sequences, primers were designed (Table 5.1.) to create probes to detect the MRFs Myf-5 (GenBank accession number Y17154), MyoD (GenBank accession number U12574), and myogenin (GenBank accession number X89007) using GeneJockey software (Biosoft, Ferguson, MO). Because of high homology between MRFs, primers were designed to amplify non-homologous regions of each MRF to ensure specificity of probe binding to the target MRF. RT-PCR was performed and products were run on a 1% (w/v) agarose gel. Products of the expected size (Table 5.1.) were extracted from the gel using a GeneClean kit (Bio101, La Jolla, CA) and blunt-end cloned into the EcoRV site of a pBluescript KS+ plasmid (see Appendix A.3.). Sequencing was performed on all clones to verify the identity and orientation of the MRF inserts. The T7 site of the plasmid was the preferred site for use in generating *in situ* probes (See Appendix A.3.). Plasmids were digested using KpnI and SstI and the resulting insert was purified and cloned into a pBluescript SK+ plasmid so that both the antisense and the control sense probes could be generated using a the T7 site (see Appendix A.3.). After cloning into the pBluescript SK+ plasmid the identity and orientation of the insert was verified through sequencing. Transcript sequences of MRFs and the sequence that was probed for are shown in figures 5.1-5.3.

In situ hybridization was performed using digoxigenin labeled probes (see Appendix A.2.). Probes were labeled with digoxigenen using DIG RNA labeling mix as per manufacturers instructions (Roche), to add DIG-labeled UTP to the riboprobe, and T7 RNA polymerase (Roche) to synthesize the riboprobe from the T7 direction of the plasmid insert.

5.2.4. Hybridization and Visualization

Tissue sections were dewaxed in 100% (v/v) toluene twice for five minutes, then rehydrated using an ethanol series (100%, 95%, 70%; v/v) for two minutes each, followed by a 2 minute wash in DEPC-treated H_2O, and two five minute washes in PBS. Slides were then post-fixed using 4% formaldehyde (w/v) for 10 minutes. An RNase treatment in 0.1% (w/v) DEPC-PBS was performed for 15 minutes twice. A proteinase K digestion step was carried out in a proteinase K solution (20mM Tris pH7.5; 5mM EDTA; 20µg/ml proteinase K) at 37°C. Proteinase K was used to digest proteins of the cell membrane, to allow better access of riboprobes into the cell. In the attempts to optimize the *in situ* hybridization protocols, the time of proteinase K treatments varied from 20 seconds to one minute. This was followed by two 10 minute washes in 5X SSC. Slides were pre-hybridized in a hybridization solution (50% (v/v) formamide; 5X SSC; 500µg/ml salmon sperm DNA) in an incubator at hybridization temperatures for two hours. Probe was denatured for five minutes at 80°C and added to the slides, and the slides were incubated at hybridization

temperature overnight. The hybridization temperatures tested ranged from 45°C to 70°C.

After hybridization, post-hybridization washes were performed. A 30 minute wash in 2X SSC at room temperature was followed by two 60 minute washes (2X SSC followed by 0.1X SSC) at the hybridization temperature. Slides were then washed in equilibration buffer 1 (10mM Tris pH 7.5; 150 mM NaCl). An anti-DIG monoclonal solution (1:2000 AP conjugated Anti-Digoxigenin-AP, Fab fragments [Roche]; 1% (w/v) blocking reagent [Roche]) containing an antibody for digoxigenin conjugated to alkaline phosphatase was applied to the slides and incubated at room temperature in the dark for two hours. Two 15 minute washes in equilibration buffer 1 was performed after this incubation, followed by a 5 minute wash in equilibration buffer 2 (100mM Tris pH 9.5; 100mM NaCl; 50mM $MgCl_2$).

In order to visualize hybridization of probes to MRFs, hybridized slides were incubated in a solution of nitroblue tetrazolium (NBT; Roche) and 5-bromo,4-chloro,3-indolylphosphate (BCIP; Roche) in buffer 2. These colourless reagents react with the alkaline phosphatase to produce a visible purplish product localized to regions of probe hybridization. Slides were counterstained in a 0.5% (w/v) methyl green solution for two minutes, and rinsed three times in distilled H_2O. This was followed by a dehydration series of washes in ethanol (35%, 50%, 75%, 95%, 100%; v/v) and toluene for two two-minute washes. Coverslips were then mounted to slides using DPX mounting media (Sigma).

5.3. RESULTS

An example of a successful hybridization is shown in Figures 5.4 and 5.5. Figure 5.4 shows an example of a tissue section probed with a MyoD antisense probe. Staining can be seen in the somites and in the developing muscles of the back. Figure 5.5 shows a control sense probe and no staining above background levels were observed, indicating a specific binding of the antisense probe. Due to the low expression of MRF genes it was necessary to increase incubation periods to allow for colour development of target transcripts. Unfortunately however, in most cases a general over-staining occurred through-out the tissue sections, showing no specific staining when probed.

5.4. DISCUSSION

After several attempts using various modifications described in the protocol above, detection of MyoD, Myf-5, and myogenin was not successful. There were certain instances where the hybridization did succeed, but results were not consistent enough to derive any meaningful results. In most cases the problem appeared to be due to a general background staining of whole sections that were similar when probed with either a sense or antisense probe of the target mRNA. Thus background staining likely masked any specific expression in expected regions (somites and muscles of the back).

There may be several reasons that, alone or in combination, were responsible for the lack of success with this technique. MRF genes are expressed at relatively low levels, and the digoxigenin labeled riboprobe may not have had the required sensitivity to show specific staining of mRNA transcripts in the presence of high levels of non-specific staining. Previous studies using *in situ* hybridization to detect Myf-5, MyoD, and myogenin in the mouse (Sassoon et al., 1989; Ott et al., 1991), and MyoD in the pig (Chang et al., 1995), used radioactively-labeled probes. These probes provide a greater sensitivity target transcripts, as radioactivity provides strong signals that can be visualized by a autoradiography film. However, because no direct visualization of the tissue itself can be seen with the expression of the transcripts, the resolution in the quality of the section reported in these studies was quite low. Another means of increasing the sensitivity of digoxigenin may be to increase the length of the probe to the full length of the mRNA target, instead of the size of the probes used here. However, it is not clear whether this would significantly increase the chemical signal from DIG-labelled probes given the low abundance of the transcripts. Furthermore, because of high sequence homology among the MRF genes, it is unknown what the potential for non-specific binding to non-targeted MRFs might be with a full-length probe. A recent study highlights these issues with regard to the sensitivity of *in situ* hybridization, and with MyoD in particular (Gerhart et al., 2004). The authors suggest an approach using fluorescently-labeled dendrimers as accurate and sensitive method to detect low-level transcripts (see Appendix A.4.).

Due to the difficulty in establishing a functional protocol to detect MRFs using *in situ* hybridization, it was decided to use real-time PCR to help determine if changes in the expression of MRFs occurs at day 30 of gestation. In chapter three, it was observed that the relatively higher states of uterine crowding produced in the current study also resulted in the reduction in the expression of the MRF, myogenin. Therefore, there is a potential link between the expression of MRFs at day 30 of gestation and the reduction in secondary muscle fiber numbers.

