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Achievements of research in reproduction sciences

Robert A. Cushman, Mark F. Allan and Larry A. Kuehn

USDA, ARS, U.S. Meat Animal Research Center, Clay Center, NE 68933-0166, USA
Email: Bob.Cushman@ARS.USDA.GOV

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Introduction

Genetic applications to improving fertility in farm animals have been limited due to the nature of the phenotypes. Most reproductive phenotypes are sex-limited and many are binomial in nature, making the calculation of genetic parameters difficult. However, in recent years, the genetic parameters of a number of reproductive traits with economic importance to cattle producers have been reported (Table 1), and chromosomal regions associated with some of these traits have been identified (Table 2). At the U.S. Meat Animal Research Center (USMARC), the use of genetic selection for ovulation rate and twinning rate to improve a reproductive trait (prolificacy) in cattle was successful over more than twenty years (Cushman *et al.*, 2005; Echternkamp *et al.*, 2007a; Echternkamp *et al.*, 2007b). This project demonstrated that genetic selection could be used to improve reproductive traits, and that chromosomal regions associated with reproductive traits could be identified (Kappes *et al.*, 2000; Allan *et al.*, 2007). However, most producers will improve their herds by purchasing genetics instead of using genetic selection, because genetic selection of appropriate sires is too slow due to the number of years required. Furthermore aggressive culling of open cows has not led to an increase in reproductive efficiency, most likely due to large environmental influence and low heritability of fertility traits. Therefore, the use of modern molecular technologies (i.e. functional genomics, marker assisted management, and pharmacogenetics) to improve

Table 1. Heritability of traits associated with reproductive efficiency in cattle.

Trait	h^2	References
Age at puberty	0.10 ± 0.09	Smith *et al.* (1989a)
	0.61 ± 0.18	MacNeil *et al.* (1984)
	0.28 ± 0.05	Mialon *et al.* (2001)
Calving ease	0.21	Cubas *et al.* (1991)
	0.43	Bennett and Gregory (2001)
	0.13	Hickey *et al.* (2007)
Follicle diameter	0.16 ± 0.03	MacNeil *et al.* (2006)
Heifer pregnancy rate	0.28 ± 0.15	Thallman *et al.* (1999)
Ovulation rate	0.07	Van Vleck and Gregory (1996)
	0.10	Gregory *et al.* (1997)
	0.08	Allan *et al.* (2007)
Postpartum interval	0.28	Darwash *et al.* (1997)
	0.35 ± 0.06	Mialon *et al.* (2000)
Scrotal circumference	0.39	Smith *et al.* (1989b)
	0.25	Gregory *et al.* (1997)
	0.57 ± 0.09	Kealey *et al.* (2006)
Stayability	0.22 ± 0.03	Silva *et al.* (2006)
Twinning	0.03	Van Vleck and Gregory (1996)
	0.09	Gregory *et al.* (1997)
	0.10	Allan *et al.* (2007)

Table 2. Chromosomal regions associated with reproductive traits in cattle.

Trait	Chromosomes	References
Age at puberty	29	Casas *et al.* (2004)
Calving ease	2, 6, 8, 9, 10, 13, 14, 15, 16, 17, 18, 23, 24, 26, 27, X	Casas *et al.* (1998), Kuhn *et al.* (2003), Ashwell *et al.* (2005), Schnabel *et al.*(2005), Holmberg and Andersson-Eklund (2006), Kaupe *et al.* (2007)
Conception rate	1, 7, 10, 20, 21	Boichard *et al.* (2003)
Non-return rate	1, 7, 9, 10, 14, 15, 18, 20, 27, X	Kuhn *et al.*(2003), Ron *et al.*(2004), Schnabel *et al.*(2005), Muncie *et al.* (2006), Holmberg *et al.* (2007), Kaupe *et al.* (2007)
Ovulation rate	5, 7	Kappes *et al.* (2000)
Paired testes weight	29	Casas *et al.* (2004)
Pregnancy rate	2, 9, 11, 15, 18, 28, 29	Boichard *et al.*(2003), Holmberg and Andersson-Eklund (2006), Muncie *et al.* (2006)
Twinning	5, 7, 8, 10, 12 14, 21, 23, 29	Lien *et al.* (2000), Cruickshank *et al.* (2004), Cobanoglu *et al.* (2005)

reproductive management may provide an additional tool that will allow producers to enhance reproduction through genetic selection or management interventions based on individual genetics.

