

ABSTRACT

BLOWE, CHARLOTTE DAWN. Characterization of Type I molecular markers in a line of pigs selected for increased litter size. (Under the direction of Eugene Eisen and Joseph Cassady.)

Direct selection for increased litter size was practiced for eleven generations in a Large White-Landrace composite line of pigs. Litters were standardized at birth so that no replacement gilts were reared in a litter with more than ten pigs. A contemporary control line was maintained. In generation nine, the estimated mean breeding value for litter size was 0.63 pigs greater in the select than control line. The objective of this research project was to test associations between Type I markers and response to selection. A candidate gene approach was employed to search for markers, which may explain some of the difference in litter size between the two lines. Two novel markers were discovered within the follistatin gene, which have shown associations with litter traits. The estrogen receptor marker was not segregating in the population of pigs used in this study. The retinol binding protein marker was segregating in the population studied; however the magnitude of allele frequency change was relatively small. Polymorphisms were not detected in other candidates tested.

**Characterization of Type I molecular markers in a line of pigs
selected for increased litter size**

By

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Personal Biography

Charlotte Dawn Blowe was born on February 7, 1979, during a snowstorm in the small town of Ahoskie, North Carolina. She was raised in the loving home of Michael and Bonnie Blowe along with her younger sister, Christy. She has had a love for animals and a curiosity about life for nearly her whole life. Her love of biology began in the tenth grade under the instruction of Mrs. Catherine Allen. Charlotte graduated salutatorian from Hertford County High School in May 1997. Later that year she began her undergraduate training in marine biology at the University of North Carolina-Wilmington. She graduated from UNCW in May 2000 with a Bachelor of Science degree in marine biology. She moved to Raleigh, NC to pursue her Master of Science degree in animal science in 2001. She is currently engaged to Brian Stanley and has been since 1998.

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INTRODUCTION

Genetic improvement is reliant on the efficiency of reproduction. The current market economy exerts pressure for amplified productivity in animal breeding. World population growth and increased income throughout the world is increasing demand for animal products, which can only be met by intensifying animal breeding (Soller 1998).

In swine, there exists a store of within-breed genetic variation for most economically important traits. There is a larger supply of genetic variation across breeds (Soller 1998). Increasing costs related to production of extra pigs are negligible (Rothschild and Bidanel 1998). Therefore, considerable gains can be accomplished by enhancing number of pigs weaned per sow.

Breeder capability to recognize individuals that are carrying favorable alleles or allelic combinations is often very limited. Phenotypes may not be easily measured or even expressed, making phenotypic selection hard. Even when phenotype is expressed, it is an inaccurate indication of genotype (Falconer and Mackay 1996). This is due to the fact that genetic variation depends on allelic variation at a variety of loci, and gene expression at these loci is greatly influenced by environmental factors. Due to reproductive traits being late onset, sex-limited and lowly heritable, traditional selection methods have produced limited improvement in these traits.

Technological and scientific developments have the ability to be combined into a method for identifying genotypes of production traits directly at the DNA level, without going through phenotypic expression. This opens up an avenue for development and implementation of genetic improvement programs that are based on DNA-level selection. In programs of this nature, DNA variants at certain chromosomal locations serve as

markers for the presence of alleles having positive or negative effects at the quantitative trait loci (QTL) affecting trait expression (Soller 1998). When a locus exhibits a prevalent action and explains a significant portion of variation of a trait, it is termed a QTL. In DNA sequences, there are non-coding regions, called introns, within and between genes. Some introns regulate gene activity. They are spliced out of messenger RNA during transcription. Polymorphisms occurring in promoters or intron-splice sites could alter gene activity and thus, phenotypic expression. Non-coding polymorphisms that occur in an intron or outside a gene could be associated with an altered phenotype if they are positioned close to a functional mutation.

Reproductive success is dependent upon a number of complex physiological events in both sexes. If genetic markers can be found which are linked to QTL affecting economic traits in swine, these markers could be used to increase selection accuracy (Hayes *et al.*2001). The objective of this research project was to examine known genetic markers and explore novel genetic markers for an association with litter size traits.

LITERATURE REVIEW

Traditional improvements, economics and nature of litter size

Improving reproductive traits in livestock species has become an increasingly important endeavor (Vincent *et al.* 1998). Production efficiency of livestock is greatly influenced by reproductive success. Litter size (LS) is one of the most important and easy to measure of reproductive traits. A lot of effort has been made to improve litter size. Modest gains in litter size can mean large economic returns, improving overall efficiency of the pork industry (Rothschild *et al.* 2000). At present litter size varies greatly from as little as two to as many as 20 pigs per litter, with means from nine to 11. Phenotypic standard deviations range between 2.5 and 3 pigs (Johnson *et al.* 1999). There is enough genetic variation to increase litter size (Linville *et al.* 2001).

Sow productivity is limited by number of piglets weaned per sow per year (Ashworth 1998). Herd management improvements have been successful in reducing farrowing interval and pre-weaning mortality (Legault 1985). Better environmental conditions, like temperature and light, influence embryo survival (Ashworth 1998). Also, ample space and reduced stressors have positive influences on embryo survival (Hernsworth and Barnett 1990). Proper maternal nutrition has been shown to be associated with litter size improvements. Management of weaning and mating times also influence litter size (Ashworth 1998).

Traditionally, producers made genetic progress by using phenotypic information on individuals available for selection and information on their relatives (Visscher and Haley 1995). Analyzing phenotypic performances of individuals mainly uses breeding values. Advanced statistical methods, like best linear unbiased prediction (BLUP) are

utilized to divide genetic and environmental effects (Henderson, 1984). However, phenotype cannot always be recorded for an individual due to sex limitations and expense. Litter size heritability is low (0.1) (Haley *et al.* 1988). It is sex limited and cannot be measured until sexual maturity is reached. If direct selection in both sexes could be done for genes affecting expression of this trait, selection response would be enhanced (Linville *et al.* 2001). Polymorphisms have begun to be investigated, which can be linked to differences in production or other economically important traits.

Genetic differences among lines and breeds can be most effectively taken advantage of by utilizing crossbreeding programs. Crossbreeding has been shown to be an effective means of improving reproductive performance. This improvement comes from increasing heterozygosity. Rothschild and Bidanel (1998) reported that crossbred sows have a reduced age at puberty, higher conception rates, increased ovulation rate and increased litter size. Crossbreeding results have been integrated into crossbreeding programs at the producer level (Rothschild and Bidanel 1998). Pork producers practice crossbreeding by using specialized maternal and paternal genotypes. The Meishan breed has been examined as a maternal line because of its increased conception rate and larger number of ova produced (Rothschild and Bidanel 1998). Meishan sows have higher ovulation rates and increased prenatal survival (Haley *et al.* 1995).

Many genes in both sows and piglets control litter traits (Rothschild and Bidanel 1998). There have been some unsuccessful attempts to directly increase litter size (Ollivier and Bolet 1981, Bolet *et al.* 1989, Lamberson *et al.* 1991). Hyper-prolific selection is a way to increase litter size. This type of selection is an intense selection of sows over several parities. It also involves backcrossing their sons to sows with similar

high prolificacy. Hyper-prolific sows have been found to have more piglets born alive and increased ovulation rates (Rothschild and Bidanel 1998).

There is some substantiation that genetic improvement of litter size can be increased by genetically acting on its component traits, like age at sexual maturity, fertility, prolificacy and piglet viability, or their causal physiological processes (Rothschild and Bidanel 1998). Ovulation rate, prenatal survival and uterine capacity are components of litter size (Tess *et al.* 1983, de Vries 1989, Bennett and Leymaster 1989). Because heritabilities of litter size components, like ovulation rate, embryo survival and uterine capacity, are higher than that of litter size, the components are being used to indirectly select for litter size (Rothschild and Bidanel 1998). There has been some success with this approach (Lamberson *et al.* 1991). Selection indices have been utilized to combine information from several traits in efforts to maximize genetic progress in the overall breeding goal, which probably consists of a mixture of traits (Visscher and Haley 1995). It was proposed that using a selection index with ovulation rate and embryo survival would be an effective way to increase litter size (Johnson *et al.* 1984). Johnson *et al.* (1999) reported some success with this method.

Traditional methods of genetic selection are most often employed to improve livestock species. Litter size in swine has improved over the last several decades due to change in management strategies, using superior dam lines and by crossbreeding (Rothschild 1996).

Biotechnology influences on the swine industry

Limited improvement in reproductive traits made by selection and crossbreeding has promoted the search for single genes affecting litter size. Advances in molecular

genetics have made it possible to search for major genes and QTL as well as to study candidate genes that might control reproductive traits. Progress made in the past few years in the swine genetic map allows for the systematic search for loci affecting economically important quantitative traits. Newly developed molecular techniques provide an opportunity for direct selection of animals based on genes that control litter size, as long as such genes or linked genetic markers can be identified. Use of genetic markers will likely contribute to more efficient genetic improvement of reproductive traits that are late onset and sex-limited (Rothschild and Bidanel 1998). Identification of individual genes, with large effects on quantitative traits, provides opportunity to improve selection accuracy (Rothschild *et al.* 1996). Molecular techniques allow opportunity to examine genetic variation directly (Visscher and Haley 1995).

Animal breeding is a field of science that is related to a wide array of biotechnologies. Impact of a particular biotechnology can be measured by the influence it has on genetic progress. Depending on the type of biotechnology used, different components of genetic progress may be affected, like prediction accuracy, generation interval, selection intensity, and genetic variation (Gengler and Druet 2001).

Quantitative Trait Loci (QTL)

One group of biotechnologies summarized by Gengler and Druet (2001) can improve determination of an animal's genetic merit. These techniques relate to QTL, their detection and use. The basic idea around QTL is that a specific gene is responsible for a portion of genetic variation. A primary feature of this type of technology is its early availability in life of the individual, thus allowing an earlier, more accurate selection. It is believed that QTL will play a major role in animal breeding, especially if their use in

future breeding programs can be optimized. Examination of how marker technology can influence the genetic gain formula reveals several things. Enhancing reproductive efficiency can increase selection intensity, and providing additional information can increase accuracy of individual evaluations. Reducing genetic diversity could reduce genetic variance, and allowing selections to be made earlier could decrease generation interval. There are some major advantages to incorporating QTL or marker usage in breeding programs. The first advantage is increase in accuracy of selection due to addition of information directly related to genotype. A second advantage is the possibility of reducing generation interval by allowing selection to be made at an earlier age because this technique is not sex or age dependent. Initial QTL detection is done in families or small populations. Therefore integration of usage of a QTL into a whole population must be done carefully or restricted to families where the alleles are known to segregate. Because QTL detection is done on a single trait basis, pleiotropic effects on other traits must be studied carefully. Use of genetic markers is associated with some extra costs and risks due to poor QTL position estimates and effects (Visscher and Haley 1995).

There are two methods used to determine if a locus is a marker: the genetic marker approach and candidate gene approach. The candidate gene approach involves detecting markers for unknown QTL and directly using potential candidate genes as QTL (Gengler and Druet 2001). It uses a known gene as a candidate for the QTL. If a polymorphism is thought to be merely a marker for a DNA region containing the QTL, then the method is referred to as a genetic marker approach (Georges *et al.* 1995).

Genetic Marker Approach

The genetic marker approach, also referred to as linkage mapping, genome scan or positional cloning, involves systematically scanning an entire genome with evenly-spaced, highly polymorphic DNA markers, whose exact position is known. Kwon and Goate (2000) summarized this molecular approach. By using families, researchers can locate genetic regions “in linkage” with the trait by observing that affected family members share certain marker alleles located in regions more frequently than is expected by chance. These regions can then be isolated or cloned for supplementary characterization and analysis of responsible genes. The main advantage of linkage mapping is that experimenters need no prior knowledge of the biology or physiology underlying the trait being studied. The genetic marker approach is based on a known DNA polymorphism, but the polymorphism is usually nonfunctional. Use of genetic markers to detect chromosomal regions responsible for a fraction of a trait’s genetic variability is a very different approach to detecting QTL as pointed out by Parmentier *et al.* (2001). Detection using genetic markers makes use of the fact that two DNA sequences located close to each other on the same parental chromosome are likely to be transmitted together. Even though the marker is not involved in the genetic aspects of the trait, it can detect the QTL via linkage. Efficiency of positional cloning depends mainly on marker characteristics. Markers are best when distributed uniformly across the entire genome because QTL could be located anywhere. To characterize and trace parental chromosomes, the marker must have at least two distinct alleles. Determination of marker genotypes should be easy and inexpensive whenever possible (Parmentier *et al.*

2001). Markers linked with QTL can be found by a genome scan, which are very expensive (Hayes *et al.* 2001).

While linkage mapping is an impartial search of the entire genome without any presumptions about a certain gene's role, the candidate gene approach allows researchers to investigate the validity of a hypothesis about the genetic basis of a trait (Kwon and Goate 2000).

Candidate Gene Approach

General Description

A commonly used technique to identify genetic factors for complex traits is the candidate gene approach, also summarized by Kwon and Goate (2000) and Parmentier *et al.* (2001). A candidate gene approach directly tests effects of genetic polymorphisms of a potentially contributing gene in an association study. The candidate gene approach involves assessing associations between a certain allele of a gene and the trait itself. This type of association study addresses the question “Is an allele of a candidate gene more frequently seen in subjects with the phenotype in question than in individuals without the phenotype?” The candidate gene approach consists of examining various genes potentially involved in the physiological process of interest and determining whether an allele of that candidate gene locus results in the phenotype of interest. Classification of polymorphic sites and evaluation of allelic variations of candidate genes, among individuals with differing phenotypic merits, offer opportunity for easy identification of genetic markers associated with phenotypic worth.

Kwon and Goate (2000) describe the candidate gene process. The first step in conducting candidate gene studies is choosing a suitable candidate gene. Once a

candidate gene has been chosen, experimenters must find or choose a polymorphism that will be useful in testing in an association study. Ideally researchers must locate existing gene variants and determine which of those variants results in proteins with altered functions that may influence the trait being examined. Unfortunately, in most cases, the researcher knows a gene's sequence but knows nothing about functional variations in that gene. Functional mutations result in amino acid changes in the resulting protein or because it occurs in DNA regions controlling the gene's activity. To be useful in a candidate study, the genetic variant should occur with sufficient frequency to allow detection of differences among individuals with and without the trait in question. Unfortunately, not all genes have an easily identifiable common functional variant that can be exploited in association studies. In many instances, researchers have located only changes in individual nucleotides (single nucleotide polymorphisms, SNPs), which have no known functional significance. Nonetheless, SNPs can have potentially useful roles in narrowing a linkage region. They may show statistically significant associations with genes if they are located within or near that gene by virtue of linkage disequilibrium. These SNPs can be particularly useful in studies of complex traits for which many potential candidate genes exist. Once a candidate gene is chosen and a polymorphism found, investigators test the role of the candidate in a sample of affected and unaffected individuals.

Advantages and Disadvantages

Researchers, Kwon and Goate (2000) reported several advantages of the candidate gene approach. Candidate gene studies can be executed relatively quickly and inexpensively. They allow the possibility of locating genes with small effects. In

contrast with linkage mapping, studies of candidate genes do not require large families with affected and unaffected individuals. Instead it allows use of unrelated individuals or small families. Candidate gene studies are also better suited for detecting underlying common and more complex traits where the association with any given candidate gene is rather small (Collins *et al.* 1997). The candidate gene approach is useful for quickly determining association of a genetic variant with a trait and for locating genes of modest effect (Kwon and Goate 2000).

However, Kwon and Goate (2000) also listed some disadvantages to this method. It is limited by how much is known about the biology of the trait being investigated. The major issue with the candidate gene approach is that in order to select candidate genes, experimenters must possess an understanding of mechanisms underlying phenotypic expression. The candidate gene approach is dependent completely upon prior knowledge of genetics and physiology. Genes and their nucleotide sequences must be known. Selection of the correct gene is then based on good knowledge of its role in a metabolic pathway. Then additive effects for the different alleles for selected candidates are compared to establish allele substitution effects (Gengler and Druet 2001).

Utilization

A practical search for QTL involves a search that narrows with each stage. The first step is identifying chromosomal areas containing QTL of interest, followed by recognizing the pattern of QTL within these regions. Identification of tightly linked markers within these areas is done next. Finally, potential candidates would be chosen from this region (Parmentier *et al.* 2001).

Biotechniques

Polymerase Chain Reaction (PCR)

One procedure having a large effect on molecular genetics is polymerase chain reaction (PCR). The process of PCR involves exponentially amplifying a region of DNA. It involves using specifically designed, short segments of DNA, called primers, individual nucleotides, a thermo-stable polymerase and a special buffer to stabilize the reaction. During PCR, DNA is heated so that it is denatured into single strands first. Then the temperature is reduced so that primers can anneal to genomic DNA. Finally, the temperature is raised so polymerase can attach at the primers and incorporate free nucleotides to complement the single strand. These three steps are repeated many times.

