GENOME-WIDE TRANSCRIPTIONAL AND DNA METHYLATION PROFILING OF THE BOVINE ENDOMETRIUM

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Abstract

The aim of this research was to identify key molecular mechanisms regulating early pregnancy events in dairy cattle. Genome-wide gene expression and DNA methylation profiles were characterised in the endometrium of fertile and sub-fertile dairy cows at day 17 of pregnancy and the oestrous cycle. Gene expression data in combination with QTL data was then used to identify candidate genes for genetic analysis. The results of this study identified several biological processes likely to be important contributors to pregnancy success. In particular, genes classified as having roles in immune response were up-regulated in pregnant animals and down-regulated in sub-fertile animals. Additionally, gene expression was correlated with DNA methylation in several genes in these pathways. Lastly, QTL for fertility traits were identified on chromosome 9. Genes located in the QTL region were identified and cross-referenced with the genes identified as differentially expressed in fertile and sub-fertile dairy cows. The most differentially expressed gene was chosen as a candidate gene for genetic analysis. However, no association of polymorphisms in this gene with fertility phenotypes was detected. The results of this research have identified several genes in biologically relevant pathways that are influenced by the presence of the embryo. Further, many of these genes demonstrate differential expression in fertile and sub-fertile dairy cows. Modulation of the maternal immune system is highlighted as a potentially important process required for pregnancy success. The expression profiles of genes in these pathways suggest that insufficient tolerance to the embryo may contribute to pregnancy loss in the sub-fertile dairy cow strain. The differential regulation of these genes could be the consequence of genetic or epigenetic regulation. For example, there is evidence that DNA methylation may contribute to regulation of gene expression in response to the embryo and could account for some of the differences identified in the two strains studied. Alternatively, genetic variation could underlie these differences. This study has identified several important biological pathways and potential regulatory mechanisms that warrant further investigation.

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Chapter 1. INTRODUCTION

Over the past three decades, genetic selection in dairy cows has focused on milk production at the expense of other traits such as fertility. Milk production capacity of the modern dairy cow has increased dramatically, but the fertility of these cows has steadily declined (1). The estimated calving rate from conception is only around 50%, and even lower in higher producing animals. In a study by Evans et al in 2006, it was estimated that 50% of the improved profitability acquired through genetic selection for milk production was lost due to expenses associated with declining fertility (2). Reduced fertility results in longer inter-calving intervals and higher replacement rates (3). There is also the environmental cost of poor fertility, as more cows are required to sustain a given replacement rate, increasing fertilizer usage and methane production (2, 4).

1.1. Calving rate

Bovine fertilization rates do not differ significantly between high-producing and low-producing lactating cows or heifers, given the same quality of semen and correct timing of artificial insemination relative to oestrus. Fertilization rates in cattle are relatively high, at around 90% (5). The discrepancy in bovine reproductive performance comes in calving rate.

Calving rate for heifers or low to moderate-producing lactating cows is around 55%, with an embryonic and foetal mortality rate of 40%. This is in contrast to high producing cows, where calving rate is less than 40% and foetal mortality is closer to 60%. It is estimated that 70-80% of these losses occur during the pre-implantation period of embryonic growth, between days 8 and 16 (6).

The causes of embryonic loss during this period include chromosomal abnormalities, inheritance of embryonic lethal alleles, poor embryo quality or development, asynchrony between the developing embryo and maternal endometrium, lack of sufficient maternal-embryonic signalling, and an adverse uterine environment or poor receptivity of the endometrium to the embryo (1, 7-10).

Table 1 Timing of embryonic and foetal loss in dairy cattle (5, 11).

	Day 0	Days 7 - 21	Until Day 90
Low - Moderate Producers	Fertilization rate: **90%**	Pregnancy rate: **60-65%**	Pregnancy rate: **50-55%**
High Producers	Fertilization rate: **90%**	Pregnancy rate: **45-50%**	Pregnancy rate: **40-45%**
	Failure in embryonic development	Failure of the embryo to prevent luteolysis	Late embryonic losses

1.2. Negative energy balance

Cattle undergo a period of negative energy balance during the period immediately preceding parturition when energy demands from the foetus increase exponentially, and immediately post-calving when energy demand for milk synthesis is high. During such times, net energy requirements exceed net energy intake (12). The degree of negative energy balance is more severe in high-producing cows, which have been selected for their ability to maintain high levels of milk production, often at the expense of body reserves.

Dairy cows in negative energy balance have low circulating glucose and insulin concentrations, and high glucagon and growth hormone (GH) concentrations. This results in stimulation of gluconeogenesis from amino acids, release of glycogen from muscle, mobilization of adipose tissue for oxidative metabolism through lipolysis (1), release of non-esterified fatty acids (NEFA) from adipose tissue and ketone bodies from the liver. Levels of NEFA and ketone bodies are related to the degree of negative energy balance.

Negative energy balance is also a time when the somatotrophic axis is uncoupled (13). Growth hormone receptor expression is down-regulated in the liver, resulting in low circulating concentrations of insulin-like growth factor (IGF1), despite high GH concentration. This promotes the utilization of mobilized substrate to maintain homeostasis rather than for growth and cellular proliferation. GH promotes the mobilization of lipids and a reduction in lipid accretion. During a period of under-nutrition or reduced nutrient intake, growth hormone receptor (GHR) expression in the liver (but not adipose tissue) is reduced (14). Several studies have linked the degree of negative energy balance (often associated with high milk production) and the subsequent alterations in tissue mobilization and circulating hormone concentrations with various measures of reproductive performance. The resumption of oestrous cycles post-partum in animals in severe negative energy balance (still maintaining a high level of milk production) is reportedly delayed, compared with animals in a less severe negative energy balance (15-17).

Resumption of luteinizing hormone (LH) pulses in early lactation is necessary for the growth of the pre-ovulatory dominant follicle, and for ovulation to occur. The physiological state of negative energy balance is not conducive to LH secretion and hence ovulation. Negative energy balance is also associated with reduced ovarian responsiveness to LH. This is thought

to occur through the regulation of LH receptors on the follicle by insulin and IGF1. LH receptor expression on granulosa cells is dependent on oestradiol and FSH and the production of these are reduced when insulin and IGF-1 are low as occurs during negative energy balance (18-20).

1.3. Metabolic state and diet

The metabolic state of the cow can also affect luteal cell function. The rate of increase in progesterone production by the corpus luteum is lower in high-producing dairy cows. The rate of increase in progesterone concentration has in turn been correlated with pregnancy rate (21).

Nutritional status has also been reported to influence the oocyte's developmental competence. This may occur through alterations in the reproductive system during times of negative energy balance and/or when animals are fed different dietary constituents (22-24). In particular, Sinclair et al 2000 (25) demonstrated that diets high in rumen-degradable nitrogen can lead to increased concentrations of ammonia and urea in follicular fluid, resulting in reduced developmental competence.

The effects of diet on oocyte quality can depend on the metabolic state of the animal. Animals that are thin can benefit from high-energy and/or protein diets, while detrimental effects have been seen with the same diets in animals that are in good condition (26). It has also been reported that diets which are beneficial for follicular development may be detrimental to oocyte competence (23).

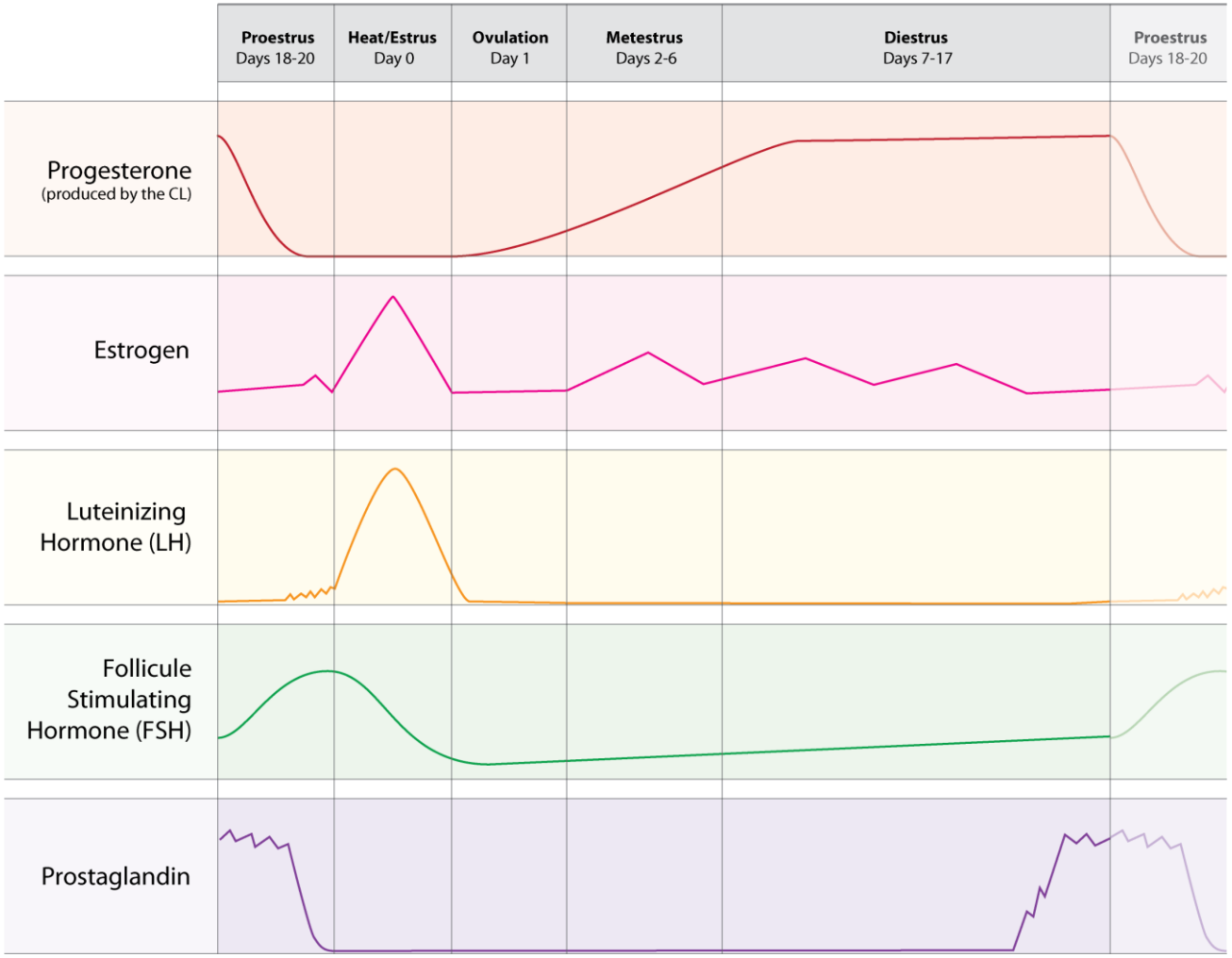
The metabolic status of the cow during follicular and embryonic development may affect uterine function through subtle alterations in the steroid hormones oestrogen and progesterone, which regulate gene expression and the secretion of proteins by the endometrium. It is therefore vital to better understand the molecular events associated with the pre-implantation phase of the bovine reproductive cycle.

1.4. The oestrous cycle

The reproductive cycle in placental mammals such as bovines is referred to as the oestrous cycle. Unlike the menstrual cycle occurring in humans and other primates, the functional

layer of the endometrium is reabsorbed rather than being shed during each cycle. The bovine is a non-seasonal polyestrus animal, with a normal oestrous cycle of 18-24 days that can be divided into luteal and follicular phases, or further into pro-estrus, oestrus, met-estrus and di-estrus (Figure 1).

Figure 1. The bovine oestrous cycle.

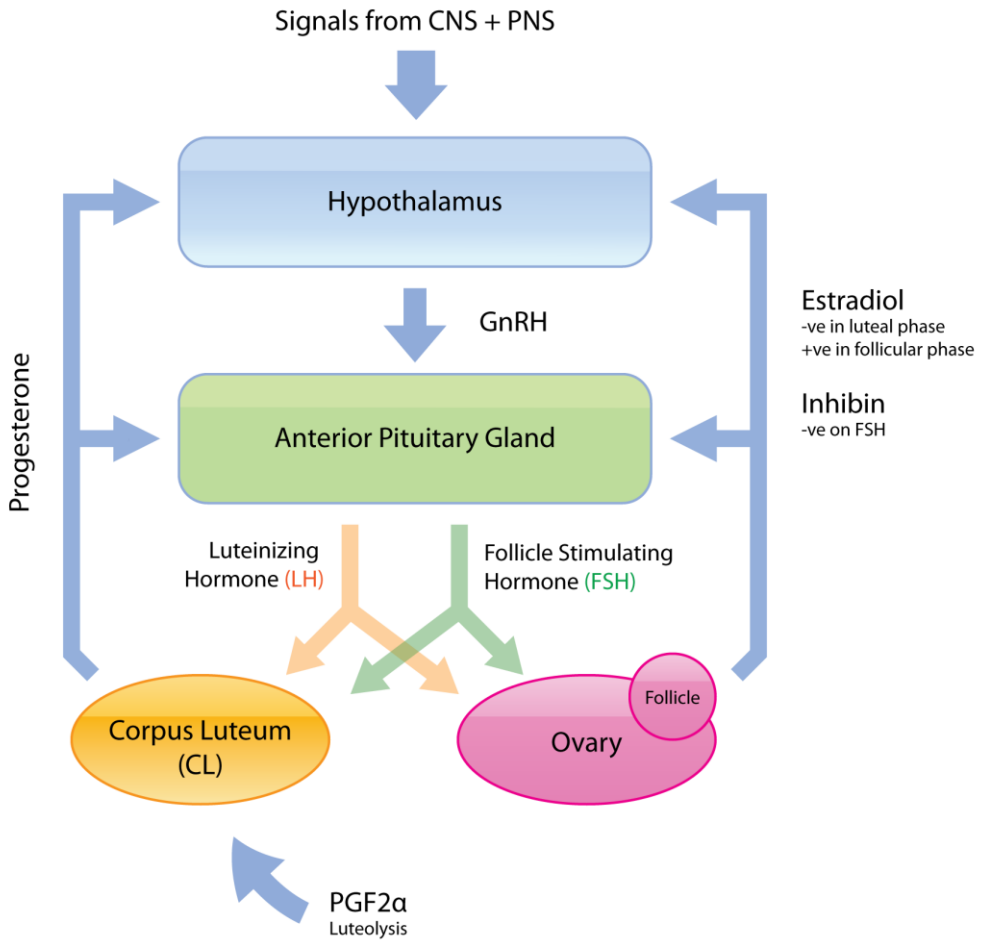

Proestrus
Days 18-20
Heat/Estrus
Day 0
Ovulation
Day 1
Metestrus
Days 2-6
Diestrus
Days 7-17
Proestrus
Days 18-20
Progesterone
(produced by the CL)
Estrogen
Luteinizing
Hormone (LH)
Follicule
Stimulating
Hormone (FSH)
Prostaglandin

Pro-estrus is the time when a follicle in the ovary becomes dominant, and is stimulated by follicle stimulating hormone (FSH) and luteinizing hormone (LH) to produce increasing amounts of oestrogen. High concentrations of oestrogen result in behavioural oestrus - the time when the female bovine is sexually receptive, lasting between 8 and 24 hours. This is followed by induction of the LH surge necessary for ovulation of the oocyte from the follicle. During the met-estrus period, luteinisation takes place. This involves differentiation of the ruptured follicle into the mature corpus luteum (27). The corpus luteum produces progesterone through the di-estrus period until the onset of luteolysis. The regulation of events occurring during the oestrous cycle is discussed below.

1.4.1. Endocrine regulation

The oestrous cycle is regulated by the hypothalamic-pituitary-gonadal axis (Figure 2). Gonadotropin-releasing hormone (GnRH) secreted from the hypothalamus acts on the anterior pituitary gland, thereby regulating the secretion of LH and FSH into circulation. GnRH pulse frequency is coincident with LH pulse frequency. GnRH and LH are regulated by a negative feedback effect of oestradiol in combination with high progesterone in the luteal phase. GnRH, LH and FSH on the other hand are regulated by positive feedback through oestradiol (without progesterone) from the pre-ovulatory follicle during the follicular phase (28-30). FSH is also negatively regulated through inhibin, which is thought to be produced by the dominant pre-ovulatory follicle (31).

Figure 2. Hypothalamic-pituitary-gonadal axis.


Signals from CNS + PNS
Hypothalamus
GnRH
Anterior Pituitary Gland
Progesterone
Estradiol
-ve in luteal phase
+ve in follicular phase
Inhibin
-ve on FSH
Luteinizing Hormone (LH)
Follicle Stimulating Hormone (FSH)
Corpus Luteum (CL)
Follicle
Ovary
PGF2α
Luteolysis

1.4.2. Follicular growth

During the oestrous cycle, there are two to three waves of follicular growth, during which FSH causes a cohort of between two and five antral follicles to emerge and grow to about 5mm in diameter. From this group of follicles, a single follicle is selected as the dominant follicle (32). The dominant follicle continues to grow, while other follicles in the cohort undergo atresia. The mechanism that allows one dominant follicle to grow while other follicles undergo atresia is thought to involve the development of LH receptors on the granulosa cell layer of the dominant follicle, thereby enabling LH-dependent growth. Also, a reduction in insulin-like growth factor binding proteins (IGFBPs) in the dominant follicle leads to an increase in the bioavailability of insulin-like growth factor (IGF1) (33). The dominant follicle also produces inhibin, which negatively regulates FSH production, thereby preventing other smaller follicles from growing (34). The dominant pre-ovulatory follicle also produces oestrogen, which when in sufficient concentrations, causes oestrous signs and the final LH surge prior to ovulation. The dominant follicle is either ovulated or undergoes atresia if there is insufficient LH amplitude to initiate ovulation (35, 36).

1.4.3. Ovarian steroid secretion

LH and FSH also control ovarian steroid secretion. Oestradiol is predominantly secreted by the dominant follicle and its concentration increase during the follicular phase, reaching a peak at the time of the LH surge. Oestradiol concentrations have been correlated with the occurrence of each follicular wave. LH controls the secretion of androgens from the thecal cells, with Estradiol-17 beta being produced through the aromatization of testosterone. This provides the necessary increase in oestradiol (without progesterone) which acts via positive feedback on the hypothalamus and leads to the LH surge that initiates ovulation. The LH surge causes follicular cells to differentiate and initiates inflammatory events that are necessary for the follicle wall to rupture, thereby facilitating ovulation (35).

1.4.4. Corpus luteum

The corpus luteum (CL) is a temporary structure formed from the pre-ovulatory follicle left behind after ovulation that produces the steroid hormone progesterone. LH stimulates the formation of luteal cells from the theca and granulosa cells of the pre-ovulatory follicle (37).

The cells of the corpus luteum are either small (when derived from granulosa cells) or large (when derived from theca cells). As these luteal cells mature their progesterone production capacity increases, accounting for the increase in progesterone concentration during the luteal phase. As the CL matures it becomes unresponsive to LH and develops prostaglandin receptors. Large luteal cells are also thought to be responsive to growth hormone (GH) (38).

Progesterone produced by the corpus luteum produces the negative feedback effect on gonadotropin release as well as priming the uterus, making it receptive to embryo implantation. The corpus luteum begins to regress on day 16 of the reproductive cycle, in response to pulsatile release of prostaglandin F2 alpha (PGF2α) from the endometrium.

Continued exposure of the uterus to high levels of progesterone leads to the down-regulation of progesterone receptors in the luminal and superficial glandular epithelium (39). In sheep, the loss of PGR expression combined with increased oestradiol concentration allows for ERα (oestrogen receptor alpha) induction and increased expression of the OXTR (oxytocin receptor) (40). Oxytocin acting through the OXTR then induces the pulsatile release of PGF2α, allowing the luteolytic mechanism to occur (41). Transport of PGF2α from the endometrium to ovary is facilitated by the prostaglandin transporter (PGT) protein in ruminants. At least 80% of PGF2α release is facilitated by a PGT mediated mechanism and 20% is facilitated by PGT independent simple diffusion in endometrial luminal epithelium cells (42). In cattle, OXTR increases independently of ERα increase. Increased oestradiol increases hypothalamic oxytocin pulsatile release (increased frequency and low amplitude), as well as increasing OXTR. Oxytocin released from the posterior pituitary binds to endometrial OXTR, which causes sub-luteolytic concentrations of PGF2α to be released. Luteal oxytocin release is then thought to amplify the pulsatile release of PGF2α and initiate luteolysis.

Interferon tau (IFNτ) produced by the embryo in cattle is thought to suppress the expression of OXTR, and potentially inhibits the pulsatile release of PGF2α through induction of the endometrial prostaglandin synthesis inhibitor (EPSI), linoleic acid (43). Linoleic acid is a competitive inhibitor of arachidonic acid for prostaglandin synthesis. This acts to prevent the conversion of arachidonic acid into PGF2α by prostaglandin synthase, thereby blocking luteal regression (44, 45).

In sheep, IFNτ secreted by the embryo suppresses transcription of OXTR gene in the luminal epithelium through the interferon regulatory factor 2- ERα-OXTR pathway. In pregnant sheep, the pulsatile release of PGF2a is inhibited and basal release is increased compared to non-pregnant sheep (42). Further, IFNτ inhibits the PGT mediated release of PGF2α in the luminal epithelium. However, in cattle, the prostaglandin carrier MRP4 is stimulated by oxytocin but not influenced by IFNτ (46).

1.5. The journey from ovulation to implantation

During ovulation, the oocyte is released from the dominant follicle into the oviduct. This occurs approximately 24-48 hours after the LH surge (47). Mammalian oocytes derive nutrition for growth and development from the yolk material contained within their cytoplasm. Fertilization by either natural or artificial insemination occurs in the oviduct. Sperm penetrates the zona pellucida and cytoplasm, followed by fusion of the nuclei. By the morula stage, there are insufficient nutrients in the yolk material and the embryo must obtain materials from the uterine fluid to metabolize. During this time the zygote, which is still contained within the zona pellucida, doubles the number of blastomeres it contains at each cleavage. The first cleavage events occur whilst the zygote is traveling down the oviduct towards the uterus - a journey that takes between 71 and 98 hours (48, 49). In these early stages the embryonic genome is mostly repressed, and the mRNA formed during oocyte growth is recruited for the majority of protein synthesis. In bovines, activation of the embryonic genome is thought to occur at the 4 cell stage, with the zygote entering the uterus at the 8-16 cell stage (totipotency is lost at the 16 cell stage), immediately preceding the transition from the morula to blastocyst stage (48, 50, 51).

Fluid begins accumulating between the zygote's cells, and these spaces unite to form a fluid-filled cavity called the blastocoele. A few cells differentiate into a compact group forming the inner cell mass, while those cells surrounding the cavity form the trophoectoderm. At around 2 week’s gestation, the zona pellucida ruptures and the blastocyst hatches. Once this occurs, the blastocyst is no longer constrained by the zona pellucida and is able to further increase in size. The spherical blastocyst maintains its shape for several days, until the trophoectoderm (outer embryonic membrane/chorion) extends to become oval in shape and undergoes a rapid expansion, becoming a long filamentous structure of about

25cm by day 17. Following this elongation and extension into both uterine horns, the embryo begins to have a fixed position in the uterine lumen. (52). Some trophectoderm cells undergo consecutive nuclear divisions without cytokinesis, becoming multinucleated. Binucleate cells (BNC) fuse apically with the luminal epithelium and form syncytia of trinucleate cells, further migration and fusion eventually results in the formation of syncytial plaques that are linked by tight junctions. In sheep, these multinucleated cells eventually cover the entire caruncule surface forming the placentome of the synepitheliochorial placenta (53, 54).

In bovine and other ruminants, the endometrium contains intercaruncular and caruncular regions. The intercaruncular region of the endometrium contains uterine glands that secrete nutrients and signalling molecules for the growing embryo. The caruncular endometrium (which does not contain any glands) is the site of embryo attachment, and will eventually form placentomes which are capable of small molecule and gaseous exchange (55).

1.5.1. Embryo development

The developing embryo depends on the oviductal and uterine environment for its nutrient supply prior to implantation. Nutrients, such as ions, amino acids and glucose, as well as growth factors such as inulin like growth factors (IGF1 and IGF2), are controlled in part through maternal diet and metabolic status. For example, nutrition is a major regulator of components of the IGF system. The IGF system is made up of the ligands IGF1 and IGF2, their receptors and six binding proteins which modulate the bioavailability of these ligands. The oviductal expression patterns of IGFBP2 and IGFBP6 are altered for cows in negative energy balance (56). This may cause an alteration in IGF signalling (57, 58). Pushpakumara et al 2002 (57) showed up-regulation of IGF1 in response to oestradiol, as well as differential expression of IGFBPs in a time and region-specific manner, hypothesized to create a gradient of IGF1 along the oviduct. Alteration in the expression of components of this system, as may occur during negative energy balance, might be detrimental to embryonic development, perhaps providing a mechanism for reduced fertility in these animals.

Expression of the IGF system has also been investigated in the uterus during the oestrous cycle and in early pregnancy (58). Robinson et al 2000 (58) showed that components of the IGF system were differentially expressed in the uterus, and that factors that influence the

expression of this system may alter uterine function through modulation of uterine glandular activity and development of uterine caruncules. The pre-implantation embryo expresses IGF receptors, and both IGF1 and IGF2 are present in uterine fluids. In addition, IGF1 and IGF2 have been reported to promote IFNτ production in vitro (59). Robinson et al showed that IGF1 was localized to the sub-epithelial stroma (with highest expression occurring at oestrus) and IGF2 was localized to the caruncule stroma. The authors did not find any pregnancy-associated differential regulation of IGF1 and IGF2, but did, however, show that IGFBP1, IGFBP2 and IGFBP3 were influenced by pregnancy status.

1.6. Regulation of endometrial receptivity

The mammalian uterus undergoes cyclical changes in response to the ovarian steroids oestrogen and progesterone throughout the reproductive cycle. These changes are necessary to establish and maintain pregnancy. For pregnancy to be established, the developmentally normal blastocyst stage embryo must be recognized and nourished by the maternal system, and be able to implant itself into a receptive endometrium to begin the process of placentation. In order for this to occur, the endometrium must allow the allogenic embryo to develop, grow, and eventually become receptive to the formation of a close physical association with the maternal system. There is a limited period of time during the oestrous cycle in mammals when this can occur, known as the window of implantation (60).

A key feature of a successful pregnancy is synchrony between the developing conceptus and the uterine environment. This is achieved in part through maternal-embryonic signalling. The ruminant conceptus grows to a relatively large size before it forms a close contact relationship with the maternal endometrium and placentation of the synepitheliochorial placenta begins. Placentation in the ruminant is relatively non-invasive compared with humans and mice, with no direct contact between the maternal system and the conceptus (61). The uterine secretions from the endometrial glands provide not only nutrients for the conceptus but also a communication channel for the growing conceptus, with the initial recipient of conceptus signalling being the endometrial epithelium. This signalling system enables the conceptus to communicate with the maternal system, announcing its presence and preventing luteal regression (55).

1.6.1. Progesterone and oestrogen

Progesterone produced by the corpus luteum during the luteal phase of the oestrous cycle plays a vital role in the development of endometrial function important to pregnancy. The role of oestrogen and progesterone has been investigated through receptor deletion studies. Lubahn et al 1993 (62) reported that the oestrogen receptor was not essential for prenatal sexual development, but was important for reproductive function in the mouse. Lydon et al 1995 (63) reported that mice lacking the progesterone receptor had reproductive disorders.

Progesterone and oestradiol regulate PGR, ER and OXTR expression. Pre-ovulatory oestradiol is important for the development of cellular morphology and secretory organelles, as well as being involved in the regulation of steroid receptors and cellular proliferation. Progesterone receptors (PGR) are present on the pre-implantation bovine embryo. Expression of PGR is low and there is no reported data on their functional capacity (64). Results from experiments where progesterone was added to embryo culture media are inconclusive. There does not appear to be any direct benefit of progesterone addition to culture (64, 65). However, many studies culture embryos under oil which is inappropriate when adding lipophilic steroids like progesterone, because the oil overlaying the culture media absorbs the steroids (66, 67). There is no data supporting a direct influence of progesterone on embryos; however, there is currently no data on post-transfer embryos. To test this directly a progesterone receptor knockout or knockdown would be required.

The action of progesterone is regulated through its binding to progesterone receptors on the maternal endometrium. Progesterone, acting through its receptor, initiates changes in the expression of progesterone responsive genes, as well as initiating changes in uterine secretions.

The expression of epithelial progesterone receptors varies during the reproductive cycle in humans, with higher concentration in the follicular phase and lower concentrations in the luteal phase. The expression of PGR in the luminal and glandular epithelium decreases in the progesterone dominated luteal phase , while in humans it has been shown to maintain expression in the stroma (68). Maximal levels of PGR expression occur between days 4 and

10 in the bovine (69). Oestradiol is also involved in regulation of PGR expression, and is thought to initiate up-regulation in the follicular phase and beginning of the luteal phase.

The concentration of progesterone during the early luteal phase has been associated with embryonic survival in cattle (70, 71). For example, supplementation of progesterone in the early luteal phase has been reported to result in embryos of increased size, whereas progesterone supplementation later in the cycle has had variable results. The amount of progesterone produced by the corpus luteum has been associated with embryo size and also the level of IFNτ produced by the embryo (72-74). Satterfield et al 2006 (75) reported increased progesterone concentrations led to increased embryo size as well as uterine histotroph production in the ewe. Robinson et al 2006 (72) reported that the elongating trophoblast does not produce more IFNτ mRNA per cell. However, as the trophoblast elongates and hence the number of cells increases, the overall production of IFNτ increases.

Progesterone concentration is thought to affect the growth and development of the embryo through mediation of nutrient secretions released from the uterine glands and increased uterine receptivity (73, 74). Further, progesterone supplementation alters gene expression in the uterus in cattle which can influence embryo development and competence (76, 77). The embryo's growth depends on its ability to produce enough IFNτ to prevent luteolysis from occurring. If progesterone concentration is not optimal during the pre-implantation stage, this may negatively impact the production of uterine secretions causing abnormal growth, and possibly, insufficient IFNτ to prevent OXTR up-regulation and subsequent luteolysis at day 16.

1.6.1.1. Structural changes regulated by progesterone

In a study by Wang et al 2007 (78), high levels of circulating progesterone on day 8 of the reproductive cycle were reportedly associated with an increased number of uterine glands in the cow, whereas a high progesterone concentration on day 5 was associated with decreased gland duct size on day 8 (78). The authors suggest that cyclic changes including changes in gland duct density occurring in the endometrium are regulated through progesterone concentration. The importance of the rate of progesterone rise rather than the concentration at a particular point in time is highlighted in this study. Thus, progesterone may affect embryo development through modification of endometrial glands,

leading to changes in the histotroph secretions that provide nourishment for the developing embryo.

Elongation of the embryo is dependent on uterine gland secretions and the signals that are contained in them. Evidence for this comes from the fact that embryos do not elongate in culture and embryos do not grow beyond the pre-implantation stage in uterine gland knockout (UGKO) studies. In a study by Gray et al 2001 (79), endometrial gland duct density was positively correlated with conceptus survival in sheep. Interestingly, the study indicated that blastocysts recovered between days 6 and 9 were developmentally normal, while obvious differences between UGKO and control sheep blastocysts were apparent at day 14. They also noted that day 14 concepti recovered from UGKO sheep resembled a normal day 11 blastocyst that is yet to undergo elongation, providing further evidence for the importance of uterine gland secretions during the elongation stage of development. Uterine gland secretions provide not only the nutrients, but also growth factors and cytokines for the promotion of cell division, proliferation, morphogenesis and differentiation.

The luminal epithelium has been shown to undergo ultra-structural changes specific to cycle stage and pregnancy (80, 81). Of particular interest are structures called pinopodes, which are bleb-like protrusions found on the apical surface of the endometrium in most mammals during the implantation period. In bovines, protrusions are present during the luteal phase of both cycling and pregnant animals. However, after day 18 they are only present in the endometrium (both caruncular and intercaruncular) of pregnant animals suggesting they are regulated at least in part though progesterone.

In humans, endometrial pinopodes are thought to provide an embryo attachment site free of anti-attachment molecules such as mucins, which are highly expressed during the luteal phase. This is in contrast to the mouse and sheep, where mucin expression is decreased during the window of implantation (82, 83).

1.6.2. Interferon tau (IFNτ)

Interferon tau (IFNτ) acts to block luteal regression and maintain pregnancy through its action on the endometrial epithelium, with production peaking prior to the attachment phase of implantation. The mechanism by which IFNτ prevents luteolysis is not well

understood in bovines. It has been reported, however, that IFNτ produced by the embryo binds to endometrial type-1 interferon receptors, leading to the activation of the JAK-STAT (Janus kinase-signal transducer and activator of transcription) pathway (84). The synthesis of interferon-stimulated proteins is then thought to inhibit the cascade for synthesis of PGF2α.

Two potential mechanisms are postulated for the inhibitory effect of IFNτ on PGF2α. The first is conjugation of interferon stimulatory proteins to cytosolic proteins important to PGF2α synthesis. The second is stimulation of transcriptional repressors, which then act on genes important to PGF2α production. In the ovine for example, IFNτ induces expression of the transcriptional repressor *IRF1*, which is then able to bind to interferon response elements, such as those contained in the promoter of the ovine oestrogen receptor gene, and inhibit its transcription. In bovines there is up-regulation of transcriptional repressors *IRF1* and *IRF2*, and the *OXTR* gene promoter contains IRE elements that can bind transcriptional repressors, thus providing a potential pathway for luteolysis (85).

Studies using purified or recombinant IFNτ indicate that administration of IFNτ to non-pregnant animals can prevent luteal regression at day 16, and prolong corpus luteum lifespan and production of progesterone to at least day 50 of pregnancy. While IFNτ can prevent luteal regression and maintain a "pregnant" state, other factors are required for pregnancy maintenance beyond day 50 (86). Candidates for these other factors involved in the prevention of luteolysis and maintenance of pregnancy include placental lactogens, trophoblast Kunitz domains and pregnancy associated glycoproteins.

IFNτ is unique to ruminant ungulates, and unlike other type 1 interferons it is not induced by viral infection (87). Gel mobility shift assays and cell transfection have been used to detect important promoter regions in the IFNτ gene. These approaches have identified regions in the promoter that are required for constitutive expression of IFNτ (88). Expression of the bovine IFNτ gene (Figure 3) is regulated by specific promoter regions referred to as distal and proximal promoter regions. The promoter region of the IFNτ gene contains transcription factor binding sites, including sites for ETS2, AP1 and DLX3. These transcription factors act in a combinatorial manner to modify the expression of IFNτ. It has been proposed that factors in uterine secretions binding to trophoectoderm receptors activate key signalling pathways that modify the expression of interferon tau (89-91).

Figure 3. Bovine interferon tau (IFNτ) gene. Transcription factor binding sites for AP1, ETS2, DLX3, and OCT4.


AP1 like site
AP1 like site
Octamer like Binding site
Distal Promoter region
Octamer like binding site
AP1 like site
Octamer like binding site
AP1 like site
ETS2 motif
Octamer like binding site
Octamer like binding site
Core ets2
AP1 like site
DLX3 Binding Site
DLX3 Binding Site
Proximal promoter region
IFNT
Start
IFNT
IFNT
IFNT
IFNT
IFNT
IFNT
IFNT
IFNT
IFNT
Stop
IFNT
IFNT
IFNT

1.6.2.1. ETS2 and AP1

Deletion of the *ETS2* gene results in embryonic lethality in the mouse due to placental defects. *ETS2* contains a DNA-binding domain that binds to a specific sequence motif containing a GGAA core. *AP1* transcription factor binding sites contain a TGAC sequence. Gene regulatory activity of ETS2 requires phosphorylation of Thr72 in its pointed domain (90). Activation of the MAPK pathway is therefore necessary to achieve the activated phosphorylated state. Ezashi and Roberts 2004 (90) investigated the roles of ETS-2 and AP1 in the expression of *IFNτ* as well as the effects of growth factors that are known to stimulate the MAPK pathway on the expression of *IFNτ*. Results indicate that the Ras/MAPK signal transduction cascade controls expression of *IFNτ* through the regulation of ETS2 and AP1 transcription factors. A more recent study by Das et al 2008 (89) provided evidence for a possible role of protein kinase A (PKA) acting in synergy with the transcription factor ETS2 in the regulation of *IFNτ* expression in choriocarcinoma cells. They also suggest that growth factors contained within the maternal uterine secretions are likely to trigger this cascade, allowing the coordination of *IFNτ* expression with the physiological state of the mother.

1.6.2.2. Distal-less 3 (DLX3)

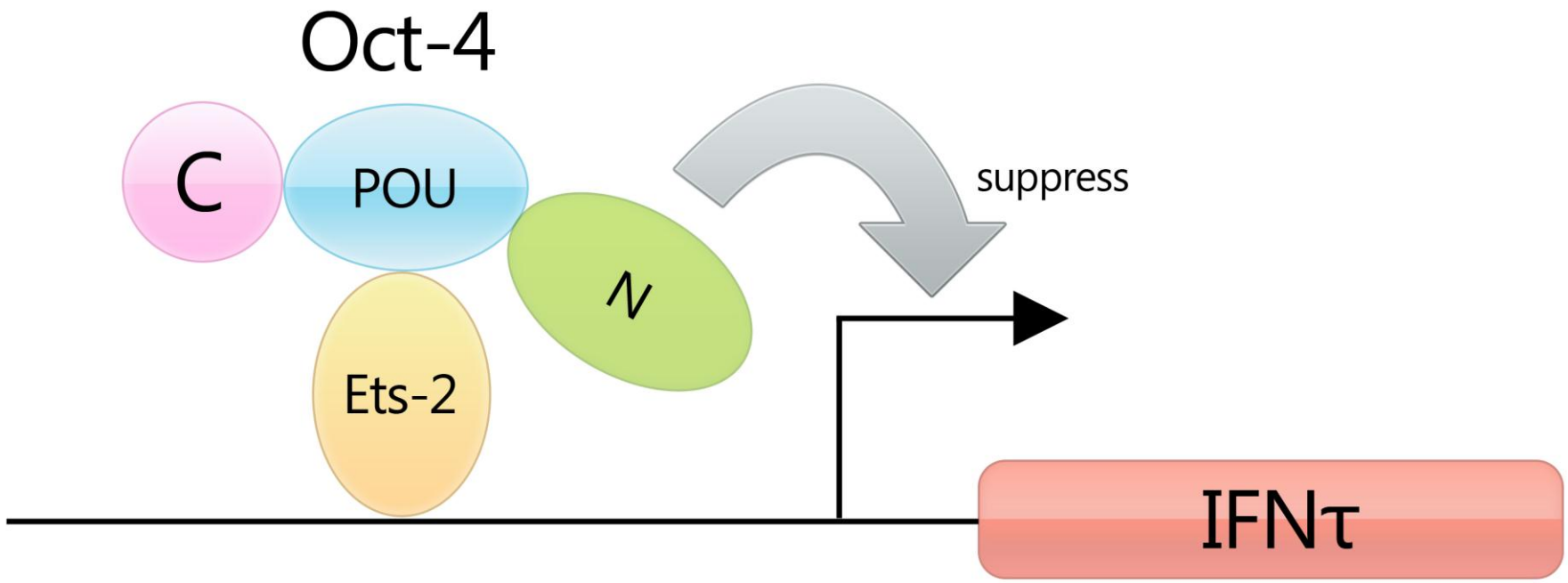
DLX3 is a homeodomain transcription factor that has been investigated by Ezashi et al 2008 (91). It had previously been reported that *DLX3* is expressed in trophoblast cells of mice and humans with Dlx3-/- mice not surviving beyond day 9.5 to 10 due to placental defects. The DLX3 protein binding domain recognizes a specific sequence with a TAAT core motif (Figure 3). The authors established a regulatory role for DLX3 by showing that *DLX3* silencing, using siRNA in a trophoblast cell line (CT-1), resulted in a decrease in the expression of *IFNτ*. They also showed that DLX3 and ETS2 had a greater-than-additive effect on the expression of *IFNτ*.