Transcripts as biomarkers of fertility

The first challenge in implementation of functional genomics for identification of biomarkers of fertility was development of methods that allowed large numbers of samples to be analysed in a timely manner. Real-time RT-PCR has lead to faster and relatively less expensive methods of measuring transcript levels (Bustin, 2000, 2002). Measuring transcript levels by real-time RT-PCR has provided the ability to run a larger number of samples (i.e. 96-well plates) in a 2-3 hour period as compared to Northern blots, which took several days to process. Under these conditions, the utility of transcripts as biomarkers of fertility has been increased, because processing time and efficiency using real-time RT-PCR is comparable to radioimmunoassays and ELISAs historically used by physiologists for measuring proteins and steroids.

The next consideration in the implementation of functional genomics in production agriculture was the identification of easily obtainable relevant biological samples that could be collected from large numbers of animals in production settings without interrupting normal management practices. Two types of samples that seem obvious under these stipulations are peripheral blood leukocytes (PBL) and sperm. Peripheral blood leukocytes can be collected easily from males and females at any time they are being handled as part of normal management practices. Semen is collected as part of standard breeding soundness exams before breeding, and excess sperm can be processed for transcript profiling. Other potential biological samples for transcript profiling in reference to reproductive status of domestic farm animals includes embryos (collected by flushing cows), oocytes (collected by ultrasound guided oocyte aspiration), uterine biopsies (Katagiri and Takahashi, 2006), ovarian biopsy (Aerts *et al.*, 2005), liver biopsy (Radcliff *et al.*, 2003a,b), adipose biopsy (McNamara and Valdez, 2005), and muscle biopsy (Wang *et al.*, 2005). These samples are less conducive to collection because more technical skill and time per animal are required and, for the most part, the animals almost certainly need to be handled at times beyond the typically normal management practices. Therefore, these samples are more useful in a research setting than they are in a production setting.

Finally, the greatest challenge when working with mRNA is ensuring the quality of the sample and the physiological status of animals at the time of collection. Transcript levels within a sample could vary due to age, gender, nutritional status, health status, season, time of day samples were collected and many others. With large enough numbers of samples collected in a relatively synchronous manner and a high level of quality control, superfluous factors become less of a concern, but when one is validating transcript levels as biomarkers, concerns about quality and consistency of samples must be a continual consideration.

Peripheral blood leukocyte expressed transcripts for pregnancy diagnosis

To date, one of the best examples of PBL transcript technology in animal production agriculture is the identification of PBL-expressed transcripts associated with early pregnancy in sheep and cows (Yankey *et al.*, 2001; Han *et al.*, 2006; Gifford *et al.*, 2007; Stevenson *et al.*, 2007). Austin *et al.* (1996) first identified a ubiquitin homolog produced by the pregnant bovine uterus in response to interferon-τ that was termed interferon specific gene-15 (ISG-15). Yankey *et al.* (2001) reported that transcript levels of another interferon-τ stimulated gene (*Myxovirus* resistance) were increased in PBL of pregnant ewes, leading Han *et al.* (2006)

to use real-time RT-PCR to measure ISG-15 mRNA in PBL to diagnose pregnancy in dairy cows. Cows with high plasma progesterone concentrations between days 15 and 32 after insemination and with ISG-15 mRNA above threshold levels between days 17 and 25 after insemination were confirmed pregnant by transrectal ultrasonography at day 32 after insemination. Interestingly, in this study there were a number of cows with detectable ISG-15 at day 19 after insemination that demonstrated a decline in ISG-15 between days 21 and 32 after insemination and were not pregnant at ultrasonography, suggesting embryonic mortality. While there are issues with handling and processing leukocytes for RNA, this technology is useful for identifying differential gene expression in PBL that might be associated with early pregnancy (Gifford *et al.*, 2007; Stevenson *et al.*, 2007).