Although, the *in situ* hybridization protocol used here was not successful, *in situ* hybridization remains a potentially useful tool to help further explain differences in MRF expression determined by real-time PCR. Perhaps using a more sensitive probing method, such as that described by Gerhart et al. (2004), may prove to be more successful. Since the expression of MRFs in chapter three revealed changes due to uterine crowding, and the determination of potential delays of MRF expression, *in situ* hybridization may be used to expand observations to periods prior to day 30 of gestation. Determination of the precise timing when effects of uterine crowding on MRF expression occur may be useful in further elucidating the mechanism behind changes in MRF expression, and would be useful in determining suitable animal management strategies to minimize these effects.

Gene	Primers	Sequence 5' --> 3'	Expected Size
Myf-5	Sense	CAG AAG ATG GAC CTG ATG GAC G	
	Antisense	GTT TTC CAC CTG TTC CCT CAG C	437 bp
MyoD	Sense	GCG GAC GAC TTC TAT GAT GAC C	
	Antisense	CGC TGA TTC GGG TTG CTA	352 bp
myogenin	Sense	GCT GTA TGA GAC ATC CCC CTA C	
	Antisense	CAT TCA CCT TCT TGA GCC TGC G	289 bp

Table 5.1. Primer sequences for the generation of plasmids inserts used for the synthesis of sense and antisense probes used for hybridization.

GCCGACCCAGGCCGCCAGGCGTCTGCCCCTGTTAATTAGCAGAGCAACCGAGCAGGGAGT
TCCGCCCGCGACGTGCCCGCCCGCGGAGGCGCCAGGCCCCGGGCTTCTCCCCGATCTGAT
CTATCTCGCAGCTGCCCAGGTGCACCGCCCGCCTGTCCGCAGAAGATGGACCTGATGGAC
GGCTGCCAGTTCTCGCCTTCTGAGTACTTCTACGATGGCTCCTGCATCCCATCCCCCGAGG
GCGAGTTCGGGGACGAGTTTGAGCCACGAGTGGCTGCTTTCGGGGCGCACAAAGCAGACC
TGCCCGGCTCAGACGAGGAAGAGCACGTGCGAGCACCTACGGGCCACCACCAGGCCGGC
CACTGCCTCATGTGGGCCTGCAAAGCGTGCAAGAGGAAATCCACCACCATGGATCGGCGG
AAGGCGGCCACCATGCGCGAGCGGAGACGCCTGAAGAAGGTCAACCAGGCGTTTGAGAC
GCTCAAGAGGTGCACCACGACTAACCCCAACCAGAGGCTGCCCAAGGTGGAGATCCTCA
GGAATGCCATCCGCTACATTGAGAGCCTGCAGGAGCTGCTGAGGGAACAGGTGGAAAAC
TAATACAGCCTGCCCAGGCAGAGCTGCTCTGAGCCCACCAGCCCCACCTCCAGCTGCTCC
GACGGCATGCCTGAATGCAACAGCCCTGTCTGGTCCCGAAAGAACAGCAGTTTTGACAGT
ATCTACTGTCCGGATGTACCAAATGATATGCCACGGATAAAAGCTCCTTATCCAGCCTGG
ATTGCTTATCCAGCATAGTGGATCGGATCAGCAACTCCGAGCAACCTGGACTGCCTCTCC
AGGACCCAGCCTCTCTCTCTCCAGTTGCCAGCACCGATTCTCAGCCTGCAACTCCAGGGGC
CTCTAGTTCCAGACTTATCTATCACGTGCTATGAACTAAAAATCTAGTCTAGACCATTTCT
GCCAGGAGTGCCTATTACACAGGAGGAAGGAGGCCCAAAAGGCCCAAAAGCAAGACAAC
CTGTATATAAACATTTTTTTTCAGTTGTAAATTTGTAAATACTATCTTGCCACTTTATAAGA
AAGTGTATTTAACTAAAAAGTCACTATTGCAATTAATTCTTTATTTCTTCTTCTTTTCCTTT
GTCTTGGCATTAAATATATAGTTCCAATGATATTATTTCTTATAGGGGCAATTCATCCAAG
GGTAGCTCGTTGCAATGCTTAACTTATACTTTTTATAATATTGCTTATCAAAATATTACCTC
TGTTTAGAGCTTTATTTTTTTCCCCTTTAAAAATATTAGAACAAATACTAGAACTGGAAAT
CAAGTTATAGGGAGTTTTAAATATATTTAACTTTTTTGCTTCTCTTTAATCCTTTGGTTATA
TTGTGTTAAGTAAAAATATAACATACTGCCTAATGGTATATATTTTGATCTTATAAGAAAT
GCATCTTTTTAATGTAAGCACAAAATAGTACTTTGTGGATGATTTCAAGATGTAAGAGATT
TTGGAAATTCCACCATAAATAAAA

Figure 5.1. Sequence of porcine Myf-5 mRNA. Nucleotides in red show sequence for which antisense probes were designed to anneal to.

ATGGAGCTGCTGTCGCCACCGCTCCGCGACGTAGATTTGACGGGCCCCGACGGCTCTCTCT
GCAACTTTGCAACAGCGGACGACTTCTATGATGACCCGTGTTTCGACTCCCCGGACCTGCG
CTTCTTCGAGGACCTGGACCCGCGCCTCGTGCACGTGGGCGCGCTCCTAAAGCCCGAGGA
ACACTCGCACTTCCCTGCCGCAGCGCACCCGGCCCCGGGAGCTCGTGAGGACGAGCATGT
GCGCGCGCCCAGCGGGCACCACCAGGCGGGCCGCTGTCTACTGTGGGCCTGCAAGGCGTG
CAAACGCAAGACCACTAACGCCGACCGCCGCAAGGCCGCCACCATGCGCGAGCGGCGCC
GCTTGAGCAAAGTCAACGAGGCCTTCGAGACTCTCAAGCGCTGCACGTCTAGCAACCCGA
ATCAGCGGCTGCCCAAGGTGGAAATCCTGCGCAACGCCATCCGCTATATCGAAGGCCTGC
AGGCGCTGCTTCGCGACCAGGACGCCGCCCCCCCTGGCGCTGCAGCGGCCTTTTACGCGC
CTGGCCCGCTGCCCCCGGGCCGAGGCGGAGAGCACTACAGCGGTGACTCAGACGCATCCA
GCCCGCGCTCCAACTGTTCCGACGGCATGATGGATTATAGCGGCCCCCCGAGCGGTGCCC
GGCGGCGGAACTGCTACGACGGCACCTATTACAGCGAGGCGCCCAGCGAACCCCGGCCC
GGGAAGAATGCTGCGGTGTCGAGCCTCGACTGTCTGTCCAGCATCGTGGAGAGCATCTCC
ACCGAGAGCCCCGCCGCGCCCGCGCTTCTGCTGGCGGACACGCCGCGGGAGTCGTCTCCG
GGCCCGCAAGAGGCGGCCGCCGGGAGCGAGGTCGAGCGCGGCACCCCCACCCCTTCCCC
GGACGCCGCCCCGCAGTGCCCCGCGAGCGCGAACCCCAACCCTATCTACCAGGTGCTCTG
A

Figure 5.2. Sequence of porcine MyoD mRNA. Nucleotides in red show sequence for which antisense probes were designed to anneal to.