1.6.2.3. Oct-4

Oct-4 is a POU octamer binding domain transcription factor that is known to be a pluripotency determining factor. Oct-4 can block the effect of the transcription factor ETS2 and repress its activation of IFNτ. This is proposed to occur through a quenching

mechanism, whereby the POU domain of Oct-4 interferes with the ability of ETS2 to interact with the transcriptional machinery, as illustrated in Figure 4 below (92).

Figure 4. Proposed quenching mechanism for Oct4 suppression of IFNτ transcription (92).


Oct-4
C
POU
N
Ets-2
suppress
IFNτ

1.6.3. Molecular regulation of endometrial receptivity

Successful implantation depends on two factors: embryo quality and endometrial receptivity. The molecular mechanisms by which the bovine endometrium becomes receptive to embryo implantation are not fully understood. During the pre-implantation period the intercaruncular endometrial glands secrete uterine histotroph under the influence of the steroid hormones progesterone and oestrogen; this is vital for development and survival of the growing embryo. Components of histotroph include growth factors, cytokines, transport proteins and other substances important for embryo development and survival. In addition, it provides a communication channel to the embryo. The proteins expressed on the luminal surface of the endometrium such as adhesion molecules, which are the first point of embryo-maternal contact, must also undergo changes. Structural changes to the endometrium must also occur, including the remodelling of the extracellular matrix to support placentation. Factors that are known to be important in the acquisition of a receptive endometrium in the bovine and other species are discussed below.

1.6.4. Cellular adhesion molecules

The surface of the luminal endometrial epithelium and trophoblast contain a glycoprotein coat called a glycocalyx (60, 80). As the glycocalyx is the contact point for maternal-embryonic contact, the components and regulation of this glycoprotein coat are likely to be important in the events leading up to and including implantation. The glycocalyx is composed of several glycoproteins that are involved in cellular adhesion, including integrins, mucins, selectins and cadherins. The expression, regulation and possible roles of these are discussed below.

1.6.4.1. Integrins and their ligands

Integrins are transmembrane glycoproteins that are composed of two non-covalently linked alpha and beta subunits (60). Integrins can act as a receptor for extracellular matrix (ECM) proteins through their extracellular domains, and can interact with the cytoskeleton through intracellular domains. This enables integrins to facilitate signal transduction between the ECM and the cytosol. Complementary expression of integrins and their ligands on the surface of the endometrium and embryo are proposed to facilitate embryonic–endometrial

attachment in a sandwich model of embryonic adhesion (60). Integrin expression patterns are, however, species-specific.

Most integrins are expressed ubiquitously throughout the cycle, but some appear to have cycle- and, in particular, implantation period-dependent expression. For example, the *α1β1*, *α4β1* and *αVβ3* integrins are expressed on the apical surface of the endometrium during the window of implantation in humans (day 20-24). In particular, the β3 subunit, which is not detected prior to day 19 in the human reproductive cycle, is more highly expressed after day 19. It has been proposed that these integrins (in particular the αVβ3 integrin) act as receptors for embryonic attachment, through interaction with ligands such as osteopontin and other complementary adhesion molecules expressed by the embryo (68). The human trophoblast has also been reported to express integrins that bind to ligands expressed on the endometrium, as well as expressing ligands for integrins expressed by the endometrium. The expression of integrins and their ligands is also an important regulator of implantation in other species, although the patterns of expression are different – possibly the result of differences in reproductive cycles and placentation.

In cattle, *αVβ3* expression in endometrial tissue is present from day 0 to 15 but is absent at day 16 of the cycle in non-pregnant animals (93). In pregnant animals, however, expression is detected again on day 17 (94). The expression of the β3 subunit has been shown to be regulated by oestrogen in a cell culture system. Integrin subunits α1, α3, and α6, and the ligands collagen IV and laminin are reportedly expressed in the endometrium and trophoblast from day 18 of the reproductive cycle in pregnant and non-pregnant animals. However, α1 subunit expression is only expressed until the attachment phase. In humans, when the α1 subunit is dimerised with the β1 subunit, it attaches to laminin and is thought to be involved in trophoblast invasion into the decidua (95). The differences in human and bovine expression patterns may reflect the relatively non-invasive nature of placentation in the latter species.

Integrins and their ligands are expressed in the endometrium and embryo surface in ovine, and are hypothesised to be important regulators of implantation (83, 96-100). However, there are no implantation- or pregnancy-related expression changes in integrins (83, 100). It

has been suggested that, rather than integrin expression, ligand expression may be altered during the implantation period.

Osteopontin (OPN/SPP1) is an ECM protein that is a member of the small integrin-binding ligand N-lined glycoprotein (SIBLING) family. OPN is involved in cell-cell adhesion and communication, cell-mediated immune responses and cell survival (93). In the human endometrium, osteopontin has been shown to be up-regulated during the window of implantation. In the ewe, progesterone has been shown to induce OPN expression in the endometrial glands during the luteal phase, with greater concentrations of OPN found in pregnant versus cycling ewes (100, 101). However, during the bovine and ovine implantation period it has recently been shown that there is no complementary expression of the αVβ3 integrin and its ligand OPN. This suggests that in these species, unlike primates and rodents, the interaction of these two proteins is not involved in embryo attachment (102).

1.6.5. Selectins

Selectins are another group of glycoproteins whose expression is thought to be important in the implantation process. Selectins are known to play a role in leukocyte trans-endothelial trafficking, whereby selectins expressed on leukocytes facilitate their rolling along the vascular endothelium, which expresses selectin ligands (103). Dominguez et al 2005 (104) identified a parallel between the role of selectins and their ligands in leukocyte trafficking and their potential use in “rolling” of the blastocyst during the implantation period in humans. In support of this concept, human embryos are reported to express L-selectin and there is up-regulation of the selectin ligands *MECA79* and *HECA452* in the human endometrium during the receptive period (105).

1.6.6. Mucins

Mucins are high molecular weight glycoproteins. Members of the mucin family are reportedly involved in the regulation of implantation in various species. MUC1 is an anti-adhesive glycoprotein that contains an intracellular cytoplasmic tail and an extracellular ectoderm. It interferes with cellular adhesion though a steric hindrance (directly proportional to the length of the ectoderm) (60). In mice, *MUC1* expression is down-

regulated during the window of implantation, which is thought to facilitate embryo-endometrial interactions (82). In sheep and pigs, the expression of *MUC1* is also down-regulated at the same time as progesterone receptor down-regulation (97, 100).

However, in humans, its expression increases on ciliated cells during the window of implantation and is absent from non-ciliated cells and from uterine pinopodes during the window of implantation. This is thought to be a mechanism to direct the attachment of the embryo to uterine pinopodes. Sheddases (possibly produced by the embryo), which are capable of proteolytically degrading the mucin ectodomain, are then thought to remove the ectoderm from the site of implantation (106).

In bovines, expression of the mucin gene *MUC16* has recently reported to be down-regulated in intercaruncular endometrial tissue during the luteal phase (107). The down-regulation of *MUC16* in human pinopodes has also been demonstrated recently (108). MUC16 has a larger extracellular ectodomain than MUC1 and could, therefore, be considered to be more anti-adhesive than MUC1. Gipson et al 2008 (106) investigated the expression of both *MUC1* and *MUC16* in human endometrium biopsies, as well as assessing the effect of siRNA-mediated knockdown of *MUC1* and *MUC16* in a uterine cell line (ECC-1) on trophoblast adhesion. The results from this study indicate that MUC16 is an anti-adhesive molecule expressed on the endometrium that acts to prevent trophoblast adhesion. These data indicate that during the receptive phase *MUC16* is down-regulated and this supports trophoblast adhesion. In comparison with *MUC1*, the expression of *MUC16* was more consistently down-regulated on endometrial pinopodes (*MUC1* was expressed on pinopodes in some biopsies studied) and the siRNA knockdown of *MUC1* did not promote adhesion of trophoblast on the endometrial cell line ECC-1.

1.6.7. GlyCAM-1

Glycosylation-dependent cell adhesion molecule 1 (GLYCAM1) is a sulfated glycoprotein that acts as a ligand for L-selectin and is involved in leukocyte-endothelial cell adhesion (101). GlyCAM-1 is expressed on the ovine endometrium and trophoectoderm, and is thought to be involved in embryonic attachment as a selectin ligand.

1.6.8. Cadherins

Cadherins are glycoproteins which have a calcium dependent cell-to-cell adhesion mechanism. In the human endometrium E-cadherin is reportedly up-regulated during the luteal phase and has been suggested as being involved in the attachment of the embryo to the endometrium (109). The expression of calcitonin (which is known to increase intracellular calcium and, therefore, regulates the expression of E-cadherin) is up-regulated by progesterone during the mid-luteal phase in humans (110).

1.6.9. SERPIN

SERPIN is a progesterone-induced, strongly basic, immunosuppressive and anti-proliferative glycoprotein present in uterine secretions (111). SERPIN has been proposed to support conceptus growth through its ability to sequester the pluripotent growth factor activan A. Because of its immunosuppressive and anti-proliferative properties, it has been hypothesized to prevent foetal allograph rejection through inhibition of lymphocyte proliferation in the uterus (111).

1.6.10. Chemokines

Several chemokines and their receptors are reportedly expressed in the uterus and trophoblast, respectively, in species such as the ovine and human. The endometrium secretes several chemokines. In particular, the ovine endometrium expresses *CXCL9, CXCL10* and *CXCL11* during the pre-implantation period and the ovine trophoblast expresses the receptor for these chemokines, *CXCR3* (112). The expression of the chemokines *CXCL9* and *CXCL10* are increased by IFNτ produced by the embryo, and some chemokines are also regulated by progesterone. The expression patterns of chemokines and their receptors during the implantation period suggest that they are involved in the regulation of embryo attachment.

1.6.11. Extracellular matrix proteins

The extracellular matrix (ECM) is composed of proteins such as collagen, elastin, fibronectin, fibrilin, laminin and proteoglycans (113). Implantation and placentation in the bovine requires a certain degree of ECM remodelling to enable trophoblast bi-nucleate cells and

uterine epithelial cell fusion. Components of the ECM are regulated by the expression of matrix metalloproteinases (MMP) and tissue inhibitors of metalloproteinases (TIMP). The expression of MMPs and TIMPS has been detected in human, mouse and bovine endometrium and trophoblast tissue. Their expression is affected by several growth factors and cytokines that are thought to be important in the regulation of implantation (113).

1.6.12. Cytokines and Growth Factors

1.6.12.1. LIF and M-CSF

Endometrial expression of pleiotropic cytokine *LIF* (leukaemia inhibitory factor) is crucial for implantation to occur in the mouse. The purported role of LIF in the implantation process comes from studies where wild-type embryos failed to implant in the uterus of a LIF deficient mouse, but the phenotype could be rescued through the administration of LIF (114). LIF is present in bovine uterine secretions, and receptors for LIF have been localized on the bovine embryo. The addition of bovine LIF to culture medium has also been reported to improve the growth of bovine embryos (115). Macrophage colony stimulating factor (M-CSF) regulates the proliferation and differentiation of hemopoietic stem cells into macrophages (116).

The expression of *LIF* and *MCSF* in the bovine endometrium was investigated by Oshima et al 2003 (116). *LIF* and *MCSF* expression did not increase in the pre-implantation or implantation period in the bovine endometrium, but did increase after placentation and may, therefore, have a more important role in pregnancy maintenance rather than the implantation process itself in cattle.

1.6.12.2. Interleukins

The interleukins *IL6* and *IL1* have increased expression in the endometrium during the luteal phase, suggestive of an active role in implantation. The exact role of these interleukins is, however, unclear. For Example, *IL1* receptor blockade in the mouse is reported to prevent implantation; however, mice lacking a functional *IL1* receptor have only a slight reduction in mean litter size, with no other reproductive issue being apparent (117).

1.6.12.3. Growth Hormone

Growth hormone (GH) is a known regulator of embryonic and postnatal growth (118). The ovine and bovine embryos express both GH and the GH receptor (119). Treatment of murine embryos in culture with growth hormone improves overall embryo development, suggesting its expression in the endometrium of bovines and other species may be important for embryonic development (120).

1.6.12.4. Epidermal growth factor family

Epidermal growth factor members are transmembrane proteins that are proteolytically cleaved to release their mature form into the extracellular space. Their receptors are composed of a cytoplasmic domain and an extracellular binding domain (121). Complementary expression of members of this family in the endometrium during the window of implantation and their receptors on the trophoblast surface has been demonstrated in many species, suggesting a conserved role for this family in the implantation process.

Heparin binding-epidermal growth factor (HB-EGF) is an epidermal growth factor that is up-regulated in the human and Rhesus monkey endometrium during the window of implantation (122, 123). The receptors for this protein have been localized to the trophoectoderm of the embryo. Another member of this family, transforming growth factor (TGFα), has increased expression in the mouse and sheep uterus during the time of implantation, and the surface of the trophoectoderm expresses receptors for this factor (124, 125). In the bovine, *TGFα, HB-EGF* and EGF receptor are expressed on the blastocyst, and *TGFα* and EGF receptor are expressed on the endometrium during the pre-implantation period (124). These data are suggestive of an important role of the EGF ligands and their receptors in the process of implantation.

1.6.12.5. Fibroblast growth factor 2

Fibroblast growth factor 2 (FGF2) is secreted by the endometrium in the luteal phase (126). Michael et al 2006 (126) investigated the effect of FGF2 on the production of IFNτ in both a trophoectoderm cell line and in an *in vitro* derived blastocyst stage bovine embryo; FGF2 supplementation resulted in a dose-dependent increase in IFNτ mRNA and protein

expression. FGF2 supplementation resulted in a small increase in DNA synthesis in the CT-1 cell line, but had no effect on *in vitro* derived bovine blastocysts.

A recent study by Khatib et al 2008 (127) demonstrated that a previously defined SNP in the *FGF2* gene was associated with embryonic mortality. The SNP at this position had previously been linked to improved milk composition (GG genotype). In the current study the A allele was associated with decreased embryonic survival.

1.6.12.6. Vascular endothelial growth factor (VEGF)

Vascular endothelial growth factor (VEGF) stimulates angiogenesis, an important event that occurs during the process of implantation and placentation. *VEGF* and its receptors are expressed in the bovine endometrium and trophoblast during implantation (128).

1.6.12.7. Granulocyte-macrophage colony-stimulating-factor (GM-CSF)

Granulocyte-macrophage colony-stimulating-factor (GM-CSF) is a cytokine that has immuno-regulatory and anti-apoptotic roles, and has been reported to improve embryo yield and quality in culture. GM-CSF has also recently been reported to increase proliferation and the expression of IFNτ in a trophoectoderm cell line (129). IFNτ in turn increases the expression of *GM-CSF* (130).

1.6.12.8. Tumor necrosis factor alpha (TNFα)

TNFα stimulates PGF2α production through an increase in the expression of *COX2*, the enzyme responsible for the rate-limiting step in the conversion of arachidonic acid to the precursor of PGF2α (131). Okuda et al 2004 demonstrated that IFNτ blocked the stimulatory effect of COX2 on the production of PGF2α in the stroma. The effect of TNFα on PGF2α production was inhibited through blocking of the COX-2 enzyme. It has been hypothesized that TNFα stimulates anti-luteolytic pulses of PGF2α in the stroma, which is then capable of initiating the positive feedback loop necessary for luteolysis (131).

1.6.12.9. HOX-10

HOX genes are transcription factors that are primarily expressed during embryogenesis (132). However, they are also expressed in adulthood in the female reproductive tract and

are thought to be involved in the maintenance of developmental plasticity of the endometrium, enabling the structural and functional changes that occur during the reproductive cycle (133).

HOXA10 expression in the mouse reportedly affects the number of implantation sites (134) and has is important in pinopod development (135). Deletion studies in mice indicate that null females have an unreceptive endometrium.

HOXA10 has also been reported to induce the expression of uterine epithelial β3 integrin subunit, which, as previously mentioned, is involved in the attachment of the embryo to the endometrium and is up-regulated during the window of implantation.

1.6.13. WNTs

Wingless-type MMTV integration site family members (WNTs) are a group of secreted signalling molecules homologous to the Drosophila segment polarity gene, wingless (wg) (136). WNT signalling has been implicated in uterine receptivity and embryo implantation in the mouse (137). Expression of genes involved in WNT signalling has also been detected in cattle and human endometrium and in the embryo during the implantation period (138). In the ovine, the expression of several genes involved in WNT signalling has been investigated (136). Hayashi et al investigated the role of WNT signalling in ovine, and demonstrated that the ovine embryo expresses all essential components of the WNT signalling pathway. In particular, WNT7A was reported to be an important regulator of uterine morphogenesis, receptivity and pregnancy recognition (136). The expression of *WNT7A* in the sheep is correlated with loss of progesterone receptors in the luminal epithelium of the endometrium and the production of IFNτ by the conceptus. Hayashi et al 2009 suggest that WNT signalling is involved in maternal-embryonic interactions (136, 139). IFNτ secreted by the conceptus induces expression of *WNT7A*, which in turn acts on the conceptus to promote proliferation. *DKK1*, which inhibits WNT signalling, is expressed in the endometrium.

1.6.14. MicroRNAs

MiRNAs are small non-coding RNAs that are typically between 19 and 22 nucleotides long. The reproductive axis exhibits the second highest level of specific miRNAs in the human

body (140). Microarray analysis has identified miRNAs up-regulated during the window of implantation, suggesting they may have important regulatory roles in the implantation process. A recent report by Chakrabarty et al 2007 (140) suggests *COX2* as a potential target for these up-regulated miRNAs.

1.6.15. Epigenetics

Recently, epigenetic regulation of endometrial gene expression has been highlighted as a potential mechanism involved in uterine receptivity (109). Epigenetics is defined as heritable changes in gene expression unrelated to altered DNA sequences (141, 142). These epigenetic modifications include remodelling of chromatin, through modification of DNA methylation or post-translational modification of histone tails (143). The fundamental unit of chromatin is the nucleosome. The nucleosome is an octamer composed of four core histone proteins (H3, H4, H2A, H2B) around which 147 base pairs of DNA are wrapped. Histone modifications include; acetylation, methylation, ubiquitylation, phosphorylation and sumoylation. The most commonly studied form of histone modifications are acetylation, methylation and phosphorylation (144). DNA methylation is the covalent modification of DNA through the addition of a methyl group to the cytosine ring of the DNA strand. This addition converts cytosine to 5-methylcytosine and is catalysed by DNA methyltransferases (DNMTs). There are four DNMTs that have been identified in mammals. These are: DNMT1, which maintains DNA methylation during replication by copying the DNA methylation of the old DNA strand to the newly synthesized strand. DNMT2 characterized as having weak DNMT activity *in vitro* and has been reported to methylate tRNA. DNMT3a and DNMT3b, responsible for *de novo* methylation, as they are able to target un-methylated CpG sites (145). In mammals, DNA methylation occurs at the cytosine residue of a CpG dinucleotide. DNA methylation is generally associated with inhibition of promoter activity as it can prevent transcription factor binding and is associated with a repressed chromatin state indirectly through recruitment of methyl-CpG-binding proteins (MBPs). Methylation has been highlighted as a potential regulatory mechanism in uterine receptivity. Additionally, aberrant methylation may contribute to poor fertility. The role of methylation in regulating the expression of genes involved in uterine receptivity has recently been highlighted. E-cadherin is down-regulated in some infertile women and is down-regulated in a non-receptive endometrial cell line (109, 146). Inhibiting methylation of E-cadherin in a non-

receptive endometrial cell line results in up-regulation of E-cadherin and in the cell line becoming receptive. Another gene regulated by methylation, *HOXA10,* is expressed in the endometrium and is involved in regulating endometrial receptivity. Down-regulation of *HOXA10* in women with endometriosis has recently been associated with aberrant methylation (147).

1.7. Approaches

The process of embryo implantation requires synchronous communication between the embryo and maternal system, enabling endometrial receptivity and the development of a competent embryo. As discussed earlier, the majority of pregnancy loss in bovines occurs during the pre-implantation period, when the embryo is beginning to elongate and form a close physical contact with the endometrial epithelium.

Embryo transfer experiments control to a certain extent, the contributions of the embryo itself to reproductive failures. This allows the assessment of the maternal uterine environment effect, on the pre-implantation period along with dam metabolic status and genetic parameters. The use of embryo transfer and oestrous cycle synchronization across many species has indicated that uterine environment is often the source of reproductive failures (54). Approaches to studying the uterine environment include morphological analysis of uterine tissues, analysis of uterine flushing for protein secretions, as well as protein and gene expression analyses of endometrial and embryonic tissues.

1.7.1. Morphology

Morphological assessment of the endometrial ultrastructure and embryo provides only a limited view of endometrial function and embryo development. While it can pick up on more extreme differences, it lacks the sensitivity of other techniques. For example, morphological assessment has been reported on only moderately predictive of blastocyst viability in humans (148). Also, combining this approach with gene expression studies has indicated that tissues classed as morphologically similar can often have quite dissimilar transcription profiles (149).

1.7.2. Proteomics

By detecting protein changes in uterine tissues and flushings, proteomic analysis can provide insight into the environment created by the maternal system for embryo development. In studies comparing pregnant with non-pregnant animals, the effect of the embryo's presence on uterine secretions can also be assessed. Protein arrays in particular allow the researcher to study expression levels of multiple proteins simultaneously, a promising approach that can potentially reveal interactions, both within the protein domain and with other molecules (150, 151).

1.7.3. Methylomics

Regulation of gene expression through methylation has recently been highlighted as a potential mechanism involved in attainment of uterine receptivity. DNA methylation analysis methods can be global or gene-specific. Global methylation analysis refers to measurement of the overall level of methyl-cytosines in the genome. Whereas, gene-specific methylation, quantifies methyl-cytosines at specific locations. There are several methods used for analysis of gene-specific methylation. The most reliable and commonly used method is bisulphate conversion of un-methylated cytosine residues to uracil. Bisulphite treated and untreated DNA samples can then be sequenced to determine if and where the DNA was methylated. Recently it has been demonstrated that bisulphite treatment cannot distinguish between methylated and hydroxy-methylated DNA, and that PCR amplification of bisulphite treated hydroxy-methylated DNA can stall amplification (152). Therefore, results of experiments using bisulphite treated DNA need to be assessed with caution. Alternatively, PCR amplification with methylation specific primers could be performed to assess methylation status. Other high-throughput approaches for gene-specific methylation detection include base specific cleavage followed by MALDI-TOF mass spectrometry (153), and next generation sequencing technologies. An alternative approach to bisulphite treatment is affinity purification of methylated DNA (154). This can be achieved using a monoclonal antibody against 5-methylcytosine to isolate methylated DNA. To quantify DNA methylation, a DNA sample is split into two samples. One sample is used for affinity purification and labelled with a fluorescent dye, and the other sample is labelled with an alternative fluorescent dye. The samples can then be co-hybridized to a tilling

microarray and differences in the fluorescence can be used to determine where methylation occurs. This approach is higher-throughput than bisulphite techniques; but there are some limitations. For example, methylated CpG rich sequences give higher enrichments than methylated CpG poor sequences (155).

1.7.4. Genomics

Genomics provides an opportunity to assess the regulation of implantation at the level of the gene. There are a variety of experimental approaches to studying gene level uterine function. Commonly, candidate genes thought to be involved in a particular biological process have been investigated through quantitative real time PCR and sequencing to gain insight into a particular function. Real time PCR is highly sensitive and can detect subtle differences in transcription level, but is inherently throughput limited. In practice, only a limited number of genes can be analysed concurrently using this technique. Other gene-based approaches include genotyping assays using SNP chips. Array-based technologies like these provide the opportunity to study multiple genes at once, and are thus more powerful as a discovery tool than the candidate gene approach. More recently next generation sequencing has been utilised to quantify genome-wide gene expression. The advantage of sequencing RNA (RNA-seq) is that there is the potential to quantify all transcript variants and identify sequence variants (156).

Alternatively, QTL mapping provides a slightly different approach to selecting candidate genes.

1.7.4.1. QTL mapping

The mapping of particular traits to an area on a chromosome provides relatively precise identification of important regulatory regions for that trait. QTL analysis can be used to identify candidate genes in this way, which can then be investigated in terms of gene expression and genotyping assays in combination with association for a particular trait. More recently, QTL mapping has been combined with gene expression data to identify genetic variation that underpins gene expression. This has been termed an expression QTL (eQTL) (157-159).

1.7.4.2. Microarray technology

Microarray analysis enables the assessment of function of thousands of genes by measuring the expression of their transcripts. This tool provides a global view of gene expression in a given cell or tissue at a particular point in time under specified conditions (160). Global gene expression analysis, where gene expression of a large proportion of the transcriptome is measured, provides a powerful discovery tool compared to the more focused candidate gene approach, and can often be used in conjunction with QTL analysis to provide further focus on a particular gene or group of genes (161, 162).

1.7.4.3. Types of arrays

Microarray technology has improved significantly over the past 10 years. Current technologies are highly reproducible as confirmed by validation using the more sensitive quantitative real time PCR technologies. There are various types of microarrays including commercially available microarrays available for the study of gene expression. Currently there are two major commercially available bovine microarrays, produced by Affymetrix and Agilent respectively. The two-colour Agilent microarray system developed by Agilent and ViaLactia Biosciences uses an oligonucleotide-based printing technology, and contains 44 thousand 60-mer probes consisting of two identical 22 thousand probe sets. The Affymetrix GeneChip® Bovine Genome Array on the other hand contains 25-mer oligonucleotide probes for approximately 23,000 bovine transcripts, and was developed from publicly available sequence information. A brief explanation of available array types and considerations when choosing a particular platform follows.

1.7.4.4. cDNA versus oligonucleotide

Microarray probes are typically either synthetic oligonucleotides or cDNA fragments. Probe sequences used must be unique, and have minimal cross hybridization with related sequences. Also, the choice of probe sequences should form a comprehensive representation of the genome. cDNA fragments are commonly used in a non-commercial setting. However, cDNA isolated from bacteria or clone sets can often be compromised due to issues such as mis-annotation and contamination. Probes made from cDNA clones are also limited to the available clones (163). In contrast, oligonucleotides can be custom made

for any known sequence, and are only limited by our current knowledge of an organism's genome. Given the new high-throughput sequencing technologies, this is becoming less of a problem. Synthetic oligonucleotides are also more reliable than cDNA clones in terms of mis-annotation and contamination. Oligonucleotide probe design is one area that requires significant resources. Given that oligonucleotides are much shorter in length than a typical cDNA probe, they require careful design to minimise cross hybridisation (164). However, there are many available commercially produced microarray platforms for commonly studied species such as the bovine, and in the case of less commonly used species there are carefully designed tools that enable efficient oligonucleotide design such as Agilent's earray.

1.7.4.5. One colour versus two colour

Microarrays come in two forms with respect to hybridization of a sample – one colour and two colour arrays. One colour based microarrays rely on high quality arrays with low array-to-array variability. One example of this approach is the Affymetrix array platform, which uses a synthetic short oligonucleotide on silicon wafers for single sample hybridization.. An alternative is the two colour based microarray. This type of array is inherently comparative and gives a relative expression value. The power in this type of approach is the ability to control for array-to-array variation in the form of a reference design (for example, where all samples are hybridized to a reference sample) or to hybridize two samples to one array, making it a cost effective option. One obvious limitation of this approach is the need to design the hybridization scheme efficiently in order to answer a particular experimental question, an issue which is not relevant with one colour microarrays (165). Another issue that is sometimes cited as a limitation in two colour designs is the effect of dye bias of the two different dyes. Dye bias occurs when the incorporation of a particular dye may influence the expression reading for that dye (166). However, this problem can be somewhat rectified through the use of post labelling. The incorporation of a modified nucleotide during in vitro transcription followed by dye coupling to the modified nucleotide (where coupling is not biased towards either dye) can reduce the effects of dye bias. Other ways to reduce dye bias include normalisation procedure and the use of a reference design (167).

1.8. Conclusion

In order to understand why high-producing dairy cows have reduced fertility, it is critical to understand the regulatory mechanisms surrounding the implantation process. This review has surveyed the current state of knowledge regarding reproduction in bovines, and has highlighted gaps in this knowledge meriting further attention and research. Of particular interest are the molecular events occurring during the pre-implantation period, where high pregnancy loss rates are observed. There are various potential avenues for future research into the process of implantation, and one of the most promising is application of microarray technology to discover differences in gene expression and methylation in various reproductive tissues at different points in the reproductive process. Understanding the mechanisms behind these expression changes carries great potential for improving fertility rates in bovines and other species, with associated benefits for both farmers and the animals themselves.

Chapter 2. OPTIMISATION OF METHODOLOGIES FOR DETECTION OF GENE EXPRESSION IN BOVINE TISSUES

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I developed the methods, performed the experimental work and statistical analysis and wrote the manuscript. The other authors contributed to the development of methods and experimental design and critical analysis of the manuscript.

2.1. Background

Quantitative real-time reverse transcription PCR (RT-PCR) is an extremely sensitive technique that allows the precise measurement of gene expression across more than seven orders of magnitude (168, 169). RT-PCR is often considered the gold standard for quantifying gene expression, and is commonly used to validate techniques with greater throughput but less overall sensitivity, such as microarray analysis (170-172). RT-PCR relies on the use of fluorescent dyes to quantify transcript amplification, with the amplification cycle number at which these dyes/transcripts are detected (above background) giving an indication as to the relative abundance of the target molecules. The sensitivity of RT-PCR makes it a powerful tool for gene expression measurement, especially when sample quantities are limited or a transcript is expressed at a low level. However, this sensitivity also means that a great deal of care must be taken with regards to experimental design and implementation of the procedure.

When designing an experiment to evaluate gene expression in a group of samples, a number of critical factors must be kept constant. These include RNA extraction, DNase treatments, and cDNA synthesis. Normalisation of RT-PCR results is required to control inter-sample differences that may arise as a result of these sample processing steps, and ensure the gene expression of target transcripts are robustly quantifiable (173, 174).

The most common method for normalising RT-PCR data involves the use of one or more endogenous control genes. An ideal endogenous control gene is one that is stably expressed

within the samples to be compared, regardless of tissue differences, experimental conditions, or treatments. Choosing an endogenous control gene to normalise gene expression data is one of the most crucial steps in the experimental design. Genes used as endogenous controls in RT-PCR experiments are often chosen with little prior knowledge of their expression over the experimental conditions examined, and are often selected arbitrarily from a pool of commonly used endogenous control genes such as GAPDH, and β-actin (175). The most widely used endogenous control gene in studies of endometrial gene expression is *GAPDH* (176-179). However, the suitability of *GAPDH* as an endogenous control gene has recently come into question, especially due to its potential regulation in a wide variety of physiological states (180), making it a questionable choice for RT-PCR normalisation (181).

Over the past three decades, genetic selection for milk production has resulted in a significant decline in dairy cattle fertility (1). The fertilisation rate in dairy cattle is around 90% and does not differ between low-moderate and high-producing animals. However, the calving rate in lower producing animals is approximately 55%, whereas in high-producing animals this rate is approximately 35%. Pregnancy losses are thought to occur primarily during the pregnancy recognition/pre-implantation period (5), making studies of endometrial gene expression critical to further understanding of pregnancy establishment, recognition and maintenance within the bovine reproductive cycle.

The primary aim of this study was to identify suitable endogenous control genes for analysis of endometrial tissues from pregnant and cycling bovines. This study also aimed to investigate the potential use of microarray data analysis for identification of novel endogenous control genes, and the effect of endogenous control gene selection on the calculated expression of a target gene.

A total of 15 candidate endogenous control genes were analysed in 44 samples representing two different tissues (intercaruncular and caruncular) from 22 animals. These animals were either pregnant or cycling at day 17 of the reproductive cycle, and represented Holstein-Friesian cows from two divergent genetic backgrounds - North American (NA), and New Zealand (NZ).

Two strategies were employed to identify the candidates. Five genes were selected on the basis that they had been previously used as housekeeping genes (182-184), and an additional 10 novel genes were derived from a microarray experiment based around the same 44 samples used in the current analysis. Genespring GX software was used to generate a list through filtering on expression stability across the 44 samples. This list was subjected to GeNorm (185) and Normfinder (186) analysis to identify the 10 most suitable genes. The suitability of all 15 genes was then tested through statistical analyses, including a comparison of expression stability as determined by GeNorm and Normfinder algorithms. The effect of using these endogenous control genes was then evaluated using relative quantification of a gene known to be differentially expressed in the study.

2.2. Methods

All animal manipulations were carried out with the approval of the Ruakura Animal Ethics Committee (Hamilton, New Zealand). This work was conducted at No 5 Dairy, DairyNZ Ltd (Hamilton, New Zealand).

2.2.1. Sample information

Endometrial tissue samples (intercaruncular and caruncular) were obtained immediately post-mortem from 22 Holstein-Friesian dairy cows. The exact details for sample information and collection are described in Meier et al 2009 (187). Briefly, animals had oestrous cycles synchronised, with half of the animals receiving a blastocyst stage embryo at day 7 of the oestrous cycle. Endometrial samples were obtained post-mortem from each animal at day 17 of the reproductive cycle. The animals consisted of 12 pregnant and 10 cycling, further divided into North American (NA) and New Zealand (NZ) genetic ancestry (Table 4). This genetic strain model was chosen because NA cows have been reported to have poorer reproductive performance than NZ cows (188).

Table 2. Tissue categories**.** Two different tissue types each represented by four different groups of Holstein Friesians.

	Pregnant		Cycling	
Tissue type	**New Zealand**	**North American**	**New Zealand**	**North American**
Intercaruncular	6	6	5	5
Caruncular	6	6	5	5

2.2.2. RNA extraction

Endometrial tissue was homogenised in Qiagen buffer RLT (QIAGEN GmbH, QIAGEN, Hilden, Germany) using FastPrep Lysing Matrix D tubes in a FastPrep instrument (MP Biomedicals, Solon, OH).

Total RNA was extracted using a Qiagen RNeasy kit (QIAGEN). All samples were DNase treated using the Ambion DNA-free kit (Ambion, Austin, TX) according to the manufacturer's instructions. RNA quantity was determined by spectrophotometry in a Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, DE). RNA integrity was checked using the Agilent 2100 Bioanalyzer with a RNA 6000 Nano LabChip kit (Agilent Technologies, Palo Alto, CA).

2.2.3. cDNA synthesis

One µg of an endometrial RNA sample was used for cDNA synthesis using the Invitrogen Superscript III Supermix kit (Invitrogen Corporation, Carlsbad, California). Total RNA was reverse transcribed according to the manufacturer's instructions using a final concentration of 27µM of random pentadecamers primers. Briefly, RNA and random primers were mixed and denatured at 65 C for 5 minutes, followed by 1 minute on ice. Annealing buffer and Superscript/RNase was added to samples; these were then incubated for 10 minutes at 25 C (primer annealing), followed by 50 minutes at 50 C, and finally 5 minutes at 85 C to inactivate the enzyme. Reverse transcription (RT) negative controls were performed to test for the presence of genomic DNA contamination in RNA samples. Duplicate experimental samples were processed for cDNA synthesis as described above, but without the inclusion of the reverse transcriptase enzyme. Amplification was then tested for all genes using RT-PCR followed by assessment on a 3% agarose gel. No amplification was found in any of these samples.

2.2.4. Candidate endogenous control genes

Fifteen potential endogenous control genes were selected either from a literature search or were identified from a microarray study through statistical analysis using Genespring GX (Agilent Technologies) software in combination with the GeNorm (185) and Normfinder (186) algorithms.

Briefly, Genespring GX software was used to analyse an array data set (Agilent 44k 60-mer oligonucleotide bovine array) representing 44 bovine endometrial samples collected as described above. All array data had undergone standard quality control and statistical analysis, including filtering on flags (present or marginal in all samples) and filtering on raw expression level of 200 to obtain reliable data. A list of 27 genes were derived from this dataset by filtering on normalised expression level (upper limit 1.2 and lower limit 0.833) to obtain genes that had stable expression across all 44 samples. This dataset was further analysed using the Microsoft Excel applets GeNorm and Normfinder. These programs were used to determine the 10 most stably expressed genes in the array data set.

2.2.5. Quantitative Real-Time PCR

Real-time PCR using the Roche Lightcycler 480 (Roche) was performed on 15 candidate endogenous control genes for each of the 44 bovine endometrial samples using the Roche real-time PCR master mix (Lightcycler 480 Probes Master) in combination with Roche Universal Probe Library (UPL) assays. Assays were designed to publicly available bovine gene sequences (NCBI) using Roche UPL design software (ProbeFinder, v.2.45). All assays were designed to span an intron-exon boundary to prevent amplification of DNA. The primer and probe sequences are presented in Table 5.