Primer Design

The key to amplifying the region of DNA desired is to accurately design primers. Primer design is somewhat of an art. For this research project, introns within certain genes of the porcine genome served as the desired segments. Possible intron regions were identified by aligning porcine expressed tag sequences (EST), which represent exon sequences, with human sequences, which include the complete sequence. The ESTs were found using the TIGR database (www.tigr.org). The ESTs were matched with human sequences using the Human Golden Path program (<http://genome.ucsc.edu>). This allows alignment of sequences against the consensus sequence for the human genome. Intron-exon junctions are well conserved across species. Exon sequence is also well conserved between swine and human. Results are analyzed to find regions of non-homology, which likely represent intron regions. Homologous regions spanning non-homologous areas are then used in efforts to design primers. Because many factors must be considered when

designing primers, it is generally best to use a computer program. The program Primer3 (www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi), available on the Internet, takes into consideration GC content, melting temperature, primer length, and affinity of the primers for each other.

Restriction Enzymes

Having many copies of the segment of DNA of interest allows the researcher to perform analyses. The process of PCR is used quite often to search for polymorphisms, which are often useful. Polymorphisms in DNA are genetic variants that fall into two major classifications: those involving base-pair substitutions or small structural changes, including insertions, deletions and inversions, and polymorphisms involving variations in the number of tandem repeats of short, simple DNA motifs. There are a variety of methods used to detect polymorphisms. One method of detection uses restriction endonucleases, enzymes that recognize specific, short DNA sequences and cleave DNA at those recognition sites. Enzymes are incubated with the desired gene product at the enzyme's optimal temperature. Digestion of an amplified gene product yields various sized fragments of DNA, which can be separated by gel electrophoresis. Electrophoresis is a technique that allows molecules to be separated based on size and electric charge. A polymorphism is detected by a difference in banding pattern on the electrophoretic gel. These polymorphisms are termed restriction fragment length polymorphisms (RFLPs).

Marker Assisted Selection (MAS)

Traditional selection programs have been based on phenotypic observations. Effectiveness of phenotypic selection is reduced when the trait of interest is only measurable in one sex, late in life, or postmortem. In these situations use of genotype as a selection criterion could be a powerful tool, which can be determined early in the

animal's life and in both sexes. Discovery of mutations in genes responsible for traits of interest or in a locus marker for such genes can allow geneticists to employ the marker assisted selection (MAS) approach (Parmentier *et al.* 2001). This type of selection can be defined as utilization of DNA sequences that are linked with economically important traits to supplement phenotypic selection.

The foundation of this selection method is the highly variable nature of DNA. Because of molecular advances, it is now possible to more easily detect DNA mutations (Parmentier *et al.* 2001). Mutations can directly affect protein structure and function, RNA stability, rate and regulation of gene transcription, or amino acid sequence of the gene product. Mutations affect not only the gene itself, but also a closely linked region, which co-segregates with it. In this instance, the linked gene is generally unknown. No matter if the mutation affects functionality of the encoded protein or a closely linked region, if an allele of the polymorphic gene is associated with a production trait, it is termed a marker and can be used in MAS programs (Parmentier *et al.* 2001).

Hayes *et al.* (2001) simulated a small nucleus herd of 20 sows with 18 chromosomes each. Each chromosome was 100 cM and contained four QTL and five markers. Four traits were affected by QTL. Each nucleus piglet was genotyped for markers linked to QTL with the largest effects on a particular trait. This genotyping was done before selection. Selection decisions were made with and without marker information. Using marker information improved accuracy of selection and improved response to selection by 4-5% per generation.

Marker assisted selection is not affected by environmental factors, is not limited to a single sex, and does not require expensive or destructive testing, giving it some

advantages over phenotypic selection. It can also be done at an early age, reducing generation intervals. Programs employing MAS can include all individuals, even ones that are not born yet increasing selection intensity (Soller 1998).

Before entering into a MAS program, confirmation of a QTL's presence of must be made. Confirmation should be done using a large number of animals in each population of interest. Biological systems are intricate, and thus, implicate many genes having different influences. Interactions among loci should always be considered when contemplating use of a particular locus in MAS (Parmentier *et al.* 2001).

Genes Chosen

Many molecules, including steroid and peptide hormones, prostaglandins, cytokines and receptors, regulate preparation, initiation and progression of gestation and parturition in mammals. It is well known that reproduction in mammals is controlled by hypothalamic, pituitary and gonadal hormones (Igarashi *et al.* 1993).

Estrogen Receptor (ESR)

Biology and role

Steroid hormones, as well as their receptors, play an integral role in reproductive processes (O'Malley 1990). A prime example is a key female sex hormone, estrogen, whose function is mediated by its receptor. Estrogen receptor (*ESR*) is a good candidate gene for litter size due to its fundamental role in several reproductive pathways (Drogemuller *et al.* 2001). Estrogen synthesis by the rapidly elongating conceptus prior to pregnancy establishment in pigs increases significantly, thus implying a major impact of estrogen receptors on embryonic mortality (Pope 1994). Estrogen plays a role in maternal recognition of pregnancy (Geisert *et al.* 1990). The growing conceptus

produces estrogen, and receptors in the sow's uterus recognize this production by the conceptus (Hafez 1993).

Previous findings

The *ESR* locus has been of particular interest to swine producers since discovery of a polymorphism by Rothschild *et al.* (1996) in a *Pvu*II restriction site that shows linkage disequilibrium with a gene or genes controlling litter size in pigs of Meishan and Large White origins. They detected two *ESR* alleles. The *B* allele, originally identified in Chinese breeds, was found to be significantly associated with an increase in litter size. Results were confirmed by Short and colleagues (1997) in four commercial lines owned by the Pig Improvement Company. Rothschild *et al.* (1996) and Short *et al.* (1997) used data from approximately 10,000 litters to show that the *B* allele of *ESR* was associated with increased pigs per litter. The first estimates of litter size differences favoring the *BB* homozygote over *AA* were 2.3 and 1.2 pigs per litter in first parities of Meishan and European lines, respectively (Rothschild *et al.* 1996). Short *et al.* (1997) found differences in European lines to be 0.83 pigs per litter. They found the additive genetic effect associated with the *B* allele of the *ESR* gene was 0.42 pigs per litter in first-parity gilts.

Retinol Binding Protein 4 (RBP4)

Biology and role

Numerous proteins that are required for normal fetal pig development are secreted by the uterine endometrium (Roberts and Bazer 1988). Uterine proteins deliver nutrients to the developing conceptus. Retinol binding protein (RBP) is a uterine protein thought to transport retinol (Adams *et al.* 1981, Clawitter *et al.* 1990). Secretion of

uterine proteins, like RBP, transforms during pregnancy, and these changes are most likely related to the needs of the developing fetus. Harney *et al.* (1993) reported in gravid swine endometrium there are increasing levels of RBP4 gene expression from day 10 to 12. They indicate an important role for this transporter of vitamin A in uterine and conceptus physiology during pregnancy establishment. Yelich *et al.* (1997) reported that most embryonic death losses occur from day 10 – 18 of gestation. This is the time when trophoblasts are elongating and estrogen is being secreted by the conceptus. A major protein produced by the conceptus, which may play a role in trophoblast elongation, is RBP4 (Harney *et al.* 1990). It enhances gene expression of transforming growth factor β via retinoic acid receptors and is involved in embryonic development (Yelich *et al.* 1997). Retinol binding proteins are synthesized just prior to elongation of the trophoblast (Yelich *et al.* 1997). The role of RBP4 in embryonic development and timing of transcription make it a likely candidate affecting litter size (Linville *et al.* 2001).

Previous findings

An *MspI* PCR-RFLP test was developed to determine effects of this gene (Rothschild *et al.* 2000). Initial experiments using RBP4 have shown an additive gene effect of 0.52 ± 0.30 pigs per litter for the favorable allele in a hyper-prolific Large White line and 0.45 ± 0.43 in the control line (Ollivier *et al.* 1997). It was reported that RBP4 has an affect of 0.52 ± 0.45 for litter size in the select line and 0.32 ± 0.30 in the control line (Messer *et al.* 1996a). Rothschild *et al.* (2000) reported smaller effects for RBP4 and found the *A* allele of RBP4 to be the favorable allele. The favorable allele was found at higher frequencies than the alternative allele in three genetic lines examined, suggesting that prior selection had increased the frequency of the *A* allele. They found a significant

additive effect associated with RBP4 genotype of 0.15 pigs per litter born alive. They reported differences between alternate homozygous genotypes were approximately 0.50 pigs per litter for total number born (TNB) and 0.26 pigs per litter for number born alive (NBA). Results of the Rothschild *et al.* (2000) study demonstrate a moderate increase in number born, particularly in Landrace lines.

Follistatin (FS)

Biology and role

Follistatin (*FS*) is a cysteine-rich, monomeric, glycoprotein, which is encoded by a single gene (Knight and Glister 2001). It is subject to post-translational modification, which results in bioactive proteins of molecular weights ranging from 31-42 kDa (Robertson *et al.* 1987). Molecular analysis of different isoforms conducted by Inoye *et al.* (1991) has revealed that a solitary gene encodes *FS*. It is subject to alternative splicing. The gene for follistatin is composed of a region encoding for a short signal peptide and 5 exons. Alternative splicing occurs at the 3' end of the gene with the option of splicing out the fifth exon or keeping it. Alternative splicing generates two major species of RNA encoding proteins of 317 and 334 amino acids. After the signal peptide has been cleaved off, follistatin can be modified further by glycosylation and proteolysis.

Follistatin is functionally linked to the transforming growth factor-beta (TGF- β) superfamily by its role as a “high-affinity binding protein for activins” (Knight and Glister 2001). Binding of activin is considered to be the major functional role of follistatin because affinity of it for activin is similar to affinity of activin's receptors (Matthews 1994). Follistatin was first identified in ovarian follicular fluid through its

ability to “modulate” FSH secretion from gonadotropins *in vitro* (Knight and Glister 2001). Follistatin suppresses FSH secretion while activins enhance it. Activin β subunits are present in all reproductive tissues (Meunier *et al.* 1988). An activin receptor, ActRIIA, is necessary for folliculogenesis and therefore fertility (Matzuk *et al.* 1995a). Follistatin binds and neutralizes activin with a high affinity, thus preventing interaction with the type II receptors (Shimonaka *et al.* 1991).

Growth and development of primordial follicles through to ovulatory standing is linked with marked proliferation, recruitment and differentiation of somatic cells, and with changes in oocyte size and morphology (Knight and Glister 2001). Follicular development up to late preantral and early antral stages is independent of pituitary gonadotropins (Findlay and Drummond 1999). Activins and follistatins have been implicated as local players in folliculogenesis at this stage (Webb *et al.* 1999, Elvin *et al.* 2000). There is an abundant amount of evidence to support the opinion that activin and follistatin function as intraovarian regulatory molecules that are involved in follicle cell proliferation, steroidogenesis, oocyte maturation and corpus luteum function (Knight and Glister 2001).

Many *in vitro* studies have supported activin’s role in regulation of steroidogenesis in a variety of species. Knight and Glister (2001), Patel (1998) and Nishomori and Matzuk (1996) have summarized this. Activin suppresses theca cells’ androgen expression, enhances estrogen production, increases proliferation of granulosa cells, increases FSH receptor expression, and enhances oocyte maturation. Follistatin binds activin and thus reverses its effects. Without follistatin, activins are able to support synthesis of FSH while concurrently suppressing secretion of growth hormone (GH),

prolactin (PRL) and adenocorticotrophic hormone (ACTH), thus, allowing the individual to enter a reproductive phase. Alternatively, elevated levels of follistatin bind activin, reducing the amount of free activin. This leads to a prevention of FSH secretion while allowing release of GH, PRL, and ACTH, causing the organism to enter a quiescent stage. *In vitro* studies of isolated follicles, granulosa cells, theca cells and oocytes support the theory that follistatin, synthesized by follicular granulosa cells, and exerts local control to modulate follicle growth, gonadotropin responsiveness, steroidogenesis, oocyte maturation, ovulation, and corpus luteum function. Follistatin has also been found to reduce oocyte developmental competence and neutralize effects of endogenous and exogenous activin, which is consistent with its functional role as an activin-binding protein.

Knight (1996) reviewed follistatin, inhibin and activin roles in female reproduction. In the ovarian follicle, follistatin suppresses activin, which plays a role in aromatase expression. Evidence from *in vitro* studies using granulosa and theca cells shows that inhibins, activins and follistatins, which are synthesized and secreted by granulosa cells, exert local autocrine and paracrine actions in order to regulate follicular maturation and steroidogenesis. It has been hypothesized that at early stages of follicle development when androgen requirements are less, thecal androgen synthesis is kept in balance due to an excess of activin over inhibin and follistatin. Nonetheless, when dominant follicles approach preovulatory stage, rising levels of follistatin and inhibin expression by granulosa cells up-regulate thecal androgen emission, thus ensuring that granulosa cells receive ample supply of aromatase substrate for estradiol conversion.

Endometrial activin A may have potentially strong effects on embryonic and placental development during early stages of human implantation (Jones *et al.* 2002). In several species, including mice and *Xenopus*, activin A has been found to be involved in embryogenesis and is synthesized at a high rate with its receptors and binding proteins during early embryonic development (Smith *et al.* 1990, Thomsen *et al.* 1990, Kimelman *et al.* 1992, Albano *et al.* 1994). Activin A, manufactured by maternal endometrial epithelial and decidual cells during the period of fertilization and implantation, may play a significant part in maternal-fetal communication during embryo and trophoblast development and, therefore, could be important for the establishment of pregnancy (Jones *et al.* 2002). Activin A may also have significant roles in regulating endometrial and trophoblast development in early pregnancy. Because of activin's role in these matters, follistatin may also be a vital participant.

It has been proposed by Hillier (1991) that in immature follicles, which synthesize little estrogen, thecal androgen synthesis is low due to relative excess of activin over follistatin. As a dominant follicle approaches preovulatory status, increasing expression of follistatin in granulosa cells up-regulates thecal androgen secretion and ensures that the granulosa cells receive an adequate supply of P450 aromatase substrate to match increasing demand for estradiol synthesis. This proposal is supported by histological evidence of developmental changes in expression of activin and follistatin during folliculogenesis and by observed changes in intrafollicular concentrations of activin during follicular development (Magoffin and Jakimiuk, 1998).

In addition to activin, follistatin can also bind inhibins, members of the TGF β superfamily, but with a much lower affinity, and follistatin does not affect the bioactivity

of inhibin (Knight and Glister 2001). Follistatin has the ability to bind other members of the TGF- β superfamily, including BMPs (bone morphogenic proteins) 2, 3, and 7 (Shimonaka *et al.* 1991, Fainsod *et al.* 1997, Yamashita *et al.* 1995). Several BMPs, 2, 4 and 7 are produced in large amounts in the decidualized mouse uterus but have not yet been reported in the human endometrium (Ying and Zhao 2000, Paria *et al.* 2001).

Patel (1998) reviewed findings about follistatin expression in the body. The significance of follistatin was originally perceived to be limited to a time frame around the reproductive cycle in higher vertebrates. While follistatin was originally isolated based on the fact that it is involved in the reproductive cycle, it has been found to be expressed in a variety of embryological and adult tissues. It has been found in the pituitary, placenta, ovary, testis, brain, bone marrow, endochondral bone, pancreas and liver. Follistatin has also been found in the circulatory system and was suggested to be a transport protein analogous to steroid binding proteins in serum. But because of its low circulatory concentration, it is not likely to be involved in binding activin in the blood. Recently it was realized that follistatin is linked with cytokines, which are involved in production of the acute-phase response and inflammation (Phillips *et al.* 1998). Follistatin levels rise after surgery, and inflammation is an inducer of follistatin levels (Klein *et al.* 1996, 1993). This indicates other roles for follistatin. Variations in circulating follistatin levels during certain diseases, like chronic liver disease and renal failure, point to changes in these diseases (Sakamoto *et al.* 1996). Follistatin has been shown to accelerate liver regeneration (Kogure *et al.* 1995). How this happens is not yet understood. Follistatin is expressed in cells of developing bones and callus of repairing bones after fractures (Funaba *et al.* 1996). Follistatin is likely to have vital regulatory activities in

the prostate gland, pancreas and kidney (Thomas *et al.* 1997, Kotajima *et al.* 1995, Michael and Farnworth 1992).