ATGGAGCTGTATGAGACATCCCCCTACTTCTACCAGGAACCCCACTTCTATGACGGGGAA
AACTACCTGCCCGTCCACCTCCAGGGCTTTGAGCCACCAGGCTACGAGCGGACTGAGCTG
AGTCTGAGCCCTGAGGCCCGAGTGCCCCTGGAAGATAAGGGGCTGGGGACCCCCGAGCA
CTGCCCAGGCCAGTGCCTGCCGTGGGCATGTAAGGTGTGTAAGAGGAAGTCCGTGTCTGT
GGACCGTCGGCGGGCCGCCACGCTGAGGGAGAAGCGCAGGCTCAAGAAGGTGAATGAGG
CCTTTGAGGCCCTGAAGAGGAGCACCCTGCTCAACCCCAACCAGCGGCTGCCCAAGGTGG
AGATCCTGCGCAGCGCCATCCAGTACATCGAGTGCCTGCAGGCCCTGCTCAGCTCCCTCA
ACCAGGAGGAGCGAGACCTCCGCTACCGAGGCGGGGGCGGGCCGCAGCCAGGGGTGCCC
AGTGAATGCAGTTCCCACAGCGCCTCCTGCAGTCCAGAATGGGGCAGTGCACTGGAGTTC
GGCCCCAACCCAGGGGATCATCTGCTCACAGCTGACCCTACAGATGCCCACAATCTGCAC
TCCCTCACCTCCATCGTGGACAGCATCACAGTGGAGGATGTGGCTGTGGCCTTCCCAGAT
GAAACCATGCCCAACTGA

Figure 5.3. Sequence of porcine myogenin mRNA. Nucleotides in red show sequence for which antisense probes were designed to anneal to.

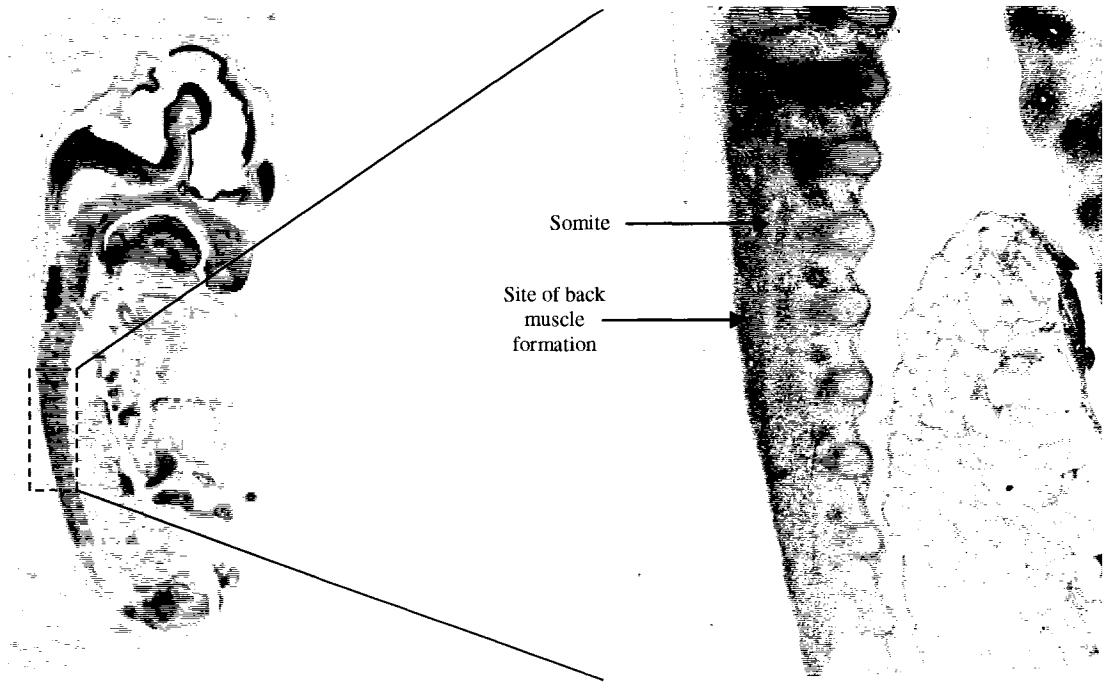

Figure 5.4. Saggital section of day 30 embryo probed with MyoD antisense probe. Deep puplish staining in the back and the somites is observed, indicating expression of MyoD in these regions.

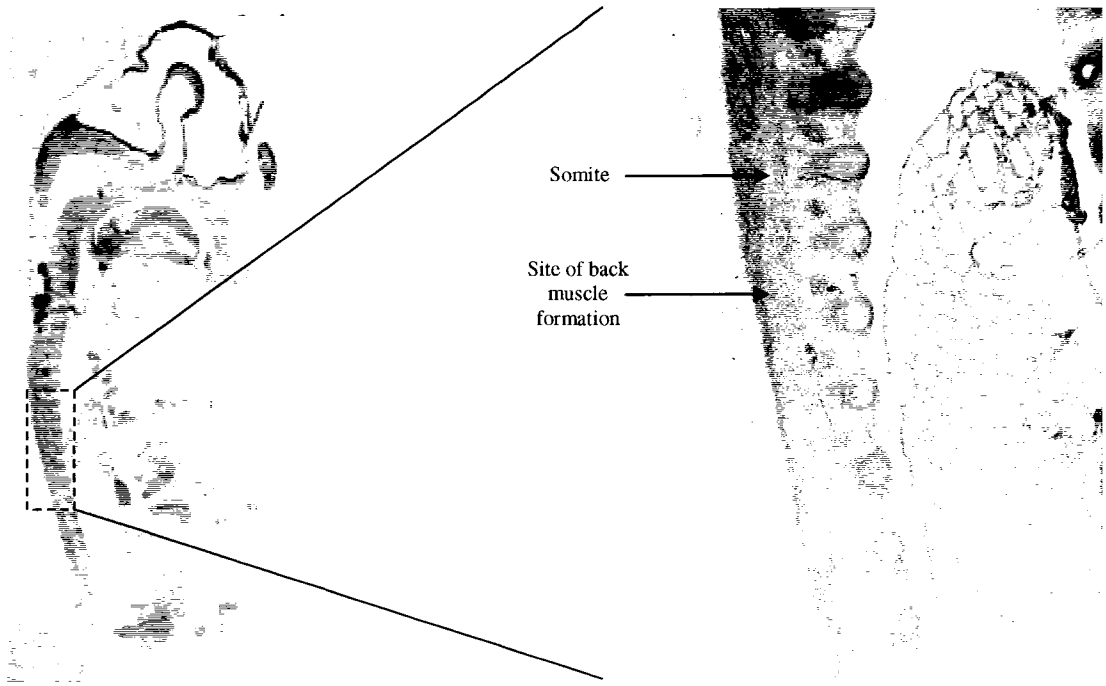

Figure 5.5. Negative control hybridization using MyoD sense probe. No colour development in the back or somites indicates specificity of antisense probe binding.