The PCR reaction volume was 10 µL consisting of 0.5 µM of each primer and 0.1 µM of probe. Standard cycling conditions were used [95 C for 10 minutes, (95 C for 10 seconds, 60 C for 30 seconds) x 50 cycles, 40 C for 40 seconds].

To quantify gene expression, cDNA was diluted 100-fold for all genes except *ACTR1A, DNAJC17, HDAC1, RANBP10*, and the target gene *OXTR* where cDNA was diluted 10-fold.

Each PCR run included a no-template control with water added instead of cDNA, as well as a RT negative control for each gene. Triplicate measurements were performed for all samples and standard curves. All samples for each gene were run on the same plate.

The Roche Lightcycler 480 software was used to perform quantification analysis of gene expression using the relative standard curve second derivative maximum analysis method, a non-linear regression line method. A six point relative standard curve of serial dilutions of

cDNA was used with an estimated starting concentration of 1.0 and final concentration of 1.6E-03.

2.2.6. Relative quantification of OXTR

The Roche Lightcycler 480 Software was used to perform advanced relative quantification analysis of *OXTR* gene expression. *SUZ12, UXT* and *GAPDH* were each used as a reference for the quantification of *OXTR* expression. Relative quantification was also performed using the normalisation factor of the two most stably expressed genes identified through GeNorm analysis (*SUZ12* and *ZNF131*). Normalisation factors were calculated by taking the geometric mean of the two genes for each sample.

2.2.7. Data analysis

Statistical analyses were performed using the Excel applets GeNorm (version 3.5) (185) and Normfinder (186) to estimate expression stability of the candidate endogenous control genes. GeNorm requires expression data to be input as concentrations determined via quantification, taking into consideration PCR efficiencies (Table 3). The program then estimates the most stable genes based upon pairwise comparisons of sample variability. The two most stable genes are identified and a normalisation factor calculated. Normfinder analyses the stability of the candidate genes taking into consideration inter-group variability. The program then ranks genes based on a stability value, with the lowest value indicating the most stably expressed gene. Significance tests were also performed on the data. Microsoft Excel was used to perform T-tests (with Bonferroni multiple testing correction applied) to test for significant differences between the means, and F-tests were used to assess differences in variance between experimental groups for each gene. ANOVA was also used to test for any significant difference in the means (GenSTAT). Pearson correlation calculation was used to assess the correlation of the microarray reported expression for the target gene (*OXTR*) and the RT-PCR reported expression.

Table 3. Characteristics of gene specific real-time PCR assays. Roche Universal probe library assays were designed for 15 candidate endogenous control genes including 10 experimentally derived genes, five commonly used genes and one target gene – *OXTR*.

Experimentally derived genes

Gene symbol	Accession			Amplicon size (bp)	PCR efficiency
DNAJC17[1]	NM_001046276	Left primer	TCTGAAGATTTCCTGGTTGGA	90	1.92
		Right Primer	CTCTCGGACACCACTGAGC		
		Probe	#57: GGCCCCAG		
HDAC1[2]	NM_001037444	Left primer	GGATGAGAAAGAGAAAGATCCAGA	76	1.53
		Right Primer	TTCTTGCTTCTCTTCCTTGGTT		
		Probe	#35:AGAAGAGGA		
RANBP10[3]	NM_001098125	Left primer	CTCAACAGCGCCATTTTAGA	94	1.81
		Right Primer	CCATGAGCCGTAGACATTCA		
		Probe	#37:TGCCCTGG		
CNOT7[4]	NM_001034312	Left primer	GATAGGACCGCAGCATCAG	111	1.82
		Right Primer	CCACAATATTTGGCATCATCA		
		Probe	#100:GCTCACAG		
ACTR1A[5]	XM_879421	Left primer	TATCTGCACCGCAGGAGAG	96	1.54
		Right Primer	CCTTCTTAGAGACCCACATCTTCT		
		Probe	#130:CTGGACAC		
BBS2[6]	NM_001038160	Left primer	GAGCAGGTCATCTGCGTGT	132	1.9
		Right Primer	TCCCCTCCTAAGAAGAAGCTGT		
		Probe	#150:TGCTGTTC		
SUZ12[7]	XM_582605	Left primer	GAACACCTATCACACACATTCTTGT	130	1.99
		Right Primer	TAGAGGCGGTTGTGTCCACT		
		Probe	#150:GAACAGCA		
ZNF131[8]	NM_001101218	Left primer	AGAAAGAAGCTTTATGAATGTCAGG	94	1.9
		Right Primer	GTTTATCTCCAGTGTGTATCACCAG		
		Probe	#33:AGCTGGGA		
SLC30A6[9]	NM_001075766	Left primer	CAGTTGGACAAACTTATCAGAGAGG	66	1.9
		Right Primer	ATGCTCATTTCGGACTTCCA		
		Probe	#58:GGATGGAG		
C2ORF29[10]	XM_582695	Left primer	CCTTCAAGAGCCCCCTGT	64	1.96
		Right Primer	GGGTCCTTTTCCAACTCTCC		
		Probe	#73:TCCTCAGC		
Literature derived genes					
RPS9[11]	NM_001101152	Left primer	TGAGGATTTCTTGGAGAGACG	126	1.97
		Right Primer	ATGTTCACCACCTGCTTGC		
		Probe	#138:ATCCACCA		
UXT[12]	NM_001037471	Left primer	AACTTCTTCGTTGACACAGTGG	130	2.01
		Right Primer	CTGTGAGGAGACTGCTCTTACG		
		Probe	#29:GGCAGAAG		
GAPDH[13]	NM_001034034	Left primer	GAAGCTCGTCATCAATGGAAA	67	1.94
		Right Primer	CCACTTGATGTTGGCAGGAT		
		Probe	#9:CATCACCA		
PPIA[14]	NM_178320	Left primer	GTCAACCCCACCGTGTTCT	99	1.94
		Right Primer	TTCTGCTGTCTTTGGAACTTTG		
		Probe	#152:GACGGCGA		
RPS15A[15]	NM_001037443	Left primer	TCAGCCCTAGATTTGATGTGC	104	1.85
		Right Primer	GCCAGCTGAGGTTGTCAGTA		
		Probe	#32:CTGCTCCC		
Gene of interest					
OXTR[16]	NM_174134	Left primer	CGTGCAGATGTGGAGTGTCT	96	1.99
		Right Primer	TTGCAGCAGCTGTTGAGG		
		Probe	#162:TCCTGGC		

[1] DnaJ (Hsp40) homolog, subfamily C, member 17.[2] histone deacetylase 1. [3] RAN binding protein 10. [4] CCR4-NOT transcription complex, subunit 7. [5] ARP1 actin-related protein 1 homolog A, centractin alpha (yeast). [6] Bardet-Biedl syndrome 2. [7] suppressor of zeste 12 homolog (Drosophila). [8] zinc finger protein 131. [9] solute carrier family 30 (zinc transporter), member. [10] LOC506268 similar to Uncharacterized protein C2orf29. [11] ribosomal protein S9. [12] Ubiquitously-expressed transcript. [13] glyceraldehyde-3-phosphate dehydrogenase. [14] peptidylprolyl isomerase A (cyclophilin A). [15] ribosomal protein S15a. [16] Oxytocin receptor

2.3. Results

2.3.1. Summary statistics

Microarray analysis of the 44 endometrial samples revealed 27 transcripts with a high degree of expression stability (filtering on expression level - upper limit 1.2, lower limit 0.833). GeNorm and Normfinder were utilised to identify the 10 most stably expressed transcripts for further analysis. For RT-PCR design, full length transcripts were identified by querying microarray probe sequences against the bovine genome (Btau3.1) using NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Gene expression levels of the candidate endogenous control genes (expressed in Cq values) are displayed in Table 4 and Figure 5. Cq values for sample replicates had very low variability with a mean intra-assay coefficient of variation (CV) of 0.41%. All genes had low overall variability, with the Cq range between 1.06 and 2.04 cycles, standard deviations ranging from 0.25 to 0.53 cycles, and CV values ranging from 0.87 to 1.48% **(**Table 4**).** Significance calculations between gene expression data for pregnant and cycling animals were performed on Cq and relative concentration values, as estimated through absolute quantification using the Roche LC480 software. No significant differences (P>0.05) in means or variances (Cq or concentration) between pregnant and cycling endometrial tissues were apparent for any candidate genes (except *BBS2*, which had a p-value of 0.04 (F-test) for variance between pregnant and cycling animals). The means and variances between the two different strains of Holstein-Friesians also lacked significance (data not shown). All genes tested except two (HDAC1 and ACTR1A) had efficiencies above 1.8.

Cqs and relative concentration variances between the two different tissue types showed no significant differences, however means were significantly different for all genes, except *RPS15A* (*P* = 0.39 (T-test), *P* = 0.032 (ANOVA)).

Figure 5. Expression levels of candidate endogenous control genes in pregnant (P) and cycling (Cy) endometrial tissue samples. Values are given as crossing point (Cq) cycle numbers. Boxes represent the lower and upper quartiles with medians; whiskers illustrate the maximum and minimums of the samples. There were no significant differences (P>0.05) between the Cq means (T-test) or variances (F-test) of pregnant and cycling animals for any genes tested (except the variance of BBS2, P=0.039).

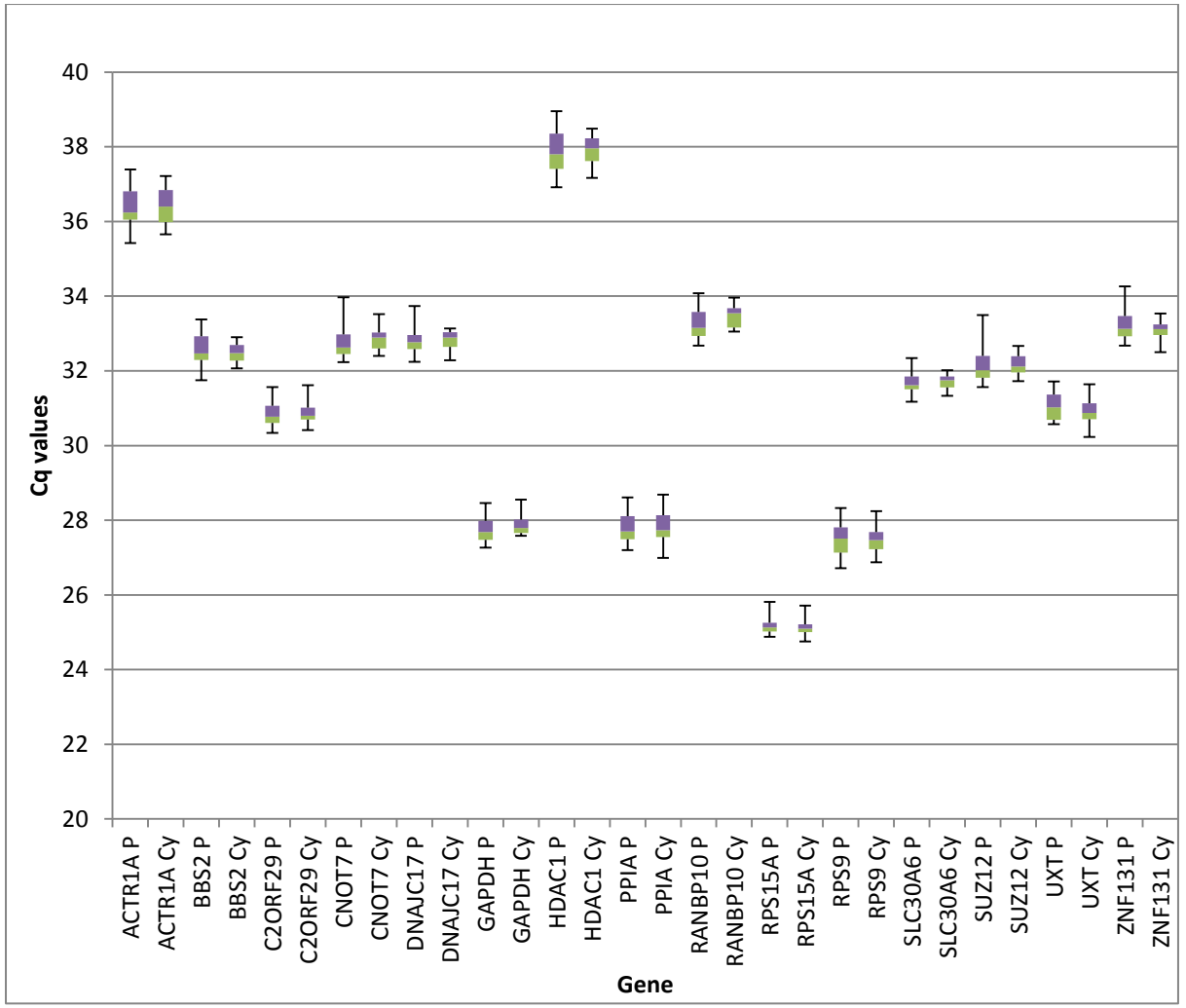

Cq values
40
38
36
34
32
30
28
26
24
22
20
ACTR1A P
ACTR1A Cy
BBS2 P
BBS2 Cy
C2ORF29 P
C2ORF29 Cy
CNOT7 P
CNOT7 Cy
DNAJC17 P
DNAJC17 Cy
GAPDH P
GAPDH Cy
HDAC1 P
HDAC1 Cy
PPIA P
PPIA Cy
RANBP10 P
RANBP10 Cy
RPS15A P
RPS15A Cy
RPS9 P
RPS9 Cy
SLC30A6 P
SLC30A6 Cy
SUZ12 P
SUZ12 Cy
UXT P
UXT Cy
ZNF131 P
ZNF131 Cy
Gene

Table 4. Crossing point (Cq) values and statistics for 15 candidate endogenous control genes assayed across 44 bovine endometrial samples.

.

Gene Symbol	Mean Cq	std dev	%CV	Min Cq	Median Cq	Max Cq	Range Cq
ACTR1A	36.4	0.53	1.45	35.42	36.27	37.39	1.97
BBS2	32.53	0.35	1.07	31.75	32.47	33.37	1.62
C2ORF29	30.84	0.3	0.98	30.34	30.78	31.62	1.28
CNOT7	32.8	0.39	1.19	32.23	32.76	33.97	1.74
DNAJC17	32.83	0.32	0.96	32.24	32.85	33.74	1.49
GAPDH	27.81	0.34	1.21	27.27	27.76	28.55	1.28
HDAC1	37.88	0.49	1.3	36.92	37.93	38.96	2.04
PPIA	27.81	0.41	1.46	26.99	27.72	28.68	1.69
RANBP10	33.36	0.37	1.11	32.68	33.33	34.08	1.4
RPS15A	25.16	0.25	1.01	24.75	25.12	25.81	1.06
RPS9	27.47	0.41	1.48	26.71	27.5	28.33	1.62
SLC30A6	31.7	0.28	0.87	31.18	31.67	32.34	1.16
SUZ12	32.16	0.39	1.21	31.56	32.1	33.49	1.93
UXT	30.99	0.35	1.12	30.23	30.96	31.72	1.49
ZNF131	33.17	0.36	1.08	32.5	33.12	34.26	1.76

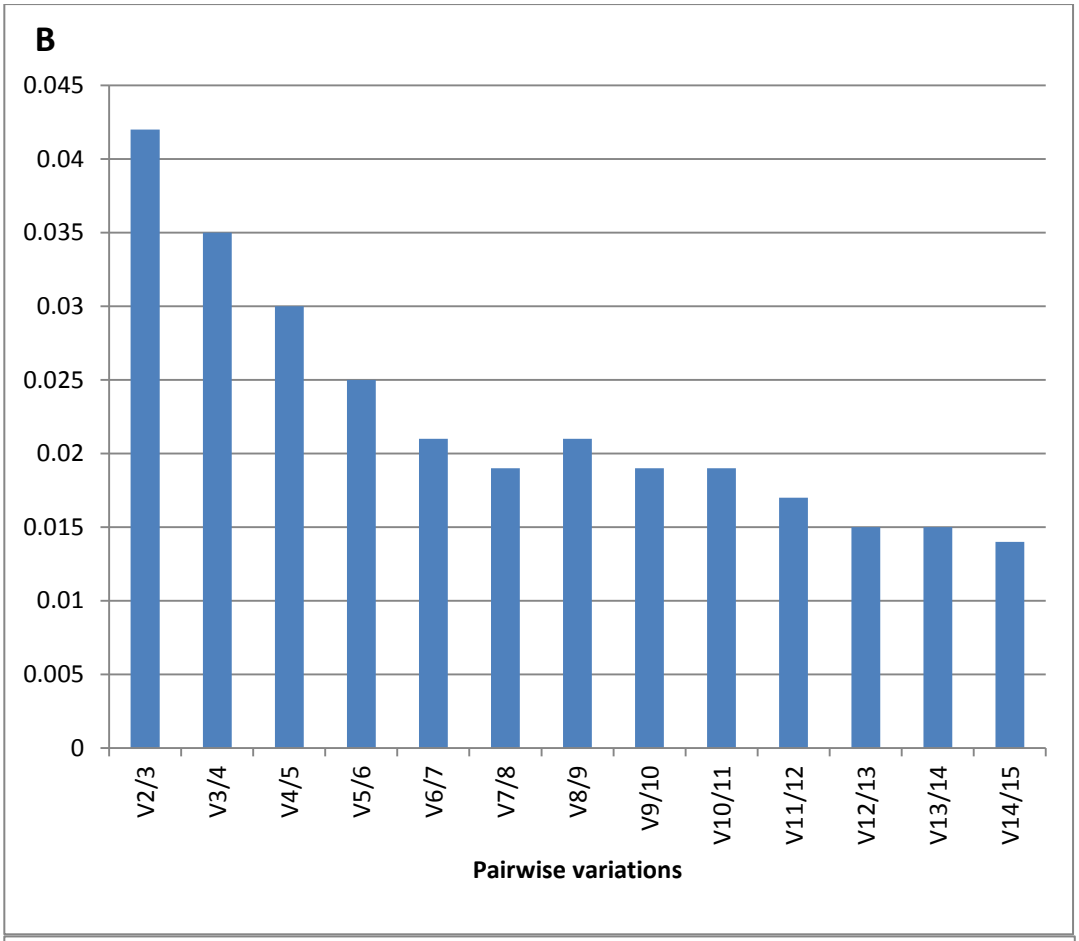
Table 5. Stability ranking of candidate endogenous control genes from Normfinder and GeNorm analyses. Normfinder rankings of genes through analysis of inter- and intra-group variability (groups defined include pregnancy status, tissue type and cow genetic strain). GeNorm rankings of genes based on pairwise analysis of expression stability.

	Normfinder		GeNorm	
Rank	**Gene Symbol**	**Stability**	**Gene Symbol**	**Stability**
1	*SUZ12*	0.043	*SUZ12*	0.12
2	*C2ORF29*	0.053	*ZNF131*	0.12
3	*HDAC1*	0.061	*SLC30A6*	0.132
4	*SLC30A6*	0.062	*C2ORF29*	0.145
5	*CNOT7*	0.066	*RPS15A*	0.157
6	*ZNF131*	0.071	*HDAC1*	0.164
7	*BBS2*	0.071	*BBS2*	0.169
8	*RPS15A*	0.077	*CNOT7*	0.173
9	*RPS9*	0.078	*RANBP10*	0.184
10	*ACTR1A*	0.08	*ACTR1A*	0.192
11	*RANBP10*	0.082	*PPIA*	0.201
12	*GAPDH*	0.083	*GAPDH*	0.208
13	*UXT*	0.084	*RPS9*	0.214
14	*DNAJC17*	0.085	*DNAJC17*	0.22
15	*PPIA*	0.086	*UXT*	0.225

2.3.2. Expression stability testing of candidate genes

To further analyse the suitability of the candidate genes for use as endogenous controls for bovine endometrial tissues, expression stability was assessed using the GeNorm (185) and Normfinder (186) algorithms. GeNorm rankings for the 15 genes tested are presented in Table 5 and Figure 6. GeNorm identified *SUZ12* and *ZNF131* as the most stably expressed genes of the 15 candidates. Four of the five most stable genes were those derived from microarray data; the other was *RPS15A*, which was selected from the literature. By contrast, four of the five least stable genes were chosen from the literature, including *GAPDH*. The most stably expressed gene identified by Normfinder was *SUZ12* (Table 5), which was also one of the two most stable genes identified in the GeNorm analysis. The best combination of genes identified by Normfinder was *SUZ12* and *C2ORF29*. The five most stably expressed genes according to Normfinder consisted entirely of microarray-derived genes, while the least stable five contained three out of five genes selected from the literature, again including *GAPDH*. The comparative ranking of all genes for both GeNorm and Normfinder algorithm analyses is displayed in Table 5**.**

Figure 6. GeNorm output. GeNorm was used to determine the most stably expressed gene for use as normalisers and how many genes needed to be used for accurate normalisation. A: Average expression stability value (M) for candidate endogenous control genes in bovine endometrial tissue samples. The M-value threshold for stability of a gene according to GeNorm is 1.5. The most stable genes as determined through analysis of the pairwise variation of each gene with all other genes were *SUZ12* and *ZNF131*. B: GeNorm output used to determine the optimal number of endogenous control genes for normalisation in bovine endometrial tissue samples. GeNorm calculates the minimum number of genes required for accurate normalisation. In this case, two genes were sufficient (V2/3 = 0.04). The number of genes necessary for accurate normalisation is defined as the point at which the addition of a gene has a non-significant effect on the calculation of the normalisation factor, the threshold for a non-significant difference being 0.15.


B
0.045
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
V2/3
V3/4
V4/5
V5/6
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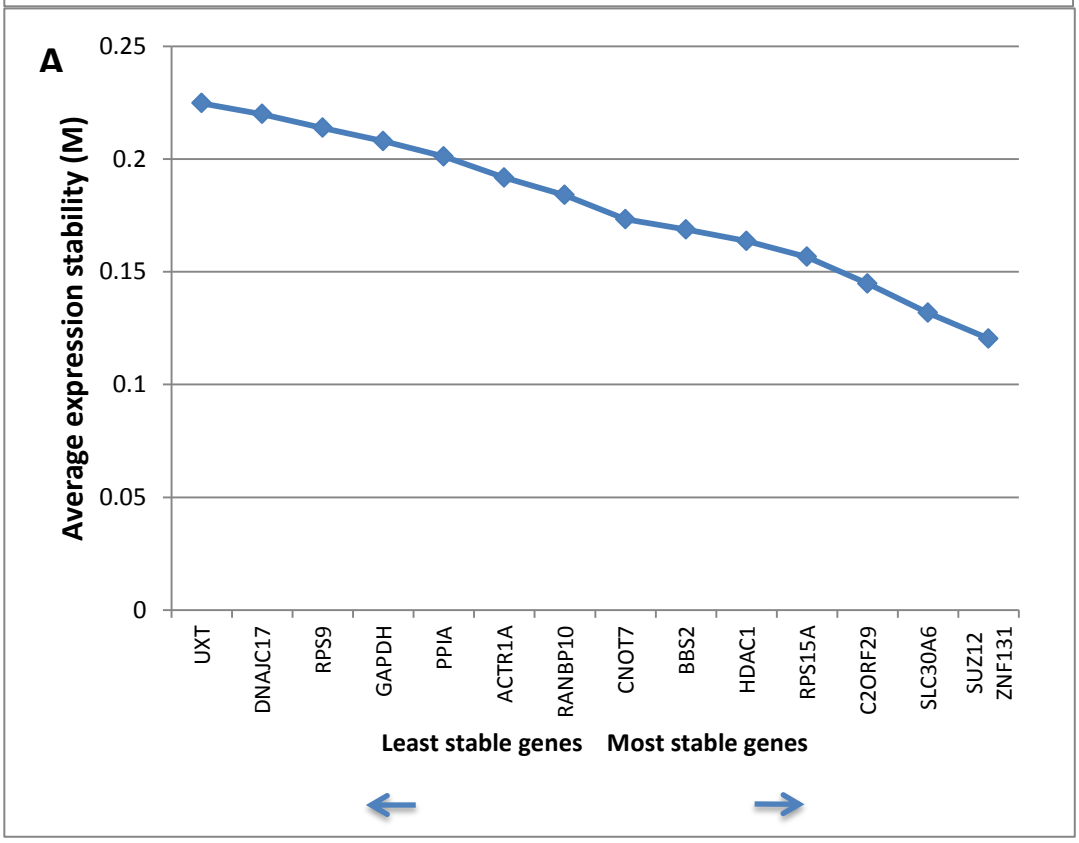
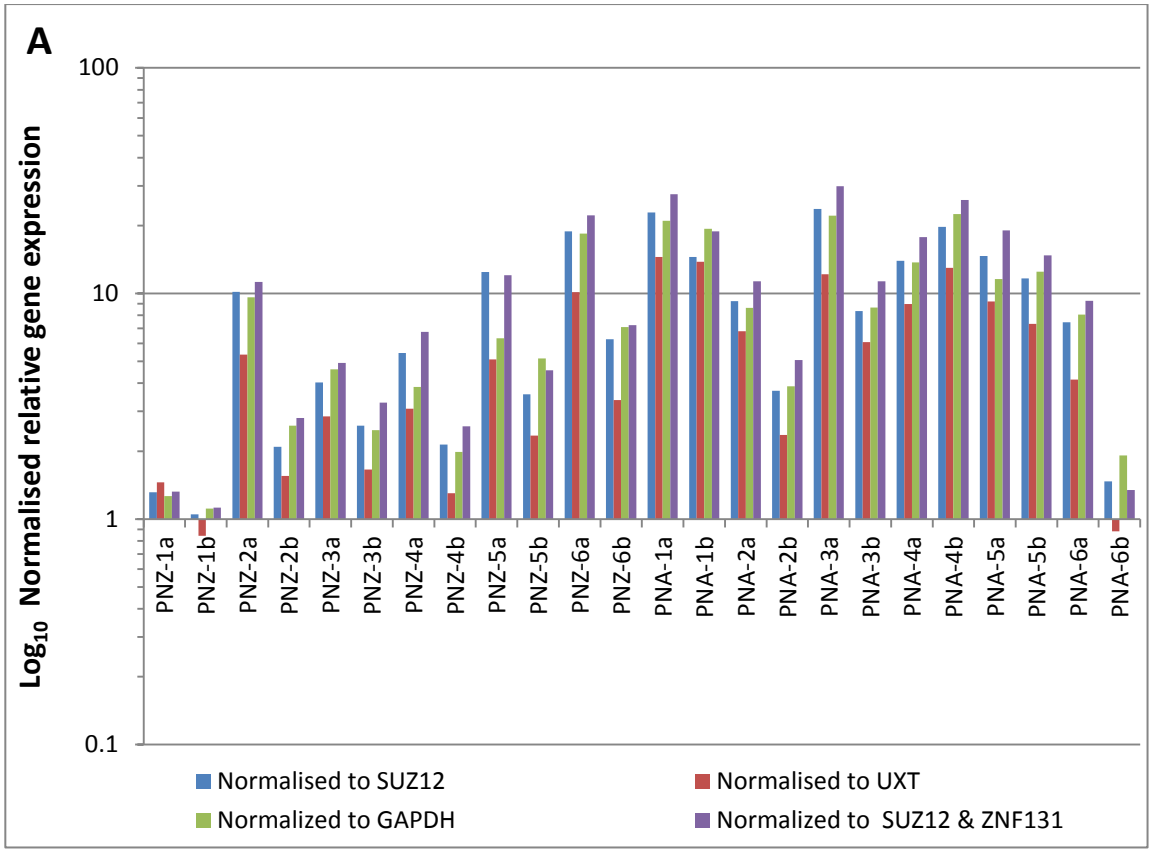
V11/12
V12/13
V13/14
V14/15
Pairwise variations

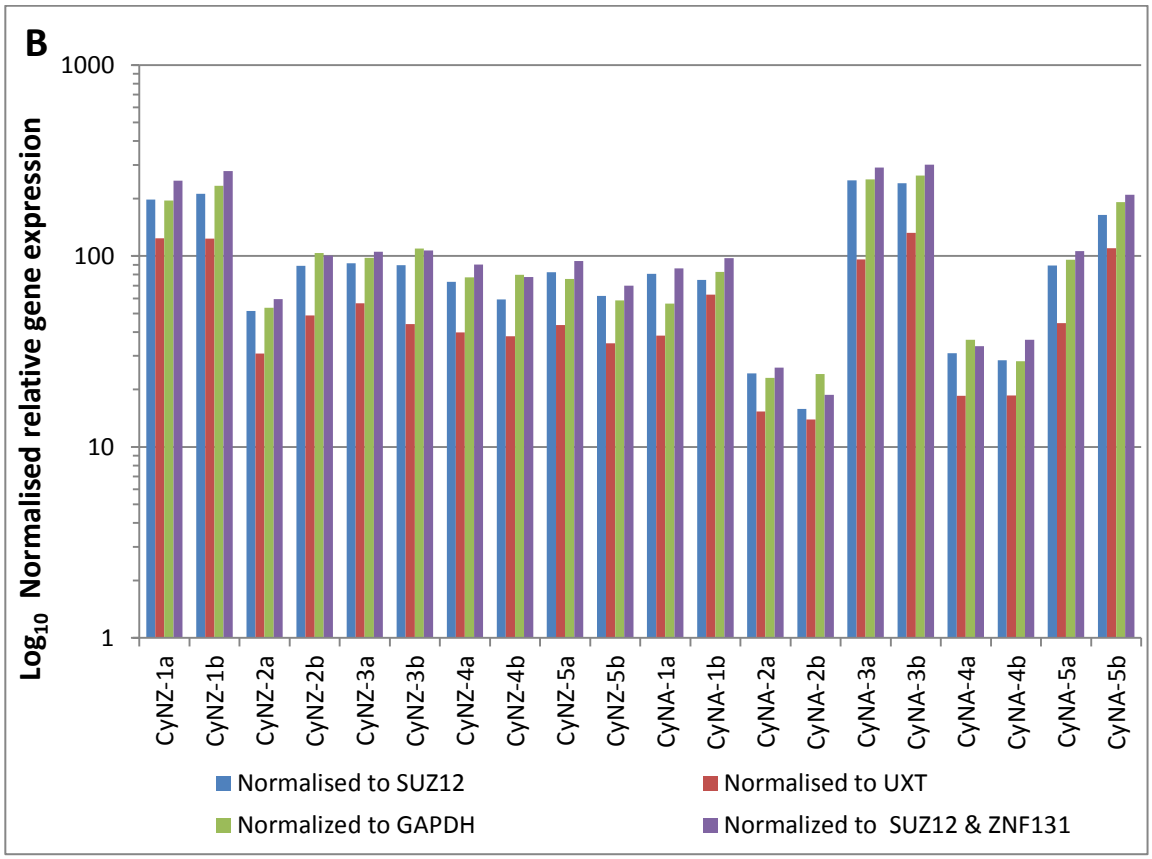

A
Average expression stability (M)
0.25
0.2
0.15
0.1
0.05
0
UXT
DNAJC17
RPS9
GAPDH
PPIA
ACTR1A
RANBP10
CNOT7
BBS2
HDAC1
RPS15A
C2ORF29
SLC30A6
SUZ12
ZNF131
Least stable genes
Most stable genes

2.3.3. Effect of endogenous control gene on target gene relative quantification

OXTR expression was significantly greater in cycling than in pregnant cows regardless of the endogenous control gene used (Figure 7, ANOVA, $P<0.01$). The use of different endogenous control genes had no effect on *OXTR* expression differences in these group comparisons. However, there were differences in the normalised *OXTR* expression values that were inconsistent across the 44 samples, depending on which endogenous control genes were employed.

Figure 7. Relative gene expression results for *OXTR* in each sample when normalised with endogenous control genes of different stability in each pregnant (A) and cycling (B) animal. These graphs show calculated relative expression values for *OXTR* when normalised to the most stable gene (*SUZ12*), two of the least stable genes (*UXT* and *GAPDH*), and to the two best genes according to GeNorm analysis (*SUZ12* and *ZNF131*), with the latter using a normalisation factor calculated from the GeNorm analysis. (Pregnant = P, cycling =Cy,caruncular=a, intercaruncular=b,)


A
100
10
1
0.1
Log10 Normalised relative gene expression
PNZ-1a
PNZ-1b
PNZ-2a
PNZ-2b
PNZ-3a
PNZ-3b
PNZ-4a
PNZ-4b
PNZ-5a
PNZ-5b
PNZ-6a
PNZ-6b
PNA-1a
PNA-1b
PNA-2a
PNA-2b
PNA-3a
PNA-3b
PNA-4a
PNA-4b
PNA-5a
PNA-5b
PNA-6a
PNA-6b
Normalised to SUZ12
Normalised to UXT
Normalized to GAPDH
Normalized to SUZ12 & ZNF131


B
1000
100
10
1
Log10 Normalised relative gene expression
CyNZ-1a
CyNZ-1b
CyNZ-2a
CyNZ-2b
CyNZ-3a
CyNZ-3b
CyNZ-4a
CyNZ-4b
CyNZ-5a
CyNZ-5b
CyNA-1a
CyNA-1b
CyNA-2a
CyNA-2b
CyNA-3a
CyNA-3b
CyNA-4a
CyNA-4b
CyNA-5a
CyNA-5b
Normalised to SUZ12
Normalised to UXT
Normalized to GAPDH
Normalized to SUZ12 & ZNF131

2.4. Discussion

Endogenous control genes identified through microarray analysis were more stably expressed than commonly used endogenous control genes. While almost all endogenous control genes were not differentially expressed between pregnant and cycling animals or between the different strains of dairy cow, the genes identified through microarray analysis were the most stably expressed. This data provides support for the use of microarray datasets to identify suitable endogenous control genes. The results also highlight the importance of endogenous control gene selection when comparing across tissue types. All genes tested were differentially expressed between tissues (except *RPS15A*). The differences in means calculated between tissue subtypes likely reflect the distinct morphological and functional differences between caruncular and intercaruncular endometrium, which relate to their respective roles in reproduction. The 'caruncules' of the endometrium are specialised projections that are the site of embryo attachment. Caruncules become highly vascularised, and are the major site for small molecule and gaseous exchange. In comparison, intercaruncular tissue is highly glandular and responsible for early nourishment of the embryo through secretions of large molecules into the uterus (55, 189, 190). The intercaruncular tissue is often thought to be more important in early pregnancy and, therefore, the majority of expression studies in pre-implantation bovine endometrium focus solely on intercaruncular tissue gene expression (107, 191, 192). There is very little reported expression analysis of caruncular endometrium and only one study comparing expression profiles of the two tissues (193). For analyses comparing the expression between tissues caution should be taken when selecting suitable endogenous control genes.

2.4.1. Endogenous control gene programs identify microarray derived genes as most stable.

Expression stability was assessed using two software programs, GeNorm and Normfinder. Both programs identified *SUZ12* as the most stably expressed gene and *GAPDH* and *UXT* as the least stably expressed genes. GeNorm calculates an expression stability value (M) for each candidate gene based on pairwise comparisons of variability. Each gene is compared to every other gene to determine the two genes that contain the least variation. The stability

value calculated for each gene is used to rank genes from least to most stable. The authors of the method give an M-value of 1.5 as a cut-off for suitability as an endogenous control gene. The principal behind the pairwise stability measure ranking is that two ideal candidate normalisation genes should have an equal expression ratio in all samples (45). In the present study, all genes are well below the stipulated 1.5 M-value. The program then calculates a normalisation factor in each sample for the most stable genes (data not presented). GeNorm also calculates the optimal number of endogenous control genes to be used in the analysis of gene expression (Figure 6B). This value is determined by locating the point where addition of the next most stable gene does not significantly affect the normalisation factor, using a cut-off value of 0.15. In this study, the value for 2 genes was 0.042, suggesting that 2 genes should be sufficient to normalise the experimental data. Normfinder is another freely available tool for the identification of stable endogenous control genes. The main point of difference between the two methods is that Normfinder takes into account both inter- and intra-group variability. The program not only identifies the most stable pair of endogenous control genes but also identifies the best overall endogenous control gene. The calculation of variability between groups is especially important in the present study considering the significant expression differences between the two tissue subtypes. The use of the two most stably expressed genes, in this case *SUZ12* and *C2ORF29* (Normfinder), should provide sufficient normalisation for tissue comparisons as Normfinder selects the best combination of genes whilst taking into account any grouping effects such as tissue type.

The differences in rankings of gene stability using the two algorithms could be due to the fact that they use very different methods to assess gene stability. GeNorm selects genes based on the pairwise variation between genes. The two most stably expressed genes are therefore those genes that share an expression profile. In contrast, Normfinder uses a model based algorithm that takes into account overall stability as well as any groups that may be present in the sample set. For example, if there are any grouping effects on gene expression a gene would be ranked lower than one that demonstrated variability not associated with any particular group.

2.4.2. Effect of endogenous control gene on target gene relative quantification

Temporal down-regulation of endometrial oxytocin receptor (*OXTR*) expression is a hallmark of early pregnancy, with embryonic interferon tau (IFNτ) inhibiting its expression (194, 195). Given the expectation of differential OXTR expression between pregnant and cycling animals, the effect of control gene stability on gene expression values of *OXTR* in the 44 endometrial samples was tested. Figure 7 presents relative OXTR expression levels when normalised with endogenous control genes of varying stability - the most stable gene identified by Normfinder (*SUZ12*), the two most stable genes identified by GeNorm (*SUZ12* and *ZNF131*), and two of the least stably expressed genes identified by both Normfinder and GeNorm (*GAPDH* and *UXT*). Normfinder identified *SUZ12* and *C2ORF29* as the best combination of genes, but the relative expression was not different from that calculated using only *SUZ12* or a combination of *SUZ12* and *ZNF131*. Correlation of normalised RT-PCR data to *OXTR* microarray expression data was not affected by choice of normalisation strategy. When compared to microarray reported expression, all calculated expression values had correlation coefficients of 0.79.

OXTR expression was significantly greater in cycling than in pregnant cows regardless of the endogenous control gene used (Figure 7, ANOVA, $P<0.01$). Notably, *OXTR* expression in pregnant animals was greater on average and more variable in NA animals (which have lower fertility in general (188) than for NZ animals (Figure 7). The use of different endogenous control genes had no effect on *OXTR* expression differences in these group comparisons.