Clearly, using real-time RT-PCR to measure transcript levels in PBL has great potential in a production setting. Further research in this area has potential to identify biomarkers for very early pregnancy (day 7 or less), for reproductive status (cycling or anestrous), and for identifying animals with a high propensity to conceive during a given breeding season. Because of the strong environmental influences on reproduction, these biomarkers may have greater value in identifying animals destined to be sub-fertile for a given breeding season than traditional genetic markers. In support of this, only about 1% of the cows at USMARC fail to conceive in two consecutive breeding seasons (Maurer and Echternkamp, 1985). The application of global transcript profiling to blood samples from farm animals of differing reproductive status could be a helpful tool in identifying these biomarkers in research herds, and real-time RT-PCR would allow the implementation in large numbers of animals.

Gamete transcriptome profiling

Global transcript profiling such as microarrays and suppression subtractive hybridisation has lead to the identification of a number of transcripts and microRNAs in male and female gametes that may be associated with fertility (Wang *et al.*, 2004; Patel *et al.*, 2007; Murchison *et al.*, 2007; Ro *et al.*, 2007ab; McDaneld *et al.*, 2007). While active transcription is limited or non-existent in later stages of gamete development, it is believed that transcripts are synthesised and stored during the earlier stages of development of both oocytes and sperm (Miller and Ostermeier, 2006; Patel *et al.*, 2007). These transcripts are translated to proteins that are required during the earliest stages of embryonic development before activation of the embryonic genome. Therefore, environmental insults to the animals such as nutrient restriction or temperature extremes that influence the early stages of spermatogenesis and folliculogenesis may have long lasting repercussions for fertility by altering the levels of transcripts, required for early embryonic development, that are stored in the gametes for weeks or months in males and even years in females.

However, there may be genetic components to the ability to withstand these environmental insults (i.e. adaptability). Ultrasound guided oocyte aspiration has been used to examine the repeatability of fertilisation rates in vitro within cows (Tamassia *et al.*, 2003) and, while the number of cows used in this study was low, there was a good repeatability within cow of the percent of oocytes fertilised and percent of blastocyst development in vitro. The high repeatability may be due to genetic or permanent environmental effects. In support of a genomic contribution, a recent study observed an association between decreased fertilisation and embryonic survival in vitro and mutations in the signal transducer and activator of transcription 5A (STAT5A) gene in dairy cows (Khatib *et al.*, 2008).

Genetic parameters for total sperm number, sperm motility and sperm quality have been reported for several breeds of bulls (Kealey *et al.*, 2006; Gredler *et al.*, 2007), and Kealey *et al.*, (2006) reported genetic correlations between sperm defects and scrotal circumference. Similarly non-return rates in artificially inseminated cows have been reported to be influenced by the bull used (Madrid-Bury *et al.*, 2005). These results suggest that, as with oocyte quality, there is a genetic component to sperm quality and that transcript profiling may provide biomarkers of sperm quality and fertility that would aid in the identification of high fertility bulls.

Lalancette *et al.* (2008) used suppression subtractive hybridisation to compare transcript profiles between sperm from dairy bulls with high (≥71%) and low (≤65%) non-return rates. They identified 55 differentially expressed transcripts in the samples from the bulls with high non-return rates and confirmed by real-time RT-PCR that both LHX2 and TGB1 mRNAs were expressed at greater levels in the high fertility bulls as compared to the low fertility bulls. While transcriptome profiling in the sperm of sub-fertile men has progressed much further (Wang *et al.*, 2004), this study is the first indication that transcript profiles may be useful biomarkers of fertility in bulls. Potentially, transcript profiling could be added to breeding soundness exams for identifying sub-fertile bulls among those that would pass a test using the traditional endpoints of scrotal circumference, percent normal sperm, and percent motile sperm.