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CHAPTER SIX

6.1. GENERAL DISCUSSION

The size of a litter is of considerable relevance in commercial swine production and, all other things being equal, increased prolificacy can be economically beneficial. However, the potential for increased prolificacy to lead to undesired detrimental effects on the development of offspring may serve to offset any gains made through increased litter size. One such trait of commercial importance is the development of muscle. A study by Town et al. (2004) analyzing muscle cellularity at day 90 of gestation indicated that the determination of secondary muscle fiber numbers was adversely affected in fetuses from relatively more crowded uterine environments, a scenario likely under good management conditions using modern commercial phenotypes.

Although the formation of secondary fiber numbers is complete by around day 90 of gestation (Wigmore and Stickland, 1983), evidence from maternal nutrition studies (Dwyer et al., 1994) suggests that the determination of muscle fiber numbers occurs between day 25 and 50 of gestation, prior to the onset of secondary muscle formation. A study into the effects of the administration of porcine somatotropin on muscle fiber formation also indicated that the effects were limited to periods prior to day 50 of gestation (Rehfeldt et al., 1993). Therefore, the effects of uterine crowding might also act early in gestation. Evidence from chapter three show a decrease in myogenin

expression in relatively crowded uterine environments, and suggests that the effects of uterine crowding on secondary muscle fiber numbers acts through effects at day 30 of gestation.

Because the effects of uterine crowding were observed at day 30 of gestation, strategies to mitigate the effects of uterine crowding may need to be applied to the very early stages of gestation. Increased nutrition may be of use in preventing reduction in muscle fiber numbers. Dwyer et al. (1994) showed that in increase in maternal nutrition during early gestation might result in increases in muscle fiber numbers. An analysis of the effects of uterine crowding at day 30 of gestation, also revealed a negative effect on average placental weight in more crowded uterine environments (Town et al., 2004) suggesting that access to nutrients may be compromised. Evidence from guinea pigs show that maternal undernutrition also results in a decrease in muscle fiber number (Dwyer et al., 1995) and this decrease may be mediated through a decrease in placental size (Dwyer et al., 1992).

However, evidence from chapter three indicates that no direct relationship exists between placental weight and MRF expression. Thus, although a decrease may be seen from undernutrition, nutrition levels over the required levels for proper muscle fiber development may not be able to exceed limits on the determination of muscle fiber numbers dictated by the development conditions in the presence of increased embryo numbers.

The potential lack of effect of increased nutrition on muscle fiber numbers may be due to the relatively low nutritional needs of the embryo at early stages of gestation. It may be that the supplementation of factors that stimulate muscle development, such as insulin-like growth factors (IGFs) or porcine somatotropin (pST) may be of use. As shown in chapter three, the effect of uterine crowding on myogenin expression, but lack of effect on MyoD, suggest differentiation of existing myoblasts rather than the determination and proliferation of cells along the myogenic lineage may be affected. IGFs have been implicated in both the proliferation and differentiation of myoblasts (reviewed by Oksbjerg et al., 2004). If the embryos themselves act as a 'sink' for IGFs, competition between embryos may be a causative factor in the reduction of secondary muscle fibers. Due to increased competition for these factors in the presence of higher numbers of embryos, the change in the levels of these factors resulting from increased nutrition may not be sufficient to compensate for the reduction in myogenin expression.

Porcine growth hormone/somatotropin (pST) has also been shown to increase muscle fiber numbers (Rehfeldt et al., 1993) and may act through increased proliferation of myoblasts (Rehfeldt et al., 2001). It has been suggested that pST might act by increasing the availability of IGF, and its related factors, and nutrients to the embryo, thereby promoting muscle cell proliferation (reviewed by Rehfeldt et al., 2004). Thus supplementation of growth factors, above levels provided solely by increased nutrition may be useful in increasing MRF expression during early myogenesis.

In addition to a lack of a direct relationship between MRF expression and placental weight, a direct relationship between MRF expression and embryo weight was also not observed in the data discussed in chapter 3. Thus, it appears that myogenesis is more sensitive to changes caused by uterine crowding at early stages of development than the overall growth of the embryo as reflected in weight gain. This result further supports the idea that nutrient availability alone may not be a critical limiting factor in muscle fiber determination.

In chapter three, a direct relationship between increased viable embryo numbers and a decrease in the expression levels of myogenin was shown, supporting the contention that competition for some critical factors may be involved. However, there is also the possibility that embryos cause this inhibition of the growth and development of their neighbors through direct interactions.

Kaminski et al. (1996) suggested that the secretion of estrogen is one factor by which more advanced embryos may restrict the development of less developed embryos. This may be of relevance to the development of muscle as well. As previously discussed in section 2.5.6, there may be a link between estrogen secretion and MyoD expression through the gene Wnt7a. The secretion of estrogen may, in effect, cause the restriction in survival of less developmentally advanced embryos and may be advantageous to the survival of the litter as a whole, particularly during the preimplantation stages of development (as discussed by Geisert and Schmitt, 2002). However, selection for increased litter size in modern swine genotypes may be

increasing the number of embryos that survive into the post-implantation period of development (Foxcroft, 1997). Therefore, the proposed restrictive nature of one embryo on another, due to estrogen secretions, may also extend further into the post-implantation period during the critical windows for the determination of muscle fiber numbers.

The difference in estrogen secretion between genders may be a factor involved in this potential effect, and differences in gender response to uterine crowding may help identify mechanism behind changes in MRF expression. Wise and Christenson (1992) observed that embryos of one sex could impact the development of other embryos of the opposite sex within a litter, and it has been suggested that endocrine interactions between day 20 and 40 of gestation may be the basis for these effects. The purpose of the study in chapter 4 was to reevaluate the expression of MRFs based on the gender of the embryos within a litter. Results from this study indicated that only males were significantly different in myogenin expression between the treatment groups. Female embryos were not statistically different in myogenin expression between CTR and LIG groups. Furthermore, the correlation between myogenin expression and viable embryo numbers was lost in female embryos, while male embryos were more highly correlated with myogenin expression levels than the litter as a whole. Whether or not this translates into differences at later stages of gestation is unknown, and further study into muscle fiber numbers at day 90 of gestation with respect to gender would help to substantiate these results.

Overall, it appears that the effects of uterine crowding may be of more consequence to males than to females. It is also possible that a mechanism is in place that specifically protects female embryos from any negative effects of uterine crowding on early myogenesis. In any case, strategies to minimize the effects of litter size on muscle development that focus more specifically on responses by males may be advantageous, as only males appear to be sensitive to uterine crowding at early during early myogenesis. Identifying potentially sensitive factors that are different between males and females may help elucidate potential mechanisms underlying negative consequences of uterine crowding. For instance, if estrogen is indeed a factor involved in muscle fiber reduction, studies could be carried out to address whether or not males are more sensitive to elevated estrogen levels.