Findings in other species

Follistatin has been characterized in numerous mammalian species, chickens and *Xenopus*. It is an extremely conserved protein, with 83% amino acid homology over all species and 95% homology in mammals (Phillips *et al.* 1998). Thus follistatin must serve an important function in all species examined.

Members of the TGF- β superfamily are produced by the human endometrium. They have been implicated in regulating angiogenesis, decidualization, implantation and immunomodulation at the maternal-fetal interface (Godkin and Dore 1998). The human endometrium undergoes decidualization during the secretory phase in preparation for trophoblast invasion in the event that pregnancy occurs (Jones *et al.* 2002). In the uterus of humans and rodents observed expression patterns of genes, which encode follistatin, activin and activin receptors, are consistent with a function in decidualization (Jones *et al.* 2000, Jones *et al.* 2002, Gu *et al.* 1995).

Activin has been shown to promote FSH receptor expression in undifferentiated rat granulosa cells (Hasegawa *et al.* 1988, Xiao *et al.* 1992). This finding may explain how a follicle at late preantral to early antral stage progresses from gonadotropin-independent to gonadotropin-dependent stage of development (Knight and Glister 2001). Because of follistatin's ability to bind and neutralize activin, the proposed mechanism of activin promoting FSH receptor expression would work best in absence of follistatin. It has been seen that undifferentiated rat granulosa cells express very little follistatin when compared with cells from more advanced follicles when compared with cells from more

advanced follicles (Shimasaki *et al.* 1989, Nakatani *et al.* 1991). Other studies have shown that activin enhances P450 activity and estradiol production while inhibiting progesterone secretion in rat and bovine granulosa cells (Hutchinson *et al.* 1987, Miro *et al.* 1991, Shukovski *et al.* 1991). In more advanced follicles activin has an anti-steroidogenic action (Cataldo *et al.* 1994). Follistatin reverses the effect of activin on progesterone secretion by human granulosa lutein cells but has no effect on those cells in absence of activin. Follistatin can also suppress P450 activity and increase progesterone secretion in rats (Xiao *et al.* 1992). Activin accelerates *in vitro* meiotic maturation of monkey oocytes (Alak *et al.* 1996), rat oocytes (Sadatsuki *et al.* 1993) and human oocytes (Alak *et al.* 1998). In all three species activin accelerated maturation was inhibited by follistatin.

Hemmati-Brivanlou and workers (1994) implicated follistatin as a key player in embryogenesis, causing a lot of interest in follistatin among developmental biologists. Follistatin has been proven to prevent ventralisation by the BMP 4 and thus, allows the ectoderm to follow a neural fate, as is the default state (Patel 1998). Matzuk *et al.* (1995b) used techniques involving homologous recombination to delete the murine follistatin gene. Even though the phenotype is lethal, mice survive to term. This proves that deletion of follistatin does not prevent neural induction. Mice that lack follistatin are smaller than heterozygous littermates. They have less muscle in their diaphragm, and their intercostal muscles are smaller. The mice fail to breathe and die soon after birth. Follistatin knockout mice also show some skeletal abnormalities as well as improper tooth and whisker development. These defects demonstrate the wide range of tissues in which follistatin functions during normal development. Germline deletion of follistatin

leads to death soon after birth and suggests that it is of major importance during early life (Patel 1998).

On the other hand, mice over-expressing follistatin did not die in a study conducted by Guo *et al.* (1998). These mice were of normal size, but had shiny irregular fur. No other abnormalities were noticed except in lines with strong over-expression. Fertility was reduced and correlated with extent of over-expression. Males had smaller testis, and females had smaller ovaries and thinner uteri. Reduced FSH levels were seen in one of the five over-expressing lines. Folliculogenesis in several of the over-expressing lines was arrested between primary and secondary follicle stages, while follicular atresia was present and almost no corpora lutea were observed. These data suggest that follistatin has a critical role during sexual development.

Why it is a good candidate

Synthesis of activins and inhibins in the endometrium varies during different stages of menstruation and early pregnancy (Otani *et al.* 1998, Leung *et al.* 1998, Jones *et al.* 2000, Petraglia *et al.* 1990). Synthesis of follistatin increases significantly during early pregnancy, in concert with increased production of activin subunits by decidualized stromal cells (Otani *et al.* 1998, Jones *et al.* 2000). Endometrial epithelial glands and decidualized stromal cells produce follistatin (Jones *et al.* 2002). Work has also shown that follistatin plays a crucial role during embryological development (Patel 1998). The drop in activin and follistatin within days of delivery after elevation during gestation suggests that these peptides play a vital but undefined role in initiation of parturition. Based on expression patterns of follistatin, location of follistatin products and

implications from studies involving follistatin function, follistatin may play an important role in determining litter size.

Prolactin (PRL)

Biology and role

Prolactin (*PRL*) is a hormone produced by the anterior pituitary. It is involved in many different endocrine activities and is necessary for reproductive success (Barker *et al.* 1992). There are in excess of 300 separate actions of prolactin reported in various vertebrate species. Included in these actions are effects on water and salt balance, growth and development, endocrine function and metabolism, brain and behavior, and immune regulation and protection (Bole-Feysot *et al.* 1998). A large percentage of these actions are in some way associated with reproduction, whether directly or indirectly (Van Rens *et al.* 2003). Several studies have concluded that PRL has a considerable affect on the ovaries and uterus (Rolland *et al.* 1976, Daniel *et al.* 1984, Jammes *et al.* 1985). Production of progesterone and relaxin from the corpora lutea is affected by prolactin (Yangfan *et al.* 1989).

Previous findings

Korwin-Kossakowska and colleagues (2003) found that *PRL* genotype had a significant affect on TNB and NBA for sows with two or more parities.

Prolactin Receptor (PRLR)

Biology and role

Prolactin receptor (*PRLR*), as the name implies, is a receptor for prolactin, which is an anterior pituitary peptide hormone that is necessary for reproductive success (Vincent *et al.* 1998). All actions of prolactin are mediated by the prolactin receptor (Van

Rens *et al.* 2003). The receptor has been identified in numerous tissues including brain, ovary, placenta and uterus in mammals (Kelly *et al.* 1991). Swine ovaries and endometrium contain PRLRs, which are distributed in a pregnancy-dependent way (Rolland *et al.* 1976, Jammes *et al.* 1985, Young *et al.* 1989). Endometrial prolactin receptor numbers increase on day 12 of pregnancy. The increase is stimulated by conceptus estrogen production, which allows for redirection of PGF_{2α} (prostaglandin F_{2α}) secretion to sustain corpus luteum function (Pope 1994). Thus, prolactin may play a vital part in establishing pregnancy in pigs. Mice having null mutations in PRLR, which eliminates functional receptors, are sterile because of a failure of embryonic implantation. They also show irregular cycles, lower fertilization rates and defective embryo development (Ormandy *et al.* 1997). This implies a potential role of PRLR in preparing and maintaining a proper environment for pregnancy.

Previous findings

An *Alu I* polymorphism was discovered at the PRLR locus in swine (Vincent *et al.* 1997), and ever since this finding PRLR has been considered as a candidate gene for litter size (Rothschild *et al.* 1998, Vincent *et al.* 1998). Gene frequencies were found to differ among breeds for PRLR; frequencies for the *A* allele were: Chester White = .25, Duroc = .79, Hampshire = .05, Landrace = .72, Yorkshire = .37, Meishan = .56, European Large White = .32 (Vincent *et al.* 1997). Genotype at the PRLR locus has been shown to explain a significant portion of variation in litter size in Large White, Meishan and Landrace based lines (Vincent *et al.* 1998). The PRLR locus was found to be associated with number born alive (NBA) and total number born (TNB) in four genetic lines tested (Rothschild *et al.* 1998, Vincent *et al.* 1998, Van Rens and Van der Lende, 2002).

Vincent *et al.* (1998) observed the *A* allele as having a significant association with an increase in litter size in three out of five commercial populations. Effects of the *A* allele ranged from 0.2 to more than one pig per litter differences in Large White and Landrace populations over opposite homozygous individuals (Southwood *et al.* 1999). Korwin-Kossakowska and colleagues (2003) found the effect of PRLR to be significant on NBA for first parities. The frequency of the *A* allele was 0.20. Litter sizes of *AA* sows were significantly lower than that of *BB* sows. Van Rens and van der Lende (2002) conducted a study to determine the effects of PRLR polymorphism on reproductive traits in Large White x Meishan F2 gilts. The polymorphism at PRLR tended to affect litter size in this study with *AA* gilts having larger litters. Ovulation rate was found to be significantly affected by PRLR genotype in a study by Van Rens *et al.* (2003). They found that number of implantation sites, number of embryos, and number of vital embryos were not significantly affected by genotype. The trend did exist for *AA* gilts to have higher numbers in each category than *BB* gilts. This study demonstrates that PRLR polymorphism affects pig ovaries, uterus and placenta in a manner, which may lead to litter size differences. In contrast, Drogemuller *et al.* (2001) found an additive effect on NBA in Durocs for the *B* allele at the PRLR locus. Isler *et al.* (2000) also found the *B* allele to be favorable. They found it to significantly influence the number of fetuses per uterine horn, average fetal weight and total fetal weight in Yorkshire x Large White crossbred pigs.

Preprolactin

Biology and role

When the preprolactin gene is transcribed, it includes the sequence, which codes for the functional form of prolactin, plus some other regions that get eliminated in post-translational modifications.

Why it is a good candidate

Because preprolactin is the precursor of prolactin, it may have some important roles in reproduction.

Leptin (LEP)

Biology and role

Leptin (*LEP*), a recently discovered 16 kDa protein that consists of 146 amino acids, is secreted by fat cells in response to changes in body weight or energy (Barb *et al.* 2001, Barb 1999). It has been implicated in the regulation of feed intake, expenditure of energy, and the neuroendocrine axis in rodents and humans (Barb 1999, Houseknecht and Portocarrero 1998). Fertility in mammals entails sufficient nutrition and metabolic fuel reserves (Van der Spuy 1985). Nutritional perturbations hinder the onset of puberty and interfere with normal estrous cycles by altering endocrine function in the gilt (Armstrong and Britt 1987, Barb *et al.* 1991, Barb *et al.* 1997), heifer (Houseknecht *et al.* 1988, Short and Bellows 1971) and ewe (I'anson *et al.* 2000, Sartin *et al.* 1988, Estienne *et al.* 1990).

Leptin acts on the brain, which in turn acts on the pituitary to increase leutinizing hormone (LH), growth hormone (GH) and PRL (Barb *et al.* 2000). Leptin treatment has been shown to advance sexual maturation in feed restricted animals (Barash *et al.* 1996, Ahima *et al.* 1997, Cheung *et al.* 1997). It was first identified as the gene product found

deficient in obese mice (Zhang *et al.* 1994). Administration of leptin to the obese mice led to improved reproduction as well as reduced feed intake and weight loss (Pellymounter *et al.* 1995, Campfield *et al.* 1995, Barash *et al.* 1996). Chronic leptin treatment restored fertility in leptin deficient mice (Barash *et al.* 1996). Data support the idea that leptin might act as a metabolic gate that permits activation of the reproductive axis and onset of puberty (Barb *et al.* 2000). Barash and workers (1996) proposed that leptin could signal the reproductive axis about the body's nutritional status, thus permitting reproduction to proceed if enough metabolic reserves are available and inhibiting reproduction if reserves are low. They found that leptin does stimulate the reproductive endocrine system. They also found leptin to stimulate gonadal function. Ovarian weight was found to increase subsequent to leptin treatment. Ovarian histology revealed increased amounts of follicular development. It seems apparent that the trophic action of leptin on gonadal function leads to an elevation in sex steroid production, shown by increased uterine weight.

An escalating number of studies have reported specific roles of leptin in control of reproductive functions (Houseknecht *et al.* 1998). Injection of recombinant leptin into Lep(*ob/ob*) mice restored secondary sex organ weight and function as well as, fertility (Barash *et al.* 1996). It is generally accepted that leptin acts on reproductive functions through its effect on the hypothalamic-pituitary axis (McCann *et al.* 1998). New evidence suggests novel, vital roles for leptin during pregnancy. It was discovered that circulating maternal levels in rats and humans increased during pregnancy and then fell around parturition (Chien *et al.* 1997, Hardie *et al.* 1997). The finding of leptin in human placenta, amnion, amniotic fluid, chorion, and chorionic villi, in addition to presence of

leptin and its receptor in pig trophoectodermal layer suggest a direct role for leptin in maternal-fetal crosstalk (Masuzaki *et al.* 1997, Ashworth *et al.* 2000). Leptin is more abundant in placenta that is supplying fetuses of normal growth than in those from growth-retarded fetuses. This suggests that leptin might be involved in growth and development of the fetus in humans and pigs (Ashworth *et al.* 1998).

Previous findings

Guay and workers (2001) found that the increase of 1.4 fetuses in a Meishan crossed line was associated with a significant increase in leptin expression in embryonic tissues. Dyer *et al.* (1999) reported similar results in purebred Meishan. Henson and Castracane (2000) proposed that leptin is involved in conceptus growth and development, fetoplacental angiogenesis and embryonic hematopoiesis.

Leptin Receptor (LEPR)

Biology and role

Leptin receptor (*LEPR*), as its name implies, is the receptor site of the protein leptin. Leptin receptor was first identified by expression cloning and has been classified as a member of the class one-cytokine receptors because of structural homology with IL-6 receptors and mutual signaling pathways (Houseknecht and Portocarrero 1998 , Tartaglia 1997). Leptin receptor is localized in brain and pituitary of pigs (Houseknecht and Portocarrero 1998). It appears that the hypothalamus is the main site of action for leptin, because leptin receptors are located within hypothalamic areas associated with control of appetite, fertility and growth (Dyer *et al.* 1997, Lin *et al.* 2000, Tartaglia *et al.* 1995).

Why it is a good candidate

Receptors of steroid hormones play an integral role in reproductive processes (O'Malley 1990). Presence of leptin receptor in pigs' trophoectodermal layer and presence of leptin expression in other reproductive tissues and fluids suggest a direct role for leptin, and thus its receptor, in maternal-fetal crosstalk (Masuzaki *et al.* 1997, Ashworth *et al.* 2000). Stratil *et al.* (1998) found polymorphic sites within the leptin receptor gene. Preliminary studies have suggested an association with litter size. Guay and workers (2001) found that the increase of 1.4 fetuses in a Meishan crossed line was associated with a significant increase in leptin receptor expression, as well as leptin, in embryonic tissues. Similar results were reported previously by Dyer *et al.* (1999) in a purebred Meishan line.

Uteroferrin

Biology and role

Uteroferrin, a purple-colored, progesterone-induced glycoprotein, is secreted by the surface and glandular epithelium of the pig uterus (Roberts and Bazer 1980, Chen *et al.* 1975). Uteroferrin appears to bear a solitary iron atom (Buhi *et al.* 1979). Uterine proteins, like uteroferrin, deliver nutrients to the developing conceptus (Vallet and Christenson 1996).

Why it is a good candidate

Numerous proteins that are required for normal fetal pig development are secreted by uterine endometrium (Roberts and Bazer 1988). Uteroferrin is manufactured in large quantities during pregnancy (Roberts and Bazer 1980). It is estimated that production during pregnancy of uteroferrin may be more than 1g/day at day 60 of gestation (Basha *et*

al. 1979). Uteroferrin's role may be one of transplacental iron transport during pregnancy (Schlosnagle *et al.* 1976). Placentation in swine is of the epitheliochorial type, and there is no invasion of maternal tissue by developing trophoblasts. Thus, the conceptus relies on secretion of macromolecular products by the sow during much of pregnancy (Buhi *et al.* 1982).

One of the purposes of maternally produced proteins is nutritional, and uterine secretions have been equated with a complex embryo culture medium (Bazer *et al.* 1978). Uteroferrin is a large component of uterine secretions (Buhi *et al.* 1982). Uteroferrin is taken up by the placenta at areolae, which are specialized regions located opposite the uterine glands (Chen *et al.* 1975). Therefore, rate at which the uterus secretes uteroferrin probably influences delivery of iron to the fetus during pregnancy (Vallet and Christenson 1996). Uteroferrin accumulates in the allanotic sac of each conceptus during pregnancy (Bazer *et al.* 1975). Thus, depending on the rate of iron removal, the allanotic sac could serve as a major site of iron metabolism in fetal pigs. The allanotic sac may be an iron reservoir for hemtopoiesis during midpregnancy. Iron, freed from uteroferrin in allanotic fluid, was found to enter fetal circulation readily and accrued in organs, in which active iron metabolism was occurring, like the liver and spleen, and fetal hemoglobin (Buhi *et al.* 1982).