However, there were differences in the normalised *OXTR* expression values that were inconsistent across the 44 samples, depending on which endogenous control genes were employed. For example, in sample Pregnant NZ-5a the calculated relative OXTR expression value was 12.46 when normalised to *SUZ12*, 6.32 when normalised to *GAPDH*, 5.10 when normalised to *UXT*, and 12.04 when normalised to both *SUZ12* and *ZNF131*. Another sample (Pregnant NZ-2a), the values were 10.15, 9.60, 5.35, and 11.26 when normalised to *SUZ12*, *GAPDH*, *UXT* and both *SUZ12* and *ZNF131*, respectively.

The average calculated fold change difference between pregnant and cycling animals was not affected by the choice of normalisation gene, possibly due to the large difference in

expression level for this comparison (10-fold average difference between pregnant and cycling animals). However, the choice of reference gene could be important when normalising genes that exhibit more subtle variation between experimental groups, given the considerable variation in expression shown between individual samples.

2.5. Conclusion

This study provides the first reported assessment of endogenous control genes for use in expression studies in bovine endometrium. Normalisation is a critical factor in reporting RT-PCR expression data, providing a necessary control for error associated with sample preparation. Normalisation using endogenous control genes provides a means of controlling this error, provided the gene(s) used are stably expressed across all samples under investigation. The study described here tested 15 candidate reference genes across 44 bovine endometrial samples representing a range of physiological states and tissue subtypes. This study evaluated the suitability of both commonly used and novel experimentally derived reference genes for use in normalisation of RT-PCR data. Candidates derived via microarray analysis were superior to existing, commonly used endogenous control genes, demonstrating the suitability of using microarray data for deriving novel endogenous control genes. This study also highlighted the importance of accurate normalisation with a stable endogenous control gene, by demonstrating relative expression of a differentially expressed gene when normalised using control genes of varying stability. SUZ12 was ranked first for stability across samples as determined by the statistical algorithms used in GeNorm and Normfinder, and is therefore proposed as the best gene for normalisation of RT-PCR data in the current study.

Chapter 3. MODULATION OF THE MATERNAL IMMUNE SYSTEM BY THE PRE-IMPLANTATION EMBRYO

This chapter was published as:

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I developed the methods, performed the experimental work and statistical analysis and wrote the manuscript. The other authors contributed to the development of methods and experimental design and critical analysis of the manuscript.

3.1. Background

Over the past three decades, there has been a coincidental decline in fertility associated with genetic selection for increased milk production. It is estimated that approximately 50% of the potential profitability from genetic selection for milk production is lost due to a reduction in fertility (1).

The fertilisation rate for lactating dairy cattle is around 90% and does not differ between low-moderate and high-producing animals when managed under pastoral conditions (5). However, the calving rate in lower producing animals is approximately 55%, whereas for high-producing animals, this rate is approximately 35% (5). Pregnancy losses are thought to occur primarily during the pregnancy recognition/pre-implantation period (5), making studies of endometrial gene expression critical to further understanding of pregnancy establishment, recognition and maintenance within the bovine reproductive cycle.

Successful pregnancy in mammals requires both a viable embryo and a receptive endometrium. Synchronous signalling between the endometrium and embryo during the pre-implantation period is critical for normal embryo development, implantation of the embryo, and placentation (196). The early embryo is nourished by secretions (histotroph) from the uterine glands (intercaruncular endometrium) and during implantation forms a close physical association (attachment) with the caruncular endometrium (55). Pregnancy thus represents an immunological contradiction, in that the immunologically foreign embryo

is able to form a close physical relationship with the maternal endometrium that lasts throughout pregnancy. Under normal circumstances, a foreign tissue would likely be rejected by the recipient unless the immune system was significantly suppressed or tolerant to the tissue. That the embryo can survive in the presence of the maternal immune system has led to the hypothesis that the uterus is an immunologically privileged site (197).

Several mechanisms have been proposed to account for the ability of the embryo to survive in the maternal environment including: antigenic immaturity of the conceptus (the bovine trophoblast, like other mammalian species, does not express classical polymorphic major histocompatibility complex (MHC) class 1 proteins in areas in contact with the maternal endometrium during early pregnancy (197)) and maternal immunological inertness to the conceptus or localised immune tolerance (198). The luminal and superficial glandular epithelium in sheep do not express some classical interferon stimulated genes; this is hypothesised to be due to inhibition by the transcriptional repressor IRF2. This mechanism may support suppression of the immune response to the embryo at the interface between the maternal endometrium and trophectoderm (199).

Local immunosuppression, required for establishment and maintenance of pregnancy, would leave the uterus vulnerable to infection. An increase in innate immune activity may be expected to protect the uterus from infection.

The aim of this study was to identify molecules and pathways involved in pregnancy recognition and maintenance and, in particular to characterise the local immune response that occurs in the endometrium as a consequence of pregnancy. The transcriptional response to the presence of an embryo was characterised during the pre-implantation period in dairy cows. Novel molecules and pathways potentially involved in mechanisms the embryo uses to evade the maternal immune system were identified.

3.2. Methods

3.2.1. Animals

All Procedures were undertaken with the approval of the Ruakura Animal Ethics Committee (Hamilton, New Zealand). The oestrus cycles of 22 lactating dairy cows were synchronized and 12 of these received embryo transfer on day 7 of the oestrus cycle. Embryos were at the

blastocyst stage of development and of grade 1 quality. Animals were slaughtered at day 17 of the reproductive cycle and endometrial tissues (both caruncular and intercaruncular) were sampled. There were 12 pregnant and 10 cyclic animals representing mixed New Zealand and North American ancestry Holstein Friesian dairy cows. Further details, including production data is provided in Meier et al 2009 (187).

3.2.2. RNA extraction

Tissues were homogenized in Qiagen buffer RLT (QIAGEN GmbH, QIAGEN Strasse 1, 40724 Hilden, Germany) using FastPrep Lysing Matrix D tubes in a FastPrep instrument (MP Biomedicals, 29525 Fountain Pkwy, Solon, OH 44139).

Total RNA was extracted using a Qiagen RNeasy kit (Qiagen). RNA quantity was determined by spectrophotometry using a Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, DE). RNA integrity was assessed with the Agilent 2100 Bioanalyzer with a RNA 6000 Nano LabChip kit (Agilent Technologies, Palo Alto, CA).

3.2.3. Microarray

One μg of RNA was amplified using the amino Allyl MessageAmp ™ aRNA Kit (Ambion, 2130 Woodward St, Austin TX, 78744) to generate amino allyl modified aRNA for use in microarray hybridization. The aRNA quantity was measured by spectrophotometry using a ND-1000 (Nanodrop Technologies, Wilmington, DE).

Five μg of aRNA was then vacuum dried and labelled with Cy3 and Cy5 NHS ester (Amersham Cy3 and Cy5 Mono-Reactive Dye Packs, GE Healthcare UK Ltd., Little Chalfont, Buckinghamshire). Labelled aRNA was then purified on column. Labelling efficiency was determined by spectrophotometry using the Nanodrop 1000.

825ng of Cy3 and Cy5 labelled and fragmented aRNA were added to Agilent 44k 60-mer oligonucleotide microarrays (G2514F), hybridized overnight (17 hours), washed and air dried according to the manufacturer's instructions (Agilent Gene Expression Hybridization Kit 60-mer oligo microarray protocol version 4.0). Arrays were scanned using the Agilent DNA microarray scanner.

3.2.3.1. Hybridization design

A total of 44 microarrays were used in this study, one for each tissue type of the 22 animals. A reference sample was utilized, made from equal amounts of RNA from each endometrial sample analysed (22 caruncular and 22 intercaruncular samples). This pooled sample was used as a 'reference' in each array hybridization. The reference sample was labelled with the Cy3 NHS ester dye, while each individual sample was labelled with the Cy5 NHS ester dye.

3.2.3.2. Data analysis and statistics

Agilent feature extraction software version 7.1 was used to analyse the scanned Agilent microarray. The 44 scanned microarray image files were uploaded to the feature extraction software. Using a design file (015354), the feature extraction software locates features and converts the extracted data from each feature into a quantitative log ratio. The software removes pixel outliers, performs statistical tests on the non-outlier pixels, subtracts background from features and flags any outlier features. The software was then used to perform a LOWESS (locally weighted linear regression analysis) dye normalisation and to calculate a p-value for each feature.

Data analysis was performed with Genespring GX 7.3.1. (Agilent, Palo Alto, CA, USA). Microarray data were imported into Genespring using Agilent's two-colour 'Enhanced FE' import scenario which included 'Per Spot: Divide by control channel' and 'Per Chip: Normalize to 50th percentile' normalization steps.

Filters applied to the data to improve the quality of the normalized dataset included; firstly, filtering 'on flags' to ensure any probes that were not deemed 'present or marginal' (according to feature extraction spot quality guidelines) in at least 22 of the 44 arrays were omitted from analysis; secondly, probes that did not have a minimum threshold of 80 raw intensity units in at least 22 of 44 arrays were also omitted from analysis (15,833 probes passed this filter). The raw intensity cut off value of 80 was determined based on the base over proportional (C=a/b) calculation, which is generated by plotting the standard deviation of normalized values against the control values. The point at which the curve flattens out is where the data measurement becomes reliable or where C (control strength) = a/b (where a = base and b = the proportional coefficient).

Differentially expressed probes were identified using a T-Test, including a Benjamini-Hochberg false discovery rate multiple testing correction (MTC). For probes that were not annotated, full length transcripts were identified where possible by querying microarray probe sequences against the bovine genome (Btau3,1) using NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Microarray data was submitted to NCBI gene expression omnibus (GSE19140).

3.2.4. cDNA synthesis

One µg of an RNA sample was used for cDNA synthesis using the Invitrogen Superscript III Supermix kit (Invitrogen, Carlsbad, California, USA). Total RNA was transcribed according to the manufacturer's instructions using 27µM of random pentadecamers. Briefly, RNA and random primers were mixed and denatured at 65 °C for 5 minutes, followed by 1 minute on ice. Annealing buffer and Superscript/RNase was added to samples and incubated for 10 minutes at 25°C (primer annealing), followed by 50 minutes at 50°C and 5 minutes at 85°C to inactivate the enzyme. Reverse transcription controls were performed, whereby the above process was completed without the addition of superscript enzyme.

3.2.5. Quantitative Real Time PCR

Real time PCR using the Roche Lightcycler 480 was performed using the Roche real time PCR master mix (Lightcycler 480 Probes Master) in combination with Roche Universal Probe Library (UPL) assays (Roche diagnostics, Indianapolis, IN, USA). Assays were designed using Roche UPL design software. All assays were designed to span an intron-exon boundary.

The PCR reaction volume was 10µL, consisting of 0.5µM of each primer and 0.1µM of probe. Standard cycling conditions were used [95°C for 10 minutes, (95 °C for 10 seconds, 60°C for 30 seconds) x 50 cycles, 40°C for 40 seconds].

Each PCR experiment included a reaction in which template was replaced by water, and a reaction omitting reverse transcriptase as controls. Triplicate measurements were performed for all samples and standard curves. The percent coefficient of variation (%CV) for Cqs was calculated for each sample. All samples for each gene were run on the same plate.

3.2.6. Absolute quantification

The Roche Lightcycler 480 software was used to perform absolute quantification analysis of gene expression using the standard curve second derivative maximum analysis method, which is a non-linear regression line method. A six point standard curve was used with a starting concentration of 1 and final concentration of 1.6E-03.

3.2.7. Relative quantification

The Roche Lightcycler 480 Software was used to perform quantification using the 'advanced relative quantification analysis' algorithm. Two endogenous control genes were used to normalize the data, taking the geometric mean of the normalized ratio of target gene to each reference gene (200). A calibrator sample was then used as a control, whereby each calculated expression value was normalized to the calibrator sample.

The RT-PCR results were compared to those obtained using the microarray for five differentially expressed genes (OXTR, IDO, SPP1, OAS2, and CXCL11). Correlation coefficients were calculated for all genes to compare the calculated gene expression data from qRT-PCR and the microarray data. Genes for RT-PCR were selected based on two criteria: biological significance and fold change.

3.2.8. Gene function and pathway identification

Differentially regulated genes were annotated with biological and molecular functions using the PANTHER (Protein ANalysis THrough Evolutionary Relationships) Classification System (http://www.pantherdb.org/) (201). Ingenuity pathway analysis (IPA - Ingenuity® Systems, www.ingenuity.com) was used for biological network generation and functional analysis.

Gene lists containing differentially expressed genes for each comparison (pregnant versus cyclic, caruncular pregnant versus caruncular cyclic, and intercaruncular pregnant versus intercaruncular cyclic, 1.5 fold differential expression, P-value ≤ 0.5, Benjamini-Hochberg FDR MTC) were used for analyses. The human homologue corresponding to the bovine gene representing each transcript identified as being differentially expressed was used.

For Ingenuity pathway analysis, the above gene list was uploaded and a core analysis was performed. The default background (ingenuity knowledge base) was used for all analyses.

Each gene in the uploaded list was assessed for network eligibility (determined by the representation of the gene in the Ingenuity pathway knowledge base). Each eligible gene was then mapped using the data contained within the Ingenuity knowledge base. Networks were generated based on connectivity of each of the genes, and a score (p-value calculation) was assigned based on likelihood that these genes are part of a network and did not associate due to random chance. This score was then used to rank each of the generated networks. Molecules that are known to have direct and indirect associations were used for network generation.

IPA was also used to identify significant 'biological functions'. A Fischer's exact test was used to calculate the probability that the assigned biological function was not due to random chance.

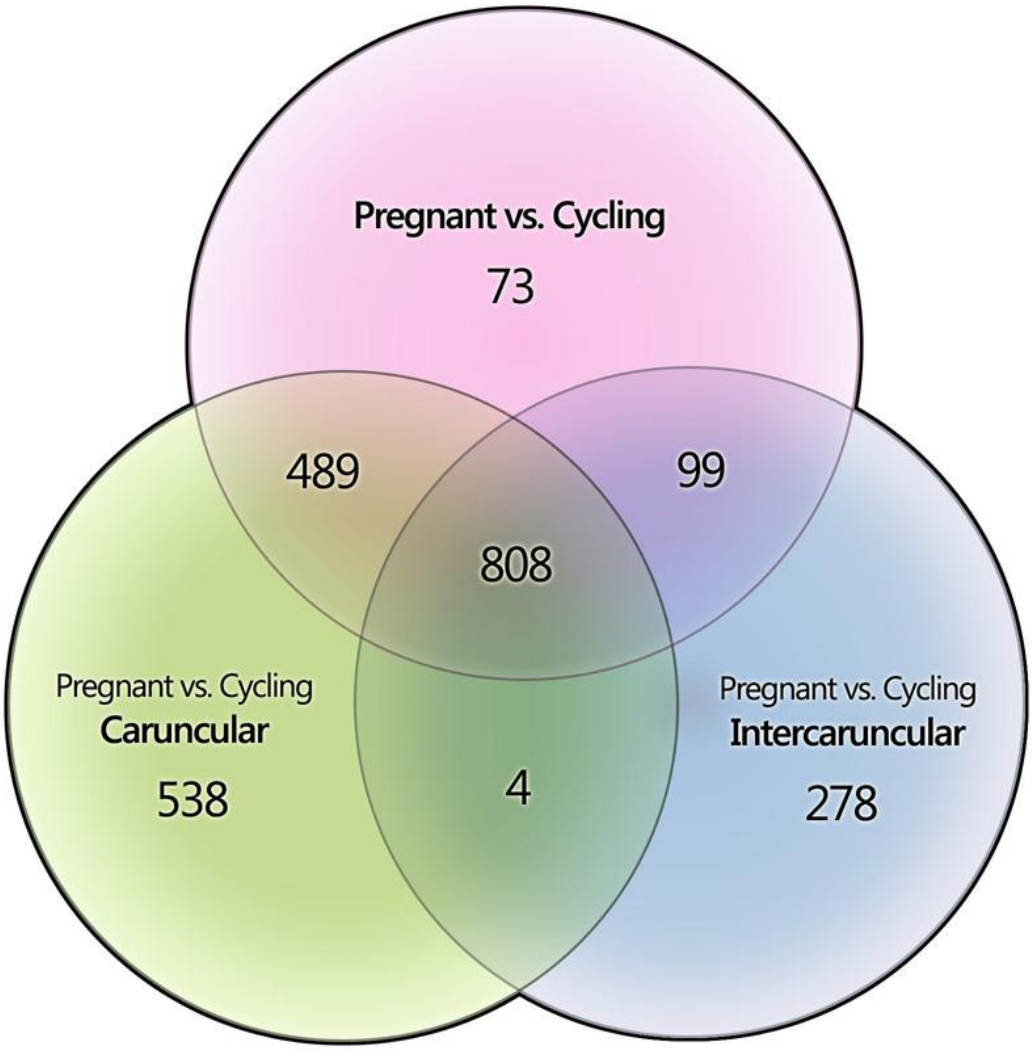
Canonical pathways contained within the IPA database were used to identify pathways that were significantly enriched in the dataset. The ratio of genes associated with each canonical pathway to the total number of genes in that pathway was calculated, and a Fisher's exact test was used to test the probability that the association of these genes was significant.

3.3. Results

3.3.1. Differentially expressed genes

Microarray analyses revealed 1,839 and 1,189 differentially expressed transcripts between pregnant and cyclic animals (with ≥1.5 fold change in expression; P-value < 0.05, MTC Benjamini-Hochberg) in caruncular and intercaruncular endometrium respectively (Additional file 1, table 1). The majority of transcripts were up-regulated in pregnant animals (1,027 in caruncular, 633 in intercaruncular). Some genes were differentially expressed between pregnant and cyclic animals in either the caruncular or intercaruncular tissues. Of these, 1,027 (480 up-regulated in pregnant) differentially expressed transcripts were identified in caruncular endometrium only, and a further 377 (86 up-regulated in pregnant) differentially expressed transcripts were identified only in intercaruncular tissue (Figure 8).

Figure 8. Number of genes differentially expressed between pregnant and cyclic animals in both and individual tissue types. Genes with ≥1.5 fold change in expression; P-value < 0.05, Benjamini-Hochberg MTC.


Pregnant vs. Cycling
73
489
99
808
Pregnant vs. Cycling
Caruncular
538
4
Pregnant vs. Cycling
Intercaruncular
278

3.3.2. Molecular and biological function and pathway analysis

Ingenuity pathway analysis (IPA) revealed molecular networks and pathways associated with genes that were differentially expressed in pregnant versus cyclic animals. 1,499 individual genes were contained within the Ingenuity database and were used for analysis. The four most statistically significant canonical pathways identified by IPA were interferon signalling (Figure 9), complement system (Figure 10), Role of pattern recognition receptors in the recognition of bacteria and viruses, and antigen presentation (Additional file 1, table 2). The networks generated by IPA that contained the most significant number of genes with direct relationships (molecules that directly affect one another) were:

- **Network 1** - Cell death, haematological disease, immunological disease (score=49, 28 molecules)
- **Network 2** - Infection mechanism, antimicrobial response, cell signalling (score=45, 26 molecules)
- **Network 3** - Infectious disease, cell morphology, cellular assembly and organization (score=43, 26 molecules)
- **Network 4** - Cellular growth and proliferation, connective tissue development and function, cell cycle (score=23, 16 molecules)
- **Network 5** - Lipid metabolism, molecular transport, small molecule biochemistry (score=21, 15 molecules).

'Functional groups' and biological processes associated with pregnancy and oestrous cycle progression (cycling animals) in the dairy cow during the pre-implantation period were identified. Genes were assigned to molecular and biological functions using the PANTHER (Protein Analysis Through Evolutionary Relationships) classification system (http://www.pantherdb.org/). The biological process "immunity and defence" contained the most genes for this analysis. (Additional file 1, table 3).

Figure 9. Interferon signalling cascade. Genes differentially expressed between pregnant and cyclic dairy cows in the study reported are shaded red (up-regulated in pregnant) and green (down-regulated in pregnant). The bovine embryo produces IFNτ which binds to type-1 interferon receptors, leading to the activation of the JAK-STAT (Janus kinase-signal transducer and activator of transcription) pathway and the synthesis of a range of interferon stimulated gene products.

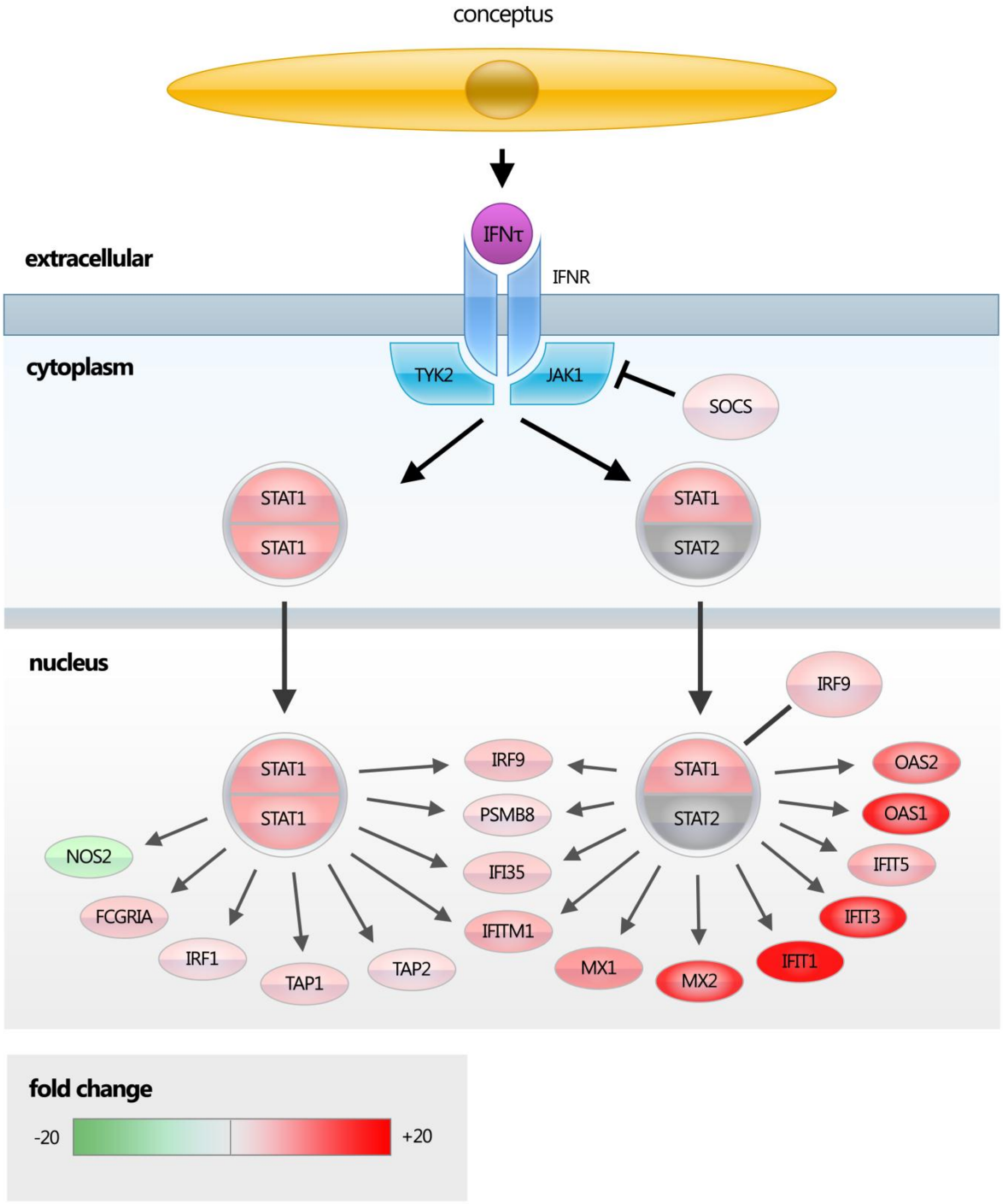
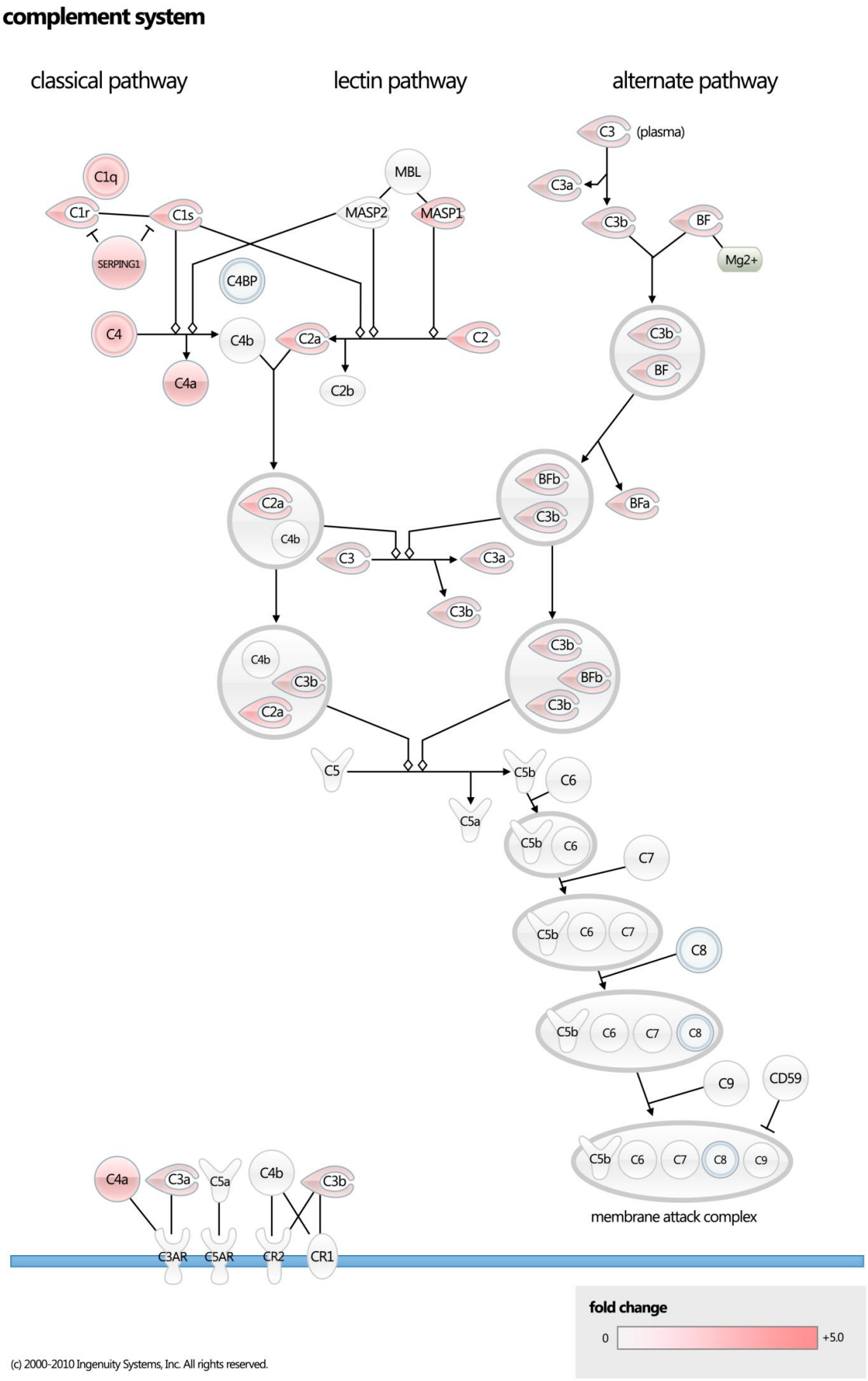

conceptus
IFNτ
extracellular
IFNR
cytoplasm
TYK2
JAK1
SOCS
STAT1
STAT1
STAT1
STAT2
nucleus
IRF9
STAT1
STAT1
IRF9
PSMB8
IFI35
IFITM1
STAT1
STAT2
OAS2
OAS1
IFIT5
IFIT3
IFIT1
NOS2
FCGRIA
IRF1
TAP1
TAP2
MX1
MX2
fold change
-20
+20

Figure 10. Complement pathway. Genes up-regulated in pregnant dairy cows are shaded red. The complement system is part of the innate immune response and can be activated in three ways: classical pathway, alternative pathway and lectin pathway, all of which converge at the level of C3 convertase. The Classical pathway begins with activation of the C1 complex (6xC1q, 2xC1r and 2xC1s) through the binding of C1q to antigen bound antibodies (IgG or IgM) or directly to the surface of a pathogen. Binding causes a conformational change in C1q which leads to activation of C1r and C1s (serine proteases), this leads to cleavage of C4 and C2. The cleavage products of C4 and C2 form C3 convertase cleaves C3, leading to the formation of C5 convertase that cleaves C5 and results in formation of the membrane attack complex (202).


complement system
classical pathway
lectin pathway
alternate pathway
C1q
C1r
C1s
SERPING1
MBL
MASP2
MASP1
C4BP
C4
C4a
C4b
C2a
C2b
C2
C3
(plasma)
C3a
C3b
BF
Mg2+
BFb
BFa
C5
C5b
C6
C5a
C7
C8
C9
CD59
membrane attack complex
C3AR
C5AR
CR2
CR1
fold change
0
+5.0
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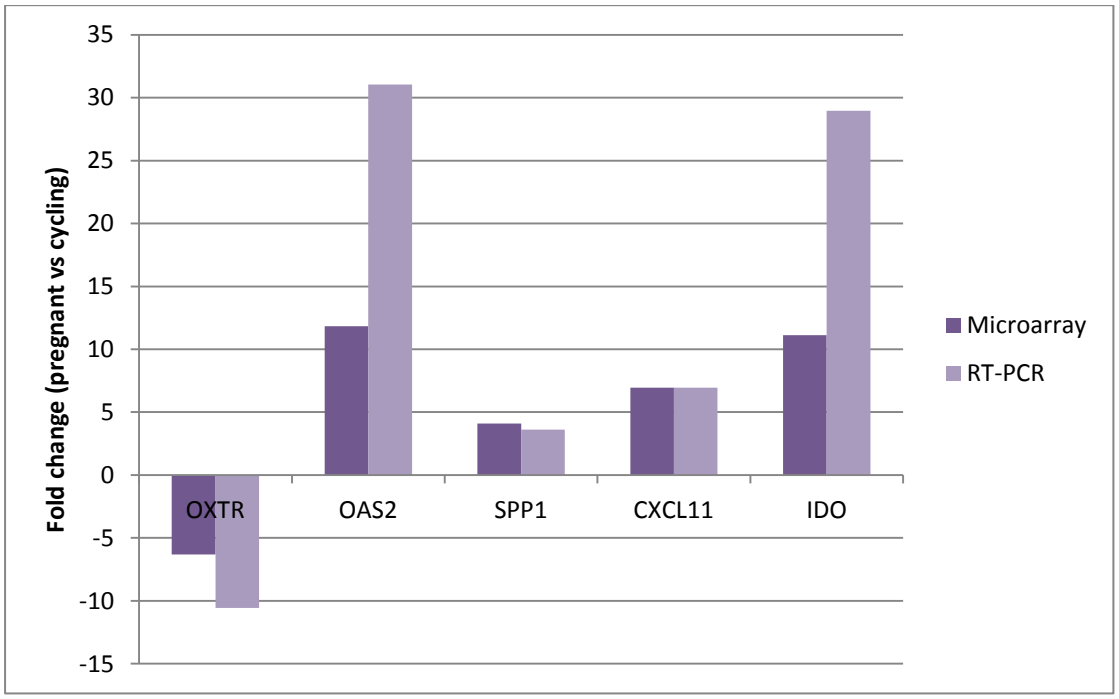
3.3.3. Relative quantitative real time PCR confirmation of microarray results

Relative qRT-PCR was used to confirm microarray results. 5 genes of interest (*OXTR*, *IDO, SPP1, OAS2,* and *CXCL11*) that were differentially expressed according to microarray analysis were quantified, and the correlation between qRT-PCR and microarray (Pearson correlation coefficient) calculated exceeded 0.75 for all genes tested (Table 6, Figure 11).

Table 6. RT-PCR confirmation of microarray

Fold change Pregnant vs Cyclic				**Primer Sequences**		
	Microarray	**RT-PCR**	**Correlation**	**Forward**	**Reverse**	**Probe**
OXTR	-7.26	-10.55	0.9	CGTGCAGATGTGGAGTGTCT	TTGCAGCAGCTGTTGAGG	UPL#162
OAS2	10.94	31.05	0.93	TGGACGGTCAACTACAGTTTTG	CTGGGTCCAAGATCACAGG	UPL#69
SPP1	3.41	3.6	0.75	AAGTTCCGCCGATCTAACG	CCTCACTCTCTATGTGTGATGTGAA	UPL#82
CXCL11	5.23	6.95	0.83	TGCTGCAATTGTTCAAGGTT	TCTGCCACTTTGACTGCTTTT	UPL#81
IDO	15.25	28.97	0.76	ACGTAGGCTTTGCTCTTCCA	GAGATCCAGGCATCATAAGGA	UPL#65

Figure 11. RT-PCR confirmation of microarray.


35
30
25
20
15
10
5
0
-5
-10
-15
Fold change (pregnant vs cycling)
OXTR
OAS2
SPP1
CXCL11
IDO
Microarray
RT-PCR

3.4. Discussion

3.4.1. Genes up-regulated in pregnant animals

In this study, endometrial expression profiles of day 17 pregnant and cyclic dairy cows were characterized and several genes and pathways that were differentially expressed between the two states were identified, providing insight into the molecular mechanisms active during this time. Genes and pathways involved in the maternal immune response to the presence of the embryo appear to be particularly important in early pregnancy, as these were some of the most up-regulated genes in pregnant animals. The immune response to pregnancy may be one of the key regulators of pregnancy maintenance, and deregulation of the immune response may be responsible, at least in part, for the large number of pregnancy losses that occur during this time. Both innate and adaptive immune system genes were differentially expressed during early pregnancy. Many genes of the adaptive immune response that were up-regulated may function to induce immune tolerance to the embryo, while genes of the innate immune response (in particular, antimicrobial genes) may function to protect the uterus against infection during a time of local immune suppression.

3.4.2. Immune response and interferon signalling

Interferon stimulated genes (ISG) were among the most up-regulated group of genes in pregnant animals; this is consistent with maximal production of the pregnancy recognition signal interferon tau (IFNτ) by the embryo during this time. The most statistically significant networks up-regulated in pregnant animals were involved in the immune response, and immunity and defence were identified as the most abundant gene ontology terms.

Several ISGs were identified as being differentially expressed in pregnant and cyclic animals, in agreement with previous studies (107, 191, 195, 203, 204). The current study, however, has identified several additional ISG not previously identified in the bovine endometrium (Figure 9, Additional file 1, table 2). Many of these genes may function to provide localized immune system suppression to allow the embryo to survive within the uterus. For example, IFITM1 has recently been demonstrated to act as an immune suppressive molecule in gastric cancer cells (205). The up-regulation of *IFITM1* in cancer cells was associated with an increase in migration and invasive capacity, and its action in gastric tumour cells was linked

to suppression of NK cells (205). Peptide Transporter 1 (*TAP-1*) and *TAP-2* were up-regulated in pregnant animals in agreement with another study (206) along with MHC I A, and MHC I G. Up-regulation of TAP proteins may be involved in local immune suppression, as up-regulation of TAP1 has been associated with the capacity of natural killer cells to be non-cytotoxic (207). Kalkunte et al 2009 demonstrated that the non-cytotoxic phenotype of uNK cells is achieved through the expression of vascular endothelial growth factor-C (VEGF-C), which enhances the resistance of trophoblast and endothelial cells to lysis through induction of the TAP-1 protein (207). They reported that VEGF-C protected target endothelial and trophoblast cells from cytotoxic NK cells directly through induction of TAP-1.

Additionally, in agreement with a local immune suppression environment, *IFIT1, OAS1* and *OAS2* are up-regulated in the autoimmune disease systemic lupus (208). OAS up-regulation during early pregnancy is also involved in regulating the production of osteopontin (SPP1) (86, 209), that was also up-regulated in pregnant animals. Up-regulation of SPP1 in pregnant animals may have several functions, including promoting adhesion of the trophoblast to the endometrium, stimulating morphological changes in the trophoblast (98), and regulating the immune response. SPP1 is expressed and secreted by immune cells including T-lymphocytes, monocytes, macrophages and NK cells. SPP1 is presumed to regulate the TH1/TH2 balance and apoptosis (98). *SPP1* polymorphisms are associated with many immune-mediated inflammatory diseases (210) consistent with a local immune suppressive state. Quantitative trait (QTL) analyses in pigs have identified SPP1 as a candidate gene for reproductive performance (211).

Alternatively, up-regulation of these genes may be an important mechanism to enhance the response to potential viral pathogens that the uterus may encounter during the time of local immune suppression that occurs in response to the embryo. This hypothesis is supported by the up-regulation of *MX1* and *MX2*, both of which are up-regulated in response to viral infection (212, 213). *MX2* expression is also up-regulated in peripheral blood leukocytes during pregnancy (214), suggesting the innate immune system is active during early pregnancy.

Several of the above genes are also thought to be important regulators of luteolysis (212). For example, OAS inhibits prostaglandin F2α synthesis, possibly through alteration of

arachidonic acid metabolism (215). Interestingly, *MX2* expression is lower in the uterus of pregnancies with cloned embryos than in embryos produced by IVF.

3.4.2.1. Cytokines, chemokines, and growth factors

Pregnancy requires a delicate balance between pro-inflammatory and anti-inflammatory molecules to maintain maternal immune system integrity, while preventing rejection of the embryo. Expression patterns of chemokines and their receptors during the implantation period suggest that they are involved in the regulation of embryo attachment as well as having immuno-modulatory properties. Chemokines attract leukocytes to sites of inflammation, and contribute to their local activation.