The evidence from this thesis indicates that there are changes in myogenin expression due to uterine crowding. However, the mechanisms behind the change in secondary muscle fiber numbers at day 90 is far from comprehensive. Further insight into the mechanisms through which uterine crowding acts would also be helpful in developing strategies to minimize the effects. As discussed in section 3.4, changes in myogenin expression present many possible scenarios to explain the eventual effect seen on secondary fiber numbers. Additionally, although changes in MyoD expression were not seen at day 30 of gestation, the methods used in this study may not have been sensitive enough to detect changes of this low-abundance transcript. Furthermore, whether or not a delay may have occurred in MyoD expression cannot be determined at this time.

It was hoped that *in situ* hybridization could be used to examine whether any developmental delays in expression occurred, and these studies are described in chapter 5. Unfortunately, the application of this method was not successful in being able to visualize spatiotemporal delays in MRF expression. Use of improved methods not only at day 30, but also at developmental periods prior to this time, might still be useful in determining the overall mechanism by which myogenesis is altered.

It therefore appears that breeding strategies geared towards increasing prolificacy may not necessarily be beneficial in terms of muscle accretion. This may be of particular relevance as the levels of crowding in this thesis may only represent a modest level of crowding when compared to potental littersizes of modern commercial dam-lines. Furthermore, new management strategies that are focused towards early periods of gestation, and those that specifically target males may help minimize the effects of uterine crowding on muscle development, and further study into the precise mechanism of myogenic effects may help to develop these strategies. Changing of animal breeding selection criteria away from an emphasis on increased prolificacy towards more optimal litter sizes for ensuring uniform high-muscle producing offspring may be most beneficial. Since it appears that MRF expression is related to the number of viable embryos at day 30, it may be also beneficial to control the number of viable embryos during this critical window in the determination of muscle fiber numbers. Thus, strategies, such as increasing the ovulation rate to

maximize the potential number of offspring in a litter, should instead be more tightly regulated so that the number of embryos present post-implantation during the critical window of myogenesis is approximately the same as the number of offspring born.

In summary, there is evidence to suggest that the effects of uterine crowding on the determination of secondary muscle fiber numbers (Town et al., 2004) are mediated through changes in early myogenesis by at least day 30 of gestation. In this experimental paradigm this effect also appears to be more pronounced in males than in females, although further studies are needed to see if this effect persists through later stages of gestation. These results provide new avenues for study to help maximize the potential for muscle accretion in commercial swine production.

6.2. REFERENCES

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APPENDIX

A.1. REAL-TIME RT-PCR

Real-time RT-PCR is a semi-quantitative method to determine the relative abundance of a target mRNA, by measuring the amounts of amplification product generated during each cycle of a PCR reaction. Measuring fluorescence given off, as each amplicon is amplified allows for the relative quantification of the amount of amplicons present (summarized in figure A.1.). A target mRNA is first converted into a cDNA, which is then used as a template for real-time PCR. In addition to primers for a standard PCR reaction, a probe is also annealed to the target cDNA. A reporter dye and a quencher molecule are chemically linked to either side of the probe. While the probe is intact (both the reporter dye and the quencher are present) no fluorescence is given off. As the PCR amplification extends the primers the exonuclease activity of the polymerase cleaves the bound probe into nucleotides. Once separated from the quencher, the reporter dye fluoresces. Thus, as each amplicon is generated, a certain amount of fluorescence is derived.

The fluorescence signal is measured at each cycle (see figure A.2.). At any given cycle, the higher the fluorescence (ΔR_N) produced correlates with a greater number of copies of the amplicon that are present at that cycle. This also means that at any given ΔR_N, the cycle at that point represents a given amount of products at that cycle. Therefore, the earlier, in terms of cycle numbers that a given ΔR_N is reached, the

greater the amount of initial template that must have been present for amplification to occur.

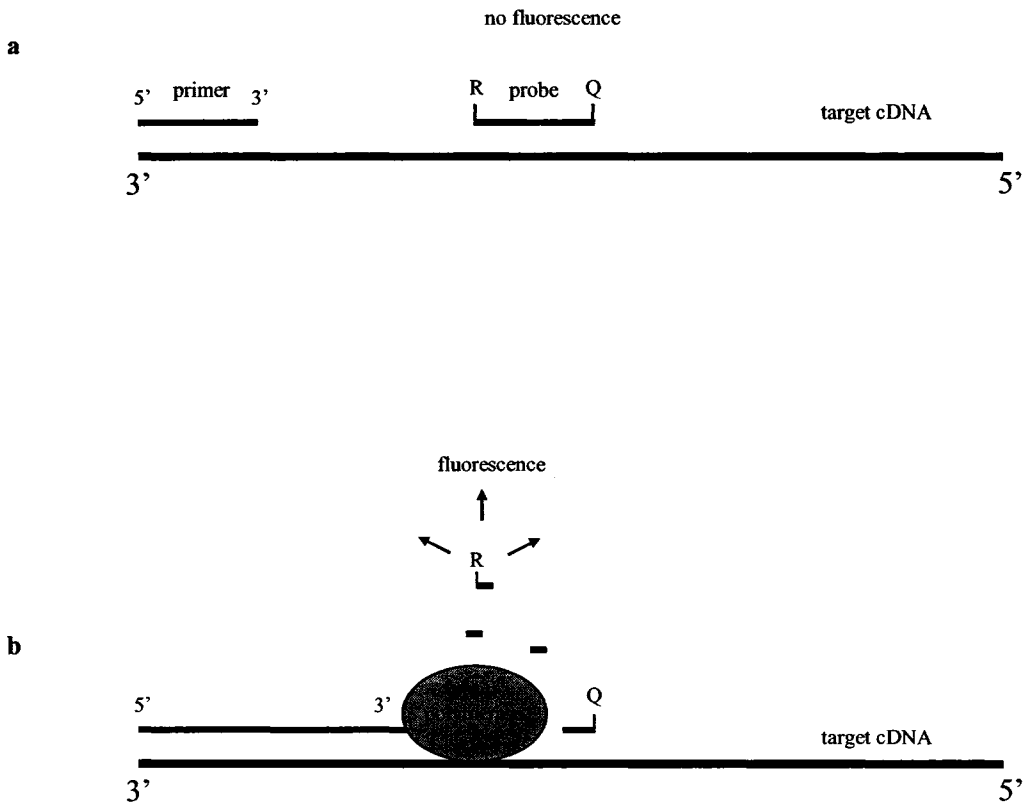


Figure A.1. Summary of real-time reaction. (a) Prior to amplification the quencher (Q) prevents flurescence of the reporter dye (R). (b) As amplification occurs, the polymerase cleaves the probe into nucleotides. The reporter dye is now able to fluoresce and is detected.

