Deciphering the pig genome to understand gamete production

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The field of livestock genomics has made considerable advances in the past decade. In the area of pig reproduction, a number of genome scans have identified several genomic regions associated with variation in reproductive measures ranging from ovulation rate, litter size and testis size. Additionally, several candidate genes have been associated with variation in litter size. These studies primarily focused on developing genetic markers to facilitate selection decisions. To date, their results have made minor contributions to commercial pig performance and our knowledge on the inheritance of complex phenotypes. With the availability of additional resources for pig, as well as from human and mouse studies, future studies should be directed to identifying genetic variation that affects biological processes. To reach this goal, teams of diversely trained scientists need to be formed that include geneticists, physiologists, molecular biologists and bioinformaticists. A diversified team of scientists equipped with all of the available research tools (genomic sequence data, expression arrays, knowledge of gene product functions, etc.) and appropriate swine populations should be able to decode the genome's hidden secrets on how it controls reproductive processes.

Introduction

The field of swine genomics is now over a decade old, and in many ways, it seems that the promises this technology were projected to hold for pig production were not fulfilled. Genomic maps were likened to a "Rosetta Stone" of life holding all of the key information necessary to easily modify the performance of a species. However, the amount of information contained in a genome has proven to be immensely larger than expected and more difficult to decipher than originally thought. As a result, we are still many years away from determining an animal's productivity solely based on its genotypic profile.

The field of swine genomics is rapidly evolving as "high-throughput" technologies are developed and a plethora of sequence and expression data accumulates. Now that the complete sequence of the human and mouse genomes are known, many sequencing projects of other species are underway (including chicken, dog and cattle) and a proposal to collect this information for the pig has been developed. New projections on the impact this information will have on pig production are under way. Certainly not all of these projections will come to fruition, but definite advances will be made and our knowledge on how the genome affects biological processes will be greatly increased.

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The focus of this review will be to evaluate the current level of knowledge on genomics of gamete production in the pig, and then, to project the approaches that will be taken in the future. Future approaches should be team based, expound upon the current knowledge base, increase our understanding of the genome and biological processes, and result in improved reproduction rates in commercial pigs.

Current status of genetic markers for reproduction

Considerable advances in pig genomics have been made, and there are a large number of examples where this information has been used to determine breeding decisions. Most notable is the removal of the deleterious "stress gene" (Fujii *et al.*, 1991) from pigs, but other examples include selection for reproduction (Rothschild *et al.*, 1996), pork quality (Milan *et al.*, 2000), growth rate (Van Laere *et al.*, 2003) and even coat color (Kijas *et al.*, 1998). Furthermore, most commercial breeding companies are using proprietary genetic markers to assist in breeding decisions (Lohuis, 2003; Plastow *et al.*, 2003; Schneider *et al.*, 2003).

Genomic studies for reproduction are quite expensive, primarily due to the number of animals that need to be measured (approximately 500 animals preferred), expense of collecting phenotypic data, and cost to collect genotypic data on all animals for 120-200 genetic markers. Therefore, not many studies have taken a genomic approach. An approach that requires fewer resources and can use commercial populations is to evaluate associations between polymorphisms in candidate genes and performance. These studies only evaluate a small portion of the genome, but if utilizing commercial populations, may include 1,000 or more animals. Bidanel *et al.* (2002) reviewed genetic markers in pigs and most of the information presented in this section was summarized in their publication.

Results from genome scans

To date, only six populations with reproductive measures have been thoroughly studied and reported across the globe. Five of these populations utilized Meishan germplasm as one of the founding breeds. Female reproductive traits were studied in five populations and male reproduction traits were studied in three. Results from the INRA Meishan-Large White population have only been reported in an abstract (Bidanel *et al.*, 2001) and popular press (Bidanel *et al.*, 2002), therefore, many of the details of this study are unavailable.

From five studies on female productive traits (Bidanel et al, 2001; Cassady *et al.*, 2001; de Koning *et al.*, 2001; Rohrer *et al.*, 1999 and Wilkie *et al.*, 1999), 24 associations, i.e. quantitative trait loci (QTL), reached the suggestive level of genome-wide significance. The only overlap was a QTL affecting age at first oestrus at the telomere of the long arm of chromosome 1 (Bidanel *et al.*, 2001; Rohrer *et al.*, 1999). Other findings included seven QTL for ovulation rate on chromosomes 3, 4, 7, 8 (two unique locations), 9 and 10; five QTL for age at first oestrus on chromosomes 7, 8, 10, 12 and 13; five QTL for litter size on chromosomes 7, 11, 12, 14 and 17; and a QTL for uterine capacity on chromosome 8.