However, unlike the male gamete that is itself easily obtainable, for biomarkers of fertility from the female gamete to be useful, the mRNA levels in oocytes would need to correlate to mRNA levels in PBL or some other easily obtainable sample, similarly to what has been demonstrated with transcript biomarkers of pregnancy (Yankey *et al.*, 2001; Han *et al.*, 2006; Stevenson *et al.*, 2007; Gifford *et al.*, 2007). Recently, Cushman *et al.* (2007a) quantified expression of mRNA for the circadian clock gene, Period 1, in the bovine and ovine oocyte. Subsequent investigation demonstrated that high levels of Period 1 mRNA in PBL of cows prior to the start of the breeding season was associated with anestrus and low fertility (Cushman *et al.*, 2007b; Fields *et al.*, 2008), suggesting that Period 1 transcript level in PBL could be a biomarker of reproductive status and fertility, although

no correlation between Period 1 transcript levels in the PBL and in the oocyte have been determined. Clearly, profiling of the mRNAs and microRNAs expressed in the gametes and gonads could provide further knowledge of the factors influencing gamete quality and reproductive efficiency, and lead to the discovery of more biomarkers and genetic markers of fertility.

Genetical genomics

The new field of genetical genomics combines the use of genetic markers and transcript profiling to identify regions of the genome associated with transcript expression levels (e-QTL; Bueno Filho *et al.*, 2006). While some of the second tier biopsy tissues (oocytes, ovarian biopsies, uterine biopsies, adipose biopsies, etc.) may not be as conducive to regular collection in a production setting for identification of biomarkers of fertility, they represent tissue samples that could be collected on fairly large numbers of animals in a research setting. These samples would be useful for genetical genomics analysis, because the identification of e-QTL associated with the transcript levels of genes involved in reproductive function could further the numbers of chromosomal regions associated with fertility (Bing *et al.*, 2005; Kadarmideen *et al.*, 2006), providing further opportunities for bringing DNA markers for fertility traits to the industry.

Pharmacogenetics

There is large variation among patients in the dose of hormones required in assisted reproductive technologies. There may be genetic markers associated with required dosage that would help to increase efficacy of treatments by tailoring hormone doses to the individual (Greb *et al.*, 2005; Marrer and Dieterle, 2007; Moron *et al.*, 2007). Similarly, there is great variation among peripubertal heifers and postpartum cows in the response to hormone treatments to synchronise estrus and induce ovulation (Goodling *et al.*, 2005). Pharmacogenetic techniques could be applied to farm animal production if we can identify genetic regions associated with the response to hormones for synchronisation of estrus and induction of ovulation. If biomarkers of reproductive status (cycling or anestrous) can be identified and subsequently used for genetical genomics analysis, they could aid in the identification of genetic markers that could help in improving hormonal doses for synchronisation of estrus and induction of ovulation in the postpartum cow.

Summary and conclusions

The applications of genetics to production agriculture and assisted reproductive technologies are increasing rapidly. In terms of application to assisted reproductive technologies, functional genomics may have more utility in the dairy industry where cows are handled more frequently and fertility has become a major issue due to the negative impact of milk production on reproduction (Dal Zotto *et al.*, 2007). However, because reproductive failure is the primary reason that females are removed from the production herd, it represents a significant financial loss to beef and pork producers. Therefore, the continued development of biomarkers and genetic markers of fertility could have significant implications for profitability in all aspects of production agriculture. Finally, while the examples used here were directed toward the beef and dairy industry, it should be noted that many of the same tools have been or are being developed for swine and sheep production as well.

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