Aromatase

Biology and role

Cytochrome P-450 aromatase is a product of the CYP 19 gene (Graddy *et al.* 2000). Aromatase P-450 (P-450arom) is the terminal enzyme that catalyzes the formation of estrogens from androgens in a diverse range of tissues including the

placenta, gonads, brain, liver and adipose tissue (Simpson *et al.* 1994). Aromatase is the rate-limiting enzyme in the production of estrogen in granulosa cells (Rao *et al.* 1993).

Aromatase in swine is quite unique. Expression of aromatase in swine is similar to that of other mammals, but unlike other species the isoform may vary with tissue type. Type I is predominantly expressed in ovary, type II in mid to late pregnancy endometrium and placenta, and type III in peri-implantation conceptus (Choi *et al.* 1996, Choi *et al.* 1997, Ko *et al.* 1994). Grady *et al.* (2000) presumed that these three forms show differences in substrate specificities, expression levels, activity and mode of regulation. They confirmed existence of three aromatase isoforms in the porcine genome.

Why it is a good candidate

Endocrine and paracrine signals mediated by estrogen are critical to the biological functions and/or proper development of multiple tissues ranging from embryonic gonad to adult brain in diverse vertebrate species (Simpson *et al.* 1994, Conley *et al.* 1996).

Grady *et al.* (2000) reported that aromatase is synthesized by ovary, endometrium, placenta and peri-implantation embryos in pigs and other mammals (Graddy *et al.* 2000). Because of the organs, which synthesize it, they suggested that aromatase may have a functional role in pregnancy. Mice that had their aromatase function knocked out were completely infertile (Fisher *et al.* 1998).

Graddy and colleagues (2000) found that changes in aromatase types expressed in uterine endometrium are a function of pregnancy stage. Existence of three distinct genes encoding aromatase isoforms suggests significant differences in the mechanisms regulating porcine aromatase. Transitions in aromatase isoforms may be correlated with uterine development necessary for pregnancy events (Graddy *et al.* 2000). The

significance of embryo-derived estrogens is in the initiation and formation of maternal-embryo communication, which occurs to a larger degree in pigs than any other mammals (Roberts *et al.* 1993).

Retinoic Acid Receptor Gamma (RAR γ)

Biology and role

Retinoic acid receptors (RAR) bind to retinoic acid, a very important hormonal signal. Vitamin A or retinoic acid (RA) is a product derived from endogenous retinol (Rothschild *et al.* 2000). Retinoic acid's main role is believed to be coupled with its action as a morphogen during embryogenesis (Zingg *et al.* 1993). It is essential in vertebrate species for controlling processes involved in embryonic patterning and organogenesis (Sporn *et al.* 1994, Chambon 1996). Mice with mutations in RARG gene often died in utero. Those that did not were markedly growth-deficient and had external deformities (Lohnes *et al.* 1994). This reflects early roles of retinoic acid signaling in axial rotation, segmentation and hindbrain closure. Wendling and workers (2001) concluded from their studies that signaling through RA receptors is necessary to establish proper hindbrain segmentation in mice.

Previous Findings

It has been shown that supplementing the diet of pregnant sows with vitamin A can increase litter size (Brief and Chew 1985). Preliminary studies by Messer *et al.* (1996b) reported effects on litter size for RARG.

Epidermal Growth Factor (EGF)

Biology and role

Epidermal growth factor (*EGF*), a polypeptide consisting of 53 amino acids, has been found to encourage cell growth (Vaughan *et al.* 1992). It is in the uterine fluid and may control conceptus and endometrial development during early pregnancy (Kim *et al.* 2001). It plays a role in physiology of reproduction (Hadley 1996). This protein and other factors act to regulate granulosa cell function (Chang *et al.* 1993). Epidermal growth factor has been implicated as a local player in folliculogenesis (Web *et al.*, 1999). It is produced by the conceptus and in the sow uterus. This factor stimulates growth and proliferation of skin epithelia in fetuses, and in neonates it stimulates pulmonary epithelia to grow and mature (Hadley 1996). Kim *et al.* (2001) reported that its expression is elevated before and during conceptus elongation. They found it decreases as elongation nears conclusion. Therefore, it is possible that EGF plays a role in conceptus elongation (Kim *et al.* 2001). They showed that EGF expression is associated with areolae development between day 30 and 40 of pregnancy. Areolae are responsible for uptake of uterine products, and they develop over uterine gland openings from day 30 to 40. So it is possible that variations in EGF expression may play some part in areolae development.

Previous Findings

Epidermal growth factor and its receptor mRNA expression have been reported in the pig conceptus by reverse transcription PCR (Vaughan *et al.* 1992). It has also been found in uterine fluid, and receptors have been found in the endometrium (Brigstock *et al.* 1996, Wollenhaupt *et al.* 1997). The location of receptors and tissues showing EGF expression suggest that EGF plays a role in the development of the porcine conceptus and

endometrium (Kim *et al.* 2001). Uterine capacity is a trait that contributes to litter size in pigs (Christenson *et al.* 1987). Direct selection for uterine capacity has increased uterine capacity significantly (Christenson *et al.* 2000). A QTL for uterine capacity was found on chromosome 8 near the known area of the location of the gene for EGF precursor (Mendez *et al.* 1999). Because of its location and its possible effect on conceptus or endometrial growth, EGF is a candidate gene.

Estrogen Sulfotransferase (EST)

Biology and role

Estrogen sulfotransferase (EST) is a cytosolic enzyme, which catalyzes sulfonation of estrogens. A sulfonyl group is added to estrogens at the 3' hydroxyl position (Qian *et al.* 1998). Sulfated estrogens do not bind to estrogen receptors, and thus, they are hormonally inactive (Brooks *et al.* 1978). Addition of the sulfonyl group to estrogens makes them water-soluble. The balance of this enzyme with its opposing enzyme, sulfatase help to determine estrogen sensitivity in estrogen target tissues. Because EST is found in the same tissues as ESR, it is believed that EST might act as a regulator to attenuate local estrogen response.

Why it is a good candidate

Enzymes whose job is steroid transformation are increasingly recognized as vital modulators of target tissue sensitivity to steroid hormones (Qian *et al.* 1998). The reaction that EST carries out may play a part in controlling estrogen levels in the endometrium. During female reproductive cycles EST is up regulated in the secretory phase of the endometrium (Pack and Brooks 1974). Porcine genome mapping revealed a region on chromosome 8, which is associated with uterine capacity, a component of litter

size (Kim *et al.* 2002). Estrogen sulfotransferase was mapped to chromosome 8 in swine near the region that is associated with uterine capacity (Kim *et al.* 2002). Expression of EST was found to be elevated from day 15 to 20 of pregnancy in the pig endometrium (Kim *et al.* 2002). Its expression levels significantly decreased from day 30 to 40 (Kim *et al.* 2002).

Relaxin

Biology and role

Relaxin is the term given to the protein, which was first shown to create relaxation and softening in the pubic ligaments in the guinea pig (Hisaw 1926). Kimura *et al.* (1994) and Sherwood (1994) summarized the properties and functions of relaxin. Relaxin was first identified 75 years ago. It is a peptide hormone, belonging to the insulin family of hormones, which is produced by the corpus luteum during pregnancy in pigs and rats. Relaxin has a major part during pregnancy in promoting connective tissue remodeling, including cervical ripening and relaxation of the pubic ligament. It acts on the connective tissues of the reproductive tract to aid in maintaining pregnancy, delivery facilitation and mammary gland preparation.

Why it is a good candidate

Relaxin has been reported to play a role in preventing premature birth in some species (Kimura *et al.* 1994). The corpus luteum is the major source of relaxin (Bagnell *et al.* 1993). Relaxin binding sites have been discovered in reproductive tissues like uterus and cervix (Tan *et al.* 1998, Weiss *et al.* 1982). During pregnancy, relaxin was found to increase growth of the uterus in pigs (Min *et al.* 1997). Experiments in rats where circulating relaxin was removed have suggested that relaxin in combination with

estrogen are necessary for normal parturition. (Downing and Sherwood 1985). Without circulating relaxin, gestation and labor were prolonged or prevented, and fetal mortality was increased. Relaxin knockout mice are fully fertile; however protracted labor was observed in these mice (Kimura *et al.* 1994). Because of the location of relaxin expression, its influence on uterine size, and its critical role in safe delivery of young, relaxin is a good candidate.

Prostaglandin Endoperoxide Synthase 2 (PTGS2)

Biology and role

Prostaglandin endoperoxide synthase 2 (*PTGS2*) is also called cyclooxygenase 2 (*COX2*) (Gladney *et al.* 1999). It is a rate-limiting enzyme in formation of prostaglandins (Lim *et al.* 1997). Prostaglandins are a class of powerful regulatory molecules, which are synthesized by many tissues (Richards *et al.* 1993). A null mutation in the *PTGS2* gene in mice yielded multiple reproductive failures, including incomplete oocyte maturation, non-extrusion of the first polar body and failure of wild type blastocysts to implant (Lim *et al.* 1997). Richards *et al.* (1993) reported that prostaglandin endoperoxide synthase is a hormone that is differentially regulated as follicles develop by FSH and LH. Prostaglandins are quickly induced by elevated gonadotropins just prior to ovulation.

Previous Findings

A RFLP was found in PCR products of *PTGS2* using *Mse I* (Gladney *et al.* 1999). Linville and workers (2001) found an allele frequency difference between lines selected for increased ovulation rate and a control line using this RFLP.

Conclusions

Optimal reproductive function is a necessary process for maintaining a species. Reproduction must be under precise genetic regulation. Breed differences and within breed genetic variability implies that genetic improvement in reproductive traits in pigs is possible (Rothschild and Bidanel 1998). Conventional animal breeding is characterized by utilization of existing genetic variability rather than artificially creating it and by detection of superior individuals using phenotype and advanced statistical methods, which allow genetic and environmental effects to be separated (Gengler and Druet 2001). However, utilization of crossbreeding programs, selection indices, and changes in management has only made minimal improvements in increasing litter size in swine. Because of this limited success, much effort has been employed recently to explore the use of molecular genetics to improve litter size.

Selection to increase frequency of favorable alleles has been initiated to improve reproductive traits in pigs (Rothschild 1998). Selection of individuals carrying favorable alleles at QTL based directly on DNA evaluation is called marker assisted selection (MAS) and has the potential to improve traits like litter size (Vincent *et al.* 1998). Markers are being sought in various ways, which show associations with economically important traits. Two major ways of detecting these markers are genome scans and candidate gene approaches. Each type comes with its own set of pros and cons. However, markers can be utilized in animal breeding programs. The candidate gene approach relies on identifying potential markers based on known biology and gene function (Rothschild and Soller 1997). The process is summarized by Rothschild *et al.* (2000). Polymorphisms are then identified within candidate genes. As soon as a

polymorphism is found, association studies are conducted to determine if a relationship exists between the marker and traits of interest. The studies do not require any crossbreeding experiments and can be applied directly to a commercial herd.

Because reproductive traits are so multifaceted, researchers are able to consider many different facets of the organism's biology to come up with candidate genes. Discovery of distinctive genes influencing litter size, followed by MAS, has the capability of hastening rate of genetic improvement in this economically important trait (Rothschild *et al.* 2000). A combination of MAS and traditional selection methods is effective for traits with a late age of onset, sex-limitations, or low heritabilities, like litter size (Soller 1994). Genetic markers allow early identifications of both males and females carrying favorable alleles, thus, improving selection accuracy, reducing generation interval and speeding up genetic progress (Drogemuller *et al.* 2001). Rothschild (1998) stated as more associations between markers and traits are identified, applications of MAS will increase. It is a technology that is very promising for reproductive and fertility traits like litter size.

Many genes can be examined for possible use in a MAS program. This is especially true when considering such a multi-faceted trait like litter size. All of the genes summarized previously could serve as candidates in a candidate gene search for litter size. There are countless others which could also be examined.

Grengler and Druet (2001) discussed the influences of biotechnology on animal breeding. Widespread use of biotechnology will result in major impacts on genetic progress. Biotechnologies, like candidate genes and genetic markers, can enhance determination of genetic values of individuals. Much research has been focused on

detection of QTL. The fundamental goal of the search is to improve accuracy of predicting genetic merit. Regardless of these large research efforts, these techniques will likely never explain all genetic variation. Because of this they need to be integrated into quantitative genetic evaluations.

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CHARACTERIZATION OF A LINE OF PIGS SELECTED FOR INCREASED LITTER SIZE FOR SEVERAL GENES USING THE CANDIDATE GENE

APPROACH

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Abstract

The purpose of this study was to characterize changes in allelic frequencies in a line of pigs selected for increased litter size using a candidate gene approach. Pigs were selected from the largest litters where litter size was based on number of fully formed pigs. Litter sizes were standardized at birth so replacement gilts were reared in litters of ten or fewer pigs. A contemporary control line was maintained. In generation nine, the estimated mean breeding value for litter size was 0.63 pigs greater in the select line than the control line. Regions of follistatin (*FS*), estrogen receptor (*ESR*) and retinol binding protein 4 (*RBP4*) were amplified using PCR. Two different RFLPs were identified for *FS*. One was characterized by *Msp* I (*FS* 1), and the other was characterized by *Fnu* 4HI (*FS* 2). Both lines were fixed for the *A* allele for the *ESR* marker. Frequencies of the *A* allele for *RBP4* were 0.266 and 0.207 in the select and control lines, respectively. Allele frequencies were not significantly different between lines. Frequencies of the *B* allele of *FS* 1 in generation ten were 0.339 and 0.588 in the control and select lines, respectively. Frequencies of the *B* allele of *FS* 2 were 0.3 and 0.577 in the control and select lines, respectively. Marker assisted selection has the potential to be highly advantageous in selection for lowly heritable and sex-limited traits, such as litter size. Changes in litter

size observed in this population were not associated with the *ESR* locus. However, follistatin may be responsible for changes in litter size observed between lines.

key words: candidate gene, litter size, swine, follistatin

Introduction

Improving reproductive traits in livestock species has become an increasingly important endeavor (Vincent *et al.* 1998). Production efficiency of livestock is greatly influenced by reproductive success. Litter size is economically important and easily measured traits. A lot of effort has been made to improve litter size (Johnson *et al.* 1999). Modest gains in litter size can mean large economic returns in overall efficiency (Rothschild *et al.* 2000).

Traditional methods of genetic selection are most often employed to improve livestock species. Litter size in swine has improved over the last several decades due to change in management strategies, using superior dam lines and by crossbreeding (Rothschild 1996). However, improvement in reproductive efficiency has lagged compared to that observed in growth and backfat.

At present litter size varies greatly from as few as two to as many as 20 pigs per litter, with means from nine to 11. Phenotypic standard deviations range between 2.5 and 3 pigs (Johnson *et al.* 1999). Enough genetic variation exists to increase litter size (Linville *et al.* 2001); however, litter size is lowly heritable (0.1) (Haley *et al.* 1988), sex limited and measurable only after sexual maturity is reached. If direct selection, in both sexes could be done for genes affecting litter size, selection response would be enhanced (Linville *et al.* 2001).

Expansion of porcine genome maps offers opportunities to locate individual genes that control reproduction (Rothschild 1998). Newly developed molecular techniques provide an opportunity for direct selection of animals based on genes that control litter size, as long as such genes or linked genetic markers are identified. Identification of

individual genes with large effects on quantitative traits provides opportunity to improve selection accuracy (Rothschild *et al.* 1996). Selection to increase frequency of favorable alleles has been initiated to improve reproductive traits in the swine industry (Rothschild 1998). Marker assisted selection (MAS) has potential to be used as a tool to improve response to selection for traits like litter size (Vincent *et al.* 1998). The candidate gene approach is used to identify potential markers and is based on biological function of particular genes (Rothschild and Soller 1997). Polymorphisms are then identified within candidate genes (Rothschild *et al.* 2000). As soon as a polymorphism is found, association studies are then conducted to determine if a relationship exists between the marker and traits of interest. Candidate gene studies do not require any crossbreeding experiments and can be applied directly to a commercial herd.

Reproduction is a complex quantitative trait. Thus, a number of potential candidate genes exist. Discovery of distinctive genes influencing litter size followed by MAS has the capability of hastening rate of genetic improvement in this economically important trait (Rothschild *et al.* 2000). Combined with traditional selection methods, MAS is effective for traits like litter size which have a late age of onset, are sex-limited, and have low heritabilities (Soller 1994). Genetic markers allow early identification of males and females carrying favorable alleles, thus improving selection accuracy, reducing generation interval and speeding genetic progress (Drogemuller *et al.* 2001). Rothschild (1998) pointed out that as more associations between markers and traits are identified, applications of MAS will increase, making it a technology that is very promising for reproductive and fertility traits like litter size.

The objective of this study was to determine if known polymorphisms in *ESR*, *RBP4*, and *FS* could explain response to eleven generations of selection for increased litter size.