Several chemokines were up-regulated in pregnant animals in agreement with other studies (112, 216-218). The CXCR3 ligands *CXCL9*, *CXCL10* and *CXCL11* were all up-regulated in pregnant animals, a difference that is more pronounced in the caruncular compared with intercaruncular tissue. CXCR3 is preferentially expressed on TH1 cells, and the expression of ligands for this receptor suggests there may be an influx of TH1 cells into the uterus. Up-regulation of the CXCR3 receptor ligands and the influx of TH1 cells have been associated with allograft rejection (216, 219). However since *CXCR3* was not up-regulated, it suggests that either TH1 cell numbers were not increased in the uterus of pregnant animals, or that they were not expressing this receptor. CXCR3 is also expressed in human uterine natural killer cells (220), so up-regulation of these chemokines may function to attract the trophoblast and/or uNK cells. Several chemokines that were up-regulated in pregnant animals are known to attract immune tolerance promoting leukocytes, including TH2 and NK cells. For example, *CCL11*, which was up-regulated in both intercaruncular and caruncular tissue in pregnant animals attracts CCR3-expressing TH2 cells (221), and *CCL2*, which was only up-regulated in caruncular tissue, attracts leukocytes expressing its receptor CCR2 (221, 222). Consistent with this is the association of *CCL2* up-regulation with immune tolerance in endometriosis, a mechanism suggested to act through its action on the FAS ligand, inducing apoptosis of T lymphocytes (223). The FAS ligand, along with *FAS* and the downstream effector molecules *FADD* and caspase were all up-regulated in pregnant animals in both tissue types. Another NK cell attracting chemokine up-regulated in pregnant animals was *CCL8* (224). This chemokines was up-regulated 17-fold in the caruncular

endometrium, and 9-fold in the intercaruncular endometrium. In addition to attracting NK cells, CCL8 can attract monocytes, lymphocytes, eosinophils, and basophils through its capacity to bind to the CCL2 receptor CCR2 as well as CCR1, CCR3 and CCR5. Both *CCR1* and *CCR5* were up-regulated in caruncular and intercaruncular tissues of pregnant animals. Co-expression of proteases that can convert CCL8 to CCL8(6-75) results in an anti-inflammatory response, as CCL8(6-75) can inhibit other chemokines through its ability to act as a receptor antagonist (225).

Several interleukins that were up-regulated in pregnant animals may function to increase the presence of immune tolerance promoting T-regulatory (T-reg) cells in the uterus, as well as shifting the inflammatory balance towards an anti-inflammatory response. T-reg cells require low levels of some cytokines in order to differentiate from naive CD4+ T cell precursors, with high levels blocking suppression. In particular, *IL-15*, which was up-regulated 2 fold in the caruncular endometrium of pregnant animals. IL-15 also induces proliferation of T-reg cells (226). Another cytokine that was up-regulated in the pregnant endometrium, *IL-7* , is considered a growth and survival promoting factor for T-reg cells (227). Interleukin1β (*IL-1β*) and interleukin 18 (*IL-18*) are pro-inflammatory cytokines that were up-regulated in pregnant animals. Caspase 1, which proteolytically cleaves the IL-1β precursor to its active form, was also up-regulated in the caruncular endometrium of pregnant animals. Inhibitors of these cytokines, *IL-1RN* and *IL-18BP* were also up-regulated in pregnant animals. *IL-1RN* has recently been reported to be up-regulated in the pregnant equine endometrium (228), and down-regulated during the window of implantation in the cyclic human (229).

The TGFβ superfamily is a large functionally diverse protein family which includes several subfamilies including activins, bone morphogenic proteins (BMPs) and growth differentiation factors (GDF) (230). Members of this family were differentially regulated with respect to pregnancy in the current study including TGFβ1, 2 and 3, which were all down-regulated in pregnant animals. The TGF-β superfamily members have immuno-modulatory/inflammatory actions, some of which may be important during pregnancy recognition and maintenance. However, in the current study *TGFβ1*, *2*, and *3*, *TGFβ1I1*, and *BMP6* were all down-regulated in pregnant animals, while *TGFβI* was up-regulated in pregnant animals in caruncular endometrium. Other members of this family that were

down-regulated in pregnancy include; myostatin, inhibin β A and inhibin β B, this is coupled with increased expression of follistatin (2.8 fold), an inhibitor of both activins and myostatin. While activins have immuno-modulatory actions (231) the GDF, myostatin, has been proposed to have a role in glucose metabolism (232) in the placenta (233), and in an endometrial cell line (234). Myostatin may be an important regulator of glucose availability to the embryo through regulation of glucose in endometrial histotroph secretions, and later for foetal development through regulation of placental glucose transport. Reduced myostatin accompanied by increased follistatin indicates that this pathway is under tight control during early pregnancy.

3.4.2.2. Antigen presentation and complement related genes

Complement pathway genes are some of the most commonly up-regulated genes in this and previous studies of endometrial gene expression during the window of implantation (195, 235-237). In particular, several molecules involved in activation of complement via the classical pathway were up-regulated in pregnant animals (Figure 10). Up-regulation of complement may serve to provide the developing embryo with protection against pathogens and/or immune complexes and apoptotic cells, an important adaptation given the local immune suppressive state necessary for successful embryo implantation and placentation. Molecules that protect against complement mediated cells are expressed in the endometrium and trophoblast (238). Consistent with this observation, pentraxin 3 (*PTX3*) was up-regulated in pregnant animals, particularly in the caruncules. PTX3 binding to the C1q complex can activate or inhibit the classical complement pathway depending on the nature of the interaction (239, 240). Up-regulation of *PTX3* may function to control the complement cascade in the pregnant animal and prevent excessive inflammatory reactions. Another gene that was up-regulated and may function to inhibit complement mediated immune responses is *SERPING1*. SERPING1 is a progesterone-induced, immunosuppressive and anti-proliferative glycoprotein contained in uterine secretions (111). SERPING1 can inhibit complement-mediated immune responses through inactivation of C1s. Up-regulation of *SERPING1* may function to suppress a complement-mediated immune response to foetal antigens, and may also be important for embryonic growth. SERPING1 has been proposed to support conceptus growth through its ability to sequester the pluripotent growth factor activin A. Because of its immunosuppressive and anti-proliferative properties, it has been

hypothesized to prevent foetal allograph rejection through inhibition of lymphocyte proliferation in the uterus (111). Other SERPIN's that were differentially regulated include *SERPINA9, SERPINA1 and SERPINB11*.

Other genes that were up-regulated in pregnant tissue encode immuno-proteasome subunits *PSMB8* and *PSMB9*, and antigenic peptide transporters *TAP1* and *TAP2* (60). These results identify modulation of the extent and specificity of antigen presentation as a mechanism likely required for pregnancy success.

MHC class 1 and class 2 molecules along with *B2M* were identified as being up-regulated in pregnant animals. Up-regulation of MHC class 1 and *B2M* in response to pregnancy and interferons has been demonstrated (199, 241). Down-regulation of classical MHC class 1 molecules in trophoblast tissue is a well characterized mechanism proposed to prevent immunological attack of the embryo (197). The expression of MHC molecules in uterine tissue during pregnancy is poorly characterised. It has been proposed that up-regulation of MHC class 1 molecules and B2M in the endometrium may compensate for loss of mucin expression in the endometrial luminal epithelium (241). Mucin-1 (MUC-1) forms part of the glycocalyx barrier that provides innate immune protection against bacterial infections; down-regulation of MUC-1 is thought to be required for embryo attachment as the ectoderm tail presents a steric hindrance to attachment (60). *MUC-1* was down-regulated in pregnant animals in the study reported here, suggesting this mechanism may be active in the bovine endometrium also. Furthermore, the non-classical MHC class 1 molecule *HLA-G* was up-regulated in pregnant animals, perhaps to support an immunosuppressive action on the maternal immune system.

3.4.2.3. Antimicrobial response genes

Several antimicrobial genes are up-regulated in the endometrium of pregnant animals. *LBP* and *BPI,* encode proteins that bind bacterial endotoxin. Both were up-regulated in pregnant animals, 19 fold and 6 fold respectively, as was the antibacterial gene *LYZ1* which was up-regulated 2.4 fold in pregnant animals. Increased expression of these genes in pregnant animals may confer innate immune protection against potential bacterial infection during a time of local immune suppression, as occurs during pregnancy (242).

3.4.2.4. Other immune response related genes

Indoleamine -2,3 dioxygenase (*IDO*) and tryptophanyl-tRNA synthetase (*TTS /WARS*) were up-regulated 5.7 fold and 2.4 fold respectively in endometrium of pregnant cows in the current study. Up-regulation of *IDO* has been reported in other species (243-245) and is likely to have an immunosuppressive function in the cow. Munn et al (1998) described a novel mechanism by which the maternal system prevents foetal rejection through increased IDO expression, specifically through the suppression of T-cell activity. Dendritic cell expression of IDO causes increased catabolism of the essential amino acid tryptophan, and increased concentration of the tryptophan metabolites 3-OH-Kynurenine and 3-OH-anthranilic acid. Tryptophan catabolism reduces the amount of tryptophan available in the microenvironment for protein synthesis, and can thus prevent expansion of T-cells. In addition, tryptophan metabolites have immunosuppressive properties such that they cause T-cell apoptosis. Cells expressing IDO are protected against tryptophan starvation, possibly through expression of TTS/WARS. TTS is the only known aminoacyl-tRNA synthetase induced by interferon gamma. The formation of a tryptophan-tRNA complex results in the generation of a reservoir of tryptophan that may function to protect IDO expressing cells from tryptophan depletion. Up-regulation of these genes suggests this mechanism is active in the pregnant bovine endometrium and is likely an important contributor to pregnancy success in dairy cattle.

Several galectins were differentially expressed between pregnant and cyclic animals. Galectin 9 was up-regulated 2.8 fold in pregnant cows while galectin 8 and galectin 3 binding protein (caruncular only) were up-regulated 1.7 and 2.2 fold respectively. In contrast, galectin 12 was down-regulated in pregnant animals (1.6 fold) compared to cyclic animals. Galectins are a family of lectins that bind to beta-galactoside motifs in ligands such as laminin, fibronectin, and mucins in bivalent or multivalent ways, to modulate cellular adhesion (246). Galectin 3 binding protein (Lgals3bp) can bind to Galectin -1, -3 and -7, promoting cell-cell adhesion through bridging between galectin molecules bound to ECM components (247, 248). It can also self-assemble to form high molecular weight complexes that promote cell adhesion through binding of integrins, collagens and fibronectin independently of Galectin 3 (248). It has been demonstrated that Galectin-9 administration improves the survival of allogenic skin grafts in mice, possibly through induction of host

cytotoxic CD8a+ T cell apoptosis (249). Galectin-9 inhibits the secretion of TH1 and TH17 type cytokines, and promotes the synthesis of TH2 type cytokines in vitro (250). Galectin-9 is potentially involved in modulation of the immune system during early pregnancy through its ability to induce apoptosis of immune cells, including activated CD4+ and CD8+ T cells through Ca2+ calpain caspase 1 pathway (251).

Some of the most highly expressed genes in the pregnant animals in this study were guanylate binding proteins (*GBP*), which belong to a family of GTPases that also includes Mx proteins (up-regulated in pregnant animals). GBPs regulate inhibition of proliferation, invasion of endothelial cells and cell survival. GBPs bind to guanine nucleotides, they contain two binding motifs. *GBP-1* is up-regulated in the human endometrium during the window of implantation (110), and *GBP-1* and *GBP-3,-4,-5* are up-regulated in the bovine endometrium at day 18 of pregnancy (191). Consistent with this premise, *GBP-1,-2,-3,-4* and *GBP-6-7* were up-regulated in pregnant animals in the current study. GBPs are up-regulated in patients with inflammatory bowel disease (IBD) and also found to be associated with epithelial tight junctions, where down-regulation of GBP through siRNA causes an increase in barrier permeability (252). It has recently been suggested that the functional significance of GBP up-regulation is to protect cells against pro-inflammatory cytokine induced apoptosis (252).

3.4.2.5. Difference in caruncular and intercaruncular endometrial tissue

The majority of genes differentially expressed between pregnant and cyclic animals are in both caruncular and intercaruncular tissues. However, the magnitude of differential expression between pregnant and cyclic endometrium differs in the two tissues, and there are some genes that are only differentially expressed in one of the two tissue types. The caruncules of the endometrium are specialised projections that are the site of embryo attachment. Caruncules become highly vascularised, and are the major site for small molecule and gaseous exchange (55). In comparison, intercaruncular tissue is highly glandular and responsible for early nourishment of the embryo through secretions of large molecules into the uterus (55, 189, 190). In addition, there are a greater number of stromal cells in the caruncular endometrium. The magnitude of some of these gene expression changes differs considerably between the two tissue types. *RSAD2* is the most differentially expressed gene (23 fold up-regulated in pregnant animals) in the comparison of pregnant

versus cyclic animals. In this comparison, *RSAD2* is 3 fold more differentially expressed in caruncular than it is in intercaruncular tissue. This difference occurs in most of the genes discussed. The larger difference in gene expression seen in the caruncular tissue (indicating a greater response to pregnancy) may reflect the role of this tissue in implantation. The caruncules, being the site of embryo attachment may demand more extreme gene expression changes that promote tolerance to the embryo, allowing it to form a closer physical relationship without immunological attack.

3.5. Conclusion

The maternal immune system is actively surveying the uterine environment during early pregnancy. The embryo modulates this response, inducing expression of molecules in the endometrium that function to suppress the immune response and/or promote tolerance to the embryo. The current study demonstrates this response with widespread up-regulation of immune response pathways. During this period of immune suppression the endometrium would be expected to be susceptible to infections; the endometrium must, therefore, actively express specific molecules for defence against foreign pathogens. up-regulation of genes of the innate immune response including antimicrobial response genes support this hypothesis. This system requires intricate control through expression of protective inhibitors in the endometrium, and raises the question of whether the embryo expresses these same inhibitory molecules.

Chapter 4. ENDOMETRIAL GENE EXPRESSION DURING EARLY PREGNANCY DIFFERS BETWEEN FERTILE AND SUB-FERTILE DAIRY COW STRAINS

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I developed the methods, performed the experimental work and statistical analysis and wrote the manuscript. The other authors contributed to the development of methods and experimental design and critical analysis of the manuscript.

4.1. Introduction

On average, high producing dairy cattle have poor reproductive performance compared with low to moderate producing dairy cows (253). They present with differences in oocyte and embryo quality, greater embryo loss, and lower pregnancy rates compared with lower-producing dairy cows (188, 253, 254). It is estimated that 60-70% of pregnancy losses occur in the first three weeks of pregnancy, the period of pregnancy recognition (5, 255). Successful pregnancy recognition and maintenance requires both a viable embryo and a receptive endometrium (60). Several factors have been implicated in pregnancy success, including embryo-maternal communication, inhibition of luteal regression, the immune response to pregnancy, and preparation of the uterus for implantation (256), which, when compromised, can lead to pregnancy failure. The uterine environment is likely, therefore, to be a key determinate of reproductive success. In this study, Holstein Friesian dairy cow strains characterized as having, on average, fertile (Holstein-Friesian cows with New Zealand ancestry) and sub-fertile (Holstein-Friesian cows with North American ancestry) phenotypes (188, 253) were used to test the hypothesis that endometrial gene expression patterns differ between dairy cow strains classified as fertile and sub-fertile (188, 253). High-quality blastocyst-stage embryos were transferred to oestrus-synchronized dairy cattle from fertile and sub-fertile strains. Endometrial tissue was obtained at day 17 of pregnancy and microarrays used to characterize differences in transcriptional profiles.

4.2. Methods

4.2.1. Animals and treatments

All procedures were undertaken with the approval of the Ruakura Animal Ethics Committee (Hamilton, New Zealand). The oestrus cycles of 14 lactating dairy cows were synchronized (at 58.8 (SEM 3.77) and 60.2 (SEM 1.51) days post calving in dairy cows of sub-fertile and fertile strains, respectively) and received a single embryo transferred on day 7 of the oestrus cycle (257). The oestrous cycles of the animals were synchronized using a controlled intra-vaginal drug-release device containing progesterone (1.38 g, CIDR-B™ Pfizer Animal Health Group, Auckland, New Zealand) and 2 ml oestradiol benzoate i.m (2 mg, CIDIROL Bomac Laboratories Limited, Auckland, New Zealand) for 8 days (day of insertion day -8) with all animals receiving two 2 ml injection of sodium chloprostenol (500 µg, EstroPlan, Parnell Laboratories NZ Ltd, Auckland, New Zealand) 6 days (an injection in the morning and afternoon) after CIDR insertion. All animals received a 2.5 ml injection of a GnRH analogue buserelin (10 µg, Receptal Intervet Limited, Auckland, New Zealand), given 24 h after CIDR device removal. The day after GnRH injection was Day 0 of the synchronized oestrous cycle. Embryos were at the blastocyst stage of development and of grade 1 quality. Embryos were produced from oocytes recovered from ovaries collected at the abattoir. The origin and reproductive history of the donor cows were unknown. Animals were slaughtered at day 17 of the reproductive cycle and endometrial tissues (both caruncular and intercaruncular) were sampled. Selection criteria for the study included strain and calving date, and health post-calving was an exclusion criterion (cows with severe uterine infections or mastitis were excluded before being enrolled in the embryo transfer round). Cows in each strain were matched for calving number and age. A total of 12 pregnant animals out of the 14 enrolled in the study were utilized, due to the associated costs of slaughtering the cows. These animals represented fertile (six Holstein-Friesian cows with New Zealand ancestry/≤30% North American genetics, n=6, NZ) and sub-fertile (six Holstein-Friesian cows with >87% North American ancestry, n=6, NA) phenotypes of Holstein-Friesian dairy cows (188, 253, 258, 259). Briefly, Holstein-Friesian dairy cow strains of NA ancestry have poorer oocyte and embryo quality, lower conception rate to first and second services, lower 6-week pregnancy rate and overall lower pregnancy rate compared with NZ strain Holstein-Friesian dairy cows. Gene expression differences between the two strains in non-reproductive tissues have been

published previously (260). Detailed methods and production data for the cows used in this study have been published previously (187).

4.2.2. Uterine flushings

Each horn of the uterine tract was flushed with 20 mL of saline and the uterine luminal fluid (ULF) recorded as being ipsilateral or contralateral to the ovary containing the corpus luteum (CL). Trophoblast (embryo) recovered from the uterine flushing's were measured using a confocal light microscope and a ruler. The elongated trophoblast tissues were not tangled during flushing and were therefore easily measured. Embryo viability was not tested directly. The ULFs were centrifuged to remove cellular debris then lyophilized and reconstituted with distilled water containing a cocktail of protease inhibitors (Complete, Roche, USA) before being dialyzed. The protein concentration was measured (261) and samples stored at –20 °C until analysed.

4.2.3. Western blotting

Western blotting procedures as previously described (262) were used to quantify protein. Briefly, ULF samples containing either 20 or 100 µg of total protein and 2µl human serum control in 50% glycerol, 0.5M Tris-HCL, pH 6.8 loading buffer were applied to 12% or 15% SDS-polyacrylamide gels and subjected to electrophoresis then electroblotted onto re-enforced nitrocellulose membrane (BioTrace NT, Pall, USA). Protein was detected using anti-IFNτ (gift from Dr RM Roberts, University of Missouri, MO) at 1:2000. Bound antibody was visualized by chemiluminescence (luminol, Sigma-Aldrich) or SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL) and bands quantified using Quantity One software (Bio-Rad Laboratories, Hercules, CA).

4.2.4. Statistical analyses

To test for possible differences in embryo size, IFNτ concentration, and progesterone concentration across the 17 days of the experiment, ANOVA was utilized. To test for a possible interaction effect of day and strain on progesterone, it was necessary to log transform the data because of heterogeneity of the variance on different days. Log_{10} progesterone was analysed in a repeated measures analysis using mixed models including day, strain and the interaction of day with strain as fixed effects and cow as a random effect,

modelling the within cow repeated measurements with a compound symmetry covariance structure.

4.2.5. RNA Extraction

Caruncular and intercaruncular endometrial tissues were homogenized in Qiagen buffer RLT (QIAGEN Hilden, Germany) using FastPrep Lysing Matrix D tubes in a FastPrep instrument (MP Biomedicals, Solon, OH).

Total RNA was extracted using a Qiagen RNeasy kit (QIAGEN. Cat# 74104). RNA quantity was determined by spectrophotometry using a Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, DE). RNA integrity was assessed with the Agilent 2100 Bioanalyzer with a RNA 6000 Nano LabChip kit (Agilent Technologies, Palo Alto, CA. Cat# 5067-1511).

4.2.6. Microarray

One μg of RNA was amplified using the amino Allyl MessageAmp ™ aRNA Kit (Ambion, Austin TX. Cat# AM1751) to generate amino allyl modified aRNA for use in microarray hybridization. aRNA quantity was measured by spectrophotometry using a ND-1000 (Nanodrop Technologies, Wilmington, DE).

Five μg of aRNA were then vacuum dried and labelled with Cy3 and Cy5 NHS ester (Amersham Cy3 and Cy5 Mono-Reactive Dye Packs, GE Healthcare UK Ltd., Buckinghamshire, UK. Cat# PA23031 and PA25031). Labelled aRNA was then purified on column and labelling efficiency was determined by spectrophotometry using the Nanodrop 1000.

Eight hundred and twenty five ng of Cy3 and Cy5 labelled and fragmented aRNA were added to Agilent 44k 60-mer oligonucleotide microarrays (G2514F), hybridized overnight (17 hours), washed and air dried according to the manufacturer's instructions (Agilent Gene Expression Hybridization Kit 60-mer oligo microarray protocol version 4.0). Arrays were scanned using the Agilent DNA microarray scanner.

4.2.6.1. Hybridization design

A total of 25 microarrays were used in this study, one for each tissue type of the 12 animals and one technical replicate. A reference sample was used, made from equal amounts of RNA from the 24 endometrial samples analysed in this study and an additional 10 from a

related study (263). This pooled sample was used as a 'reference' in each array hybridization. The reference sample was labelled with the Cy3 NHS ester dye, while each individual sample was labelled with the Cy5 NHS ester dye.

4.2.6.2. Data analysis and statistics

Agilent feature extraction software version 7.1 was used to analyse the scanned Agilent microarray. The 25 scanned microarray image files were uploaded to the feature extraction software. Using a design file (015354), the feature extraction software locates features and converts the extracted data from each feature into a quantitative $\log_2$ ratio. The software removes pixel outliers, performs statistical tests on the non-outlier pixels, subtracts background from features and flags any outlier features. The software was then used to perform LOWESS (locally weighted linear regression analysis) dye normalization and to calculate a p-value for each feature.

Data analysis was performed with Genespring GX11. (Agilent, Palo Alto, CA, USA). Microarray data were imported into Genespring using Agilent's two-colour 'Enhanced FE' import scenario, which included 'Per Spot: Divide by control channel' and 'Per Chip: Normalize to 50th percentile' normalization steps.

Filters applied to the data to improve the quality of the normalized dataset included; firstly, filtering 'on flags' to ensure any probes that were not deemed 'present or marginal' (according to feature extraction spot quality guidelines) in at least 12 of the 24 samples were omitted from analysis; secondly, probes that did not have a minimum threshold of 80 raw intensity units in at least 12 of 24 samples were also omitted from analysis. The raw intensity cut off value of 80 was determined based on the base over proportional (C=a/b) calculation, which is generated by plotting the standard deviation of normalized values against the control values. The point at which the curve flattens out is where the data measurement becomes reliable or where C (control strength) = a/b (where a = base and b = the proportional coefficient). Comparisons between fertile and sub-fertile strains were carried out using ANOVA with significance thresholds set at $P<0.05$; multiple testing corrections (Benjamini-Hochberg), and post hoc tests (Tukey) were also applied. The four parameters used for ANOVA analysis of fertile and sub-fertile animals were caruncular pregnant with a large embryo, caruncular pregnant with a small embryo, intercaruncular

pregnant with a large embryo, and intercaruncular pregnant with a small embryo. Small embryos were classified as those being less than 20 cm (mean: 10 ± 2.6 cm) in length and weighing less than 40 mg (mean: 19 ± 5.9 mg, 5 embryos). Large embryos were classified as those being longer than 20 cm (mean: 36 ± 4.9 cm) and weighing more than 75 mg (mean: 158 ± 33 mg, 7 embryos). The genes with P-value <0.05 for the above comparisons were extracted from the Genespring Tukey comparison table. These lists were then used for GeneGO functional analysis. Probes with a P-value <0.001 from this gene list were used for clustering analysis. Hierarchical clustering was used to cluster the probes and slides and the results are shown in a heatmap. The R software package was used for this analysis (264). The microarray data were uploaded to NCBI Gene expression omnibus (GSE19140). For probes that were not annotated, full length transcripts were identified where possible by querying microarray probe sequences against the bovine genome (Btau5.1) using NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

4.2.7. Gene function and pathway identification

Functional analysis was performed using MetaCore (GeneGo Inc), and GSEA. Gene lists containing differentially expressed genes for each comparison tested in the ANOVA were used for analyses with MetaCore to identify significantly enriched pathway maps. All entities in the microarray were used for GSEA analysis. Twenty pathway maps exported from MetaCore that were predicted to be enriched were submitted for analysis (Additional file 2). GSEA ranks genes based on the correlation between their expression and the class distinction. Given an *a priori* defined set of genes, GSEA determines whether the members of the gene set are randomly distributed throughout the ranked gene list or are primarily found at the top or bottom of the list. An enrichment score is calculated (ES) that reflects the degree to which the gene set is overrepresented. The statistical significance (nominal P-value) of the ES is estimated using a phenotype based permutation test. The estimated significance is adjusted for multiple hypothesis testing by normalising the enrichment score (NES) and then calculating the false discovery rate (FDR) corresponding to each NES. The GSEA user manual recommends an FDR cut-off of 25% for significant enrichment of a particular gene set using the phenotype permutation based method.

4.2.8. cDNA Synthesis

One µg of an RNA sample was used for cDNA synthesis using the Invitrogen Superscript III Supermix kit (Invitrogen Corp. Carlsbad, CA.Cat# 11752). Total RNA was transcribed according to the manufacturer's instructions using 27 µM of random pentadecamers. Briefly, RNA and random primers were mixed and denatured at 65°C for 5 min, followed by 1 min on ice. Annealing buffer and Superscript/RNase was added to samples and incubated for 10 minutes at 25°C (primer annealing), followed by 50 min at 50°C and 5 min at 85°C to inactivate the enzyme. Reverse transcription controls were performed, whereby the above process was completed without the addition of superscript enzyme.

4.2.9. Quantitative Real-time PCR

Real-time PCR using the Roche Lightcycler 480 was performed using the Roche real-time PCR master mix (Lightcycler 480 Probes Master) in combination with Roche Universal Probe Library (UPL) assays. Assays were designed using Roche UPL design software (Roche Diagnostics, Mannheim, Germany. Cat# 04887301001 and 04683633001). All assays were designed to span an intron-exon boundary, to prevent amplification of DNA during real-time PCR.

The PCR reaction volume was 10 µL, consisting of 0.5 µM of each primer and 0.1 µM of probe. Standard cycling conditions were used [95°C for 10 min, (95°C for 10 sec, 60°C for 30 sec) x 50 cycles, 40°C for 40 sec]. All reactions were performed with a 1/10 dilution of cDNA.

Each PCR experiment included reactions in which the cDNA template was replaced by water, and reactions omitting reverse transcriptase were used as negative controls. Triplicate measurements were performed for all samples and standard curves. The percent coefficient of variation (%CV) for Cqs was calculated for each sample. All samples for each gene were run on the same plate.

4.2.10. Absolute quantification

The Roche Lightcycler 480 software was used to perform absolute quantification analysis of gene expression using the standard curve second derivative maximum analysis method, which is a non-linear regression line method. A six point standard curve of serial dilutions of

a cDNA sample (from an unrelated endometrial sample) was used, with a starting concentration of 1 and final concentration of 1.6E-03 relative units.

4.2.11. Relative quantification

The Roche Lightcycler 480 Software was used to perform quantification using the 'advanced relative quantification analysis' algorithm. Two endogenous control (*SUZ12* and *ZNF131*) genes were used to normalize the data, taking the geometric mean of the normalized ratio of target gene to each reference gene. These genes have been previously demonstrated to be suitable for normalizing in endometrial tissue in the samples used in this study (265). A calibrator sample was then used as a control, whereby each calculated expression value was normalized to the calibrator sample.

The RT-PCR results were compared to those obtained using the microarray for several genes of interest. A previous publication on the same study had confirmed microarray results for several genes that were not differentially expressed according to microarray analysis (265). Gene expression of nine genes of interest *(ARG1, CXCL10, CXCL11, IDO, OAS2, OXTR, PTX3, SLC2A1*, and *SPP1*) was quantified for this study. Correlation coefficients were calculated for all genes to compare the calculated gene expression data from qRT-PCR and the microarray data.

4.3. Results

4.3.1. Differentially-expressed genes

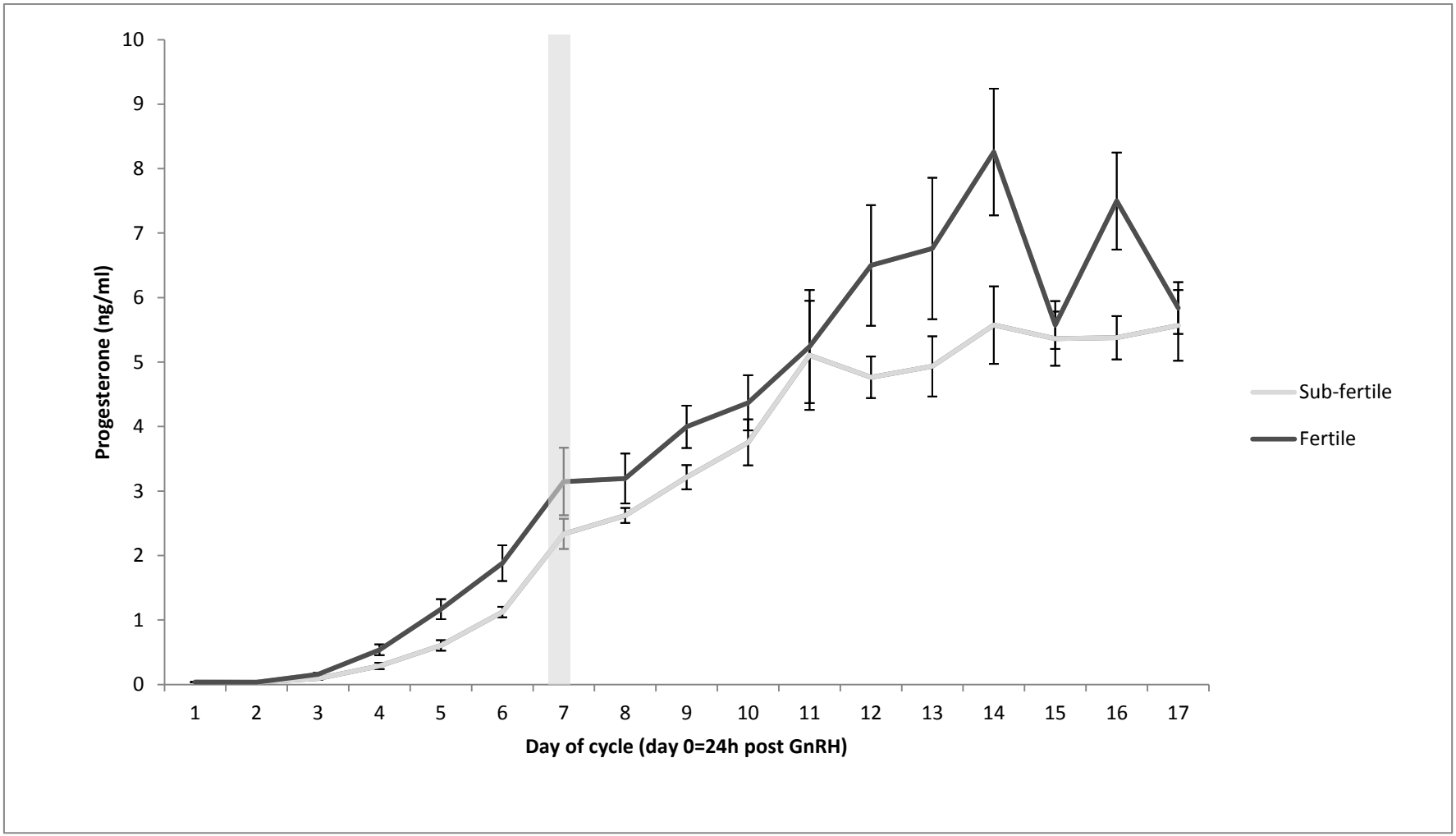
A total of 482 and 1,021 differentially-expressed transcripts (corrected P-value < 0.05) were identified in intercaruncular and caruncular tissue, respectively, in the fertile and sub-fertile dairy cow strains (Additional file 3).

4.3.2. Trophoblast growth, plasma progesterone concentration, and uterine fluid IFNτ concentration.

Embryo length at day 17 of pregnancy was not affected by dairy cow strain. However, there was greater variability in the length of embryos recovered from the sub-fertile strain of dairy cow (mean±SEM fertile: 21±3cm; sub-fertile: 28±9cm). Plasma progesterone concentration was not significantly different between the fertile and sub-fertile groups at

day 17 of pregnancy. However, average daily progesterone during the experimental period (day 1-17 of pregnancy) was greater ($P < 0.05$) in fertile cows (fertile: 3.8 ng/mL; sub-fertile: 3.0 ng/mL, SED 0.37 ng/mL: (Figure 12). There was no significant interaction effect ($P=0.53$) of strain with day. Uterine fluid IFNτ concentration did not differ in fertile and sub-fertile dairy cow strains (Table 7, t-test, P-value=0.41).

Figure 12. Daily plasma progesterone concentration (ng/ml) during the oestrous cycle leading to endometrial tissue collection in fertile (n=6) and sub-fertile strain dairy cows (n=6). The data represent the raw means (+/- SEM). The time of embryo transfer is indicated by the grey bar. Average daily progesterone during the experimental period (day 1-17 of pregnancy) was greater ($P < 0.05$) in fertile cows (fertile: 3.8 ng/mL; sub-fertile: 3.0 ng/mL, SED 0.37 ng/mL). There was no significant interaction effect ($P=0.53$) of strain with day.


Progesterone (ng/ml)
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16
17
Day of cycle (day 0=24h post GnRH)
Sub-fertile
Fertile

Table 7 Embryo weight and length, IFNτ, and P4 data. The average, standard error and t-test p-value for weight (mg) and length (cm) of embryos, IFNτ total (O.D units per 40µg protein), and P4 (ng/mL) in fertile and sub-fertile dairy cows

.

	Fertile		Sub-fertile		
	Average	**SE**	**Average**	**SE**	**T-test**
Weight	75.4	24.5	125.3	50.8	0.41
Length	21	3	28	9	0.7
IFNT total	68	22.4	106.9	39.3	0.41

4.3.3. Molecular and biological function and pathway analysis.

Functional analyses utilising GeneGO MetaCore and Gene Set Enrichment Analysis (GSEA) revealed several pathways that were significantly enriched in the comparison of fertile and sub-fertile animals. In caruncular endometrium, several pathway maps relating to the immune response were identified by GeneGO. Additionally, GSEA analysis revealed enrichment for several immune response pathways in the caruncular endometrium of fertile animals. In the intercaruncular endometrium, pathway maps relating to signal transduction and development processes were identified by GeneGO. There was no significant enrichment for pathways identified in intercaruncular endometrium using GSEA. Results from these analyses are presented in Table 8, Table 9,Table 10 and Table 11.

Table 8. Functional analysis of differentially expressed genes in caruncular endometrium. Significantly enriched functions identified through MetaCore functional analysis in the caruncular endometrium of fertile and sub-fertile animals.