The QTL detected are dependent upon the founding breeds or lines used for the project. Since most studies utilized Meishan versus occidental breeds, and the Meishan has very unique reproductive characteristics, more overlap between studies would be expected. However, the studies that used Meishan germplasm and measured litter size or uterine capacity had fewer than 250 observations (de Koning *et al.*, 2001; Rohrer *et al.*, 1999; Wilkie *et al.*, 1999), thus, their results may not be robust. Researchers typically attempt to have a minimum of 500 phenotyped animals for QTL studies. The only experiment that exceeded 500 records for ovulation rate or age at puberty was that of Rohrer *et al.* (1999).

An unexpected similarity across studies was a preponderance of QTL where Meishan QTL alleles decreased ovulation rate. This was evident in all three journal articles that included Meishan germplasm (de Koning *et al.*, 2001; Rohrer *et al.*, 1999; Wilkie *et al.*, 1999). One possible explanation is that Chinese pigs evolved with a unique set of genes regulating ovulation rate. Together as a whole, they result in a superior number of ova shed per oestrus, but the effect of an individual gene when placed in an occidental germplasm has negative epistatic effects resulting in fewer ova shed. If this is the case, it is a strong justification for studying the entire biological process involved in ova recruitment and ovulation rather than just the impact of a single genomic region to accurately understand the mechanisms behind these QTL.

The only occidental population studied compared a cross between animals from a selected line for increased ovulation rate and litter size with animals from the control line of the same experiment (Rathje *et al.*, 1997). These lines were originally developed from a Large White by Landrace cross. The initial results from the first third of the population were reported by Rathje *et al.* (1997) and the results from the complete project were presented by Cassady *et al.* (2001). All of the QTL associations detected by Rathje *et al.* (1997) were not supported in the full data set (Cassady *et al.*, 2001), indicating the need for large populations for QTL detection studies.

Genome-wide QTL studies for male reproduction traits are limited to three Meishan cross populations and only a few traits studied. Bidanel *et al.* (2001) indicated that they found QTL on chromosomes 4 and 7 for male sexual development traits (as referenced by Bidanel *et al.*, 2002). The only traits reported by Sato *et al.* (2003) were testis weights and they identified QTL on chromosomes 3 and X. Rohrer *et al.* (2001) scanned the genome for QTL affecting serum FSH concentration in boars. Four QTL were detected on chromosomes 3, 8, 10 and X. They then tested these four regions for associations with testes weight and identified a significant association with the X chromosome (Rohrer *et al.*, 2001).

The only common finding for male reproduction QTL is the testes weight QTL on the X chromosome (Rohrer *et al.*, 2001; Sato *et al.*, 2003). In a subsequent generation of the population studied by Rohrer *et al.* (2001), Ford *et al.* (2001) confirmed the effects of the X chromosome on serum FSH concentration and testes size, as well as determined that this region affected pubertal serum LH serum concentration and color of the parenchymal tissue of the testes. The authors currently believe that the effect on parenchymal color of the testes is a result of higher ferritin in the Leydig cells caused by elevated FSH and FSH inducement of iron transport (Wise *et al.*, 2003). Recently, Nonneman *et al.* (2005) reported a polymorphism in the thyroxine binding globulin (TBG) gene that affects binding of thyroxine to the TBG molecule and showed it was significantly associated with mature testes weight. They provided circumstantial evidence that thyroxine controls the length of the proliferative phase of Sertoli cells in developing testes, resulting in a difference in mature testes size. In addition, rats that were transiently hypothyroid during this critical phase of development had significantly larger mature testes and decreased levels of serum FSH and LH (Kirby *et al.*, 1992; 1997). Further research is necessary to definitively prove that this polymorphism actually causes the observed effect on mature testes size.

Candidate gene associations

In total, only six candidate genes have been associated with reproduction traits in pigs. Five studies evaluated litter size and the sixth study analyzed ovulation rate. Typically, these studies involved a larger sample of phenotyped animals than genome scans and often animals tested were from commercial populations. Associations of oestrogen receptor alpha (ERα) and prolactin receptor (PRLR) were most thoroughly evaluated.

The association of ERα genotypes with litter size was first reported by Rothschild *et al.*

(1996) and later confirmed by Short *et al.* (1997a). Follow-up studies in other laboratories resulted in inconsistent results indicating that either the polymorphism genotyped is not causative or that considerable epistatic interactions exist with this locus and other gene(s) located elsewhere in the genome. However, this marker did not affect components of litter size in two independent resource populations (Bidanel *et al.*, 2001; Rohrer *et al.*, 1999) comparable to the first population studied by Rothschild *et al.* (1996), and the effects were opposite in a comparable population studied by van Rens *et al.* (2002a). Additionally, three studies were unable to detect a significant association of ERα genotype with litter size (Drogemuller *et al.*, 2001; Isler *et al.*, 2002; Linville *et al.*, 2001) or ovulation rate (Isler *et al.*, 2002; Linville *et al.*, 2001). van Rens *et al.* (2002b) did identify a significant interaction between ERα genotype of the sow and ERα genotype of the fetus on placental efficiency which may affect foetal survival.