Materials and Methods

Population Establishment

Direct selection for increased litter size was practiced for eleven generations in a Large White-Landrace composite population. Holl *et al.* (2003) reported results from the first nine generations of selection. Briefly, pigs were selected from the largest litters where litter size was based on number of fully formed pigs. In order to minimize maternal effects on litter size, litters were standardized at birth so that no replacement gilts were reared in a litter greater than 10 pigs. A control line was maintained using random selection. The following traits were recorded in generations ten and eleven: number of fully formed pigs at birth (NFF), number of pigs born alive (NBA) and number of mummified fetuses (MUM).

Sample Collection

Buffy coat

Ten ten-milliliter samples were collected from each individual in generation ten using vacutainers containing Tris EDTA. Blood samples were centrifuged at 4°C for 20 minutes. The buffy coat was removed from each sample and placed into a labeled micro centrifuge tube. Blood samples were stored at -20°C.

Tissue

Tail tissue was collected from each individual in generation eleven at birth. Tissues were stored in labeled containers at -20°C. Any individual needing to be re-sampled had ear tissue collected. Ear tissue was treated in the same manner as tail tissue.

DNA Extraction

Buffy coat

Modifications were made to Gentra's (Minneapolis, MN) protocol for the PureGene extraction kit. A mixture of two parts distilled water and one part buffy coat was put through the modified protocol, which involved extended incubations and centrifugation steps. Red blood cells were lysed first. Then white blood cells were collected and lysed to release DNA. Samples were treated with RNase A to degrade RNA. Proteins were then precipitated out of the samples. The DNA was washed and hydrated. Samples were stored at 4°C. Concentrations of each individual were determined. Any sample with a concentration less than 25 ng/μL was re-extracted.

Tissue

Modifications to the Gentra (Minneapolis, MN) protocol were also necessary for DNA extraction from tissue samples. Tissue pieces weighting 0.005 – 0.01 g were minced and added to Cell Lysis Solution (Gentra Systems, Inc.). Protease was added to each tissue sample to degrade the proteins. Protein Precipitation Solution (Gentra Systems, Inc.) was added to each sample to separate proteins from DNA. Then DNA was precipitated with cold isopropanol and washed with 70% ethanol. After precipitation, DNA Hydrating Solution (Gentra Systems, Inc.) was added to each sample to resuspend

DNA. Samples were stored at 4°C until further use. Concentrations were found and any samples needing re-extracting were run through this protocol again.

PCR

Designing and Choosing Primers

Expressed Tag Sequences (EST) for desired genes were found using the pig gene index on TIGR's website (www.tigr.org). Then ESTs were "blasted" against the Human Golden Pathway (<http://genome.ucsc.edu>). Regions of homology spanning non-homologous regions of one kilobase or less were chosen. Homologous regions were plugged into Primer3 (www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) in order to look for possible primer pairs. Primer pairs were chosen based on whether they would span the non-homologous regions, which were likely to be introns and thus, more likely to contain polymorphisms.

Some primers were ordered based on success of previously published sequences. For primer sequences refer to Table 1.

Optimizing Primers

Twenty-four PCR reactions were conducted using a pool of DNA composed of ten or more individuals. At least 25 ng/μL of DNA was used per reaction along with 1X Taq reaction buffer (with MgCl₂), 1 μL dNTPs (100 mM), 0.5 μL of each primer (0.1 μg/μL), 1 unit of Taq and sterile water to bring reaction volume up to 25 μL. Two sets of twelve reactions were run through a temperature gradient of 50°C - 65°C on MJ Research's (San Francisco, CA) Opticon to determine optimal annealing temperatures. Products from PCR were visualized using a 1% agarose gel with ethidium bromide to determine which temperature yielded the best product.

PCR

Refer to Table 2 for appropriate annealing temperatures for each primer pair.

Restriction Digest

Digest trials

A DNA pool for each PCR product was digested with a panel of restriction enzymes to identify cutters. Manufacturer's temperature recommendations were followed for each enzyme. Twenty microliter digests were conducted using 10 μ L of PCR product, 7.7 μ L of sterile water, 2 μ L of buffer supplied with the enzyme, 0.2 μ L BSA and 0.1 μ L of enzyme. Digests were allowed to incubate at the enzyme's optimal temperature for three hours. Digest results were visualized using a 3% agarose gel with ethidium bromide. Digest results were run alongside uncut PCR product to determine if the enzyme cut effectively.

All enzymes deemed to be cutters were further tested to see if they would produce polymorphisms. This was done by using PCR from 24 individuals. Products from these individuals were then digested by each enzyme deemed to be a cutter for that particular PCR product. Digest results were visualized on a 3% agarose gel with ethidium bromide to see if resulting banding patterns differed among individuals tested.

Digestions

Any enzyme producing a visual polymorphism was tested on generation ten individuals following the appropriate PCR. Table 1 lists restriction enzymes used to detect polymorphisms in each PCR product.

Genotyping

Following digestion, bands were visualized using a 3% agarose gel with ethidium bromide. Individuals were genotyped, and results were recorded. A couple of weeks later gel images were again scored to check for genotyping accuracy. Questionable genotypes were PCR'd again. Allele frequencies were calculated for the control and select lines. Whenever there was at least a ten percent difference in allele frequencies between lines, generation 11 was tested.

Sequencing PCR products

Sequencing PCR

The PCR products, in which no polymorphism was detected using available enzymes, were cleaned using a Qiagen (Valencia, CA) clean up kit. Clean PCR products were run through a sequencing PCR protocol using big dye. These PCR products were then sent to the Genomics Research Lab at North Carolina State University for sequencing.

Data interpretation

Sequence data were analyzed using two computer programs – Consed and Polyphred (University of Washington). Each sequence was examined for good quality scores. Sequences were inspected for single nucleotide polymorphisms (SNPs). If a SNP was found in a region of sequence with good quality scores, a restriction enzyme database was searched for enzymes, which would differentially cut at those SNPs. Once enzymes were located, they were tested to see if in fact they actually yielded a visible polymorphism by following the procedure described previously for digest trials. Any enzyme, which yielded a polymorphism, was run through the generation ten individuals following the digest protocol described previously.

Statistical Analysis

Allele frequencies were calculated by dividing the total tally of an allele in a line by twice the number of observations in that line. If at least a ten percent difference in allele frequencies was observed between lines in generation ten, then individuals in generation eleven were also genotyped. A standard chi-square analysis was done to determine possible deviations from Hardy-Weinberg equilibrium. A 2x2 contingency table was used to test for significant differences between lines. Changes in allele frequency were determined by difference between the control and select lines. A variance was calculated for each difference in gene frequency using the formula

$$V_{p_s-p_c} = \frac{p_{gs}g_{gs}}{2N_{gs}} + \frac{p_{gc}q_{gc}}{2N_{gc}}, \text{ where } p \text{ and } q \text{ are allele frequencies, } s \text{ and } c \text{ are lines, } g \text{ is}$$

generation and N is number of individuals with genotypic data per line. Variances for allele frequency differences that accounted for drift were also calculated using a formula from Linville *et al.* (2001). The formula was

$$V_{p_s-p_c} = p_{gc}q_{gc} \left(\frac{1}{2N_{gc}} + F_{gc} \right) + p_{gs}q_{gs} \left(\frac{1}{2N_{gs}} + F_{gs} \right), \text{ where } F \text{ represents average}$$

inbreeding in that generation. Variances were converted to standard errors by taking the square root of each variance.

Data were analyzed using PROC GLM (SAS Inst., Inc., Cary, NC). The model for follistatin included fixed effects of farrowing season, line, genotype and genotype*line. Dependent variables were breeding values for NFF, NBA and MUM in generations ten and eleven, which were estimated using an animal model in MTDF-REML. The genotype*line interaction was deleted from the model for *RBP4*. Least square (LS) means were calculated for each trait. Additive (a) and dominance (d) effects

were also calculated using estimate statements with orthogonal contrasts of solutions for genotypic effects. Additive effect is defined as half the difference between LS means of the two homozygotes. Dominance effect is the heterozygote LS mean minus the average of the two homozygote LS means.

Results

A summary of breeding value data for litter traits for both generations examined for each line is presented in Table 3. The change in breeding values when comparing the two lines has increased from generation ten to eleven for all litter traits. Inbreeding coefficients in both lines are increasing.

Allele frequencies for *FS 1*, *FS 2*, *ESR*, and *RBP4* are presented in Table 4. Only two alleles were found, except in the case of *ESR*. The *A* allele was designated as the favorable allele for *RBP4*, while *B* was designated as the favorable allele for both follistatin markers.

All markers were tested for Hardy-Weinberg equilibrium. Results of those tests can be found in Table 5. Markers were not expected to be in Hardy-Weinberg equilibrium due to violation of the assumptions of no selection and no genetic drift. The select line has undergone several generations of direct selection. The other infringement on the Hardy-Weinberg assumption of no random drift is violated in both lines due to the small population sizes. It is impossible to avoid random genetic drift in small populations. Deviations from an ideal population in Hardy-Weinberg equilibrium were not significant for any markers in the control line. However, the deviation was significant for *RBP4* in the select line and *FS 1* in the select line in generation eleven.

Allele frequency differences for *FS* markers and *RBP4* were tested for significance using a contingency table. All differences were found to be significantly different from zero (*FS* 1 $P = 0.0335$, *FS* 2 $P = 0.00165$, *RBP4* $P < 0.001$). Allele frequency differences along with two measures of standard error are presented in Table 6.

Additive and dominance effects are listed in Table 7. Follistatin 2 was not examined because this marker yielded the same genotype as *FS* 1 in all but two pigs. Additive effects for all three traits were found to significantly differ from zero in the control line for *FS* 1. Additive effects for NBA were found to differ significantly from zero for the select line when examining the same marker. Additive effects tended to differ from zero for *RBP4* when examining breeding value for number of mummified fetuses.

The LS means for all three genotypes within each line are presented in Table 8. Each trait was analyzed. For *FS* 1, the favorable genotype appears to be line dependent, with *BB* being favorable in the select line and the opposite being true in the control line. For *RBP4*, the *AA* individuals were superior for NFF and NBA.

When examining follistatin 1, the genotype*line interaction term was found to be significant in the model for breeding values of NFF, NBA and MUM ($P = 0.0047$, 0.0088 , <0.0001 , respectively). In the same models, genotype tended to be significant for NFF and was significant for MUM ($P = 0.0941$, <0.0001 , respectively). When looking at pair wise differences of LS means for each genotype by line, *AA* differed significantly from both *AB* and *BB* in the control line ($P = 0.0251$, 0.0037 , respectively), and *AB* tended to differ from *BB* ($P = 0.0951$) for NFF breeding values. In the case of NBA breeding values, *AA* tended to differ from *AB* ($P = 0.08$) and differed from *BB* ($P =$

0.028). In the select line for this same trait, *BB* differed from *AA* ($P = 0.03$) tended to differ from *AB* ($P = 0.09$). When comparing genotypes in the control line for MUM breeding values, *AA* was found to be significantly different from *AB* and *BB* ($P = <0.0001$, <0.0001). The *BB* genotype was also found to differ from *AB* ($P = 0.0396$).

When examining *RBP4*, the genotype term was found to be significant in the model for MUM breeding values ($P = 0.0455$). Genotype was not significant in any other model for *RBP4*. Pair wise differences were only found significant in the case of MUM. The *BB* genotype tended to differ from *AA* ($P = 0.10$) and differed from *AB* ($P = 0.03$).

Discussion

Differences for *FS* in frequencies between the two lines were anywhere from approximately 0.19 to 0.28 depending on marker and generation. Effects of selection, differing frequencies in the founder population, and drift could explain differences in allele frequencies observed in the *FS* markers. Drift is a non-directional force, which acts to alter allele frequencies. When no selection is present, amount of drift depends on allele frequencies and effective population size. Once drift begins, it is most likely that an allele's frequency will continue to change in the same direction (Falconer and Mackay 1996). With selection, amount of drift in a population depends on approximately the product of effective population size and the selection differential at the locus in question (Hartl and Clark 1989). Knowing allele frequencies in the base generation would help discern changes due to drift versus selection. When attempting to account for drift, by incorporating inbreeding coefficients into variances of allele frequency differences among lines, standard error estimates increased greatly and thus, differences were non-

significant (Table 6). Differences between lines for follistatin markers may be due in part to sampling and or background genetics of each line. This is not expected to be the causative mutation because the polymorphic site is located in an intron.

The additive effect of the A allele for *RBP4* tended to be significant when investigating MUM breeding values. The effect was positive, which is not ideal in the case of MUM. Additive effects for all three traits were found to be significant or highly significant from zero for *FS 1* in the control line. These effects were found to be negative for NFF and NBA. This means that the favorable allele is different in the control line than that of the select line. When examining MUM, however, the favorable allele is like that seen in the select line. Additive effects for the B allele were found to significantly differ from zero in the select line for NBA.

Data were analyzed using a model with an interaction term. The interaction between follistatin genotype and line was found to be a significant term when considering NFF, NBA and MUM breeding values. Genotype was also found to be significant for NFF and MUM. The genotypes examined within line were all found to be significantly different from each other for NFF in the control line. For NBA in the same line *AA* was found to be different from the other two genotypes. This indicates that *AB* is more similar to *BB*, than to *AA*. In the select line *BB* was found to differ from the other genotypes for NBA. This points toward *AB* being more like *AA*. In the control line, all three genotypes differed from each other for MUM. It is odd that in the control line for NBA, the heterozygote is more like the *AA* homozygote, while it is the opposite in the select line. This is probably due to the low occurrence of *BB* individuals in the control line.

When analyzing the data by line, follistatin genotype was found to be significant for NFF, NBA, and MUM breeding values in the control line. Follistatin genotype tended to be significant for NBA breeding value in the select line. This analysis revealed the *AA* genotype to be most favorable in the control line for NFF and NBA, while *BB* was found to be most favorable in the select line for the same traits. This result could be due to the small numbers of *BB* individuals in the control line. It could also be due to differing epistatic effects between the two lines.

Follistatin markers identified in this study were also found to be segregating in a purebred Duroc population and a Hampshire-Duroc composite population (unpublished data). This indicates that the markers are not unique to Large White or Landrace breeds.

Follistatin is a cysteine-rich monomeric glycoprotein, which is encoded by a single gene and is functionally linked to the TGF- β superfamily by its role as a “high-affinity binding protein for activins” (Knight and Glister 2001). Binding of activin is considered to be the major functional role of follistatin. Affinity of *FS* for activin is similar to affinity of activin’s receptors (Matthews 1994). Follistatin was first identified in ovarian follicular fluid through its ability to “modulate” FSH secretion from gonadotropins *in vitro*; *FS* suppresses FSH secretion while activins enhance secretion (Knight and Glister 2001). Activin β subunits are present in all reproductive tissues (Meunier *et al.* 1988). An activin receptor, ActRIIA, is necessary for folliculogenesis and therefore, fertility (Matzuk *et al.* 1995a). Follistatin binds and neutralizes activin thus, preventing interaction with the type II receptors (Shimonaka *et al.* 1991).

Growth and development of primordial follicles to ovulatory standing is linked with marked proliferation, recruitment and differentiation of somatic cells, and changes

in oocyte size and morphology (Knight and Glister 2001). Follicular development to late preantral and early antral stages is independent of pituitary gonadotropins (Findlay and Drummond 1999). Activins and follistatins have been implicated in folliculogenesis at this stage (Webb *et al.* 1999, Elvin *et al.* 2000). There is abundant evidence to support the opinion that activin and follistatin function as intraovarian regulatory molecules that are involved in follicle cell proliferation, steroidogenesis, oocyte maturation and corpus luteum function (Knight and Glister 2001).

Many *in vitro* studies, summarized by Knight and Glister (2001), Patel (1998) and Nishimori and Matzuk (1996), have supported activin's role in regulation of steroidogenesis in a variety of species. Activin suppresses theca cells androgen expression, enhances estrogen production, increases proliferation of granulosa cells, increases FSH receptor expression, and enhances oocyte maturation. Follistatin binds activin and thus, reverses activin's effects. Without follistatin, activins are able to support FSH synthesis while concurrently suppressing secretion of growth hormone (GH), prolactin (PRL) and adenocorticotrophic hormone (ACTH), thus, allowing the individual to enter the reproductive phase. Conversely, elevated levels of follistatin bind activin. This leads to a prevention of FSH secretion while allowing release of GH, PRL, and ACTH, causing entrance into a non-reproductive stage. *In vitro* studies of isolated follicles, granulosa cells, theca cells and oocytes support theory that follistatin synthesized by follicular granulosa cells exerts local control to modulate follicle growth, gonadotropin responsiveness, steroidogenesis, oocyte maturation, ovulation and corpus luteum function. Follistatin has also been found to reduce oocyte development

competence and neutralize effects of endogenous and exogenous activin, which is consistent with its functional role as an activin-binding protein.