GeneGO pathway maps (Caruncular endometrium)

Entrez GeneID (bovine)	ANOVA Corrected P-value	Fold Change	Gene symbol (human)	Gene name (human)	Object type
Signal transduction_PKA signaling P-value 9.7E-06 (up-regulated in sub-fertile)					
281793	1.48E-02	1.6	*GNAS*	GNAS complex locus	G alpha protein
505611	1.18E-03	2	*GNA13*	guanine nucleotide binding protein (G protein), alpha 13	G-alpha
788151	1.16E-03	1.7	*ADCY4*	adenylate cyclase 4	Generic enzyme
282459	1.05E-04	1.7	*PPP1R1B*	protein phosphatase 1, regulatory (inhibitor) subunit 1B	Generic binding protein
282322	1.88E-02	1.4	*PRKACA*	protein kinase, cAMP-dependent, catalytic, alpha	Protein kinase
536561	1.39E-02	1.3	*GSK3A*	glycogen synthase kinase 3 alpha	Protein kinase
Development_PDGF signaling via STATs and NF-kB P-value 4.6E-05					
512484	3.51E-03	1.2	*TYK2*	tyrosine kinase 2	Protein kinase
525246	2.02E-03	-1.6	*JAK2*	Janus kinase 2	Protein kinase
347700	5.06E-03	-2.5	*EIF2AK2*	eukaryotic translation initiation factor 2-alpha kinase 2	Protein kinase
510814	1.16E-03	-2.7	*STAT1*	signal transducer and activator of transcription 1, 91kDa	Transcription factor
504531	1.15E-02	1.6	*PIK3CD*	phosphoinositide-3-kinase, catalytic, delta polypeptide	Lipid kinase
281073	4.24E-02	1.2	*CHUK*	conserved helix-loop-helix ubiquitous kinase	Protein kinase
Immune response_Antiviral actions of Interferons P-value 5.25E-05 (down-regulated in sub-fertile)					
512484	3.51E-03	1.2	*TYK2*	tyrosine kinase 2	Protein kinase
347700	5.06E-03	-2.5	*EIF2AK2*	eukaryotic translation initiation factor 2-alpha kinase 2	Protein kinase
509855	8.04E-03	-1.8	*IRF9*	interferon regulatory factor 9	Transcription factor
510814	1.16E-03	-2.7	*STAT1*	signal transducer and activator of transcription 1, 91kDa	Transcription factor
529660	1.86E-03	-2.6	*OAS2*	2'-5'-oligoadenylate synthetase 2, 69/71kDa	Generic enzyme
282534	8.40E-03	-4.1	*HLA-DQA2*	major histocompatibility complex, class II, DQ alpha 2	Generic receptor
Immune response _IFN gamma signaling pathway P-value 1.3E-04 (down-regulated in sub-fertile)					
525246	2.02E-03	-1.6	*JAK2*	Janus kinase 2	Protein kinase
511779	5.79E-03	1.3	*MAP3K4*	mitogen-activated protein kinase kinase kinase 4	Protein kinase

534492	6.33E-03	1.2	*MAPK14*	mitogen-activated protein kinase 14	Protein kinase
504531	1.15E-02	1.6	*PIK3CD*	phosphoinositide-3-kinase, catalytic, delta polypeptide	Lipid kinase
510814	1.16E-03	-2.7	*STAT1*	signal transducer and activator of transcription 1, 91kDa	Transcription factor
509855	8.04E-03	-1.8	*IRF9*	interferon regulatory factor 9	Transcription factor
347700	5.06E-03	-2.5	*EIF2AK2*	eukaryotic translation initiation factor 2-alpha kinase 2	Protein kinase
				Immune response_IL-23 signaling pathway P-value 1.5E-04	
525246	2.02E-03	-1.6	*JAK2*	Janus kinase 2	Protein kinase
281073	4.24E-02	1.2	*CHUK*	conserved helix-loop-helix ubiquitous kinase	Protein kinase
504531	1.15E-02	1.6	*PIK3CD*	phosphoinositide-3-kinase, catalytic, delta polypeptide	Lipid kinase
510814	1.16E-03	-2.7	*STAT1*	signal transducer and activator of transcription 1, 91kDa	Transcription factor
512484	3.51E-03	1.2	*TYK2*	tyrosine kinase 2	Protein kinase
				Development_A2A receptor signaling P-value 2.6E-04 (up-regulated in sub-fertile)	
281793	3.20E-03	1.6	*GNAS*	GNAS complex locus	G alpha protein
282322	1.88E-02	1.4	*PRKACA*	protein kinase, cAMP-dependent, catalytic, alpha	Protein kinase
511779	5.79E-03	1.3	*MAP3K4*	mitogen-activated protein kinase kinase kinase 4	Protein kinase
504531	1.15E-02	1.6	*PIK3CD*	phosphoinositide-3-kinase, catalytic, delta polypeptide	Lipid kinase
534492	6.33E-03	1.2	*MAPK14*	mitogen-activated protein kinase 14	Protein kinase
281073	4.24E-02	1.2	*CHUK*	conserved helix-loop-helix ubiquitous kinase	Protein kinase
				Development_Leptin signaling via PI3K-dependent pathway P-value 4.2E-04 (up-regulated in sub-fertile)	
525246	2.02E-03	1.6	*JAK2*	Janus kinase 2	Protein kinase
504531	1.15E-02	-1.6	*PIK3CD*	phosphoinositide-3-kinase, catalytic, delta polypeptide	Lipid kinase
281073	4.24E-02	-1.2	*CHUK*	conserved helix-loop-helix ubiquitous kinase	Protein kinase
536561	1.39E-02	-1.3	*GSK3A*	glycogen synthase kinase 3 alpha	Protein kinase
282322	1.88E-02	-1.4	*PRKACA*	protein kinase, cAMP-dependent, catalytic, alpha	Protein kinase
281677	1.11E-03	-1.9	*CEBPA*	CCAAT/enhancer binding protein (C/EBP), alpha	Transcription factor
				Development_Beta-adrenergic receptors regulation of ERK	
281793	3.20E-03	1.6	*GNAS*	GNAS complex locus	G alpha protein
788151	1.16E-03	1.7	*ADCY4*	adenylate cyclase 4	Generic enzyme
281797	2.43E-03	-2.6	*GNGT2*	guanine nucleotide binding protein (G protein), gamma transducing activity polypeptide 2	G betta/gamma

530001	4.94E-02	1.8	*PIK3CG*	phosphoinositide-3-kinase, catalytic, gamma polypeptide	Lipid kinase
282322	1.88E-02	1.4	*PRKACA*	protein kinase, cAMP-dependent, catalytic, alpha	Protein kinase
282320	5.70E-03	-1.6	*PPP2CA*	protein phosphatase 2, catalytic subunit, alpha isozyme	Protein phosphatase
Development_Angiotensin signaling via STATs P-value 5.0E-04 (down-regulated in sub-fertile)					
281797	2.43E-03	-2.6	*GNGT2*	guanine nucleotide binding protein (G protein), gamma transducing activity polypeptide 2	G betta/gamma
282320	5.70E-03	-1.6	*PPP2CA*	protein phosphatase 2, catalytic subunit, alpha isozyme	Protein phosphatase
512484	3.51E-03	1.2	*TYK2*	tyrosine kinase 2	Protein kinase
525246	2.02E-03	-1.6	*JAK2*	Janus kinase 2	Protein kinase
510814	1.16E-03	-2.7	*STAT1*	signal transducer and activator of transcription 1, 91kDa	Transcription factor
509855	8.04E-03	-1.8	*IRF9*	interferon regulatory factor 9	Transcription factor
Immune response_IL-22 signaling pathway P-value 5.8E-04 (down-regulated in sub-fertile)					
282534	8.40E-03	-4.1	*HLA-DQA2*	major histocompatibility complex, class II, DQ alpha 2	Generic receptor
407098	6.50E-03	1.2	*CD4*	CD4 molecule	Generic receptor
512484	3.51E-03	1.2	*TYK2*	tyrosine kinase 2	Protein kinase
525246	2.02E-03	-1.6	*JAK2*	Janus kinase 2	Protein kinase
534492	6.33E-03	1.2	*MAPK14*	mitogen-activated protein kinase 14	Protein kinase
510814	1.16E-03	-2.7	*STAT1*	signal transducer and activator of transcription 1, 91kDa	Transcription factor

Table 9. Functional analysis of differentially expressed genes in intercaruncular endometrium. Significantly enriched functions identified through MetaCore functional analysis in the intercaruncular endometrium of fertile and sub-fertile animals.

GeneGO pathway maps (Intercaruncular endometrium)

Normal and pathological TGF-beta-mediated regulation of cell proliferation P-value 2.0E-06 (up-regulated in sub-fertile)					
540106	2.44E-03	2.2	*PDGFB*	platelet-derived growth factor beta polypeptide (simian sarcoma viral (v-sis) oncogene homolog)	Receptor ligand
282382	2.81E-03	3.5	*TGFBR1*	transforming growth factor, beta receptor 1	Receptor with enzyme activity
516010	2.65E-04	1.2	*SMAD2*	SMAD family member 2	Transcription factor
504357	4.54E-03	1.4	*AXIN1*	axin 1	Generic binding protein
539003	2.27E-03	1.8	*CTNNB1*	catenin (cadherin-associated protein), beta 1, 88kDa	Generic binding protein
Cell cycle_Regulation of G1/S transition P-value 1.0E-04 (up-regulated in sub-fertile)					
282382	2.81E-03	3.5	*TGFBR1*	transforming growth factor, beta receptor 1	Receptor with enzyme activity
516010	2.65E-04	1.2	*SMAD2*	SMAD family member 2	Transcription factor
518880	1.51E-02	1.5	*RBX1*	ring-box 1, E3 ubiquitin protein ligase	Generic enzyme
510618	9.76E-03	1.3	*CDK4*	cyclin-dependent kinase 4	Protein kinase
Development_HGF-dependent inhibition of TGF-beta-induced EMT P-value 1.1E-03 (up-regulated in sub-fertile)					
282382	2.81E-03	3.5	*TGFBR1*	transforming growth factor, beta receptor 1	Receptor with enzyme activity
535916	3.73E-03	-1.7	*SMAD7*	SMAD family member 7	Generic binding protein
516010	2.65E-04	1.2	*SMAD2*	SMAD family member 2	Transcription factor
Signal transduction_Activin A signaling regulation P-value 1.2E-03 (down-regulated in sub-fertile)					
507663	2.50E-03	-1.2	*NCOR1*	nuclear receptor corepressor 1	Generic binding protein
535916	3.73E-03	-1.7	*SMAD7*	SMAD family member 7	Generic binding protein
516010	2.65E-04	1.2	*SMAD2*	SMAD family member 2	Transcription factor
Signal transduction_PKA signaling P-value 1.1E-03					
535017	3.17E-03	-3.2	*ADCY8*	adenylate cyclase 8 (brain)	Generic enzyme
505611	1.18E-03	2.5	*GNA13*	guanine nucleotide binding protein (G protein), alpha 13	G-alpha

Table 10. Summary statistics for the functional analysis of immune response genes using Gene Set Enrichment Analysis. Gene sets that were significantly enriched for in the comparison of fertile animals. Gene sets with a FDR<25% were considered significantly enriched.

Gene set enrichment analysis

Pathway maps GSEA enriched in fertile (caruncular endometrium)	**ES**	**NES**	**NOM p-val**	**FDR q-val**
Development GM-CSF signaling	0.500	1.755	<0.001	0.024
Immune response Signaling pathway mediated by IL-6 and IL-1	0.543	1.665	<0.001	0.107
Immune response classical complement pathway	0.550	1.571	<0.001	0.143
Immune response IL-6 signaling pathway	0.491	1.522	<0.001	0.216
Immune response alternative complement pathway	0.469	1.450	0.074	0.259
Immune response lectin induced complement pathway	0.509	1.450	0.073	0.225
Immune response TREM signaling pathway	0.407	1.417	<0.001	0.231
Immune response TLR3 and TLR4 induce TICAM1 specific signaling pathway	0.498	1.412	0.051	0.211
Pathway maps GSEA enriched in sub-fertile (caruncular endometrium)	**ES**	**NES**	**NOM p-val**	**FDR q-val**
Development WNT signaling pathway degradation of beta catenin in absence of WNT	-0.638	-1.644	<0.001	0.090

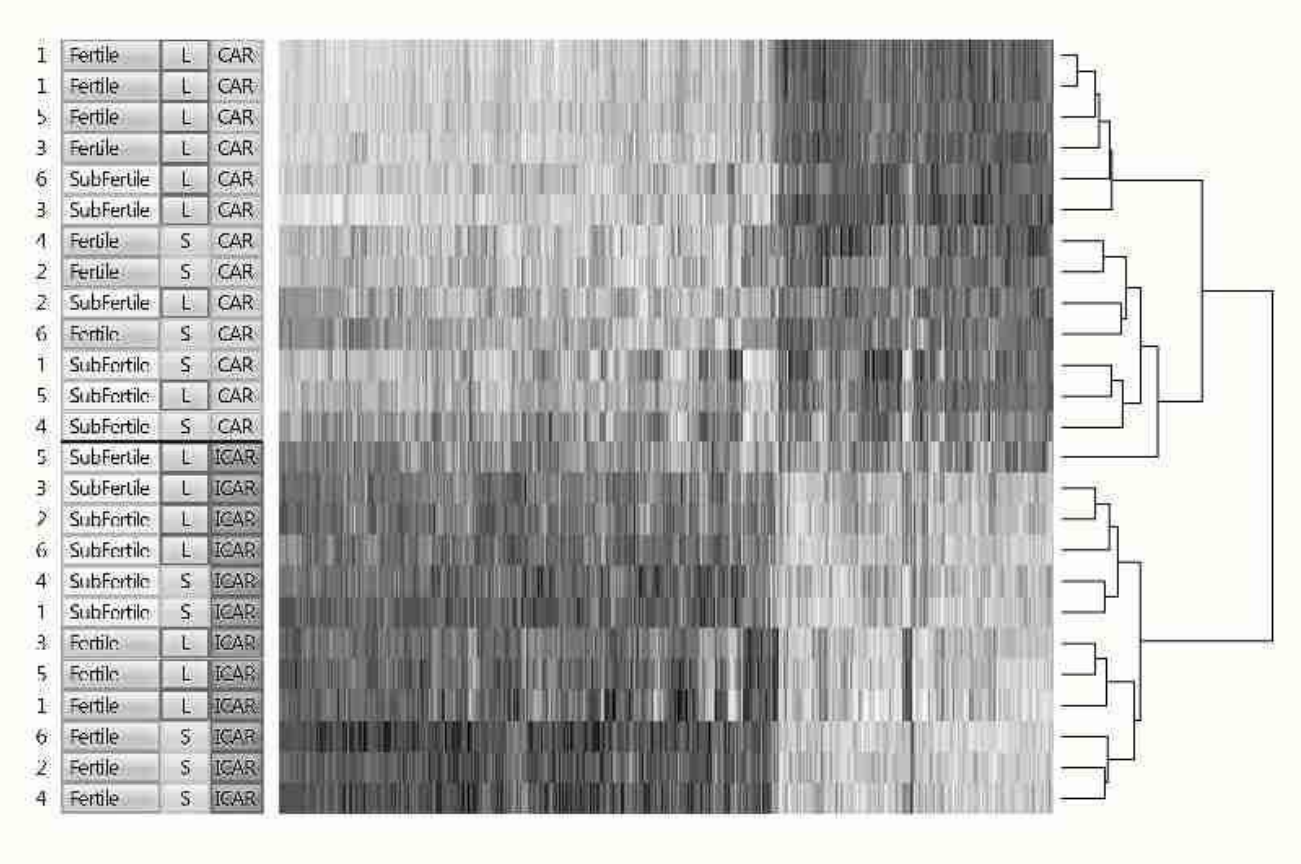
Table 11. Functional analysis of immune response genes using Gene Set Enrichment Analysis. Gene sets that were significantly enriched in the caruncular endometrium in the comparison of fertile animals. Gene sets with a FDR<25% were considered significantly enriched. Core enrichment genes are in bold. These genes contribute most to the enrichment result.

Gene set enrichment analysis
Pathway maps GSEA enriched in fertile (caruncular endometrium)
Development GM-CSF signaling
CCND, MAP2, RELA, PIM1, CCL2, MYC, JAK2, CSF2, LYN, NFKB2, MCL1, *AKT2, PIK3R1, BCL2L1, STAT5A, GNB2L1, MAP2K2, NFKBIA, CISH, AKT3, IKBKB, CASP3, STAT5B, SOS2, CREB1, EGR1, SOS1, STAT3, MAPK3, BAD, MAPK1, SHC1, CHUK, PIK3CD, PIK3R2, YWHAZ*
Immune response Signaling pathway mediated by IL-6 and IL-1
MAP2K1, RELA, IL6, SOCS1, IRAK1, NFKBIB, RELB, NFKB2, *IRAK2, IL1A, MAP2K2, NFKBIA, ILST, IKBKB, SOS2, ECSIT, MAP3K1, SOS1, MAPK3, MAP2K14, IL1R1, MAPK1, IL6R, SHC1, CHUK*
Immune response classical complement pathway
C3, CFI, SERPING1, C2, C4A, C1S, C7, C5, C6, *C5AR1, C3AR1, CRP, C1QB, ITGB2, C4BPA, C1QA, CLU, C8A, C8B, C9, CD55, CR2*
Immune response IL-6 signaling pathway
MAP2K1, RELA, IL6, RELB, NFKB2, NR3C1*, JUND, JUN, ADAM10, MAP2K2, ILST, CEBPD, SOS2, CREB1, SOS1, FOS, STAT3, MAPK3, ADAM17, MAPK1, IL6R, SHC1*
Immune response alternative complement pathway
C3, CFI, CFB, C7, C5, C6*, C5AR1, C3AR1, ITGB2, CLU, C8A, C8B, C9, CD55, CR2,CFP*
Immune response lectin induced complement pathway
C3, CFI, SERPING1, C2, C4A, C7, C5, C6, MASP1, C5AR1, MBL2, *C3AR1, ITGB2, C4BPA, CLU, C8A, C8B, C9, MASP2, CD55, CR2*
Immune response TREM signaling pathway
MAP2K1, RELA, IL6, IL12B, CCl2, IL8, BCL10, CSF2, TLR4, NFKBIB, PIK3CA, TREM1*, AKT2, IL2, PIK3R1, JUN, ITPR1, TLR2, IPR3, PPP3CB, UBA52, CCL3, MAP2K2, NFKBIA, AKT3, IL1B, IKBKB, RPS6KA1, SOS1, FOS, TNF, MAPK3, IKBKG, ITPR2, TYROBP, BAD, MAPK1, CHUK, PIK3R3, PIK3CD, PIK3R2, PPP3CA, PLCG1*
Immune response TLR3 and TLR4 induce TICAM1 specific signaling pathway
RELA, RIPK3, TLR4, TLR3, RELB, CD14, NFKB2*, TICAM2, TICAM1, BECN1, IKBKG, LY96, IFNA16, RIPK1, TIRAP*
Pathway maps GSEA enriched in sub-fertile (caruncular endometrium)
Development WNT signaling pathway degradation of beta catenin in absence of WNT
TLE3, CUL1, UBA52, TCF7L1, GSK3B, APC, ***RBX1, CSNK1D, HDAC1, SKP2, DAB2, AXIN1, TLE2, CTNNB1***

4.3.4. Clustering analysis

The microarray data for each sample was clustered initially into caruncular and intercaruncular tissue types. The caruncular tissue then clustered into large and small embryo size, and within each of these, fertile and sub-fertile cows (with two exceptions). The intercaruncular tissue clustered into sub-fertile and fertile cows, and then within each of these, large and small embryo size. The results of this analysis are presented in Figure 13.

Figure 13. Clustering analysis heatmap. Caruncular (CAR), intercaruncular (ICAR), large embryo (L), small embryo (S).


1 Fertile L CAR
1 Fertile L CAR
5 Fertile L CAR
3 Fertile L CAR
6 SubFertile L CAR
3 SubFertile L CAR
1 Fertile S CAR
2 Fertile S CAR
2 SubFertile L CAR
6 Fertile S CAR
1 SubFertile S CAR
5 SubFertile L CAR
4 SubFertile S CAR
5 SubFertile L ICAR
3 SubFertile L ICAR
2 SubFertile L ICAR
6 SubFertile L ICAR
4 SubFertile S ICAR
1 SubFertile S ICAR
3 Fertile L ICAR
5 Fertile L ICAR
1 Fertile L ICAR
6 Fertile S ICAR
2 Fertile S ICAR
4 Fertile S ICAR

4.3.5. Relative quantitative real-time PCR confirmation of microarray results

Relative qRT-PCR was used to confirm microarray results. Candidate genes were chosen to represent genes with known functional importance in pregnancy, and genes that were identified as being differentially expressed in the microarray analysis. Expression levels of nine genes of interest (*ARG1, CXCL10, CXCL11, IDO, OAS2, OXTR, PTX3, SLC2A1*, and *SPP1*) were quantified, and the correlation between qRT-PCR and microarray calculated (Table 12). All qRT-PCR gene expression data were positively correlated with microarray data and the magnitude of expression differences were consistent between the two measures of gene expression.

Table 12. RT-PCR primers and correlation with microarray data. The correlation coefficient between qRT-PCR and microarray data. Primer Sequences and UPL probe used for qRT-PCR assay.

	Primer Sequences					
	Forward	**Reverse**	**Probe**	**Exon boundary**	**Correlation**	**P-value**
OXTR	CGTGCAGATGTGGAGTGTCT	TTGCAGCAGCTGTTGAGG	UPL#162	exon1/2	0.32	0.06
OAS2	TGGACGGTCAACTACAGTTTTG	CTGGGTCCAAGATCACAGG	UPL#69	exon9/10	0.78	<0.001
SPP1	AAGTTCCGCCGATCTAACG	CCTCACTCTCTATGTGTGATGTGAA	UPL#82	exon5/6	0.71	<0.001
CXCL11	TGCTGCAATTGTTCAAGGTT	TCTGCCACTTTGACTGCTTTT	UPL#81	exon1/2	0.75	<0.001
IDO	ACGTAGGCTTTGCTCTTCCA	GAGATCCAGGCATCATAAGGA	UPL#65	exon1/2	0.76	<0.001
PTX3	TTTGGACAACGAAATAGACAATG	TCGGAGTTCTCACGACTGC	UPL#58	exon1/2	0.88	<0.001
SLC2A1	GACCCTGCACCTCATAGGC	CCTACCTGAGCATTGTGG	UPL#37	exon8/9	0.82	<0.001
CXCL10	TGAAAGCAATTAACAAGCAAAGG	CACAGGGAAACAGTTGTAGAGGT	UPL#126	exon3/4	0.97	<0.001
ARG1	ATGTGGACCCTGGGGAAC	ATGTTTCTTCCATCACCTTGC	UPL#48	exon5/6	0.8	<0.001
Reference genes						
SUZ12	GAACACCTATCACACACATTCTTGT	AGTGGACACAACCGCCTCTA	UPL#150	exon13/14		
ZNF131	AGAAAGAAGCTTTATGAATGTCAGG	CTGGTGATACACACTGGAGATAAAC	UPL#33	exon6/7		

4.4. Discussion

The hypothesis that endometrial gene expression patterns differ between dairy cow strains classified as fertile and sub-fertile was tested. An unsupervised approach was undertaken to identify pathways enriched in the dataset, irrespective of their established role in early pregnancy. In addition, the hypothesis that endometrial gene expression patterns in biological processes associated with fertility differ between dairy cow strains classified as fertile and sub-fertile was tested. Functional analysis revealed enrichment for several pathways. Some of these pathways have been characterized as being important regulators of early pregnancy events, while others are less well established in the context of early pregnancy in the ruminant.

Pathways implicated in the process of immune tolerance to the embryo were enriched in fertile cows, as were genes that have roles in preventing luteolysis and embryo signalling through the secretion of histotroph. These results provide evidence that endometrial physiology may be contributing to the inferior reproductive performance of the sub-fertile dairy cow.

4.4.1. Immune response to pregnancy

Functional analysis revealed enrichment for several pathways relating to the immune response in the comparison of fertile and sub-fertile animals in the caruncular endometrium. Pathways that regulate the immune response were down-regulated in sub-fertile animals, suggesting that down-regulation of this response could be a contributing factor to their sub-fertile phenotype. Co-ordinated modulation of the immune response is essential for successful pregnancy. Genes involved in the immune response to pregnancy and in particular those stimulated by interferons are common features of several recently published studies investigating gene expression during the pre-implantation period in cattle (191, 193, 195, 204, 213). In addition, enrichment of pathways relating to the immune response were restricted to the caruncular endometrium, consistent with a previous study comparing significant biological functions in caruncular and intercaruncular endometrium (193).

The contribution of the immune response to pregnancy success has recently been highlighted in several studies in *Bos taurus*. Salilew-Wondim et al 2010 (217) investigated differences in endometrial gene expression at day 7 of pregnancy in animals that were receptive or non-receptive to pregnancy. Results indicated early pregnancy loss may be the result of immune rejection, as evidenced by a decrease in expression of immune system related genes in the receptive endometrium. Beltman et al 2010 (266) also noted the importance of the immune response in early embryo loss. In their study, endometrial gene expression was investigated in relation to the production of normal or retarded embryos at day 7 of pregnancy. Genes involved in the immune response were differentially expressed in animals that produced retarded or normal embryos.

There are several mechanisms that enable the immunologically foreign embryo to survive in the maternal environment, including the expression of genes that promote immune tolerance to the embryo (267, 268). In this experiment, embryos were transferred from donor oocytes and, therefore, the embryo was completely foreign to the maternal system rather than being only semi-allogenic. This could have implications within the context of the immune response to pregnancy. Several genes involved in this process were down-regulated in the sub-fertile cows, including the amino acid metabolizing enzymes *ARG1* and *IDO.* These genes are up-regulated during pregnancy in cattle (263), where it is hypothesized they are acting to prevent an adverse immune response. In mice, expression of IDO prevents an adverse immune response to the embryo through depleting the microenvironment of essential amino acids, and thereby suppressing T-cell activity (269). Cells expressing IDO are protected against possible tryptophan starvation through expression of TTS/WARS (270). This gene is up-regulated during pregnancy in cattle (263). IDO expressing cells may be protected against tryptophan starvation through the generation of a reservoir of tryptophan via the formation of tryptophan-tRNA complexes (270).

Other differentially expressed genes that may be regulating the immune response during early pregnancy include the chemokines *CCL16, CXCL10 and CXCL11*. The chemokine *CCL16* was up-regulated in sub-fertile dairy cows. This chemokine has been reported to enhance cytotoxicity of T cells and stimulate the production of pro-inflammatory cytokines in mice (271-273). Additionally, the chemokines *CXCL10* and *CXCL11* were down-regulated in the sub-fertile cows.

These chemokines are up-regulated in response to pregnancy, and are believed to attract immune tolerance-promoting uterine natural killer cells to the site of implantation in humans (216, 219, 221, 274). These chemokines are also hypothesized to attract the trophoblast to the endometrium and CXCL10 promotes adhesive activity of the trophoblast, through stimulation of integrin expression in ruminant species (112, 194, 275-277). Chemokines in the uterus may have multiple roles in the bovine uterus, including promoting tolerance to the embryo and regulating attachment of the embryo to the endometrium.

Several genes of the innate immune response were differentially expressed in fertile and sub-fertile animals. Up-regulation of innate immune system genes during early pregnancy may function to protect the endometrium and embryo from possible infection during a time of local immune suppression. The canonical pathways for the complement system were some of the most significantly enriched pathways in the GSEA functional analysis. Several genes of the complement system were down-regulated in sub-fertile animals. Additionally, the TLR3 and TLR4 pathway was significantly enriched in the fertile animals. Genes that prevent immune system attack on the embryo were also down-regulated in the sub-fertile cows. For example, pentraxin 3 (*PTX3*), which acts to prevent complement-mediated attack and is up-regulated in response to pregnancy in humans (240), was down-regulated in sub-fertile dairy cows. Interestingly, these genes were only down-regulated in the caruncular endometrium.

In general, expression of genes regulating the immune response were more divergent in the caruncular endometrium, with some genes differing only in the caruncular endometrium. This could be due to the differing roles of the two tissues and the difference in immune cell populations present in the two tissue types in cattle (278).

The differential expression of these genes suggests embryo loss in sub-fertile dairy cows may be caused, at least in part, by insufficient tolerance of the maternal immune system to the pre-implantation allogenic embryo.

4.4.2. Pregnancy recognition and embryo signalling

Genes expressed in response to the presence of the embryo, and genes implicated in regulating maintenance of the corpus luteum, were down-regulated in the sub-fertile cows.

During early pregnancy, the embryo signals its presence to the maternal system to prevent luteal regression and establish pregnancy. The primary signalling molecule used by the embryo is IFNτ, which signals via the JAK-STAT pathway by binding to the interferon receptors present on the endometrium (84). Although the concentration of IFNτ in the uterine fluid did not differ between fertile and sub-fertile dairy cows, genes up-regulated in response to IFNτ signalling, were differentially expressed. Several pathways regulating the response to inteferons were enriched for in the fertile caruncular endometrium. In addition, several genes that are stimulated by interferons were down-regulated in sub-fertile animals including: *IFIT3, IFITM1, IFI44, IFI44L, ISG20*, and *RSAD2*.

The most significantly enriched pathway in the caruncular endometrium was the signal transduction PKA signalling pathway. This pathway was also significantly enriched for in the intercaruncular endometrium. PKA activation regulates a diverse range of processes including oestrogen actions (279). Activators of PKA can enhance transcriptional activity of genes in response to oestrogen (279). Several genes are activated by oestrogen in the ruminant uterus, including the luteolytic OXTR (40, 280, 281), which was up-regulated in sub-fertile animals. Therefore, greater PKA activation in sub-fertile animals may be influencing pregnancy outcome through enhancing transcription of genes such as *OXTR* in response to oestrogen.

The presented expression profile suggests a dampened response to IFNτ signalling and differential regulation of eicosanoid production during early pregnancy may contribute to early embryo loss in the sub-fertile dairy cow. Differential regulation of transcription in response to interferon could be the result of differences in genetically or epigenetically regulated transcriptional efficiency in this pathway.

Several pathways identified as being enriched in the functional analysis may regulate the production and transport of histotroph to the embryo. During early pregnancy and the oestrous cycle the endometrium is under the influence of the steroid hormone progesterone. Progesterone concentration was greater in fertile cows across the 17 days. Under the influence of progesterone, the endometrium undergoes structural changes, including remodelling of the extracellular matrix, and changes in the proteins expressed on its luminal surface, such as adhesion molecules of the glycocalyx (53). Additionally, the

endometrium expresses chemotactic molecules to attract the trophoblast (256). In ruminant species, progesterone concentration can affect nourishment of the embryo through modification of endometrial gland number and function, which leads to alterations in histotroph secretion and content (101). Histotroph secretions are vital for embryo growth and development as well as providing a communication channel to the embryo. Greater progesterone concentrations are also associated with greater embryo growth in cattle (21). Several studies have implicated greater progesterone concentration during early pregnancy in promoting pregnancy success (64, 77, 282). However, the difference in progesterone between fertile and sub-fertile dairy cows in this study was small compared with studies where progesterone concentration at day 3 was elevated to concentrations typically reached at later stages of the cycle.

Pathways regulated by TGF-beta including the 'Activin A' signal transduction pathway, were enriched for in the intercaruncular endometrium. These pathways were up-regulated in sub-fertile animals. Consistent with this finding, *FST*, an antagonist of activin and other TGF-beta ligands was down-regulated in sub-fertile dairy cows (230). *FST* also regulates myostatin, a secreted TGF-beta family ligand that is down-regulated during early pregnancy (263). Myostatin may be an important regulator of glucose availability to the embryo, through regulation of glucose in endometrial histotroph secretions, and, later, for foetal development, through regulation of placental glucose transport (232-234). The glucose transporter *SLC2A1* was down-regulated in the caruncular tissue of sub-fertile dairy cows.

The differential transcriptional profile of the fertile and sub-fertile dairy cow strains indicates that failure to provide an optimal uterine environment for embryonic growth and development may contribute to the inferior reproductive performance of the sub-fertile dairy cow strain.

4.5. Conclusion

Genes essential for the successful establishment of pregnancy are differentially expressed in the endometrium of fertile and sub-fertile dairy cow strains. Genes implicated in the process of immune tolerance to the embryo, and those that have roles in preventing luteolysis and supporting embryo growth and development, were down-regulated in the sub-fertile cows.

Results suggest that endometrial physiology contributes to the poor reproductive performance of the post-ovulatory sub-fertile dairy cow.

Chapter 5. GENOME-WIDE DNA METHYLATION PROFILING OF ENDOMETRIAL TISSUES

This chapter was submitted to Biology of Reproduction as:

WALKER C.G., LITTLEJOHN M., MEIER S., ROCHE J.R., MITCHELL M.D: DNA methylation is correlated with gene expression during early pregnancy in Bos taurus.

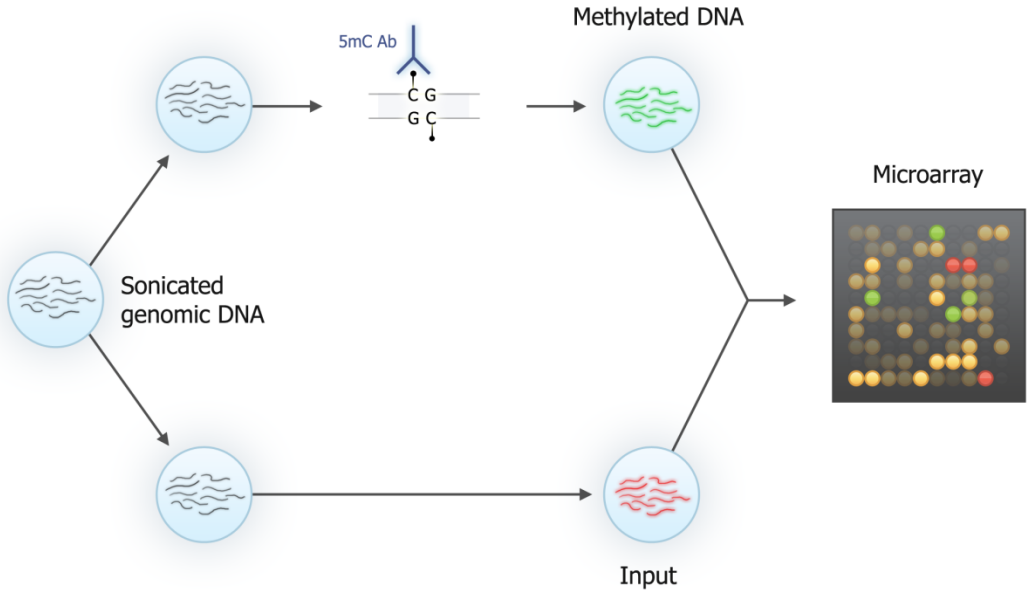
I developed the methods, performed the experimental work and statistical analysis and wrote the manuscript. The other authors contributed to the development of methods and experimental design and critical analysis of the manuscript.

5.1. Background

Synchronous development of the embryo and a receptive endometrium is critical to the establishment and maintenance of pregnancy (60). At least 30% of human pregnancies are lost prior to implantation (283) and it is estimated that up to 80% of pregnancy losses in dairy cattle occur during the pre-implantation period (5). Previous studies have indicated large differences in the endometrial transcriptional profiles of pregnant and non-pregnant dairy cows (191, 195, 263), animals with divergent fertility phenotypes (284), and animals pregnant with embryos produced from somatic cell nuclear transfer or in vitro fertilization compared with artificial insemination (204). A role for DNA methylation in regulating the expression of genes involved in uterine receptivity has recently been highlighted (109, 285). Additionally, aberrant DNA methylation in the endometrium may contribute to reproductive disorders and/or infertility (147, 286-289). For example, E-cadherin is down-regulated in the endometrium of some infertile women and is down-regulated in a non-receptive endometrial cell line (109, 146). Inhibiting methylation of E-cadherin in a non-receptive human endometrial cell line results in up-regulation of E-Cadherin and in the cell line becoming receptive. Another gene regulated by methylation, *HOXA10*, is expressed in the endometrium and is involved in regulating endometrial receptivity. Down-regulation of *HOXA10* in women with endometriosis has recently been associated with aberrant methylation (147).

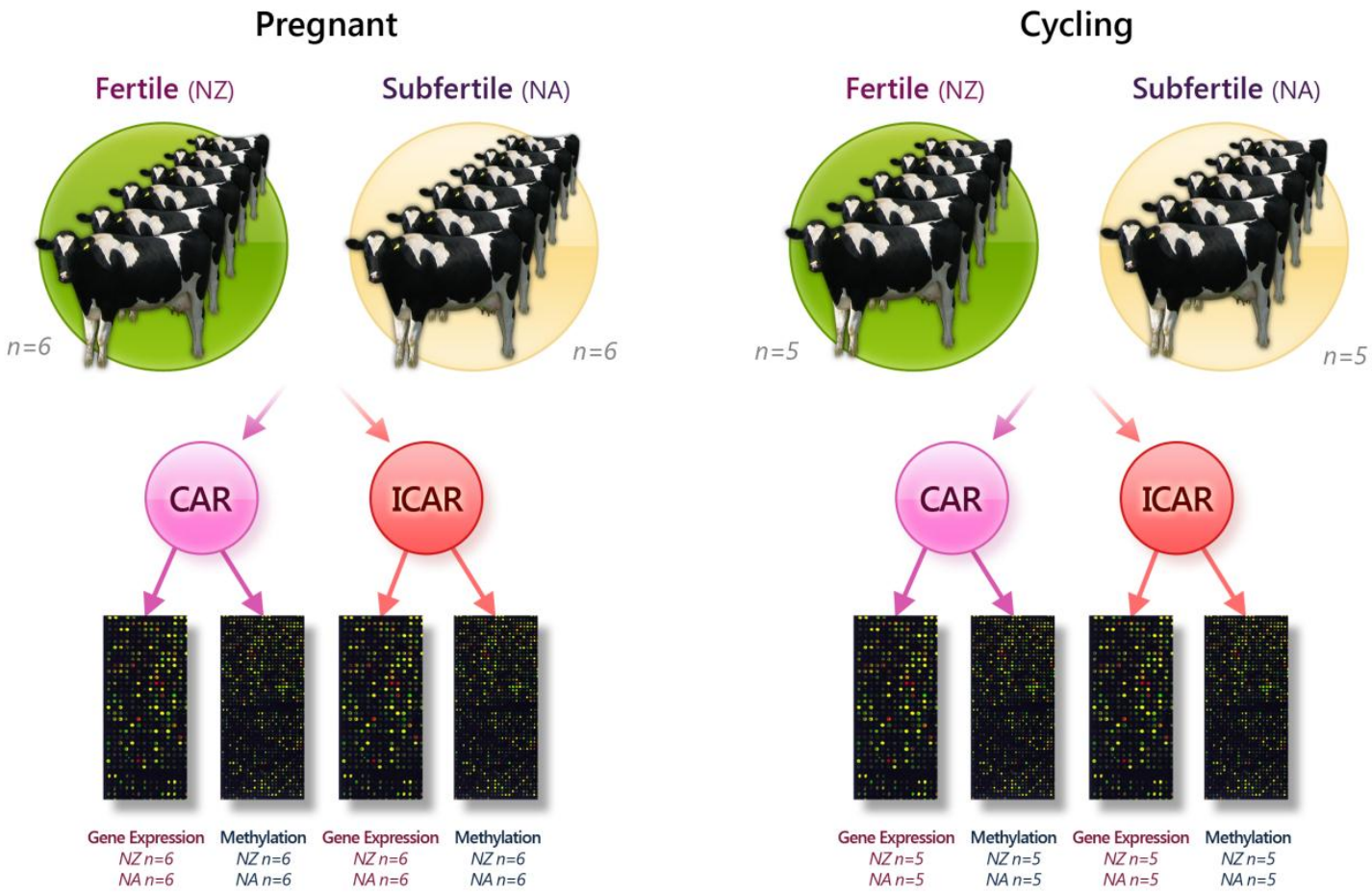
Genome-wide DNA methylation can be quantified in a high-throughput manner using methylated DNA immuno-precipatation (MeDIP) and microarrays (290). A DNA sample is divided into two aliquots and one sample is immuno-precipitated with an antibody that binds methylated cytosine, enriching the sample with methylated DNA only. The enriched sample and non-enriched sample are then labelled with different dyes, hybridised to a microarray, and the intensities quantified to determine methylation state (Figure 14).

Figure 14. Methylated DNA immuno-precipitation (MeDIP) process. A DNA sample is divided into two aliquots and one sample is immuno-precipitated with an antibody that binds methylated cytosine, enriching the sample with methylated DNA only. The enriched sample and non-enriched sample are then labelling with different dyes, hybridised to a microarray and the intensities quantified to determine methylation. state


5mC Ab
Methylated DNA
Microarray
Sonicated
genomic DNA
Input

It was hypothesised that DNA methylation regulates critical gene expression in the uterus during early pregnancy. To test this, DNA methylation was quantified in the bovine uterus at 415,000 sites in the genome in samples that had been previously assessed for genome-wide gene expression profiles (Figure 15). Gene expression was then tested for correlation to DNA methylation in probes that could be mapped to within annotated genes or 10kb 5' of genes to identify gene expression potentially regulated by DNA methylation.