The PRLR polymorphism has been studied in multiple populations. Vincent *et al.* (1998) identified a significant association between PRLR genotype and litter size in five commercial lines of pigs. Linville *et al.* (2001) was unable to detect a significant effect of this gene on litter size or ovulation rate, but Drogemuller *et al.* (2001) and van Rens *et al.* (2002c) did detect significant effects on reproduction traits. Drogemuller *et al.* (2001) found the B allele was associated with larger litters over all parities studied in German commercial pigs. To the contrary, the A allele of PRLR was associated with larger litters, greater placental efficiency, later age at puberty (van Rens et al 2002c) and increased ovulation rate (van Rens *et al.*, 2003) in a Meishan cross population.

A polymorphism in retinol binding protein 4 was associated with litter size by Rothschild *et al.* (2000). However, this finding was not confirmed by Linville *et al.* (2000). Only one study each demonstrated associations between litter size and genotypes for the genes FSH ß subunit (Li *et al.*, 1998), secreted phosphoprotein 1 (Short *et al.*, 1997b) and GnRH receptor (Jiang *et al.*, 2001).

Lessons from the past

Most candidate gene studies were designed to develop markers that could be used for breeding decisions. Typically, traits that are lowly heritable, only observed in one sex or expressed late in life were targeted since these traits are the least amenable to traditional selection procedures. Therefore, geneticists saw a need for genetic markers to improve selection for reproductive traits.

The steps necessary to reach the final objective included animal performance data, marker development, genotyping and statistical analysis. So, when "genetic" markers became available, animal breeders/geneticists began to look for associations. The first marker-phenotype association studies used blood types or serum protein polymorphisms. Jensen *et al.* (1968) detected an association with the H blood group and litter size in Hampshire and Duroc pigs. The H blood group was later used in selection decisions to remove the "stress gene allele" because close linkage with the causative gene was detected (Rasmusen *et al.*, 1976). Advancements in DNA technologies made it easier to choose a gene of interest, identify a DNA based polymorphism and genotype a population of animals. Therefore, this research was conducted by scientists trained in DNA technologies and statistical analyses without including expertise from scientists in other disciplines.

Once linkage maps, which spanned the genome, were reported (Rohrer *et al.*, 1994; Ellegren *et al.*, 1994; Archibald *et al.*, 1995), genome scans for QTL could be conducted. Due to the investment necessary to complete genome scans, the phenotypes collected were more comprehensive. The design of these resource populations included input from scientists in other

disciplines to ensure useful phenotypes were collected. Some of these resource populations have been the cornerstones of large research programs. However, all of these studies would have greatly benefited from current high-throughput "phenotyping" technologies and a greater understanding of how the genome affects performance.

What is next for genomic studies of reproduction?

The initial goal of livestock genomics (i.e., to develop genetic markers for selection) was short-sighted. An overwhelming amount of resources have gone into QTL detection in humans and mice for reasons other than marker assisted selection. These studies were conducted to understand how the genome affects biological processes. While increasing the accuracy of selection with DNA based markers will improve livestock production, a broader focus of these studies to understand biological processes is a much more valuable resource. To obtain this knowledge requires multi-disciplinary teams of scientists. Necessary team members include scientists trained in DNA technologies, physiology, molecular biology, statistical analyses and bioinformatics. Each team member is critical and it is crucial that they interact as a team. Team members need to take the lead in areas of their expertise and play a subordinate role where other members have more training. Continuous interaction between team members is also critical to successfully identify genetic variation that affects performance.

Successful examples of this approach in reproduction were demonstrated by the discovery of the causative mutations for two major genes affecting reproduction in sheep, the Booroola and Inverdale loci. Both variants were detected in the same laboratory by taking a multi-disciplinary approach (Galloway *et al.*, 2000; Wilson *et al.*, 2001). Simultaneous with Wilson *et al.* (2001), another laboratory also identified the same Booroola mutation using a similar approach (Mulsant *et al.*, 2001).