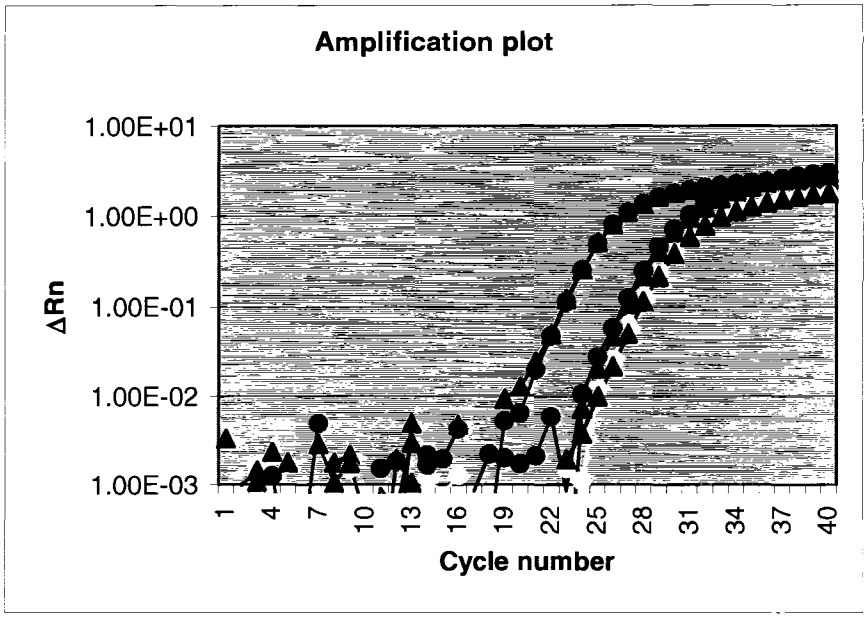


Figure A.2. Sample of an amplification plot. The lower the cycle number to achieve a given ΔRn for a target gene, the greater the initial amount of transcript present.

A.1.1. $2^{-\Delta\Delta C_T}$ comparative C_T method

The $2^{-\Delta\Delta C_T}$ comparative C_T method (Livak & Schmittgen, 2001) quantifies a target mRNA transcipt by examining expression levels of the target relative to a reference gene (normaliser) that is believed (assumed) to be expressed throughout all samples. In order to use the $2^{-\Delta\Delta C_T}$ method, the C_T of the normaliser is subtracted from the C_T of the target mRNA transcript to determine the difference (ΔC_T) between the values of the two genes. The ΔC_T of a sample is then selected as a baseline for other samples to be compared to. The difference in the ΔC_T is then calculated ($\Delta\Delta C_T$) by subtracting the ΔC_T of the sample from the baseline, and represents the difference between any two samples. Since a one C_T difference represents a 2-fold difference in

the initial amount of transcript, the mathematical equation of 2 to the power of the $\Delta\Delta C_T$ will represent the fold difference between any two samples ($2^{-\Delta\Delta C_T}$). Because a lower C_T represents a greater amount of transcript, a negative of the exponent is calculated to ensure that the fold-difference properly represents an increase or a decrease.

A.1.2. Calculating amplification efficiencies

The differences in the C_T value of any two given genes should remain constant throughout the exponential phase of amplification. However, if amplification efficiencies of any two genes differ, the ΔC_T between any two genes across may differ at different threshold levels. Therefore it is necessary to determine if the amplification efficiencies of any two genes over a range of initial starting template amounts are the same. This is done by examining the C_T values of a gene at a given threshold point over a range of dilutions (the log of the cDNA input; see Figure A.3.). If the slopes are equal, then the two transcripts being analyzed have equal amplification efficiency.

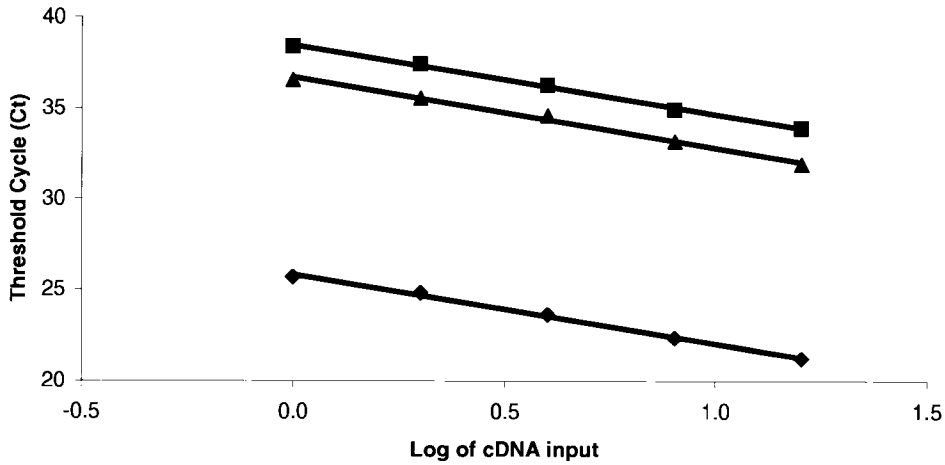
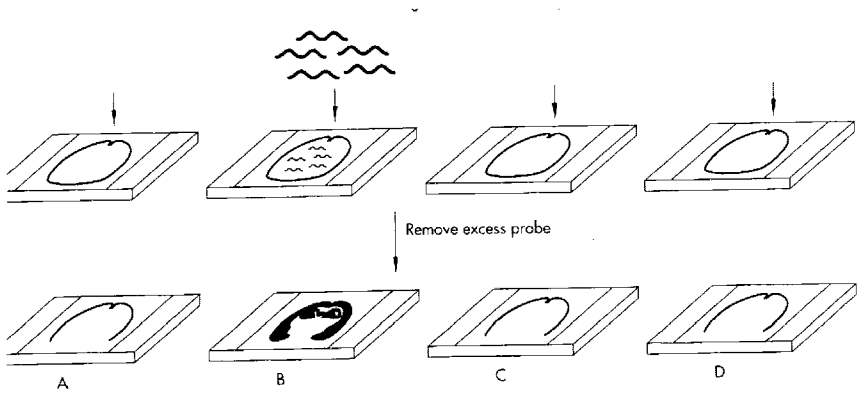


Figure A.3. Measuring the efficiency of a reaction by plotting the threshold cycle versus a log of the input of initial template amounts. Slopes are equal, and therefore the genes are suitable for comparison.

A.2. *IN SITU* HYBRIDIZATION

In situ hybridization is a technique to identify the spatial pattern of the expression of a target gene. This is done by labeling an antisense probe generated by *in vitro* transcription that can anneal to the target sense mRNA transcript of interest. In this way the target mRNA can be localized to a given region of tissue (see Figure A.4.)



From Watson et al., 1992.

Figure A.4. Summary of *in situ* hybridization. Probes for different mRNA transcripts (represented by wavy lines of varying colours) are used to probe for different targets. When visualized, targets can be seen in distinct locations of its expression (shown by corresponding colour pattern for a given coloured wavy line).

A.2.1. Non-radioactive *in situ* hybridization

In this thesis, a non-radioactive *in situ* hybridization technique using RNA probes (riboprobes) labeled with digoxigenin (summarized in Figure A.5.) was used. In this method, uracil-tri-phosphate (UTP) conjugated to digoxigenin, a plant steroid obtained from the plant *Digitalis* (Figure A.6.) was incorporated into the cRNA probes. An antibody against digoxigenin was added to slides that had been

previously incubated with the digoxigenin-labeled probes. The antibody, which is itself labeled with the enzyme alkaline phosphatase, was used to reveal the presence of any probes that had hybridized with target mRNAs. The alkaline phosphatase enzyme bound to the anti-DIG-antibody was able to hydrolyze colourless substrates to produce a coloured product. Thus, a visible colour change will occur at the location where target mRNAs are localized.