Figure 15. Experimental design. All animals had caruncular (CAR) and intercaruncular (ICAR) endometrial tissues samples at day 17 of pregnancy or the oestrous cycle. RNA and DNA were extracted and DNA methylation and gene expression quantified using Agilent microarrays.


Pregnant
Fertile (NZ)
Subfertile (NA)
n=6
n=6
CAR
ICAR
Gene Expression
NZ n=6
NA n=6
Methylation
NZ n=6
NA n=6
Gene Expression
NZ n=6
NA n=6
Methylation
NZ n=6
NA n=6
Cycling
Fertile (NZ)
Subfertile (NA)
n=5
n=5
CAR
ICAR
Gene Expression
NZ n=5
NA n=5
Methylation
NZ n=5
NA n=5
Gene Expression
NZ n=5
NA n=5
Methylation
NZ n=5
NA n=5

5.2. Methods

5.2.1. Animals

All procedures were undertaken with the approval of the Ruakura Animal Ethics Committee (Hamilton, New Zealand). The oestrous cycles of 24 lactating dairy cows were synchronized (at 58.8 (SEM 3.77) and 60.2 (SEM 1.51) days post calving in dairy cows of sub-fertile and fertile strains, respectively) and 14 received a single embryo transferred on day 7 of the oestrous cycle. The oestrous cycles of the animals were synchronized using a controlled intra-vaginal drug-release device containing progesterone (1.38 g, CIDR-B™ Pfizer Animal Health Group, Auckland, New Zealand) and 2 ml oestradiol benzoate i.m (2 mg, CIDIROL Bomac Laboratories Limited, Auckland, New Zealand) for 8 days (day of insertion day -8) with all animals receiving two 2 ml injection of sodium chloprostenol (500 µg, EstroPlan, Parnell Laboratories NZ Ltd, Auckland, New Zealand) 6 days (an injection in the morning and afternoon) after CIDR insertion. All animals received a 2.5 ml injection of a GnRH analogue buserelin (10 µg, Receptal Intervet Limited, Auckland, New Zealand), given 24 h after CIDR device removal. The day after GnRH injection was Day 0 of the synchronized oestrous cycle. Embryos were at the blastocyst stage of development and of grade 1 quality. Embryos were produced from oocytes recovered from ovaries collected at the abattoir. The origin and reproductive history of the donor cows were unknown. Animals were slaughtered at day 17 of the reproductive cycle and endometrial tissues (both caruncular and intercaruncular) were sampled. Selection criteria for the study included strain and calving date, and health post-calving was an exclusion criterion (cows with severe uterine infections or mastitis were excluded before being enrolled in the embryo transfer round). Cows in each strain were matched for calving number and age. A total of 10 cycling and 12 pregnant animals enrolled in the study were utilized, due to the associated costs of slaughtering the cows. These animals represented fertile (six pregnant and five cycling Holstein-Friesian cows with New Zealand ancestry/≤30% North American genetics, n=11, NZ) and sub-fertile (six pregnant and five cycling Holstein-Friesian cows with >87% North American ancestry, n=11, NA) phenotypes of Holstein-Friesian dairy cows (188, 253, 258, 259). Briefly, Holstein-Friesian dairy cow strains of NA ancestry have poorer oocyte and embryo quality, lower conception rate to first and second services, lower 6-week pregnancy rate and overall lower pregnancy

rate compared with NZ strain Holstein-Friesian dairy cows. Gene expression differences between the two strains in non-reproductive tissues have been published previously (260). Detailed methods and production data for the cows used in this study have been published previously (187).

5.2.2. Methylation microarray design

The microarray design targeted all CpG islands in the genome (34,021), as designated by the UCSC genome browser. CpG islands were predicted using a modification of a program developed by G. Milkema and L.Hillier (unpublished) implemented on the bosTau4.0 genome build available for download through UCSC genome browser portal. Overlapping CpG regions were merged and the regions were tiled at 10bp using Agilents eArray tool with repeat masking. Probes were designed using the criteria: 1. Tm range 60-90 degrees Celsius and 2. Mono nucleotide repeats less than 5. These probes were then BLAST against the bovine whole genome, UCSC genome build bosTau4.0 and cross hybridizing probes were removed. Criteria for identification of cross hybridizing probes included probes showing a continuous alignment stretch greater than 44bp (regardless of mismatches) at untargeted genomic locations in the bovine whole genome. Probes were designed to target all CpG islands plus a 100bp region flanking each side of all CpG island. After repeat masking, a total of 362,213 probes were designed to target all CpG islands (60bp long with an average spacing of 10bp). An additional 53,420 probes were designed to target -3kb to +1kb flanking of transcriptional start site of 1,564 transcripts of interest (with an average spacing of 50bp). The total number of probes designed for this microarray was 415,663 plus an additional 1,711 Agilent control probes.

5.2.3. DNA extraction and quality control

DNA was extracted from endometrial tissue using a Qiagen DNAeasy blood and tissue kit (QIAGEN catalogue# 69506). DNA quantity was determined by spectrophotometry using a Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, DE). Genomic DNA integrity was assessed by running it on a 0.8% Agarose Gel. Genomic DNA purity was assessed by the NanoDrop® ND-1000 UV-Vis Spectrophotometer (Nanodrop technologies, Rockland, USA). Genomic DNA with OD260/OD280>1.8 and OD260/OD230 ≥ 1.3 was used for microarray

experiments. We considered DNA to be good quality when a single clear band was seen when run against a reference genomic DNA sample.

5.2.4. Sonication

Five microgram of DNA was made up to 200 microliter with 1X PBS and was sonicated at 20% amplitude for 12 cycles at 10 ON & 10 OFF on a Sonics Vibra-Cell Sonicator (model no. VCX 130PB). 200 nanogram of this sonicated sample was run on the Agilent Bioanalyzer to check for sonication efficiency. An ideal smear size ranged between 100-700bp.

5.2.5. Immuno-precipitation of methylated regions

The sonicated DNA was then immuno-precipitated using Anti 5 methyl cytidine (Cat. no: Eurogentec Ref: MECY_0500) bound to Pan Mouse IgG Dynal beads (Invitrogen Cat no: 110.41) overnight at 4°C. Beads were washed and DNA eluted in 1% SDS solution at 65°C. One fraction of the Sonicated DNA was set aside without immuno-precipitation and this was used as the Input.

Input DNA (whole cell extract- genomic DNA) and IP DNA was precipitated with 3M Acetate after a Phenol: chloroform (25:24:1) clean up using heavy phase lock gels Cat. No. Eppendorf ref # 2302830

5.2.6. Labelling and microarray hybridization

Input DNA was labelled with Cy3 and IP DNA with Cy5 using Agilent's Genomic DNA labelling kit (Cat. no. 5190-0453). Labelled samples were cleaned and concentrated using Microcon YM-30 columns(Millipore Cat. no. 42410).

All of the labelled IP DNA (~ 3-4 ug) and 4 micrograms of labelled Input DNA were hybridized onto the 400K custom designed Agilent microarray (2 x 400K array format, AMADID and Part no. are 027994 and G4820A respectively.) for 40 hours at 65°C. Microarray was washed using aCGH wash buffer 1 and 2 for five minutes and 1 minute respectively.

5.2.7. Microarray scanning and data analysis

Hybridized arrays were scanned at 2µm resolution on an Agilent Technologies Scanner G2505C. Data extraction from Images was done using Feature Extraction software v 10.5.1.1

of Agilent. Feature extracted data was analysed using Agilent Genomic Workbench software from Agilent. Two colour hybridizations were performed by labelling set of samples with cy3 channel and set of samples with cy5 channel. Normalization of the data was done using centralization method (Centralization adds or subtracts a constant value from each log ratio measurement. This recenters the log ratio values, and makes sure that the zero-point reflects the most common ploidy state). Methylation states were obtained using Batman Algorithm (BATMAN is a visualization and report analysis that determines the absolute methylation level for CpG dinucleotides that are contained within DNA fragments queried by probes across the microarray. The algorithm begins with a calibration step, which uses a regression to fit a trend between local CpG density and either probe log-ratios, or Z-scores (**Methylation Status Detection Algorithm**). This is followed by a modelling step, which uses the calibration results to construct the conditional probability of the log-ratios (or Z-scores) given the methylation state. A sampling step occurs to generate a large number of possible methylation states. Bayesian methodology is used to invert the conditional probability and summarize the methylation state per probe. Finally, a methylation call step fits a distribution to the scores on a per- chromosome basis.

5.2.8. Methylation status detection algorithm

The methylation status detection algorithm is designed for two- colour assays, where the green (Cy3) channel is comprised of input DNA, and the red (Cy5) channel is comprised of the affinity-enriched DNA. As a result of constraints imposed on the array design, the probes that target genomic regions with varying CG content do not share a uniform melting temperature (Tm), which can result in compression of the log-ratios. The methylation detection algorithm allows normalizing the log-ratios for each probe, based on its Tm, and returns the methylation status of a probe. The algorithm first bins the probes by their melting temperature. For each bin, it applies Gaussian fits using one of three models. It fits the probe log-ratios to Gaussians, using a local searching algorithm called random hill climbing. Z-Scores and p-values derived from the Gaussian data give probabilities and confidence values for methylated and un-methylated probe populations. The algorithm then calculates a methylation log-odds, which gives the relative probability that a probe is more likely methylated than un-methylated.

To call as methylation state following parameters were taken into consider

Combined Z-score: This is the summation of the left and right Gaussian Z-scores. It reflects the location of a probe log-ratio value in relation to the Gaussian distribution(s) of probes with similar Tm. A strong positive value of the combined score is methylated, while a strong negative value indicates the probe is un-methylated.

Right Gaussian Z-score: The Z-score derived from the right Gaussian. A positive or a small negative value means that the probe is likely to be methylated.

Left Gaussian Z-score: The Z-score derived from the left Gaussian. A negative or small positive value means that the probe is likely to be un-methylated.

Log-odds: The log-odds score indicates how likely a probe is to be methylated.

Batman Call: +1 represents methylated, -1 & 0 represents un-methylated states of the probes.

5.2.9. Mapping of microarray probes to genes

Genomic co-ordinates for methylation probes were queried against RefSeq genes in the bovine genome (refFLAT file from UCSC bosTau4.0). Probes that mapped within the transcriptional boundaries or 10kb 5' of the transcriptional start site of a gene were annotated to that gene. Annotated methylation probes were then mapped to gene expression probes using the RefSeq accession as a common identifier.

5.2.10. Re-analysis of gene expression data

Gene expression microarray data was published previously (263, 265, 284) and submitted to NCBI gene expression omnibus (GSE19140). Validation of microarray data using qRT-PCR has been published previously (263, 265, 284).

5.2.10.1. Pregnant versus cycling comparison

Differentially expressed probes were identified using a 2-way ANOVA (pregnancy status x tissue), including a Benjamini-Hochberg false discovery rate multiple testing correction (MTC). Genes that were significantly different in the comparison of pregnant and cycling animals were submitted for pathway analysis using GeneGO MetaCore.

5.2.10.2. Pregnant sub-fertile versus pregnant fertile comparison

Comparisons between fertile and sub-fertile strains were carried out using ANOVA with significance thresholds set at $P<0.05$; multiple testing corrections (Benjamini-Hochberg), and post hoc tests (Tukey) were also applied. Strain was tested within both tissue types (caruncular and intercaruncular) and within sub-groups defined by the size classification of the embryo (small and large). Small embryos were classified as those being less than 20 cm (mean: 10 ± 2.6 cm) in length and weighing less than 40 mg (mean: 19 ± 5.9 mg, 5 embryos). Large embryos were classified as those being longer than 20 cm (mean: 36 ± 4.9 cm) and weighing more than 75 mg (mean: 158 ± 33 mg, 7 embryos). The genes with P-value <0.05 for the above comparisons were extracted from the Genespring Tukey comparison table. The results of this analysis have been published previously (284).

5.2.11. Correlation between DNA methylation and gene expression

Correlations between DNA methylation and gene expression data for mapped probes were obtained using linear models, including pregnancy status, tissue, strain and interactions in the model, giving a within group correlation between the 2 measurement types. A Benjamini Hochberg adjustment was made on the significance levels for the correlations.
The top 1000 correlated genes were then compared to the lists of differentially expressed genes and enriched pathways.

5.3. Results

5.3.1. Mapping

A total of 6,158 out of 13,317 unique bovine reference genes mapped to gene expression probes on the Agilent bovine specific microarray (see methods section). Thirty nine percent of DNA methylation probes mapped to within 10kb 5' of these annotated genes. A total of 190,431 unique DNA methylation gene expression probe combinations were identified. The mean number of methylation probes per gene was 24 (± 0.27) at a mean distance of 1,029 (±10) base pairs from the annotated transcriptional start site (additional file 4).

5.3.2. Correlation of DNA methylation and gene expression data

DNA methylation and gene expression data from endometrial tissue was used to assess the correlation between DNA methylation and gene expression. The 1000 most statistically significant data points were used (802 were unique genes) for subsequent analysis. Fifty two percent of these DNA methylation probes were negatively correlated with signal from gene expression probes while 48% were positively correlated. When this gene list was compared with previously reported studies of gene expression on the same tissues (263, 284), 42% of these were differentially expressed between pregnant and cycling animals and 11% were differentially expressed between pregnant fertile and sub-fertile animals (additional file 5).

5.3.3. Pathway analysis

Pathway analysis of differentially expressed genes in the comparison of pregnant and cycling animals and in fertile and sub-fertile animals used in this study has been published previously (263). Repeat analysis revealed enrichment for several pathways in agreement with the published reports. Genes that demonstrated a correlation between DNA methylation and gene expression and were differentially expressed were detected in these pathways (Table 13).

Table 13. Enriched pathways. Genes demonstrating correlation of DNA methylation and gene expression in pathways that were enriched for in transcriptional profiling of pregnant vs. cycling (PvsC) (263) and sub-fertile vs. fertile (SFvsF) (284) dairy cows.

EntrezID	ProbeID	Gene Symbol	Methylation probe	Distance	slope	Corrected P-value	P-value BH
Cell adhesion Chemokines and adhesion (PvsC)							
281043	A_73_112988	*CCL2*	GT_Bostaurus_chr19:15666153-15670153_182	810	-0.97	0.0028	0.4610
281572	A_73_115644	*VEGFA*	BT_Btaurus_CpG_93941	270	-0.41	0.0034	0.4850
281791	A_73_101527	*GNAI2*	BT_Btaurus_CpG_328804	0	0.20	0.0008	0.3445
282187	A_73_115526	*COL1A1*	GT_Bostaurus_chr19:37631830-37635830_342	0	-0.84	0.0009	0.3488
282187	A_73_115526	*COL1A1*	GT_Bostaurus_chr19:37631830-37635830_222	731	0.73	0.0011	0.3738
282187	A_73_115526	*COL1A1*	GT_Bostaurus_chr19:37631830-37635830_172	1231	-0.95	0.0002	0.2214
282308	A_73_113415	*PIK3R2*	BT_Btaurus_CpG_623928	0	-0.52	0.0007	0.3410
282308	A_73_101666	*PIK3R2*	BT_Btaurus_CpG_597003	0	-1.21	0.0023	0.4440
Cytoskeleton remodeling cytoskeleton remodeling (PvsC)							
281035	A_73_109408	*SERPING1*	GT_Btaurus_CpG_1931569	0	0.53	0.0022	0.4332
281572	A_73_115644	*VEGFA*	BT_Btaurus_CpG_93941	270	-0.41	0.0034	0.4850
282187	A_73_115526	*COL1A1*	GT_Bostaurus_chr19:37631830-37635830_342	0	-0.84	0.0009	0.3488
282187	A_73_115526	*COL1A1*	GT_Bostaurus_chr19:37631830-37635830_222	731	0.73	0.0011	0.3738
282187	A_73_115526	*COL1A1*	GT_Bostaurus_chr19:37631830-37635830_172	1231	-0.95	0.0002	0.2214
282308	A_73_113415	*PIK3R2*	BT_Btaurus_CpG_623928	0	-0.52	0.0007	0.3410
282308	A_73_101666	*PIK3R2*	BT_Btaurus_CpG_597003	0	-1.21	0.0023	0.4440
286870	A_73_115006	*EIF4G2*	GT_Btaurus_CpG_1895396	1564	0.34	0.0026	0.4538
Cytoskeleton remodeling TGF WNT and Cytoskeleton remodeling (PvsC)							
281035	A_73_109408	*SERPING1*	GT_Btaurus_CpG_1931569	0	0.53	0.0022	0.4330
281572	A_73_115644	*VEGFA*	BT_Btaurus_CpG_93941	270	-0.41	0.0034	0.4850
282308	A_73_113415	*PIK3R2*	BT_Btaurus_CpG_623928	0	-0.52	0.0007	0.3410
282308	A_73_101666	*PIK3R2*	BT_Btaurus_CpG_597003	0	-1.21	0.0023	0.4440
527124	A_73_116752	*CDKN2B*	GT_Bostaurus_chr8:23023190-23027190_62	3830	0.94	0.0030	0.4710
Immune response Antiviral actions of interferons (PvsC and SFvsF)							
282491	A_73_101550	*BOLA-DMB*	GT_Bostaurus_chr23:7459395-7463395_2	0	0.37	0.0022	0.4326
509855	A_73_116714	*IRF9*	GT_Bostaurus_chr10:21269932-21273932_282	1810	-0.36	0.0018	0.4060
512484	A_73_107438	*TYK2*	BT_Btaurus_CpG_544141	0	0.18	0.0013	0.3858

EntrezID	ProbeID	Gene Symbol	Methylation probe	Distance	slope	Corrected P-value	P-value BH
Immune response IFN alpha/beta signaling pathway (PvsC)							
509855	A_73_116714	*IRF9*	GT_Bostaurus_chr10:21269932-21273932_282	1810	-0.36	0.0018	0.4060
512484	A_73_107438	*TYK2*	BT_Btaurus_CpG_544141	0	0.18	0.0013	0.3858
Immune response IFN gamma signaling pathway (PvsC and SFvsF)							
282308	A_73_113415	*PIK3R2*	BT_Btaurus_CpG_623928	0	-0.52	0.0007	0.3405
282308	A_73_101666	*PIK3R2*	BT_Btaurus_CpG_597003	0	-1.21	0.0023	0.4440
509855	A_73_116714	*IRF9*	GT_Bostaurus_chr10:21269932-21273932_282	1810	-0.36	0.0018	0.4060
Immune response lectin induced complement pathways (PvsC)							
280678	A_73_113565	*C4A*	GT_Bostaurus_chr23:27177765-27181765_185	840	1.03	0.0008	0.3455
281035	A_73_109408	*SERPING1*	GT_Btaurus_CpG_1931569	0	0.53	0.0022	0.4332
515440	A_73_101342	*C2*	GT_Bostaurus_chr23:27224299-27228299_32	0	1.32	0.0010	0.3655
515440	A_73_101342	*C2*	GT_Bostaurus_chr23:27224299-27228299_142	410	-0.84	0.0009	0.3486
Immune response MIF the neuroendocrine macrophage connector (PvsC)							
282491	A_73_101550	*BOLA-DMB*	GT_Bostaurus_chr23:7459395-7463395_2	0	0.37	0.0022	0.4326
Signal transduction GM CSF signaling (PvsC)							
281791	A_73_101527	*GNAI2*	BT_Btaurus_CpG_328804	0	0.20	0.0008	0.3440
281791	A_73_101527	*GNAI2*	BT_Btaurus_CpG_328804	0	0.20	0.0008	0.3445
509936	A_73_115191	*ADCY6*	BT_Btaurus_CpG_383417	0	-0.38	0.0019	0.4096
618184	A_73_109298	*KDELR1*	GT_Btaurus_CpG_2357572	2953	-0.24	0.0032	0.4764
618184	A_73_109298	*KDELR1*	GT_Btaurus_CpG_2381530	6206	-0.32	0.0009	0.3482
Development_PDGF signaling via STATs and NF-kB (SFvsF)							
512484	A_73_107438	*TYK2*	BT_Btaurus_CpG_544141	0	0.18	0.0013	0.3860
Normal and pathological TGF-beta-mediated regulation of cell proliferation (SFvsF)							
504357	A_73_104663	*AXIN1*	GT_Btaurus_CpG_1063042	0	-0.85	0.0010	0.3620
540106	A_73_114389	*PDGFB*	GT_Bostaurus_chr5:117840047-117844047_42	0	-0.86	0.0000	0.0220
540106	A_73_114389	*PDGFB*	BT_Btaurus_CpG_366575	0	-1.03	0.0000	0.0380

5.4. Discussion

It was hypothesized that DNA methylation regulates the expression of genes in the uterus critical to enabling and maintaining pregnancy. Data presented support this hypothesis, demonstrating a correlation between DNA methylation and gene expression in the endometrium. Forty two percent of the correlated genes were differentially expressed between pregnant and cycling animals and 11% were differentially expressed between fertile and sub-fertile animals. This study only utilised DNA methylation probes that mapped to Reference Sequence (RefSeq) annotated genes (13,317 transcripts), utilizing less than 40% of all DNA methylation probes. It would be expected that with further annotation of RefSeq genes to the bovine genome in the future, a greater proportion of these probes would have mapped to genes and would have allowed a more comprehensive analysis. Additionally, in this study, each probe was considered when correlating DNA methylation and gene expression to identify specific sites where DNA methylation may be important rather than all probes that annotate to a particular gene.

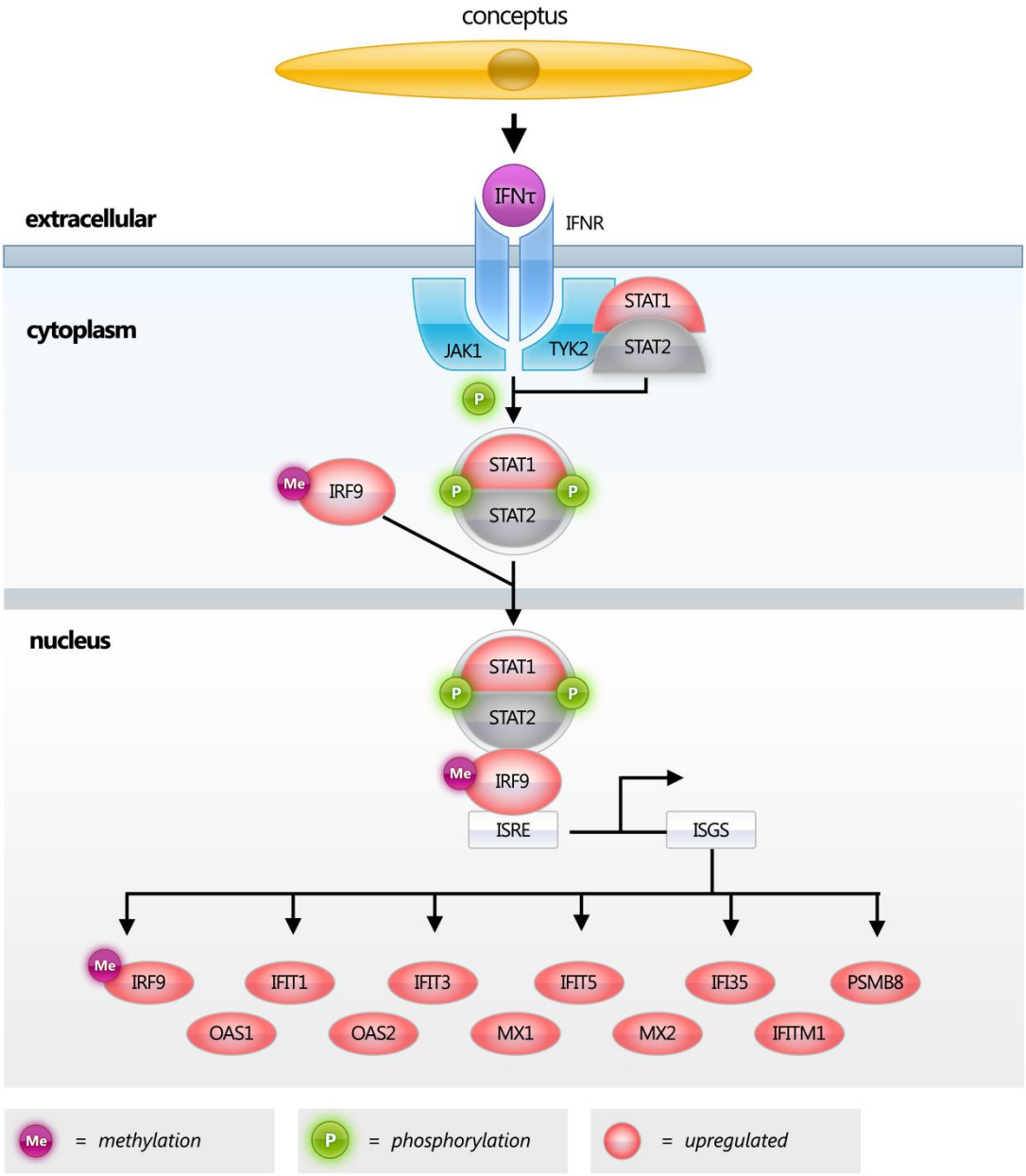
Fifty two percent of DNA methylation probes were negatively correlated with gene expression. It has been reported that DNA methylation in the promoter and intragenic regions are negatively correlated with gene expression (291-294). The correlation of DNA methylation and gene expression does not confirm that DNA methylation is regulating gene expression in the uterus in response to the embryo. Nevertheless, it is a critical step in establishing a causative relationship between DNA methylation and gene expression. These data provide support for a possible role of DNA methylation in regulating gene expression in response to pregnancy and identifies candidate genes and regions for future investigation.

5.4.1. Pathways potentially regulated by DNA methylation

DNA methylation may regulate biologically relevant pathways during early pregnancy. Pathways that were enriched in transcriptional studies were cross-referenced to DNA methylation data to identify pathways that were regulated by DNA methylation. Several enriched pathways were identified that contained genes potentially regulated by DNA methylation. Interferon signalling pathways were enriched in pregnant animals as well as fertile animal (263, 284). During early pregnancy, the embryo signals its presence to the mother to prevent luteal regression and establish pregnancy. The primary signalling

molecule used by the embryo is IFNτ. Secreted IFNτ binds to receptors on the endometrium, activating the JAK-STAT pathway (84) . Stat 1 homodimers (GAF) or Stat1-stat2 heterodimers and IRF9 (ISGF3) then bind to the promoters of interferon stimulated genes, activating their transcription (295). Several genes in this pathway were up-regulated in pregnant animals and were also down-regulated in sub-fertile animals. Interestingly, *IFR9* which is up-regulated in pregnant animals and down-regulated in sub-fertile animals had a negative correlation between DNA methylation and gene expression, suggesting that methylation may have a role in transcriptional regulation of this gene. *IRF9* is both stimulated by interferons and is part of the ISGF3 complex that hyper-activates the transcription of several interferon stimulated genes (Figure 16). This suggests that DNA methylation could be regulating the unexplained differences in the response to IFNτ in the fertile and sub-fertile animals. DNA methylation was also correlated with gene expression of several genes in other pathways predicted to be important in early pregnancy events. These included cell adhesion, cytoskeleton remodelling and cell proliferation pathways. The relevance of these pathways in early pregnancy has been previously reported (263, 284). Correlation of DNA methylation and gene expression in these pathways provides additional support for a role of DNA methylation in early pregnancy response. Genes that contribute to the enrichment of these pathways demonstrated a negative correlation between DNA methylation and gene expression. The correlation between DNA methylation and gene expression in pathways implicated in early pregnancy events and pathways differentially regulated in fertile and sub-fertile animals during this time suggests that DNA methylation in the endometrium may play a role in pregnancy success.

Figure 16. Interferon signalling pathway. Stat1-stat2 heterodimers and IRF9 (ISGF3) bind to the interferon stimulated response element (ISRE) in the promoter region of interferon stimulated genes (ISGs), activating their transcription. Genes of the interferon signalling pathway that were differentially expressed in the comparison of pregnant vs. cycling (PvsC) and sub-fertile vs. fertile (SFvsF) dairy cows. The Red shading refers to genes that were up-regulated in pregnant and/or down-regulated in sub-fertile cows.


conceptus
IFNτ
IFNR
extracellular
cytoplasm
JAK1
TYK2
STAT1
STAT2
P
Me
IRF9
nucleus
ISRE
ISGS
IFIT1
IFIT3
IFIT5
IFI35
PSMB8
OAS1
OAS2
MX1
MX2
IFITM1
= methylation
= phosphorylation
= upregulated

5.5. Conclusion

DNA methylation was correlated with gene expression in several pathways implicated in early pregnancy events. The majority of these genes demonstrated negative correlations, suggesting that DNA methylation may be contributing to endometrial gene expression changes in response to cues received from the embryo. In addition, genes that were differentially expressed in fertile and sub-fertile strains demonstrated correlation between DNA methylation and gene expression. This suggests that differential DNA methylation could be a significant contributor to the divergent fertility of these two strains of dairy cow. The results of this study demonstrated correlation between DNA methylation and gene expression only; while this provides support for a role of DNA methylation in regulating gene expression during pregnancy, it does not provide direct evidence of causation. A causative relationship between DNA methylation and gene expression during pregnancy requires further investigation. This study provides support for this mechanism and highlights candidate genes for future studies.

Chapter 6. INVESTIGATION OF CANDIDATE GENES FOR IMPROVING FERTILITY

This chapter was submitted to Journal of Dairy Science as:

WALKER C.G., MEIER S., MITCHELL M.D., SANDERS K., ROCHE J.R., SPELMAN R., LITTLEJOHN M: Polymorphisms in positional candidate gene ARG1 are not associated with fertility phenotypes in dairy cattle.

I developed the methods, performed the experimental work and statistical analysis and wrote the manuscript. The other authors contributed to the development of methods and experimental design and critical analysis of the manuscript.

6.1. Background

Early pregnancy loss in ruminants is a major contributor to poor reproductive performance. Fertilization rates in pasture-grazed dairy cows are estimated to be between 80 and 90%; yet, diagnosed conception rates are 55%, on average, and can be as low as 40% in high production cows (5). The majority of pregnancy losses occur during the pre-implantation period, when it is estimated that up to 80% of total embryonic loss occurs (5). Microarray analysis of endometrial tissue during the pre-implantation period in dairy cattle has revealed distinct transcriptional differences in dairy cow strains characterized as fertile and sub-fertile (284). In this study, the sub-fertile dairy cow strain was derived from animals of North American (NA, >87% NA genetics) ancestry, while the fertile animals were derived from a New Zealand (NZ, <30% NA genetics) strain of dairy cow. Dairy cow strains classified as sub-fertile exhibit poorer oocyte and embryo quality, lower conception rates to first and second service and lower 6-week in calf rate when compared with fertile strain dairy cows (188, 258, 259, 284, 296). This suggests genetics likely play a part in determining the divergent fertility of these two strains.

6.1.1. Fertility QTL on chromosome 9

Quantitative trait loci (QTL) for fertility traits have previously been reported in a region of chromosome 9 (297) (marker ID TGLA73 at 71.4cM). This same region was accessed in a large pedigree of Holstein-Friesian (HF) x Jersey (J) crosses. Evidence of QTL with

chromosome-wide significance for 2 year old “pregnant to first service” (F-value = 3.61, P-value = 1.96E-02) and “calved to first service” (F-value = 3.4, P-value = 3.54E-02) was detected. Peak significance was at 68cM, overlapping with the region identified by other investigators (297). To look for possible candidate genes that could underlie these QTL, map positions of genes demonstrating transcriptional differences between fertile and sub-fertile dairy cows were assessed. Using RefSeq annotations, we derived a list of 20 genes from 65 to 75Mb, UMD3.1) on chromosome 9). Of the 1,500 genes differentially expressed between the fertile and sub-fertile animals (284), two genes were in the region of interest. Of these, arginase 1 (*ARG1*), was identified as a positional candidate gene for association with fertility traits, and was prioritised for further investigation (Table 14)

Table 14. Genes identified in QTL region. Genes identified from 65to 75Mb on chromosome 9 (Build UMD3.1). Genes in bold were down-regulated in sub-fertile animals.

Chromosome: 9 Region: 65M...75M

Transcription start	Transcription stop	Gene Symbol	Orientation	Description
6.6E+07	6.6E+07	*NT5E*	-	5'-nucleotidase, ecto (CD73)
6.7E+07	6.7E+07	*MRAP2*	-	melanocortin 2 receptor accessory protein 2
6.7E+07	**6.7E+07**	***CYB5R4***	**-**	**cytochrome b5 reductase 4**
6.8E+07	6.8E+07	*THEMIS*	-	thymocyte selection associated
7E+07	7E+07	*ARHGAP18*	-	Rho GTPase activating protein 18
7E+07	7E+07	*L3MBTL3*	+	l(3)mbt-like 3 (Drosophila)
7E+07	7E+07	*SAMD3*	-	sterile alpha motif domain containing 3
7.1E+07	7.1E+07	*THBS2*	+	thrombospondin 2
7.1E+07	7.1E+07	*AKAP7*	+	A kinase (PRKA) anchor protein 7
7.2E+07	**7.2E+07**	***ARG1***	**+**	**arginase, liver**
7.2E+07	7.2E+07	*ENPP3*	+	ectonucleotide pyrophosphatase/phosphodiesterase 3
7.2E+07	7.2E+07	*CTGF*	-	connective tissue growth factor
72531450	72588110	*STX7*	-	syntaxin 7
72884792	72903384	*VNN1*	-	vanin 1
72936098	72966050	*VNN2*	-	vanin 2
73027022	73030363	*RPS12*	+	ribosomal protein S12
74207580	74210553	*TCF21*	+	transcription factor 21
74253099	74291965	*TBPL1*	+	TBP-like 1
74295221	74364337	*SLC2A12*	-	solute carrier family 2 (facilitated glucose transporter), member 12
74478548	74484105	*SGK1*	-	serum/glucocorticoid regulated kinase 1

6.1.2. ARG1 – a positional candidate gene for fertility

ARG1 was the single most differentially expressed gene in the comparison of endometrial tissues between fertile and sub-fertile dairy cows (284). The arginase isoform encoded by ARG1 is an enzyme of the hepatic urea cycle that metabolises L-arginine to L-ornithine and urea (298). Arginase can also function as an immunosuppressive enzyme through the depletion of L-arginine. Suppression of the maternal immune system through depletion of essential amino acids has been demonstrated as a signalling mechanism to avoid foetal rejection, first described by Munn et al 1998 (269). In this study, depletion of essential amino acids was a novel mechanism by which the maternal system could prevent foetal rejection through increased expression of indoleamine -2,3 dioxygenase (IDO), specifically through the suppression of T-cell activity through tryptophan deprivation Depletion of L-arginine can also suppress T cell proliferation, possibly through the down regulation of the T cell receptor (299), and is up-regulated in the term placenta and peripheral blood of pregnant women (300). In addition, *ARG1* is up-regulated at the site of implantation in guinea pigs where it hypothesised to function in an immune suppressive capacity (301). Arginine supplementation has also been reported to improve the reproductive performance of gilts through an increase in substrate material for the production of polyamines via Aginase (302). Given these roles and the observed down-regulation of ARG1 in sub-fertile animals, *ARG1* was targeted as a candidate gene for fertility.

6.2. Methods

6.2.1. Animals and trial design

The QTL trial consisted of a pedigree of 864 F2 Holstein Friesian-Jersey crossbreds (FJXB) dairy cows with a half sibling family structure (303) and has been described previously (162, 304, 305). Fertility phenotypes recorded included: “Pregnant to first service” (1 if animal was diagnosed as pregnant and the successful AB date was evaluated as the first mating, 0 otherwise) and “Calved to first service” (1 if animal calved and the successful AB date was evaluated as the first mating, 0 otherwise).

6.2.2. ARG1 sequencing

For polymorphism discovery, PCR products corresponding to all annotated ARG1 exons and 2kb of putative promoter region were amplified from the genomic DNA of the six F1 sires of the FJXB pedigree. Arginase-1 gene structure information was obtained using genomic sequence from the Btau3 reference assembly overlaid with the NCBI ARG1 RefSeq mRNA entry (NM_001046154). PCR amplification was undertaken by AGRF (Australia). Overlapping sequence reads (forward and/or reverse) were obtained for most amplicons and used to call variations.

6.2.3. Genotyping in F2 population

Genomic DNA was extracted from whole blood from the entire pedigree [18]. For QTL analysis, pedigree genotyping was conducted as described previously [18], using microsatellite markers and the Affymetrix Bovine 10K SNP GeneChip (Affymetrix). The F2 daughter animals were genotyped for ten *ARG1* markers discovered via DNA sequencing (above). Genotyping was undertaken by GeneSeek (Lincoln, NE, USA) with a matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) system.

6.2.4. Statistical analysis

Fertility traits “pregnant to first service” and “calved to first service” were used for QTL and ARG1 polymorphism association testing. To account for differences between the years over which the trial was conducted, JMP Genomics (version 8.0.2; SAS Institute Inc., Cary, NC, USA) software was used to fit a model with animal cohort treated as a fixed effect. Sire was also included in the model and treated as a random effect. Quantitative trait loci analysis was conducted using regression methodology in a half sibling model (306, 307), using the cohort and sire adjusted phenotypic residuals. Significance levels were determined at the chromosome-wide level using permutation testing (308). Following detection of the chromosome 9 QTL and subsequent polymorphism analysis of the ARG1 gene, associations between the ARG1 polymorphisms and fertility traits were tested using JMP Genomics. Data were adjusted for multiple hypothesis testing utilising Benjamini Hochberg false discovery rate, with a significance threshold of $P<0.05$. Haplotype analysis to identify polymorphisms in linkage disequilibrium was performed using Haploview (309).

6.3. Results

6.3.1. A QTL on chromosome 9 for conception rate

Analysis of fertility traits in animals revealed significant QTL on chromosome 9 (**Figure 17** and **Figure 18**). Generated under a half sibling model, peak significance was observed at 68cM for the fertility traits "pregnant to first service" (F-value = 3.61, P-value = 1.96E-02) and "calved to first service" (F-value = 3.4, P-value = 3.54E-02).

6.3.2. Sequence analysis of ARG1 reveals polymorphic variation

DNA sequencing of all exons and 2kb of putative promoter region of ARG1 in the six F1 sires and 12 animals utilised in the fertility microarray study revealed 38 polymorphisms in the ARG1 gene (Table 15). There was no clear segregation pattern identified among the sires, and therefore polymorphisms were chosen to represent a range of haplotypes (Table 16). Eight of polymorphisms were subsequently genotyped in the F2 daughter population (Table 17). Some of the polymorphisms were in LD with each other in the F2 animals (Figure 19). Success rate for the genotyping assays were greater than 95% for all assays except one. This assay was excluded from the analysis because of the high failure rate (46% failure rate).