At the U.S. Meat Animal Research Center, a multi-disciplinary approach is often taken to QTL discovery and fine-mapping. When a QTL is discovered, one of the first questions asked is "What other traits are possibly affected by the same QTL?" There are times when the answer to this question can be misleading, but it usually results in greater insight into the biology behind the QTL. One example is the work leading to the discovery of a polymorphism in TBG that was referred to earlier and is presented in greater detail in Ford *et al.*, (2005). Briefly, a QTL for fat deposition was initially identified (Rohrer *et al.*, 1998). As research into reproductive traits began, a QTL for serum FSH concentration in pubertal boars (Rohrer *et al.*, 2001) was detected with a major gene effect, followed by discovery of its effect on testes weight, serum LH concentrations, testes color and response to unilateral castration (Ford *et al.*, 2001). Eventually a hypothesis that thyroid function was possibly involved, led to the study of TBG (Nonneman *et al.*, 2005). Prior to having the biology of this QTL clearly defined, research had been directed at other "candidate" genes in the area, primarily androgen receptor.

A similar integrative approach was also taken to study other traits. Each putative QTL for ovulation rate (chromosomes 3, 8 and 10; Rohrer *et al.*, 1999) overlaps a QTL affecting serum FSH concentrations in boars (Rohrer *et al.*, 2001). Unfortunately, differences in serum FSH concentration in females were not associated with these genomic regions at either a constant age or a constant day of the oestrous cycle (Ford *et al.* unpublished data). These results suggest mechanisms behind these ovulation rate QTL are related to FSH physiology (synthesis, catabolism, feedback, etc.) that is detectable in males, but is possibly suppressed or not detectable with current techniques in females. Additional research indicated that chromosome 10 and possibly chromosome 3 QTL are partially responsible for the increased ovulation rate measured in an 11 generation ovulation rate selected line of pigs (Mousel *et al.*, 2004, 2005). This

selection line is composed of Large White, Landrace, Chester White and Yorkshire germplasm, indicating that the detected QTL are not exclusively a result of a unique Meishan allele, and they will be applicable to commercial lines.

Future studies will be fortified by the wealth of genomic information available. Data of genomic sequence and function are rapidly accumulating. Comparing the genome sequence of a wide array of organisms, as demonstrated by Thomas *et al.* (2003), facilitates discovery of new genes, regulatory elements and untranslated transcribed RNA molecules. These novel DNA features can be studied today on a genomic level by comparing the human and mouse genome sequence. Soon, these comparisons will be enriched by the inclusion of additional species (cow, chicken, dog, etc.), and once the pig genome is sequenced, these comparisons will be directly applied to the pig.

Information on expression and function of protein coding regions (genes) will fortify genome sequence information. A plethora of data for tissue specific gene expression and protein function in man and mouse continues to expand at an extraordinary rate, and much of this information can be directly applied to the pig. A multitude of human and mouse tissue specific cDNA libraries have been developed and sequenced, resulting in millions of expressed sequence tags (EST) for each species and a well documented set of genes expressed in each tissue. A large number of EST are available for the pig and are being used to determine tissue expression profiles. Gene expression arrays also provide useful insight into metabolic pathways involved in animals with different levels of performance (Caetano *et al.*, 2004). Finally, in the mouse, systematic modification of specific genes provided *in vivo* evidence of protein function of novel genes. These modifications initially utilized random mutagenesis (Wells *et al.*, 2000; Soewarto *et al.*, 2000) or targeted transgenic knock-outs of genes (Mills *et al.*, 2001). But a more recent technology, which can be applied in a wider variety of species is RNA interference (Fire *et al.*, 1998). While RNA interference could be applied in pigs, the cost to evaluate 30,000+ genes limits this holistic approach to small model organisms like the mouse. Most likely, follow up studies on the "most interesting" genes will be conducted in pigs.

There are some limitations to comparative genomics and it is necessary that information is collected in the pig. For example, the androgen receptor gene in primates and rodents has three trinucleotide repeats in the coding region that are polymorphic, and some alleles can have significant impact on the gene's function. But these three repeats were virtually undetectable in the pig, and no genetic variation exists in commercial lines (Trakooljul *et al.*, 2004; Ford *et al.*, unpublished data). Therefore, a coincidental occurrence between the human and mouse genome does not always imply a universal mammalian genomic effect. This is why the Cooperative State Research, Education and Extension Service of the U.S. Department of Agriculture (USDA-CREES) decided to partially fund a genome sequencing effort for the pig.

Unfortunately, building multi-disciplinary teams to work on each QTL requires considerable resources and restricts the number of QTL that can be simultaneously studied. However, the results may have multiple applications in the species studied (pharmaceutical, nutriceutical, management, as well as marker assisted selection), can often be applied to other species and lead to a more knowledgeable application of the information. Therefore, future research needs to involve teams of scientists with a variety of expertise so that the endpoint of the research will be "How does this region of the genome affect performance?" With this knowledge in hand an "optimal" method to utilize this knowledge can be formulated that will foresee problems with pleiotropic or epistatic effects of a locus or required changes in management to capture the value of the knowledge created.

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