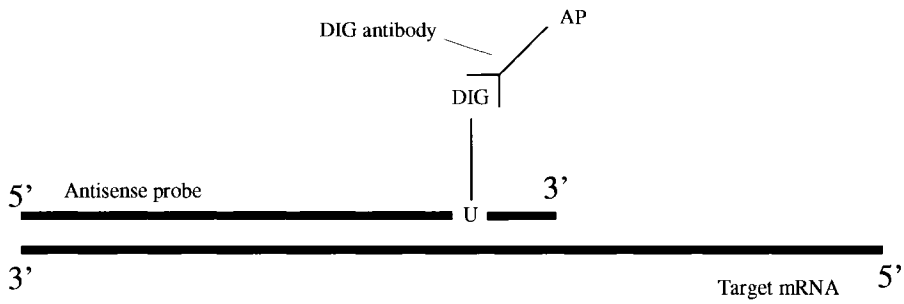


Figure A.5. An antisense riboprobe labeled with a uracil (U) conjugated with digoxigenin (DIG) is hybridized to a target mRNA. A DIG antibody is then added which binds to digoxigenin. The enzyme alkaline phosphatase (AP) is attached to the antibody, and when certain substrates are added, the alkaline phosphatase enzyme causes colour reaction.

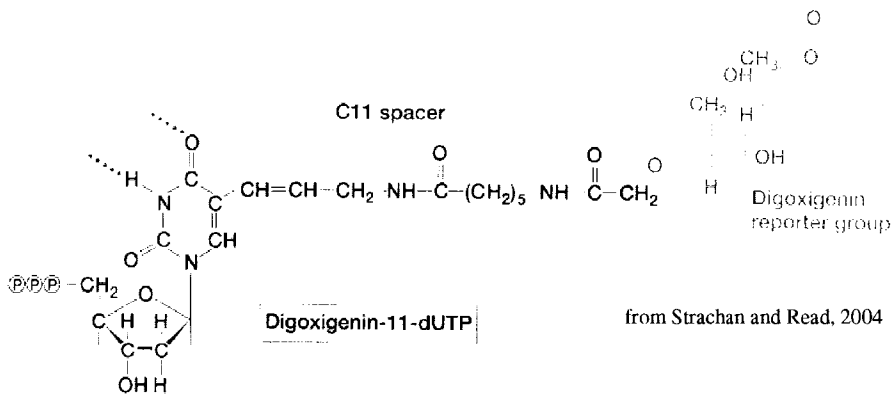


Figure A.6. Digoxigenin linked to UTP. A spacer is included between the digoxigenin molecule and UTP, in order to allow for the proper incorporation of the UTP conjugated to digoxigenin into the probe.

The sequence of a transcript from the 5' to 3' direction for a given gene is referred to as the sense sequence. In order to bind to this sequence, a probe must contain complementary base pairs to the sense sequence in the 3' to 5' direction. This complementary sequence is referred to as the antisense sequence. Since only an antisense probe is capable of specific H-bonding (base-pairing) to the target mRNA, a sense probe was also synthesized in the study described in chapter 5, to act as a negative control.

A.3. CLONING OF PCR FRAGMENTS INTO pBLUESCRIPT PLASMIDS FOR RIBOPROBE SYNTHESIS BY *IN VITRO* TRANSCRIPTION

The *in situ* hybridization protocol used in this thesis was performed using RNA probes (riboprobes). In order to synthesize riboprobes, the DNA sequence used for probe synthesis must be placed under the control of a transcription promoter to allow for RNA polymerase to transcribe the DNA. Cloning target DNA sequences into plasmids that carry such a promoter can achieve this. In this study the plasmid used was pBluescript (Stratagene; Figure A.7.). This plasmid contains a multiple cloning site (MCS) that is recognized by a variety of restriction enzymes. The target DNA is cloned into the MCS by conventional techniques of molecular biology. The plasmid also contains promoter regions for viral RNA polymerases (T7 and T3) on either side of the MCS (Figure A.8). Synthesis of both sense and antisense riboprobes from the same clone is possible by using the appropriate polymerase.

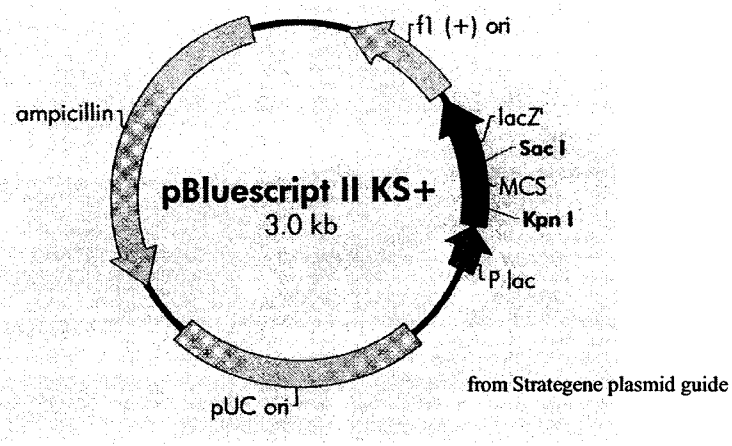


Figure A.7. The pBluescript plasmid KS, showing the multiple cloning site (MCS) into which a target DNA sequence is inserted.

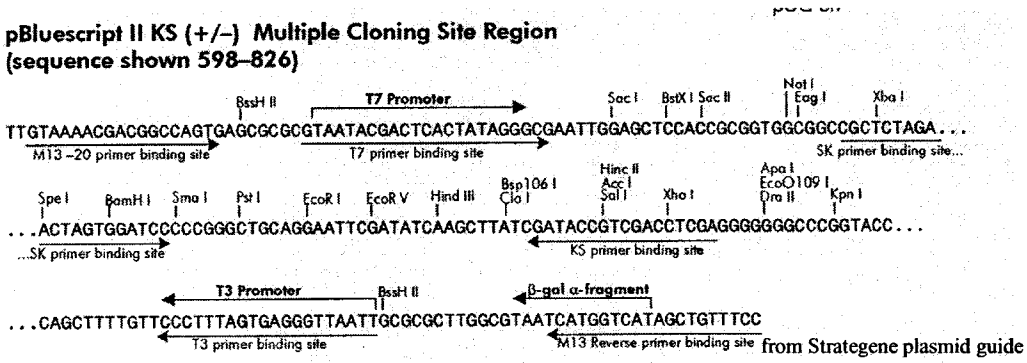


Figure A.8. Sequence of the MCS showing the T7 promoter transcribing in the Sac I to the Kpn I direction.

Since T7 and T3 are different promoters, it is possible that efficiencies may be different between them. It was decided that synthesis of both antisense and sense probe for a given gene should be done from the same promoter to allow for more consistentcy in the synthesis of both antisense and sense probes (in chapter five T7

was selected). Because the T7 promoter can only synthesize one strand it is necessary to 'flip' the target DNA so that the opposing strand can be synthesized. The availability of both the pBluescript KS (Figure A.7.) and pBluescript SK (Figure A.9.) plasmids allow this.