6.3.3. Polymorphisms in ARG1 do not associate with fertility traits.

Using modelled phenotypic data, association analyses of the genotyped SNPs revealed no significant associations with fertility traits (Table 18).

Figure 17. QTL detected on chromosome 9 for “pregnant to first service”.

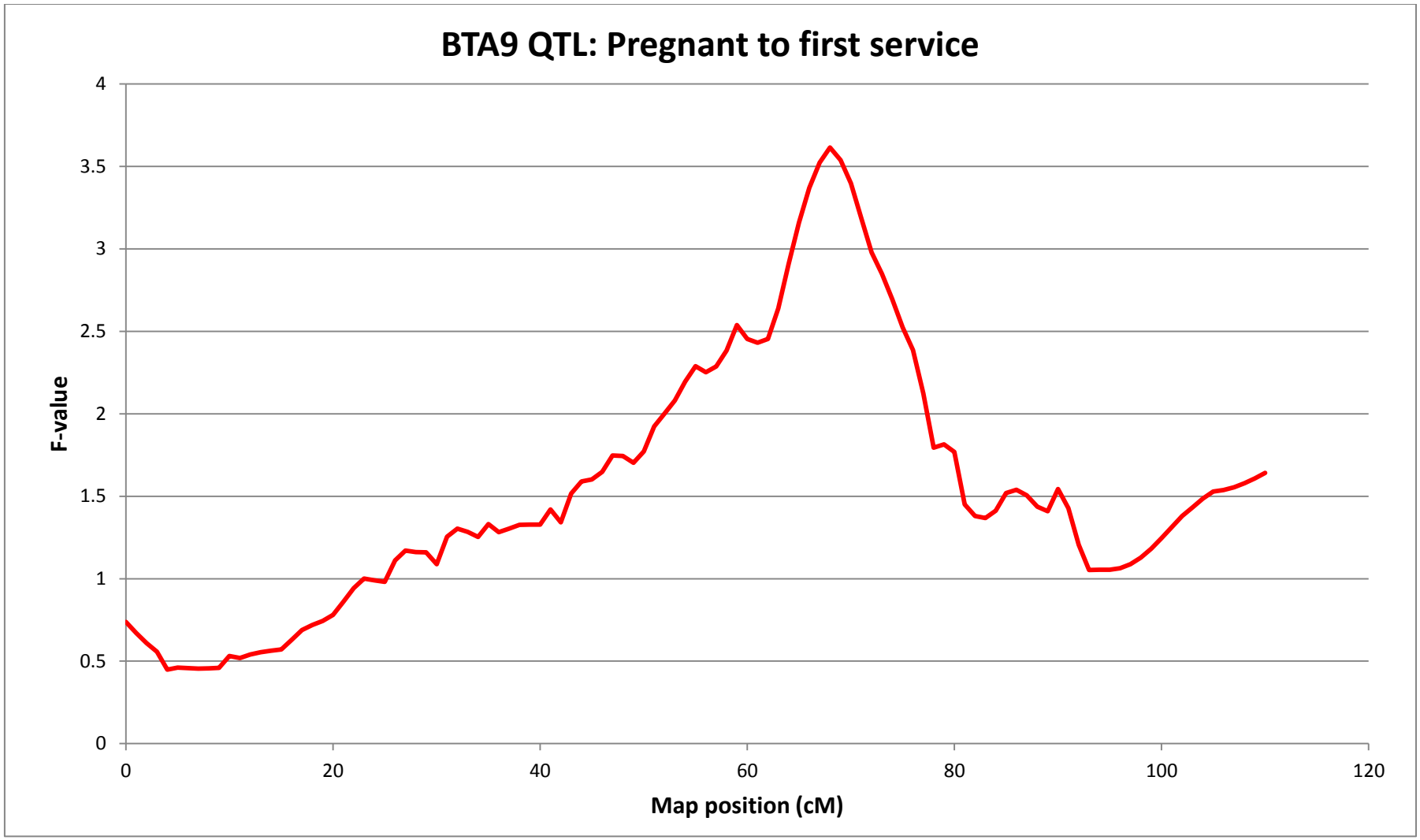

BTA9 QTL: Pregnant to first service
F-value
4
3.5
3
2.5
2
1.5
1
0.5
0
0
20
40
60
80
100
120
Map position (cM)

Figure 18. QTL detected on chromosome 9 for “calved to first service”.

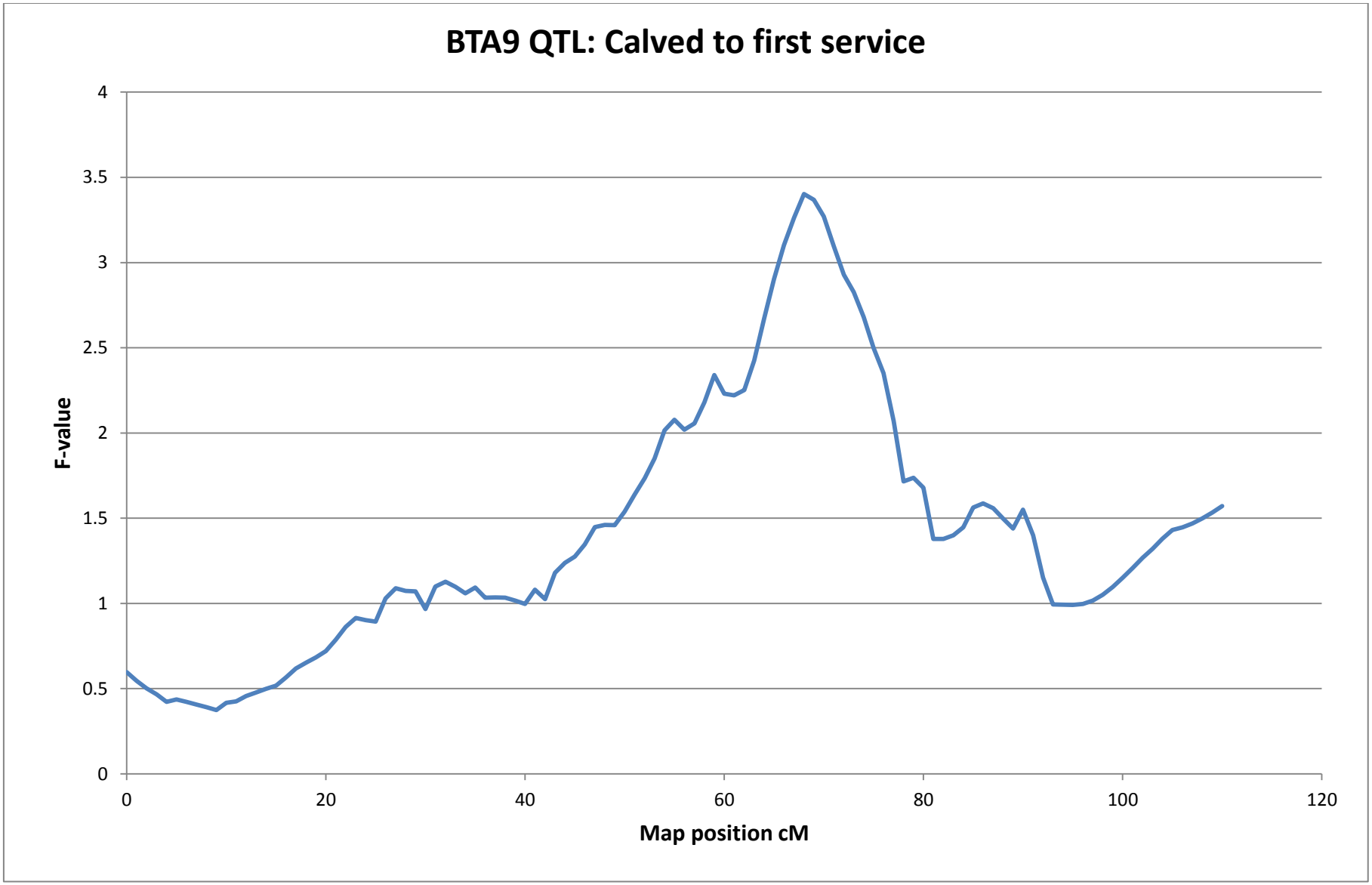

BTA9 QTL: Calved to first service
F-value
4
3.5
3
2.5
2
1.5
1
0.5
0
0
20
40
60
80
100
120
Map position cM

Table 15. Variation detected in *ARG1*. Variations detected by Sanger sequencing of the six F1 sires and 16 of the endometrial samples (E1-16) from the microarray experiment. ND - polymorphisms that were not detected/ unable to be determined.

	F1 sires						Fertile P					Fertile C			Sub-fertile P					Sub-fertile C		
Genomic bp relative to ATG	1	2	3	4	5	6	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12	E13	E14	E15	E16
-2185C>T	TT	ND	ND	CC	ND	ND	CC	ND	CC	ND	ND	CC	ND	CC	CC	ND	ND	CC	ND	ND	ND	CC
-1546G>A	GG	GG	GG	AG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	AG	AG	AG	GG	AG	GG
-1111Ains	A/-	A/-	A/-	A/A	A/-	A/A	A/-	A/-	A/-	A/-	A/-	-/-	ND	A/A	-/-	-/-	A/A	-/-	A/A	A/-	-/-	-/-
-1111A>C	AC	AC	ND	AA	AC	AA	ND	AC	ND	AC	AC	ND	ND	AA	ND	ND	AA	ND	AA	ND	ND	ND
-1110C>T	CT	CT	ND	CC	CT	CC	ND	ND	ND	CT	CT	CC	ND	CC	CC	CC	CC	CC	CC	ND	CC	CC
-897G>T	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	GT	ND	ND	ND	ND	ND	ND	ND	GG	ND	ND	ND
-896T>G	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	GG	ND	ND	ND	ND	ND	ND	ND	TT	ND	ND	ND
-861G>A	GG	ND	ND	ND	ND	ND	ND	ND	GG	GG	GG	ND	GG	ND	GG	GG	ND	ND	AG	ND	ND	GG
-774C>A	AC	AC	AC	CC	AC	ND	ND	AC	AC	AC	AC	AA	AC	CC	AA	AA	CC	AC	CC	AC	AC	AA
-485G>A	GG	GG	GG	AG	GG	GG	ND	GG	GG	GG	GG	GG	GG	GG	GG	GG	AG	AG	AG	GG	AG	GG
-447C>T	CC	CC	CC	CT	CC	CC	ND	CC	CC	CC	CC	CC	CC	CC	CC	CC	CT	CT	CT	CC	CT	CC
-411G>C	CG	CG	CG	GG	CG	GG	ND	CG	CG	CG	CG	CC	CG	GG	CC	CC	GG	ND	GG	CG	CG	CC
+91T>C	TT	TT	TT	TT	TT	CT	TT	TT	TT	TT	TT	TT	TT	TT	TT	TT	TT	TT	TT	TT	TT	TT
+107G>A	GG	GG	GG	AG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	AG	AG	GG	GG	GG	GG
+4787G>A	AG	GG	AG	AG	AG	AG	AG	GG	AG	GG	GG	GG	GG	AG	GG	GG	GG	GG	AG	GG	ND	GG
+8377G>T	GG	GG	GG	GT	GG	GT	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	ND	GG	GT	GG	GG	GG
+8530T>C	CC	CC	CC	CT	CC	CT	CT	CC	CT	CC	CC	CC	CT	TT	CC	CC	ND	CC	CT	CT	CC	CC
+8679A>T	TT	TT	TT	AA	TT	AA	TT	TT	TT	TT	TT	TT	AT	AA	TT	TT	ND	TT	AA	AT	TT	TT
+8731C>A	ND	ND	AC	CC	CC	ND	CC	ND	CC	ND	CC	CC	CC	CC	CC	CC	ND	CC	CC	CC	CC	AC
+10409T>C	TT	TT	TT	CT	TT	ND	TT	TT	TT	TT	TT	TT	TT	TT	ND	TT	CT	ND	CT	TT	CT	TT
+11212G>A	AG	AG	AG	GG	AG	ND	AG	AG	AG	AG	AG	AA	AG	GG	AA	AA	GG	AG	GG	AG	AG	AA
+11248C>G	CG	CG	CG	CC	CG	CC	CG	CG	CG	CG	CG	GG	CG	CC	GG	GG	CC	CG	CC	CG	CG	GG
+14422T>C	ND	ND	ND	CT	CT	CC	TT	CC	CT	ND	CT	CC	ND	CC	CC	ND	ND	CC	ND	ND	ND	ND
+15446T>C	CC	CC	CC	CC	CC	CC	CC	CC	CC	CC	CC	CC	CT	CT	CC	CC	CT	CC	CC	CT	CC	CC
+15471G>A	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AG	AG	AA	AA	AG	AA	AA	AG	AA	AA
+15654A>G	GG	GG	GG	GG	GG	GG	ND	GG	GG	GG	GG	GG	AG	AG	GG	GG	AG	GG	GG	AG	GG	GG

+15803C>G	GG	GG	GG	GG	GG	GG	ND	GG	GG	GG	GG	GG	CG	CG	GG	GG	CG	GG	GG	CG	GG	GG
+16124C>T	CC	ND	CC	CC	CC	CT	CC	CC	CC	CC	CC	CC	CC	CC	CC	CC	CC	CC	ND	CC	CC	CC
+16145A>G	GG	GG	AA	AA	AA	AA	GG	AA	AA	AA	AA	GG	AA	AA	AA	AA	AA	AA	ND	AA	AA	AA
+16304G>A	GG	GG	GG	GG	GG	GG	AG	GG	AG	GG	GG	GG	GG	GG	GG	GG	GG	GG	ND	GG	GG	GG
+16323G>C	CC	CC	CC	CC	CC	CC	CC	CC	CC	CC	CC	CC	CG	CG	CC	CC	CG	CC	ND	CG	CC	CC
+16393T>C	CC	CC	CC	CC	CC	CC	CC	CC	CC	CC	CC	CC	CT	CT	CC	CC	CT	CC	ND	CT	CC	CC
+16395C>G	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	CG	CG	GG	GG	CG	GG	ND	CG	GG	GG
+16435T>C	CC	CC	CC	CC	CC	CC	CC	CC	CC	CC	CC	CC	CT	CT	CC	CC	CT	CC	ND	CT	CC	CC
+16631T>G	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GT	GG	GG	GT	GG	GG	GG	GG	GG
+16646T>G	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GT	GG	GG	GT	GG	GG	GG	GG	GG
+16699A>T	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AT	AA	AT	AA	AA	AA
+16811A>G	ND	ND	ND	GG	ND	GG	ND	ND	ND	ND	ND	ND	ND	AG	ND	ND	AG	ND	GG	ND	ND	ND

Table 16 QTL effect size and segregation pattern

Pregnant_to_FRST					
Sire	Effect Size	Standard error	T-statistic	Degree of Freedom	P-value
1	0.12	0.08	1.43	146	0.15
2	-0.12	0.09	-1.25	118	0.21
3	0.17	0.08	1.99	146	0.05
4	0.21	0.10	2.17	109	0.03
5	-0.20	0.08	-2.42	146	0.02
6	-0.17	0.09	-1.87	126	0.06
Calved_to_FRST					
1	0.11	0.08	1.27	146	0.21
2	-0.10	0.09	-1.08	118	0.28
3	0.16	0.08	1.86	146	0.07
4	0.21	0.10	2.20	109	0.03
5	-0.21	0.08	-2.60	146	0.01
6	-0.15	0.09	-1.67	126	0.10

Table 17. Assay design for genotyping. The SNP to be genotyped is bold and in square brackets. Other SNP in the region are in red font.

SNP_name	Sequence	other SNPs	Note
ARG1_-411G>C	CTACCCTGGTAGTGTGTGAGGTGTGTCTTGTAGATTAACCATTAATCCTAGAGTGAAAACTGAATTCAGGGTTGTCGGGCTGGAAAAGA TGTGAGACACCATCTTGCCAGTCCCCGGTTTTTTGCCACAGGCAGATGCACAGACCTGAAA**[G/C]**AGGTAAGTACCATGCTCTGAGTTT GCACAGCTAGAACTATGGCACTTCTCCTGTGGTTATAATTAATTATGATCAGAAAAGATGACTGTGGTTAAGAAATCATGGTTGTGATTA GGAAACATTAGTGTTACCAGGTGGCTCTGTGAACTG	ARG1_-447C>T, ARG1_-485G>A	
ARG1_+91T>C	AGCTGCAGAGAGTGACGACTACCTGGAGACCTCGGGCGCCGCGGTCAGCAGTGTTGGAGCATGAGTTCCAAGCCACAGTCCATCGGG GTCATTGGAGCTCCCTTCTCAAAGGGCCAGGTGAGTCAAATTCTGGCTTTGAATAACTGCAGT**[T/C]**TATGAGAAAAGTTACGAATTTCA AAATTAGTAAGGTGTCACTGTCTGGTTAGTTAAACAGCCTGATACAACCTGACCTGAAATTTATACTAAAGTCTTTTCATCATTTTACATC ATTGTATAATTATGGTCACAAATCCACTTATAACA	ARG1_+107G>A	
ARG1_+4787G>A	TCAGCCACGAGGAGGGGTGGAAGAAGGCCCTACAGTACTTAGAAAGGCTGGTCTGCTTGAGAAACTTAAAGAACTAGGTAACTGTTTA CTTAATCAATTTTTTATTAAAGTATAGTTGATGTACAATATTATATGTTACATATTTATACA**[G/A]**TATAGTGATTCATAATTTTCAAGGTT ATACTCCATTTACAGTTATTGTAAAATGCTGGCTATATTTCCTATGCTGTACATTACATCCTTGTAGCTTATTTTATTTGTAATAGCTTGTA CCTCTCAACCCTCTACTCCTATATTGCCCCTC	N/A	
ARG1_+8679A>T	CTGATAATCTTGATGACAGTCCCTTTCAAATTGTGAAGAATCCAAGGTGTGTGGGAAAAGCAAGTGAAAAGCTGGCTGATGTGGTGGC AGAAGTCAAGAAGACTGGAAGGATCAGCCTTGTCCTGGGCGGAGACCACAGGTCTTTTTTTT**[A/T]**AATGTTTATCTCTATGGGAGTCT GGTATAAATACGGTGAAGGAAGTATAACCAAAACCATGAGAAGAGAGAAAATAGGAAAGGAAAGTATTGATCAATATTTTATATCACA TTTTCTGTCTTGTTTCTTTTAATTTTATTGATTACTACT	ARG1_+8530T>C	
ARG1_+8530T>C	TGCCTGGAAAATCCCATGGATTGAGGAGCCTGGTAGGCTACAGTCCATGGGGTCGAAAAGAGTCAGACACAACTGAGCTACTTCACTT ACTAACCTAAAACTTTTAATTATAGAGTGTGATGTGAAAGATTATGGGGACCTGTCCTTTGC**[T>C]**GATAATCTTGATGACAGTCCCTTT CAAATTGTGAAGAATCCAAGGTGTGTGGGAAAAGCAAGTGAAAAGCTGGCTGATGTGGTGGCAGAAGTCAAGAAGACTGGAAGGAT CAGCCTTGTCCTGGGCGGAGACCACAGGTCTTTTTTTTAA	ARG1_+8679A>T	Excluded from association analysis due to high fail rate
ARG1_-1546G>A	TGAAAGTTGAGTTTTCAGCCAGTTTTCTCACTCCCCTCTTTCACCCTCATCAAGAGGCTTTTTAGGTGCTCTTTAGTTCTGTAAGAGGTTA ATAATTAACTATACAAAATCCCAGAGGTAAATATCTAGGATATGCAAGCAAATCCACAA**[G/A]**TAAAAGATAGGAAGACCATGGAAAA TATTCCTCTTAAAATGGACCTTGTAAGACCTTATTGAATTTACACATTCAATAAATATTTATTGAGTGCCTATCACATGTCAACACTGCTCT GGGCACCAGGGTTACAGCAGACAAATTTCCTGC	N/A	
ARG1_-485G>A	TGGTTATTAATCAGGCAATTAATTTTTTCTTTAATTAATAATCAGAAAGGGAAGTCATATGCCTTCCCTGAGACCTACCCTGGTAGTGTGT GAGGTGTGTCTTGTAGATTAACCATTAATCCTAGAGTGAAAACTGAATTCAGGGTTGTC**[G/A]**GGCTGGAAAAGATGTGAGACACCAT CTTGCCAGTCCCCGGTTTTTTGCCACAGGCAGATGCACAGACCTGAAAGAGGTAAGTACCATGCTCTGAGTTTGCACAGCTAGAACTAT GGCACTTCTCCTGTGGTTATAATTAATTATGATCAG	ARG1_-447C>T	
ARG1_-447C>T	TAATCAGAAAGGGAAGTCATATGCCTTCCCTGAGACCTACCCTGGTAGTGTGTGAGGTGTGTCTTGTAGATTAACCATTAATCCTAGAG TGAAAACTGAATTCAGGGTTGTCGGGCTGGAAAAGATGTGAGACACCATCTTGCCAGTCCC**[C/T]**GGTTTTTTGCCACAGGCAGATGCA CAGACCTGAAAGAGGTAAGTACCATGCTCTGAGTTTGCACAGCTAGAACTATGGCACTTCTCCTGTGGTTATAATTAATTATGATCAGAA AAGATGACTGTGGTTAAGAAATCATGGTTGTGATTA	ARG1_-485G>A, ARG1_-411G>C	

Figure 19. Haplotype analysis for linkage disequilibrium (LD). Black boxes represent polymorphism in complete LD (r^2 = 1) and white represents polymorphism that are not linked (r^2 = 0). Boxes in shades of grey have r^2 values between 0 and 1.

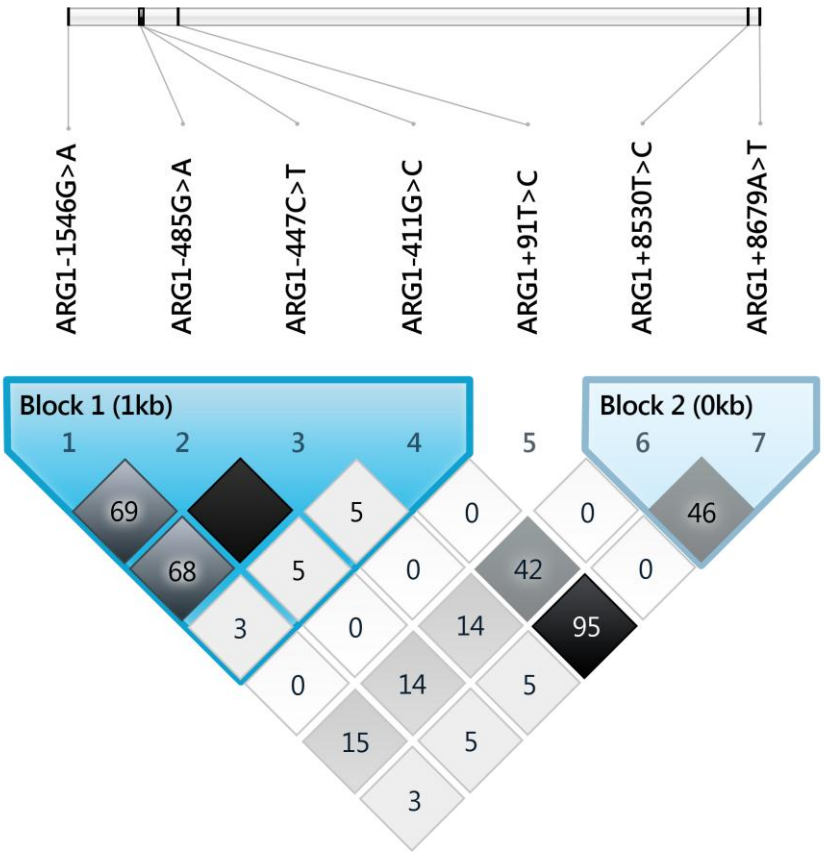

ARG1-1546G>A
ARG1-485G>A
ARG1-447C>T
ARG1-411G>C
ARG1+91T>C
ARG1+8530T>C
ARG1+8679A>T
Block 1 (1kb)
Block 2 (0kb)
1
2
3
4
5
6
7
69
68
5
5
3
0
0
0
0
0
14
14
15
5
5
3
42
95
0
46

Table 18 Association analysis among polymorphisms in *ARG1* and fertility phenotypes. Markers used for genotyping including their gene position (+/-ATG), results from the ANOVA based single marker association analysis using the “Genotype” and “Trend” tests in JMP Genomics. The “Genotype” test performs Pearson *chi*-square tests based on genotypes. The “Trend” test performs the Cochran-Armitage trend test using additive coding (0,1,2) for genotypes homozygous for the major allele, heterozygous, and homozygous for the minor allele, respectively. One marker was excluded due to high failure rate of the assay.

ARG1 SNP Name	Minor allele frequency	r_pregnant to first service				r_calved to first service			
		Genotype		Trend		Genotype		Trend	
		F-value	P-value	F-value	P-value	F-value	P-value	F-value	P-value
ARG1 -1546G>A	0.10	0.55	0.58	0.27	0.61	0.42	0.66	0.41	0.52
ARG1 -485G>A	0.14	0.94	0.39	1.42	0.23	0.83	0.44	1.65	0.20
ARG1 -447C>T	0.14	0.60	0.55	1.20	0.27	0.85	0.43	1.37	0.24
ARG1-411G>C	0.26	0.33	0.72	0.54	0.46	0.11	0.90	0.17	0.68
ARG1+91T>C	0.00	0.14	0.70	0.14	0.70	0.10	0.75	0.10	0.75
ARG1+8530T>C	0.42	1.52	0.22	0.61	0.43	1.93	0.15	0.86	0.35
ARG1+8679A>T	0.25	0.09	0.91	0.19	0.67	0.17	0.84	0.00	0.95

6.4. Discussion

It was hypothesised that variations in *ARG1* would associate with fertility traits. QTL analysis suggests that variation in chromosome 9 may underlie differential fertility. The data presented do not support a role for variation in *ARG1* in the fertility of dairy cows. Sequencing analysis of *ARG1* revealed 38 variations in the coding and putative promoter region of the gene. When a subset of these variations were tested for association with fertility traits, no significant associations were found. Further research is needed to identify the variation associated with the identified QTL.

Fertility is a key target for genetic improvement in dairy cattle. Despite low reported heritability, it is estimated that one third of the decline in pregnancy rate over the past decade is related to genetics (310). Several studies have reported QTL for fertility (297, 311, 312). However, very few causative mutations have been identified. The use of microarray data from biologically relevant tissues in combination with QTL data has enabled the identification of variations in genes regulating phenotypes in cattle previously (161). Arginase1 was the most differentially expressed gene in the endometrium of fertile and sub-fertile strains of dairy cow, and is located very closely to the reported QTL. The lack of association in this study doesn't preclude the genes involvement in fertility of dairy cows, given that intronic gene regions and other non-coding sequence were not analysed. Future research should focus on alternative candidate genes in the area of the QTL, as well as a more comprehensive assessment of the genetic variation in *ARG1.* Alternatively, whole genome association studies of larger populations with divergent fertility phenotypes may be required to identify genetic variation associated with fertility.

Chapter 7. FINAL CONCLUSIONS

7.1. SUMMARY

Fertility in dairy cows has declined significantly over the past few decades, concomitant with increased milk production (313). This infertility is an important economic issue - it is estimated that 50% of the improved profitability acquired through genetic selection for milk production is lost due to declining fertility (2).

A large proportion of failed pregnancies are a result of embryo losses during the pre-implantation period, when the developing embryo is elongating rapidly and signalling its presence to the maternal system (5). A receptive uterine environment and coordinated regulation of endometrial gene expression are essential for successful pregnancy establishment. However, the molecular mechanisms that prevent luteolysis and support embryo survival within the maternal environment are not well understood. A non-receptive uterine environment may result from adverse genetic or epigenetic regulation of gene expression. Further, DNA methylation has been highlighted as a potential contributor in regulating early pregnancy events in the uterus.

To identify why pregnancy loss occurs during the pre-implantation period, the molecular events taking place in the uterus in response to pregnancy in animals of divergent fertility were characterised. Results were used in conjunction with QTL data to identify candidate genes for genetic analysis. It was hypothesised that gene expression in uterine tissues would differ in response to pregnancy, and that the response would differ in fertile and sub-fertile strains of dairy cattle. It was further postulated that genetic variation and DNA methylation regulate the expression or function of key genes in the uterus during pregnancy. To test this, genome-wide gene expression and DNA methylation in the uterus of pregnant and cycling dairy cows with divergent fertility phenotypes at day 17 of pregnancy and the oestrous cycle were characterised. Correlation among DNA methylation and gene expression were then tested. Lastly, an association analysis of genetic variation in a candidate gene with fertility phenotypes was conducted. Analysis of fertility traits in a large cohort of animals revealed two significant QTL locating to the same locus on chromosome 9. To identify candidate genes that underlie these QTL, map positions of genes demonstrating transcriptional

differences between fertile and sub-fertile dairy cows were assessed in the QTL interval. *ARG1* was identified as a positional candidate gene, and sequencing of the F1 sires in the pedigree revealed 38 polymorphisms. A subset of these polymorphisms were genotyped in the F2 daughter population and an association analysis between genotypes and fertility phenotypes was performed.

Gene expression microarray analyses revealed large differences in the transcriptional profiles of pregnant and cycling animals. There were 1,839 and 1,189 differentially expressed transcripts between pregnant and cyclic animals (with ≥1.5 fold change in expression; P-value < 0.05, MTC Benjamini-Hochberg) in caruncular and intercaruncular endometrium, respectively. Gene ontology and biological pathway analysis of differentially expressed genes revealed enrichment for genes involved in interferon signalling and modulation of the immune response in pregnant animals. This suggests that the maternal immune system is affected by the presence of the embryo. Further analysis of microarray data revealed 482 and 1,021 differentially-expressed transcripts (P-value < 0.05) between pregnant fertile and sub-fertile dairy cow strains in intercaruncular and caruncular tissue, respectively. Additionally, functional analyses of fertile and sub-fertile animals revealed enrichment for several pathways involved in key reproductive processes, including the immune response to pregnancy, luteolysis, and support of embryo growth and development, and, in particular, regulation of histotroph composition. Genes implicated in the process of immune tolerance to the embryo were up-regulated in pregnant cows, and down-regulated in sub-fertile cows. A similar pattern was evident in genes involved in preventing luteolysis and promoting embryo growth and development. These data suggest that the sub-fertile animal is responding differently to signals received by the embryo.

To investigate the mechanisms responsible for the differences in expression between pregnant and cycling animals and fertile and sub-fertile animals DNA methylation and genetic analyses were performed. For analysis of DNA methylation and gene expression, the 1,000 most significant correlations between the two measurements were investigated further. Of these, 52% had a negative correlation between DNA methylation and gene expression suggesting possible epigenetic regulation of transcription. When this gene list was compared with lists from the previously reported gene expression studies on the same

tissues, 42% were differentially expressed when comparing pregnant and cycling animals and 11% were differentially expressed in pregnant fertile versus sub-fertile animals. DNA methylation status was correlated with gene expression in several pathways implicated in early pregnancy events in the previous analyses. These data suggest that DNA methylation may regulate the response to the embryo, and may explain some of the differences in this response in fertile and sub-fertile dairy cow strains.

Lastly, the association analyses of polymorphisms in *ARG1* with fertility phenotypes revealed no significant associations. Fertility QTL were identified on the same region of chromosome 9 for the phenotypic traits “pregnant to first service” and “calved to first service”, corresponding to the locus of a fertility QTL identified in an independent study by other researchers (297). Arginase-1 was a candidate gene based on its differential expression between fertile and sub-fertile animals and its biological function and proposed roles in early pregnancy events. The lack of association between polymorphisms in *ARG1* and fertility phenotypes in this study does not support a role for this gene in fertility in this population. However, further interrogation of intronic and non-coding sequence in *ARG1* and other genes in the QTL region is warranted given the presence of two fertility QTL in this population and fertility QTL in the same region in an independent population.

The results of these analyses highlight several key processes likely to be important regulators of pregnancy success. Furthermore, results suggest that endometrial physiology contributes to poor reproductive performance of the sub-fertile dairy cow strain.

The maternal immune system is actively surveying the uterine environment during early pregnancy. The embryo modulates this response, inducing expression of molecules in the endometrium that function to suppress the immune response and/or promote tolerance to the embryo. The results of this study demonstrate this response with widespread differential expression of immune response pathways. During this period of immune suppression the endometrium would be expected to be susceptible to infections; the endometrium must, therefore, actively express specific molecules for defence against foreign pathogens. Up-regulation of genes of the innate immune response, including antimicrobial response genes, support this hypothesis. This system requires intricate control

through expression of protective inhibitors in the endometrium, and raises the question of whether the embryo expresses these same inhibitory molecules. This study provides evidence that the endometrial gene expression profile may contribute to inferior reproductive performance of the sub-fertile dairy cow strain. The immune system of the sub-fertile dairy cows may not be sufficiently tolerant to the embryo, as suggested by reduced expression of genes that promote immune tolerance. Additionally, the sub-fertile dairy cow may have an insufficient signalling response to the embryo that may result in failure to prevent luteolysis. This is supported by the observation of greater expression of genes that promote luteolysis in the sub-fertile strain. Furthermore, DNA methylation status was correlated with gene expression in several pathways implicated in early pregnancy events. This suggests that DNA methylation is regulated by the presence of the embryo and that the DNA methylation pattern differences in fertile and sub-fertile dairy cows could explain their divergent reproductive performance. Although these data do not provide direct evidence of a causative association between DNA methylation and gene expression, they provide critical support for an effect of DNA methylation in early pregnancy events, and highlight candidate pathways for future studies.

Lastly, while no association of polymorphisms in *ARG1* with fertility phenotypes were identified, this does not preclude the gene's involvement in fertility. Given the lack of fertility QTL so far identified in any mammalian species, identification of this interval in an independent population of dairy cows in noteworthy and further interrogation of the chromosome 9 region is warranted.

7.2. **LIMITATIONS**

7.2.1. Sample size

The major limitation of this study is small sample size. Due to the ethical considerations and costs associated with slaughter studies, the discoveries are limited to a relatively small numbers of animals. The consequence of small sample size in studies utilising large scale microarrays is a reduction in statistical power. While every effort was made to control for biological variation, there is always variation between biological replicates. This variation, combined with the large number of statistical tests performed in microarray analysis may

require greater samples sizes to detect smaller effects. Endometrial biopsy to obtain reproductive tissue samples has been proposed as an alternative to slaughter of dairy cows. However, there is debate about the consequence of such a procedure. If an animal is biopsied, its ability to achieve or maintain pregnancy may be severely impacted, and potentially result in the animal being culled from the herd. Further research into biopsy techniques that do not reduce the odds of pregnancy is necessary for the advancement of our understanding in this area.

7.2.2. DNA methylation measurement

Several methods can be used to measure epigenetic marks, such as DNA methylation. Each method has advantages and disadvantages, and these need to be weighed based on the aims of the experiment. Affinity-based methods in combination with microarrays, as used in this study, constitute a relatively high-throughput approach that enables multiple samples to be processed. However, disadvantages of this approach include the inability to distinguish between CpG- and non-CpG-based methylation events, and the inability to determine methylation patterns at single base-pair resolution. Additionally, affinity-based methods can also be biased due to copy number variations and require substantial quantities of high quality input DNA. Generally, the use of array-based methods is limited to species that have a suitable array available. However, an alternative is to design a custom microarray targeting CpG and promoter regions, as was done in this experiment.

Another consideration with affinity-based approaches is that DNA samples extracted from biopsy contain multiple cell types, and each cell within a type will have a different methylation profile. Therefore, this type of analysis only gives an average DNA methylation profile for each cell and cell type that was in the tissue sample used for extraction. Bisulphite sequencing approaches can overcome this issue, when combined with high coverage next-generation sequencing approaches, but this technique will still only give the proportion of cells that are methylated and is unable to distinguish between methylated and hydroxymethylated DNA.

Another issue with large scale DNA methylation analyses is the lack of computational tools and established methodology to analyse the data. In this study, a combination of free

software and in-house computation was used. Bioinformatic tools are rapidly evolving in this area, however there is little consensus on the appropriate methodology to use when integrating array-based datasets with very few publication reporting statistical integration across “omics” platforms.

7.2.3. Bovine genome annotation

A total of 6,158 out of 13,317 unique bovine reference genes mapped to gene expression probes on the Agilent bovine specific microarray. Further, only 39% of DNA methylation probes mapped to within 10kb 5’ of these annotated genes. This resulted in a total of 190,431 unique DNA methylation gene expression probe combinations being identified. Given that the DNA methylation microarray designed contained over 400,000 probes, the majority of data has not been utilized. Further annotation of the bovine genome is required to overcome this issue.

7.2.4. Genetic analysis

Due to limited resources, only one candidate gene could be sequenced and genotyped in the F2 genetic trial population. The position, biological function and differential expression of this gene in our study made it an excellent candidate, but having likely excluded this as the causative gene underlying the fertility QTL identified in this population, further analysis of the QTL region is required. Whole genome sequencing of the F1 sires in this population has recently been completed, and these data may be utilised to identify the causative gene in the future.

7.3. FUTURE DIRECTIONS

The combination of gene expression studies and QTL data will enable the identification of candidate genes underlying fertility in the future, allowing prioritisation based on biological relevance. Given the decreasing cost and yield of DNA sequencing, whole genome sequence data of founder sires will further facilitate identification of variants for association analyses in QTL regions. Ultimately, identifying causative variants will enable integrated into breeding programs to select the most suitable cows for a particular production system.

The correlation of DNA methylation and gene expression data in this study provides impetus for future investigation in this area. Future studies should focus on in depth analysis of regions that demonstrate correlations between DNA methylation and gene expression in a greater numbers of animals. If confirmed, future studies should also focus on ways to influence reproductive outcomes through manipulation of DNA methylation profiles. Several substances have been identified that can influence DNA methylation. Therefore, the use of these substances to improve reproductive outcome could be investigated.

The combination of genetic selection and manipulation of DNA methylation provide exciting avenues for future research to improve the reproductive performance of dairy cows.

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