Knight (1996) reviewed the roles of follistatin, inhibin, and activin in the female reproductive system. In the ovarian follicle follistatin suppresses activin, which plays a role in aromatase expression. Evidence from *in vitro* studies using granulosa and theca cells, shows that inhibins, activins and follistatins, which are synthesized and secreted by granulosa cells, exert local autocrine and paracrine actions in order to regulate follicular maturation and steroidogenesis. It has been hypothesized, that at early stages of follicle development, when androgen requirements are less, thecal androgen synthesis is kept in balance due to an excess of activin over inhibin and follistatin. Nonetheless, when dominant follicles approach preovulatory stage, rising levels of follistatin and inhibin expression by granulosa cells up-regulate thecal androgen emission, thus, ensuring that granulosa cells receive ample supply of aromatase substrate for estradiol conversion.

Additionally to its projected part in assisting decidualization, endometrial activin A may have potentially strong effects on embryonic and placental development during beginning stages of human implantation (Jones *et al.* 2002). In several species, including mice and *Xenopus*, activin A has been found to be involved in embryogenesis and is manufactured vigorously together with its receptors and binding proteins during early embryonic development (Smith *et al.* 1990, Thomsen, *et al.* 1990, Kimelman *et al.* 1992, Albano *et al.* 1994). Activin A manufactured by maternal endometrial epithelial and decidual cells during the period of fertilization and implantation may have a significant part in maternal-fetal communication during embryo and trophoblast development, and therefore could be important for the establishment of pregnancy (Jones *et al.* 2002). They

concluded that activin A may have significant roles in regulating endometrial and trophoblast development in early pregnancy. Because of activin's role in these matters, follistatin may also be a vital participant.

Hemmati-Brivanlou and co-workers (1994) implicated follistatin as a key player in embryogenesis, causing a lot of interest in follistatin among developmental biologists. Follistatin has been proven to prevent ventralisation by the bone morphogenic protein 4 (BMP 4) and thus, allows the ectoderm to follow a neural fate, as is the default state (Patel 1998). Matzuk *et al.* (1995b) used techniques involving homologous recombination to delete the murine follistatin gene. Even though the phenotype is lethal, mice survive to term. This proves that deletion of follistatin does not prevent neural induction. Mice that lack follistatin are smaller than heterozygous littermates. They have less muscle in their diaphragm, and their intercostal muscles are smaller. The mice fail to breathe, and die soon after birth. Follistatin knockout mice also show some skeletal abnormalities, such as, improper tooth and whisker development. These defects demonstrate the wide range of tissues in which follistatin functions during normal development. Thus follistatin is of major importance during early life.

Mice over-expressing follistatin did not die in a study by Guo *et al.* (1998). These mice are of normal size, but have shiny irregular fur. No other abnormalities were noticed except in lines with strong over-expression. Fertility was reduced and correlated with the extent of over-expression. Males had smaller testis and, females had smaller ovaries and thinner uteri. Reduced FSH levels were seen in one of five over-expressing lines. Folliculogenesis in several over-expressing lines was arrested between primary and

secondary follicle stages, follicular atresia was present, and almost no corpora lutea were observed. Follistatin has a critical role during sexual development.

Synthesis of activins and inhibins in the endometrium varies during different stages of menstruation and early pregnancy (Otani *et al.* 1998, Leung *et al.* 1998, Jones *et al.* 2000, Petraglia *et al.* 1990). Synthesis of follistatin increases significantly during early pregnancy, in concert with increased production of activin subunits by decidualized stromal cells (Otani *et al.* 1998, Jones *et al.* 2000). Endometrial epithelial glands and decidualization stromal cells produce follistatin (Jones *et al.* 2002). Work has also shown that follistatin plays a crucial role during embryological development (Patel 1998). The drop in activin and follistatin within days of delivery after elevation during gestation suggests that these peptides play a vital but undefined role in initiation of parturition. Because of the expression patterns of follistatin, location of follistatin products and implications from studies involving follistatin function, follistatin may play an important role in litter size. Follistatin has been mapped to chromosome 16 in pigs. However, to our knowledge no QTL have been detected on chromosome 16 for litter size. This could be due in part to the fact that QTL scans only have the power to detect loci with large effects.

Steroid hormones, as well as their receptors, play an integral role in reproductive processes (O'Malley 1990). A prime example is, estrogen, a hormone in females that is involved in pregnancy. Its function is mediated by its receptor, ESR. It is a good candidate gene for litter size due to its fundamental role in several reproductive pathways (Drogemuller *et al.* 2001). Estrogen synthesis by the rapidly elongating conceptus prior to pregnancy establishment in pigs increases significantly, thus implying a major impact

of estrogen receptors on embryonic mortality (Pope 1994). Estrogen plays a role in maternal recognition of pregnancy (Geisert *et al.* 1990). The growing conceptus produces estrogen, and receptors in the sow's uterus recognize this production by the conceptus (Hafez 1993).

The *ESR* locus has been of particular interest to pig producers since discovery of a polymorphism by Rothschild *et al.* (1996) in a *Pvu*II restriction site showing linkage disequilibrium with a gene or genes controlling litter size in pigs of Meishan and Large White origins. They detected two *ESR* alleles. In that study the *B* allele, originally found in Chinese breeds, was found to be significantly associated with an increase in litter size. These results were confirmed by Short and colleagues (1997) in four commercial lines owned by the Pig Improvement Company. Rothschild *et al.* (1996) and Short *et al.* (1997) used data from approximately 10,000 litters to show that the *B* allele of *ESR* was associated with an increase in pigs born per litter. Short *et al.* (1997) found the difference in the European lines to be 0.83 pigs per litter. The additive effect associated with the *B* allele of the *ESR* gene was 0.42 pigs per litter in first-parity gilts in that study.

The favorable *ESR* allele was undetected in the control and select lines of pigs used in this study. Other investigators have reported similar findings. Short *et al.* (1997) was unable to detect the favorable *ESR* allele in pigs of Duroc origin. Drogemuller *et al.* (1999) did not find the *ESR* polymorphism in German Landrace pigs. The *B* allele for *ESR* was absent in both the COL and C lines from Linville *et al.* (2001). Other investigators have been unable to verify association of the favorable allele with reproductive traits, even when the allele is present. Effect of the *ESR* allele was not detected in a study conducted by Rohrer *et al.* (1999) or by Drogemuller *et al.* (2001).

Drogemuller *et al.* (1999) also failed to locate any evidence of a QTL influencing litter size in the region around the ESR gene. Gibson *et al.* (2002) studied the association of the ESR *PvuII* polymorphism and sow productivity traits in an F₂ population of a Meishan Large White cross. Results of this research did not confirm population wide linkage disequilibrium between *PvuII* polymorphism of ESR and sow productivity traits. Isler and colleagues (2002) and Van Rens and Van der Lende (2000) found no association of *ESR* allele with TNB or NBA.

Numerous proteins that are required for normal fetal pig development are secreted by the uterine endometrium (Roberts and Bazer 1988). Uterine proteins deliver nutrients to the developing conceptus. Retinol binding protein (RBP) is a uterine protein thought to transport retinol (Adams *et al.* 1981, Clawitter *et al.* 1990). Secretion of uterine proteins, like RBP, undergoes transformation during pregnancy, and these changes are most likely related to needs of the developing fetus. Harney *et al.* (1993) reported in gravid swine endometrium there is increasing levels of *RBP4* gene expression from day 10 to 12. Their results support an important role for this transporter of vitamin A in uterine and conceptus physiology during pregnancy establishment. Yelich *et al.* (1997) reported that most embryonic death losses occur from day 10 – 18 of gestation. This is the time when the trophoblast is elongating and estrogen is being secreted by the conceptus. Retinol binding protein 4 is a major protein produced by the conceptus, which may play a role in trophoblast elongation (Harney *et al.* 1990). Yelich *et al.* (1997) reported that it enhances gene expression of transforming growth factor β via retinoic acid receptors and is involved in embryonic development. They also reported that retinol binding proteins are synthesized just prior to elongation of the trophoblast. The role of

RBP4 in embryonic development and timing of transcription make it a likely candidate in litter size (Linville *et al.* 2001).

An *MspI* PCR-RFLP test was developed to test effects of this gene (Rothschild *et al.* 2000). Initial experiments using *RBP4* have shown an additive gene effect of 0.52 ± 0.30 pigs per litter for the favorable allele in a hyper-prolific Large White line and 0.45 ± 0.43 in a control line (Ollivier *et al.* 1997). The *RBP4* locus was reported to have an affect of 0.52 ± 0.45 for litter size in a select line and 0.32 ± 0.30 in a control line (Messer *et al.* 1996). Rothschild *et al.* (2000) reported smaller effects for *RBP4*, and found the *A* allele of *RBP4* to be favorable. In that study the favorable allele was found at higher frequencies than the alternative allele in three genetic lines examined, suggesting that prior selection had increased the frequency of the *A* allele. They found a significant additive effect associated with *RBP4* genotype of 0.15 pigs per litter born alive. They reported the difference between alternative homozygous genotypes to be approximately 0.50 pigs per litter for total number born (TNB) and 0.26 pigs per litter for number born alive (NBA). Results of that study demonstrate a moderate increase in number born, particularly in Landrace lines.

While both alleles were detected in this study, allele frequency did not differ much between the two lines for *RBP4*. Extent of allele frequency differences between select and control lines was approximately 0.06, for *RBP4* markers. Linville and workers did not detect a significant difference between the lines in their study of *RBP4*. Drogemuller *et al.* (2001) also found no significant effects on litter size in a synthetic line for the *RBP4* genotypes. From the Rothschild *et al.* (2000) study, it is difficult to

determine if *RBP4* is a major gene influencing litter size or if it is linked to the major gene(s) influencing litter size.

Short *et al.* (1997) was unable to achieve a stable estimate of 0.42 pigs per litter in first parity gilts for the *B* allele of *ESR* until over 1,000 records were used. Nevertheless, some variations did exist among lines. In initial phases of the study by Short and colleagues (1997), no significant results were found when limited data were used. As more sows were included in the experiment, effect of *ESR* genotype was found to be significant. They found that at least 1000 litter records were necessary for effect estimates to be stable. Thus, many more records may be necessary in order to achieve stable estimates of effects of follistatin markers on litter traits.

Implications

Because the *FS* markers identified in this study were found to be segregating in several populations comprised of several breeds, they have the potential to be used in populations where other litter size markers are uninformative or nonexistent. The employment of marker information in combination with traditional selection methods will allow for faster improvement of litter size; therefore, the discovery of informative markers is very important.

Table 1. Primer sequences, restriction enzymes, product sizes and chromosomal locations of each gene^a.

Candidate gene	Primer Sequence	Restriction Enzyme	Allele size, bp			Chromosomal location
			A	B	Uncut	
Estrogen receptor	CCTGTTTTTACAGTGACTTTTACAGAG CACTTCGAGGGTCAGTCCAATTAG	<i>Pvu II</i>	120	65 55	120	1
Retinol binding protein 4	GAGCAAGATGGAATGGGTT CTCGGTGTCTGTAAAGGTG	<i>Msp I</i>	190	100	550	14
Follistatin 1	GGACCGAGGAGGACGTAAAT GGCCTTCCAGGTGATGTTA	<i>Msp I</i>	220 225	425	625	16
Follistatin 2	TGCCGAATGAACAAGAAGAA CAGAAAACATCCCGACAGGT	<i>Fnu 4HI</i>	200	125 75	450	16

^aAll primer sequences are listed from 5' to 3'. Only the polymorphic bands are listed under alleles. All genes except estrogen receptor had monomorphic bands.

Table 2. Annealing temperatures for each gene^a

Candidate gene	Annealing Temperature
Follistatin 1	56°C
Follistatin 2	62°C
Retinol binding protein 4	61°C
Estrogen receptor	56°C

^aThe annealing temperatures represented in the table are the temperatures at which optimal PCR product was achieved.

Table 3. Mean breeding values for litter traits and inbreeding values by line and generation.

Trait Measured ^a	line ^b	Generation 10	Standard deviation	Generation 11	Standard deviation
NFF	c	-0.0464	0.25	-0.032	0.203
	s	0.498	0.345	0.619	0.213
NBA	c	-0.0315	0.189	-0.018	0.153
	s	0.44	0.309	0.546	0.197
MUM	c	3.35*	8.69*	5.48*	10.7*
	s	1.28*	4.26*	1.48*	5.09*
Inbreeding Coefficient ^c	c	0.136	0.025	0.174	0.0197
	s	0.181	0.0235	0.206	0.0292

^aNFF, NSB, NBA and MUM represent number fully formed, number stillborn, number born alive, and number mummies, respectively.

^bc and s represent control and select lines, respectively.

^cAverage inbreeding was calculated using all individuals in each line per generation

* $\times 10^{-8}$

Table 4. Allele frequencies overall and within control and select lines^a

Candidate gene (generation)	Allele	Overall	Control	Select
Follistatin 1 (10)	<i>A</i>	0.49	0.661	0.412
(n = 99)	<i>B</i>	0.51	0.339	0.588
Follistatin 1 (11)	<i>A</i>	0.519	0.656	0.471
(n = 187)	<i>B</i>	0.481	0.344	0.529
Follistatin 2 (10)	<i>A</i>	0.511	0.7	0.423
(n = 95)	<i>B</i>	0.489	0.3	0.577
Retinol binding protein 4 (10)	<i>A</i>	0.247	0.207	0.266
(n = 91)	<i>B</i>	0.753	0.793	0.734
Estrogen receptor (10)	<i>A</i>	1	1	1
(n = 95)	<i>B</i>	0	0	0

^aSelect and control lines are from the North Carolina State University white lines. The select line has undergone direct selection for litter size for eleven generations. The control line was maintained through random selection.

Table 5. Observed numbers of genotypes in control and select lines, chi square values for Hardy-Weinberg Equilibrium and associated P values.

Gene	Genotype	Control	χ^2	P value	Select	χ^2	P value
FS 1 (10)	<i>AA</i>	12	1.56	0.212	10	0.586	0.444
	<i>AB</i>	17			36		
	<i>BB</i>	2			22		
FS 1 (11)	<i>AA</i>	20	0.185	0.667	33	6.63	0.01
	<i>AB</i>	23			65		
	<i>BB</i>	5			41		
FS 2 (10)	<i>AA</i>	13	2.18	0.139	9	1.79	0.181
	<i>AB</i>	16			37		
	<i>BB</i>	1			19		
RBP4 (10)	<i>AA</i>	1	0.0746	0.785	8	5.51	0.0189
	<i>AB</i>	10			17		
	<i>BB</i>	18			37		
ESR (10)	<i>AA</i>	31	0	1	64	0	1
	<i>AB</i>	0			0		
	<i>BB</i>	0			0		

Generation is specified underneath gene name. The markers are follistatin (FS 1 and FS 2), retinol binding protein 4 (RBP4) and estrogen receptor (ESR).

Table 6. Allele frequency differences of the select line from the control

Candidate gene	Generation	S-C	SE w/ drift	SE w/o drift
FS 1	10	0.25	0.288	0.0134
	11	0.185	0.307	0.057
FS 2	10	0.277	0.279	0.0663
RBP4	10	0.0592	0.159	0.0664
ESR	10	0	0	0

Markers are follistatin (FS 1 and FS 2), retinol binding protein 4 (RBP4) and estrogen receptor (ESR). The select line underwent 11 generations of selection for litter size. The control line was randomly selected for 11 generations.

Table 7. Additive (a) effects of favorable allele and dominance (d) effects for breeding values of litter traits^a.

Gene ^b	a or d ^c	s or c ^d	NFF	SE	NBA	SE	MUM (x 10 ⁻⁸)	SE (x 10 ⁻⁸)
FS 1	a	c	-0.294**	0.100	-0.201**	0.0879	-10.2***	2.53
	d	c	0.0184	0.063	0.0128	0.0552	-1.01	1.59
	a	s	0.0614	0.0488	0.0918**	0.0427	-1.28	1.23
	d	s	-.0071	0.0341	-0.0109	0.0299	-0.299	0.861
RBP4	a	c & s	-0.0275	0.12	-0.0705	0.104	3.52*	2.11
	d		-0.0724	0.0874	-0.0486	0.0758	-1.26	1.55

*P value < 0.1

**P value < 0.05

***P value < 0.0001

^a Litter traits are number fully formed pigs per litter (NFF), number of pigs born alive per litter (NBA) and number of mummies per litter (MUM). The B allele was considered favorable in the case of *FS 1*, while the A allele was considered favorable in the case of *RBP4*.

^b Genes were follistatin 1 (*FS 1*) and retinol binding protein 4 (*RBP4*).