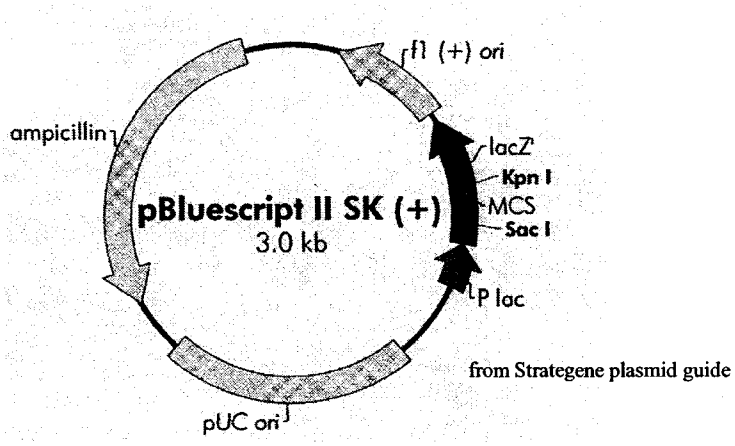


Figure A.9. The pBluescript plasmid SK, showing the multiple cloning site (MCS) into which a target DNA sequence is inserted.

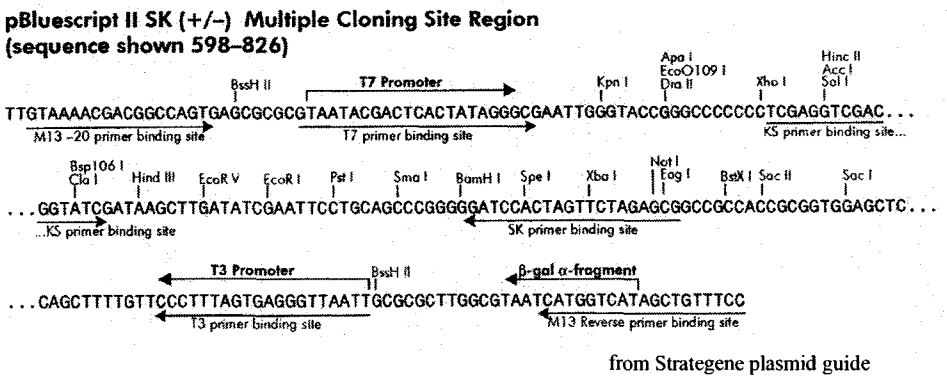


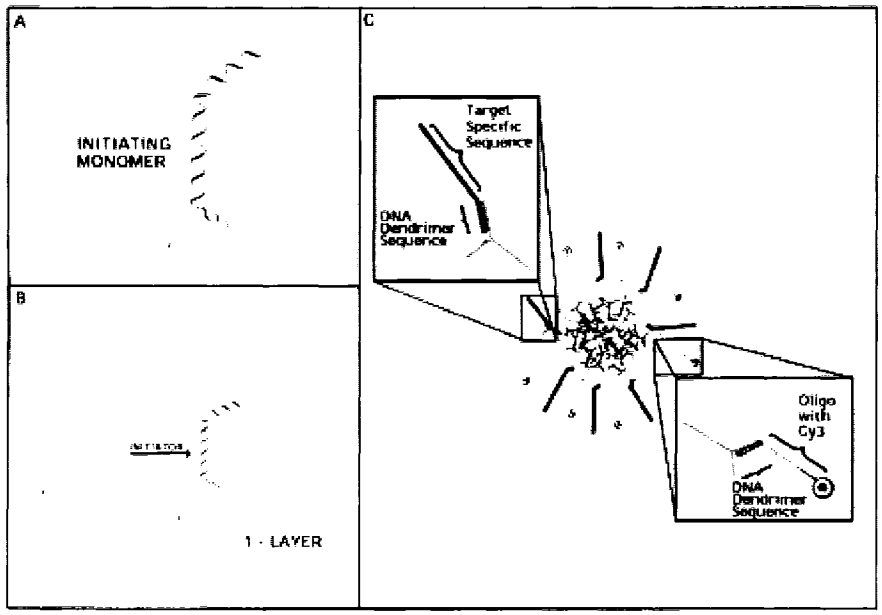
Figure A.10. Sequence of the MCS showing the T7 promoter transcribing in the Kpn I to the Sac I direction.

KS refers to the direction of two restriction enzymes (Kpn I and Sac I) in the MCS. If transcribed from the T7 promoter, transcription will go in the direction from the Sac I site to the Kpn I site (Figure A.8.). In the pBluescript SK, this is reversed, with T7 polymerase beginning transcription from the SacI side (Figure A.10). Using Kpn I and Sac I, the insert from the pBluescript KS plasmid can be inserted in the opposite direction on the pBluescript SK plasmid. This allows a target insert to be transcribed in both directions from the same T7 site, and allowing both the sense and antisense probes to be generated.

A.4. DENDRIMERS

As described by Gerhart et al. (2000; 2004), the use of dendrimers represents a potentially more sensitive approach to *in situ* hybridization of low copy number mRNA transcripts such as MyoD. The amount of labeled UTP provided by the DIG labeling Kit (Roche) allows for the incorporation of approximately one UTP-DIG nucleotide for every 20-25 nucleotides of the probe. For example, a probe of 400 bp may on average contain 16-20 UTP-DIG nucleotides. This therefore represents 16-20 copies of AP for colour development each transcript present. Higher numbers of transcripts therefore raise the amount of AP present for colour development. Conversely, low numbers of transcripts result in fewer AP present. The use of dendrimers provides a method by which the number of DIG molecules present can be substantially increased 100- to 1000-fold (Gerhart et al., 2000). This allows for greater sensitivity to low copy number transcripts.

Dendrimers are formed by hybridizing initiating monomers (see Gerhart et al., 2000). These monomers are formed by hybridizing oligonucleotides that are homologous in the middle but not on the ends. Thus each monomore contains what is termed a double-stranded 'waist' with four single-stranded arms (Figure A.11.a). The arms then hybridize to arms of other monomers (termed one-layer dendrimers; Figure A.11.b). This process is repeated until four-layer dendrimers are created (Figure A.11.c). The probe for the target transcript is annealed to the outer arms of the four-layer dendrimer. In the example showed in Figure A.11.c a fluorescent tag is used. However, if digoxigenin is preferred, within this 'ball' of dendrimers 500 molecules or more of digoxigenin can be incorporated into the dendrimers (Gerhart et al., 2000), thus greatly increasing the number of DIG molecules available for antibody binding and thereby increasing the sensitivity of detection.



from Gerhart et al., 2000

Figure A.11. Creation of a dendrimer. Monomers (a) are annealed to form one layer dendrimers (b). This is repeated until four-layer dendrimers (b) are formed. Within the four-layer dendrimer several more molecules of the tag used for detection can be added in excess of those found in an antisense probe alone

A.5. REFERENCES

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