^c Additive (a) and dominance (d) effects were estimated using orthogonal contrasts in PROC GLM (SAS, Inst., Inc, Cary, NC).

^d Lines were control (c) and select (s), where the select line was selected for number of fully formed pigs for 11 generations.

Table 8. LS means for litter trait breeding values^a

Gene ^b	Line ^c	Genotype	NFF	S.E.	NBA	S.E.	MUM (x 10 ⁻⁸)	S.E. (x 10 ⁻⁸)
FS 1	c	AA	-0.0189	0.045	-0.0323	0.039	9.26	1.1
		AB	-0.147	0.041	-0.120	0.035	2.1	1.0
		BB	-0.313	0.092	-0.234	0.081	-3.05	2.3
	s	AA	0.466	0.037	0.384	0.032	1.91	0.93
		AB	0.49	0.025	0.419	0.022	1.57	0.63
		BB	0.528	0.031	0.476	0.027	0.632	0.79
RBP4	c	AA	0.159	0.33	0.0639	0.290	8	5.8
		AB	-0.0845	0.11	-0.0507	0.091	0.9	1.8
		BB	-0.0308	0.078	-0.021	0.068	5	1.4
	s	AA	0.533	0.12	0.519	0.10	-1.88	2.1
		AB	0.468	0.081	0.444	0.07	-1.13	1.4
		BB	0.529	0.055	0.449	0.047	2.41	0.96

^aLS Means were calculated using PROC GLM (SAS Inst., Cary, NC) for number fully formed (NFF), number born alive (NBA) and number of mummies (MUM).

^bGenes were follistatin 1 (FS1) and retinol binding protein 4 (RBP4).

^cLines were control (c) and select (s).

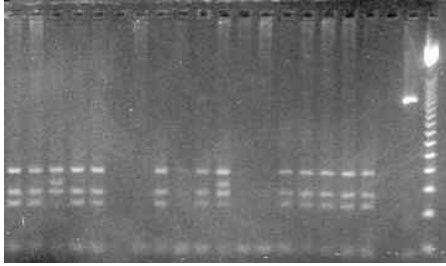


Figure 1. Retinol binding protein polymorphism. The first 2 lanes show *BB* individuals. The third lane shows an *AA* individual. An *AB* individual is in lane 11. The last two lanes show uncut PCR product and a 50 bp ladder.

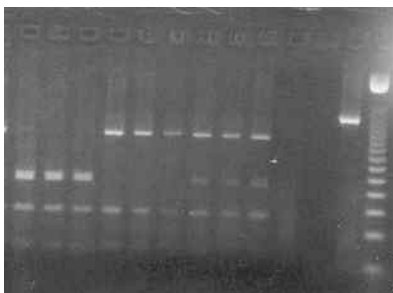


Figure 2. Follistatin 1 polymorphism. The first three lanes show *AA* individuals. The next three lanes show *BB* individuals. The next three lanes show *AB* individuals. The last two lanes show uncut PCR product and a 50 bp ladder.

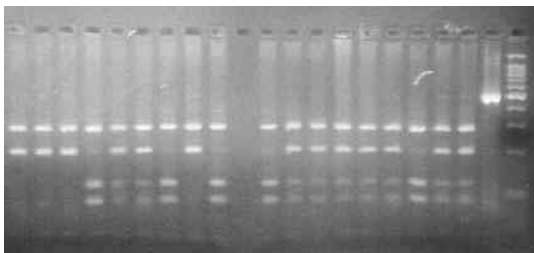


Figure 3. Follistatin 2 polymorphism. The first three lanes have *AA* individuals. The individuals in lanes 4, 7 and 9 are *BB*. Lanes 5 and 6 show *AB* individuals. The last two lanes show uncut PCR product and a 100 bp ladder.

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APPENDIX

Agarose Gels

Type	Agarose (g)	0.5X TBE (mL)	Ethidium Bromide (μ L)
3% 100 mL	3	100	2.66
3% 300 mL	9	300	8
2% 100 mL	2	100	2.66
2% 200 mL	4	200	5.33
1% 75 mL	0.75	75	2
1% 150 mL	1.5	150	4

Gels can be remelted and used again. When doing this add half the ethidium bromide as originally used.

PROTOCOLS

Sample Collection

Buffy coat

1. Using vacutainers with Tris EDTA, collect several one milliliter samples via jugular veinapuncture.
2. After collection, be sure to shake tubes to mix blood with the Tris EDTA to prevent clotting.
3. Store blood samples on ice until they can be processed.
4. Centrifuge blood samples for 20 minutes at 3000 rpm in a centrifuge cooled to 4°C.
5. Examine tubes to see if 3 layers exist. The top layer should be a clear, pinkish fluid, which is plasma. The next layer should be a thin and white. This is the buffy coat. The bottom layer should be dark red. It is the red blood cells.
6. Using a disposable pipette for each vacutainer, extract the buffy coat. This layer peels off the red blood cell layer.
7. The buffy coat should be placed into appropriately labeled micro centrifuge tubes.
8. Micro centrifuge tubes, containing buffy coat, should be stored at -20°C.
9. Disposable pipettes and used vacutainers should be discarded into an orange biohazard autoclave bag.

Tissue

1. At birth, tails are cropped from piglets.
2. These tails may be saved as sources of DNA.
3. Tails should be stored in labeled tubes at -20°C.

4. Any individual needing to be resampled, can have ear tissue collected.
5. Ear tissue should be treated in the same manner as tail tissue.

Hair

1. A pair of pliers is used to pluck hairs from individuals.
2. On average ten hairs, with intact follicles should be collected.
3. Place the hairs into bags with the appropriate identification number.
4. Be sure to clean pliers of any loose strands in between individuals.
5. Store hairs at room temperature.

DNA Extraction

DNA Extraction from unclean buffy coat (Modifications of PureGene kit from Gentra)

1. Allow n blood samples to thaw at 4°C.
2. Number n 1.5 mL micro centrifuge tubes.
3. Add 200 μL $\text{d}^2\text{H}_2\text{O}$ to each of the numbered tubes.
4. Once blood samples are thawed, vortex to mix.
5. Add 100 μL of blood sample to each of the n tubes.
6. Vortex to mix.
7. Return blood to fridge.
8. Set water bath temperature to 37°C.
9. Add 900 μL red blood cell lysis solution (Gentra) to each numbered tube containing water and blood.
10. Invert the samples several times to mix.
11. Allow the samples to incubate at room temperature for 5 minutes.
12. After five minutes invert the tubes approximately ten times to mix.
13. Allow the samples to incubate at room temperature an additional five minutes.
14. Make a master mix of cell lysis solution and RNase A according to the following:
 - 300 μL cell lysis solution for each of n samples.
 - 1.5 μL RNase for each of n samples
 - Vortex to mix.

** This is done because it is very hard to accurately pipette 1.5 μL .
15. Centrifuge samples for 35 seconds at 13,000 rpm to pellet the white blood cells.

** After this all samples should have a very visible white pellet.
16. Pipette off and discard all but ~20 μL supernatant.
17. Vortex vigorously to re-suspend the pellet in the residual fluid.
18. Add 300 μL master mix to each tube to lyse the cells.
19. Pipette the solution up and down using a large mouth pipette tip to break up any cell clumps.

** The solution should be quite viscous.
20. Incubate the samples in the 37°C water bath for an hour with shaking at 50 rpm.
21. Incubate the samples overnight at room temperature. At this point the samples are stable at room temperature for up to 18 months.

22. Examine the samples to make sure they are homogenous. If they are not then let them continue to incubate at room temp.
23. Add 100 μ L protein precipitation solution to each of the samples.
24. Vortex the samples for ~30 seconds.
25. Place samples in the fridge for 5 minutes.
26. Centrifuge the samples for 7 minutes at 13,000 rpm to pellet the proteins.
 ** The pellets should be tight, (not loose) after centrifugation. If they are not, put the samples on ice or in the fridge for 5 minutes and repeat centrifugation.
27. Set water bath temperature to 65°C
28. During the centrifugation step, number n new sterile micro centrifuge tubes.
29. Add 300 μ L isopropanol to the new set of tubes.
30. Once centrifugation is complete, pipette the supernatant (containing DNA) off and into the tubes containing isopropanol.
 ** Be careful not to get any of the protein pellet when removing the supernatant.
31. Gently invert the tubes 50 times to mix.
32. Place samples in the fridge for 5 minutes.
33. Centrifuge the samples for 7 minutes at 13,000 rpm to pellet the DNA.
34. Carefully remove and discard the supernatant from the tubes.
35. Invert tubes briefly on absorbent paper to drain.
36. Add 300 μ L 70% ethanol to each sample.
37. Centrifuge the samples for 2 minutes and 30 seconds at 13,000 rpm.
38. Carefully remove and discard the supernatant from each tube.
39. Invert the tubes and allow them to dry at room temperature for 15 – 25 minutes.
40. Add 100 μ L DNA hydrating solution to each sample.
41. Pipette the samples up and down gently with a large mouth pipette tip to dislodge the pellet from the wall of the tube.
42. Incubate the samples for 1 hour at 65°C with shaking at 50 rpm.
43. Incubate overnight at room temperature.
44. Store at 4°C when extraction is complete.

DNA Extraction from tissue samples (modifications using the PureGene kit from Gentra)

1. Number n micro centrifuge tubes.
2. Cut a piece of tissue weighting 0.005 – 0.01 g.
3. Mince the tissue piece up using a scalpel and place in the appropriate tube.
4. Add 300 μ L Cell Lysis Solution (Gentra).
5. Add 1.5 μ L Protease to each tube.
6. Incubate samples overnight at 55 °C.
7. Incubate at room temperature until most of the tissue is dissolved.
8. Transfer supernatant to clean tubes and discard the remaining tissue.
9. Add 100 μ L Protein Precipitation Solution (Gentra) to each sample.
10. Vortex samples for 20 seconds.
11. Incubate samples at 4 °C for 15 minutes.
12. Number a new set of tubes.
13. Add 300 μ L isopropanol to the new tubes and put at 4°C.
14. Centrifuge samples for seven minutes at 13,000 rpm.
15. Transfer supernatant to isopropanol containing tubes.

16. Invert tubes 50 times to mix.
17. Centrifuge samples for 3 minutes at 13,000 rpm.
18. Pour off and discard supernatant.
19. Allow tubes to drain briefly.
20. Wash pellets with 300 μ L 70% ethanol.
21. Centrifuge samples for two minutes at 13,000 rpm.
22. Pour off and discard supernatant.
23. Allow pellets to dry for approximately 30 minutes.
24. Add 100 μ L DNA Hydrating Solution (Gentra) to each sample.
25. Incubate samples for 1 hour at 65 °C.
26. Incubate samples overnight at room temperature.
27. Store samples at 4 °C.

DNA Extraction from Hair with intact follicles (modifications to Gentra's PureGene Extraction Kit)

1. Label 2 sets of n tubes with the appropriate identification numbers.
2. Add 100 μ L of Cell Lysis Solution to one set of tubes.
3. Using forceps to hold the follicle, cut the follicle from 5 hairs and add them to the tubes of Cell Lysis Solution. Be sure to cut as close to the follicle as possible.
4. Add 1 μ L Proteinase K to the tubes containing the follicles.
5. Invert the tubes 25 times to mix.
6. Incubate the samples overnight at 55°C.
7. Incubate samples at room temperature overnight.
8. Add 100 μ L of Isopropanol to the second set of labeled tubes and store at 4°C.
9. Pipette samples up and down to mix.
10. Add 33 μ L Protein Precipitation Solution to each sample.
11. Vortex samples for 20 sec.
12. Incubate samples at 4°C for 10 min.
13. Centrifuge samples for 3 min at 13,000 rpm.
14. Transfer supernatants in the samples to the Isopropanol tubes. Discard the pellets.
15. Invert tubes 50 times to mix.
16. Incubate samples overnight at 4°C.
17. 1 μ L of Glycogen Solution may be added to tubes at this time. This step is optional. If included, invert tubes 50 times and incubate at 4°C for 10 min before proceeding to the next step.
18. Centrifuge samples for 7 min at 13,000 rpm.
19. Carefully pour off supernatant.
20. Allow tubes to drain briefly.
21. Add 300 μ L 70% ethanol to tubes.
22. Centrifuge samples for 3 min at 13,000 rpm.
23. Pipette supernatant off.
24. Invert tubes on absorbent paper and allow to dry.
25. Once tubes are dry, add 20 μ L DNA Hydrating Solution to each tube.
26. Incubate at 65°C for 1 hour.
27. Incubate overnight at room temperature.

28. Pipette gently to mix before use in PCR reactions.

Creating a DNA Pool
(using DNA and water)

1. Figure out the number of PCR reactions to be run including an extra reaction. All calculations are based on this number.
2. Choose a factor greater than 4 that will evenly divide into the number of reactions (if possible). The factor chosen will be the number of individuals you will use in your pool. The answer you get when dividing the factor into the number of reactions will be the number of μL from each individual you will add.
3. Multiply the number of PCR reactions by 4, this will tell you how much water to add to the DNA pool
4. Pipette the pool up and down with a large mouth tip to mix.
5. 5 μL of pool is used in each 25 μL PCR reaction.

Example:

You want to run 35 PCR reactions, so figure on 36 reactions. Several things will divide into 36 evenly, so you must choose an option. All options are below:

<u># individuals</u>	<u>μL of each individual</u>
6	6
9	4
12	3

Obviously, the more animals the better, but you must consider how many animals you have DNA to spare from in order to choose the appropriate amount.

$36 \times 4 = 144$, so add 144 μL of water to the mix.

Creating a DNA Pool
(DNA only)

1. Figure out the number of PCR reactions to be run including an extra reaction. All calculations are based on this number.
2. Choose a factor greater than 4 that will evenly divide into the number of reactions (if possible). The factor chosen will be the number of individuals you will use in your pool. The answer you get when dividing the factor into the number of reactions will be the number of μL from each individual you will add.
3. Pipette up and down to mix.
4. 1 μL of this pool is added to a 25 μL PCR reaction.

Cleaning up DNA with the QIAquick Cleanup Kit from Qiagen

1. Add 900 μ L PB Buffer to 180 μ L of DNA sample.
2. Mix this solution well.
3. Add the solution to the provided spin column. The column should be fitted to a micro centrifuge tube before use. Be sure not to overfill the column. If the solution is too much, then steps 3-5 can be repeated as necessary.
4. Centrifuge the samples with the column for 1 min at 13,000 rpm.
5. Discard the liquid in the bottom of the tube and replace column.
6. Repeat steps 3 – 5 if additional solution remains.
7. Add 750 μ L PE Buffer to columns.
8. Centrifuge column 1 min at 13,000 rpm.
9. Discard liquid collected in the tube.
10. Centrifuge column another min at 13,000 rpm.
11. Discard collection tube with any remaining liquid.
12. Place column into a new tube.
13. Add 30 μ L EB Buffer to column.
14. Allow column to sit for 1 min.
15. Centrifuge column 1 min at 13,000 rpm. This collects the DNA into the clean tube.
16. Discard the column.
17. Store DNA at 4°C in properly labeled tubes.

Determining DNA concentration using Biomate3 Thermo Spectronic

1. Allow spectrophotometer to warm up.
2. Select “nucleic acid tests”.
3. Select “DNA (260/280)”.
4. Select “test name” to name the test (if a name is desired).
5. Select “dilution multiplier”.
6. Enter 10 μ L next to sample volume and 990 μ L next to diluent volume (or the appropriate volumes if different).
7. Select “number of samples” and enter the number of samples being speced.
8. Rinse cuvettes.
9. Add 990 μ L distilled autoclaved water to 4 or less cuvettes and 1000 μ L to 1 cuvette to serve as the blank.
10. Add 10 μ L of sample to the cuvettes.

Designing and Picking PCR Primers

1. Go to www.tigr.org
2. From the TIGR homepage, choose “tigr gene indices”.
3. Select the pig index to search.
4. From the search options, choose “gene product name”.
5. Type the name of the gene and hit enter.
6. Look at all the results received and choose the one that is the complete sequence (if possible). Also make sure that it is DNA sequence.
7. Highlight the sequence and copy it.
8. Paste the sequence into a word document being sure to label the sequence with the gene name. (Should also make a note that it is pig sequence.)
9. Go to <http://genome.ucsc.edu> to get to the human golden path.
10. From the menu bar, choose “BLAT” search.
11. Paste the pig sequence into the window and click submit.
12. If multiple results come back, look at the details of the result with the highest score.
13. In the results, scroll down to where the human chromosome sequence starts.
14. Look for regions where there is section of capitalized blue letters (these indicate homology between the pig and human sequence.) Following the capitalized blue letters, one needs to see a longer section of lower case black letters. After the lower case black letters, there should be another region of capitalized blue letters. The black lower case letters are human sequence, most likely an intron. The area from blue – black – blue should be approximately 1000 bases in size.
15. Copy the area from blue region to blue region, including the intron.
16. Past this into a new word document.
17. Go to the word document containing the pig sequence and use the find option in Microsoft Word to search for the first homologous regions (blue capitalized letters). Highlight the whole homologous section in the pig sequence. Mark the sequence in some way, by making it bold, underlined or another color. Repeat this process for the next homologous region. In between the selected regions in the pig sequence there should be a few unselected bases.
18. Once the homologous regions have been clearly marked in your pig sequence, copy the first homologous region and paste it into the window at www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi
19. Behind the sequence enter 2 slashes (//) to indicate where the intron is.
20. Copy the second homologous region from the word document.
21. Paste it into the window on the Primer3 page behind the two slashes.
22. Give the results a name (for example the gene name).
23. Click pick primers.
24. Examine the primers that the program picked and make sure that a primer lies on each side of the 2 slashes.
25. Copy the primer sequences and paste into a new word document with the gene name.
26. Order the sequences.

Diluting Stock Primers

Label tubes with primer name.

1. To create a stock solution of primer, add 1 μL sterile water per μg of primer to the tube of primer. This yields $1\mu\text{g}/\mu\text{L}$.
2. Vortex stock solutions well.
3. Add 450 μL sterile water to the newly labeled tubes.
Add 50 μL stock primer to tubes of water, being careful to add to the appropriate tubes. This yields a $0.1\mu\text{g}/\mu\text{L}$ working solution.

Master Plate

1. Type all ID numbers into a master well plate form.
2. Add 120 μL sterile distilled water to each well of a 96 well plate.
3. Add 30 μL DNA to each well according to the form.
4. Pipette the wells up and down with a large mouth pipette tip to mix.
5. Aliquot this plate out into 27 plates by removing 5 μL from the master plate and adding it to each plate.
6. Briefly centrifuge the plates at 2000 rpm.
7. Wrap the plates loosely with syran wrap.
8. Place plates in the hood to dry overnight.
9. Randomly try to pipette a number of wells in each plate to ensure that they are dry.
10. Cover each plate in parafilm and store at room temperature.

Primer Optimization

1. Prepare a DNA pool using 5 or more animals.
2. Add 1 μL DNA pool to 2 rows of a 96 well plate. (If pool is 1 part DNA to 4 parts water then add 5 μL of the pool to each well, be sure to use the amounts in () in step 3 if using the pool with water and DNA)
3. Program the opticon with a profile as follows (or find a similar program all ready on the opticon):
 - a. 94°C for 10 minutes
 - b. 94°C for 1 minute
 - c. Gradient from 50°C to 65°C for 1 minute
 - d. 72°C for 1 minute
 - e. Go to step 2 for 45 cycles
 - f. 72°C for 10 minutes
 - g. 4°C forever
 - h. end

4. Prepare enough master mix for 25 PCR reactions. Keep reagents and master mix on ice.

	<u>1 rxn</u>	<u>25 rxns</u>
d ² H ₂ O	19 (15)	475 (375)
10x Taq Buffer	2.5	62.5
DNTPs	1	25
forward primer	.5	12.5
reverse primer	.5	12.5
Taq	.1	2.5

*****Taq should be added last. Once it has been added, you must work quickly. Vortex to mix.

5. Add 24.6 (19.6) μ L master mix to wells.
6. Seal plate with adhesive plate seal. Make sure it is sealed well to minimize chance of evaporation.
7. Centrifuge plate briefly.
8. Place plate in the opticon and run the optimization program.
9. Run products on a 1 – 2% agarose gel to determine which annealing temperature worked best for the primers.

PCR

1. Add 1 μ L DNA from individuals to n number of wells in a 96 well plate.
2. Once an optimal annealing temperature has been established for a set of primers, program the thermocycler with a profile as follows (or find a similar program all ready on the thermocycler):
 - i. 94° C for 10 minutes
 - j. 94° C for 1 minute
 - k. optimal annealing temperature
 - l. 72° C for 1 minute
 - m. Go to step 2 for 45 cycles
 - n. 72° C for 10 minutes
 - o. 4° C forever
 - p. end
3. Prepare enough master mix for n+1 PCR reactions. Keep reagents and master mix on ice.

	<u>1 rxn</u>
d ² H ₂ O	19
10x Buffer	2.5
DNTPs	1
forward primer	.5
reverse primer	.5
Taq	.1

*****Taq should be added last. Once it has been added, you must work quickly.

Vortex to mix.

4. Add 24.6 μL master mix to wells.
5. Seal plate with adhesive plate seal. Make sure it is sealed well to minimize chance of evaporation.
6. Centrifuge plate briefly.
7. Place plate in the thermocycler and run the appropriate program.
8. Run 2 μL of each product on a 1 – 2% agarose gel to determine if PCR worked prior to running any other tests.

Searching for RFLPs

1. Make enzyme master mixes as follows:

	<u>per reaction</u>
sterile water	7.7 μL
buffer*	2.0 μL
BSA	0.2 μL
enzyme**	0.1 μL

*Buffer used is specific to the enzyme in the master mix.

**Each master mix has a single enzyme with its manufacturer supplied buffer.

2. 10 μL of PCR product from a pool of individuals is mixed with 10 μL of enzyme master mix.
3. Each gene product is incubated at the enzyme's optimal temperature for 3 hours.
4. Following incubation the entire digested product is loaded into a 3% agarose gel.
5. The products are visualized after 80 minutes of electrophoresis.
6. The resulting image is examined for all verified cutters.
7. Once a list of cutters is compiled for a specific gene, PCR is conducted on 24 individuals.
8. 10 μL of PCR product from the 24 individuals is then digested with 10 μL of master mix for each enzyme in the list of cutters.
9. The products are incubated for 3 hours at the enzymes' optimal temperatures.
10. The entire 20 μL of digested product is loaded on a 3% agarose gel.
11. Products were allowed to run on the gel for 80 minutes.
12. Results of the digests were visualized with UV light.
13. The results were then examined for different banding patterns among the 24 individuals for each enzyme.

RFLP

1. Make enzyme master mixes as follows for enzymes found to yield a polymorphism in any of the desired genes:

	<u>per reaction</u>
sterile water	7.7 μ L
buffer*	2.0 μ L
BSA	0.2 μ L
enzyme**	0.1 μ L

*Buffer used is specific to the enzyme in the master mix.

**Each master mix has a single enzyme with its manufacturer supplied buffer.

2. 10 μ L of PCR product from each individual is mixed with 10 μ L of the appropriate enzyme master mix.
3. Each gene product is incubated at the enzyme's optimal temperature for 3 hours.
4. Following incubation the entire digested product is loaded into a 3% agarose gel.
5. The products are visualized after 80 minutes of electrophoresis.
6. The resulting image is genotyped for each individual.
7. The genotypes are recorded for each individual.
8. Any individual with an unscorable genotype was run through the process again.
9. Allele frequencies were calculated.
10. If the frequencies differed among the lines, an additional generation of individuals was tested.

Staining a gel in Ethidium Bromide Solution

1. Add 750 μ L 0.5X TBE to a plastic container
2. Add 37.5 μ L Ethidium Bromide to the TBE.
3. Shake to container to mix.
4. Place gel into the solution for 20 – 45 minutes.
5. Rinse gel in distilled water for several minutes.
6. Store the ethidium bromide solution in the fridge in a foil covered bottle.

Appendix Table 1. Summary of candidate gene information

	Primer Sequence (5' to 3')	Published or Designed	Working?	Annealing Temp	Product size (bp)	RFLP found?	Notes
Aromatase 1	AGACGCAGGATTTTCACAGC TGCAATGAGAAACAGCATGA	designed	yes	62°C	990	no	
Aromatase 2	ATGAGGTACCAGCTGTCTGT AAGCCAAATGGCTGGAAGTA	designed	no	54°C	1200	no	double bands
Uteroferrin 1	GCTCAGTGACCGCCAGTT AGTCCTGGTGTGGTTCTGG	designed	yes	63°C	350	no	
Uteroferrin 2	CGATTGCCACAACGTGTGAAG AGGGAGGGGTGAGAAAACAC	designed	yes	63°C	350	Sau 96I	polymorphism is very rare
Relaxin	GAGCTCATATTTGCAAGCCATA CCAGAAGTGATTGCTGGAT	designed	yes	58°C	400	no	
Follistatin 1	GGACCGAGGAGGACGTAAAT GGCCTTTCCAGGTGATGTTA	designed	yes	56°C	625	Msp I, Nci I, Hpa II, Srf FI	all these enzymes cut at the same location
Follistatin 2	TGCCGAATGAACAAGAAGAA CAGAAAACATCCCGACAGGT	designed	yes	62°C	450	Fnu 4HI	
Follistatin 3	TTGGCCTATGAGGGAAGTG CTTCCTTCATGGCACACTCA	designed	yes	65°C	825	no	PCR doesn't always work
Follistatin 4		designed		56°C	450, 400	no	
Follistatin 5		designed	yes	64°C	850	no	
EGF 1	CTCTCTAGCCTTGCTCTGC AACTACCGCAACAGAAGGA	designed	yes	59°C	400	no	
EGF 2	CAGGAATGGCAATAAATTGGA ATCCAATGACACAGCTGCAA	designed	yes	55°C	700	no	
Est. Sulfo.	ATACAGAGGGCGATGTGGAA TTTTCCAAAACGAGACTGG	designed	yes	64°C	900	no	
Retinoic acid rec	GGCATGTCCAAGGAAGCTGT GTTCTCCAGCATCTCTCGGAT	published	no				Stu I published
RARG 1	CCAAGTCAAGCAAACTCCAT GCAGAGTGATCTGGTCAGCA	designed	yes	65°C			PCR doesn't always work
RARG 2	TGGTCACCAAGTGCATCATT AAGTCAGCATCGTGCATCTG	designed	no	55°C	850, 300		double bands
Preprolactin	GAAGTGAGGGGTATGCAGGA GCAAAAAGGCGAGTGTCTTC	designed	no				
Prolactin	ACCTCTCTCGGAAATGTTCA CTGTTGGGCTTGCTCTTTGTC	published	no				Bst UI published
PRLR	CCCAAAACAGCAGGAGAACG GGCAAGTGGTTGAAAATGGA	published	yes	60°C	457	Alu I	No heterozygotes
RBP4	GAGCAAGATGGAATGGGTT CTCGGTGTCTGTAAAGGTG	published	yes	61°C	550	Msp I	
ESR	CCTGTTTTTACAGTGACTTTTACAGAG CACTTCGAGGGTCAGTCCAATTAG	published	yes	56°C	120	no	Pvu II published
Prostagl.	GTGCACTACATACTTACCCACTTC AGGCTTCCCAGCTTTT(A/G)TA	published	no		1500		Mse I published
PTGS2	GGCAAAGAATGCAAAACATCA GCTCTTCTTCCTGTGCCTGA	designed	yes	63°C	600	no	PCR doesn't always work
Leptin receptor	GCATCCATATCTGAACCC CCACTTAAACCATAGCGAATC	published	no				Hinf I published
LEPR1	TGCAGTGTACTGCTGCAATG GCAAAGTGCTTCCCACAAGT	designed	yes	63°C	350	no	
Leptin	GTCACCAAGGATCAATGACAT AGCCCAGGAATGAAGTCCAA	published	no	2000			Aci I published
LEP 1	CCTGGAAGCCTCCCTCTACT TGTTGTGTGCTGTAGCCTTC	designed	yes	61°C	500	Msp I	polymorphism is very rare
LEP 2	GAAGGCTACAGGCACACACA TGCTTAGATTCAAATTCCTTGG	designed	yes	60°C	1000	no	
LEP 3	AACTGGTGCTATGGGCTCAG GCCACAAGGGAAGTCTGGTA	designed	yes	63°C		no	PCR doesn't always work

Appendix Table 2. Restriction sites within candidate genes.

	Acl I	Alu I	Bam HI	Dde I	Eco RI	Hae III	Hha I	Hind III	Hinf I	Mse I	Msp I	Nci I	Pst I	Pvu II	Sau 3AI	Sau 96I	Stu I	Sty I	Bst UI	Taq I	Hpy CH4V	SacFI
Aromatase 1	N	Y	N	Y	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	N	N	N	N	N
Uteroferrin 1	N	N	N	Y	N	Y	N	N	N	N	Y	N	N	N	N	N	N	N	N	N	Y	Y
Uteroferrin 2	N	N	N	N	N	Y	Y	N	Y	N	N	N	N	N	N	Y	N	N	N	N	Y	Y
Relaxin	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	N	N	N	N	N	N	N	N	N
Follistatin 1	N	N	N	N	N	Y	N	N	N	N	Y	Y	N	N	N	Y	N	N	Y	N	Y	Y
Follistatin 2	Y	Y	N	Y	N	Y	N	N	Y	Y	Y	N	N	N	N	Y	N	N	N	N	N	Y
Follistatin 3	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Follistatin 5	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
EGF 1	N	Y	N	Y	N	Y	Y	N	Y	N	Y	Y	Y	N	Y	N	Y	N	Y	N	Y	Y
EGF 2	N	N	N	Y	N	Y	Y	N	N	Y	Y	N	N	N	Y	N	N	N	N	N	Y	Y
Est. Sulfo.	N	Y	N	N	N	N	N	N	Y	Y	N	N	Y	N	N	N	N	N	N	N	N	N
RARG 1	Y	Y	N	Y	N	Y	N	N	N	Y	Y	Y	Y	Y	N	Y	N	N	N	N	Y	Y
RARG 2	Y	Y	N	Y	N	Y	N	Y	Y	N	Y	N	N	Y	Y	N	Y	Y	N	N	N	N
LEPR	N	N	N	Y	N	N	N	N	N	N	N	N	N	N	N	N	Y	Y	N	N	Y	Y
LEP 1	Y	Y	N	Y	N	Y	Y	Y	Y	N	Y	Y	N	Y	Y	N	Y	N	Y	N	Y	Y
LEP 2	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	N	N	N	Y	Y
LEP 3	Y	Y	N	Y	N	Y	Y	N	N	Y	Y	Y	Y	Y	N	Y	N	N	N	N	Y	Y

Y indicates that a restriction site was found within that PCR product, while an N indicates no restriction site was found.

Appendix Table 3. More restriction sites within candidate genes.

	Bcc I	Ban I	Bbs I	Bfa I	Dra I	Fru 4HI	Hpa I	Nde I	Msp1AI	Apo I	Tsp509 I
Aromatase 1	N	N	Y	N	N	Y	N	N	Y	Y	Y
Uteroferrin 1	N	N	N	N	N	N	N	N	N	N	N
Uteroferrin 2	Y	Y	N	N	N	N	N	N	N	N	Y
Relaxin	Y	N	Y	N	N	N	N	N	N	Y	Y
Follistatin 1	N	Y	Y	N	N	Y	Y	N	Y	Y	Y
Follistatin 2	N	Y	N	N	N	Y	Y	N	N	Y	Y
Follistatin 3	N	N	N	N	N	N	N	N	N	N	N
Follistatin 5	N	N	N	N	N	N	N	N	N	N	N
EGF 1	N	N	N	N	N	Y	Y	N	N	Y	Y
EGF 2	Y	N	N	N	N	N	Y	N	N	Y	Y
Est. Sulfo.	Y	N	Y	N	Y	N	N	N	N	Y	Y
LEPR	Y	N	N	N	Y	N	N	N	N	N	N
LEP 1	N	N	Y	N	N	Y	Y	Y	N	N	N
LEP 2	Y	N	Y	N	Y	Y	Y	N	Y	N	N

Y indicates that the restriction enzyme cut the PCR product while N indicates that the enzyme does not cut the product. Any product not appearing in the table was not tested due to PCR problems

Appendix Table 4. Average phenotypic values for litter traits and inbreeding values by line and generation.

Trait Measured	Line	Generation 10	Generation 11
NFF	c	9.666667	8.363636
	s	9.886792	9.193548
NSB	c	0.291667	0.333333
	s	0.226415	0.408602
NBA	c	9.375	8.030303
	s	9.660377	8.784946
MUM	c	0	0.121212
	s	0.018868	0.086022
Inbreeding	c	0.1361136	0.1743859
	s	0.1808516	0.2061295

c and s represent control and select lines, respectively. Values were calculated using records from 57 control line litters and 146 select line litters. Generation 10 was composed of 77 litters. Generation 11 was composed of 126 litters. Average inbreeding was calculated using all the individuals in each generation in each line. NFF, NSB, NBA and MUM represent number fully formed, number stillborn, number born alive, and number mummies, respectively.