

## Transcriptomic Analysis of the Bovine Endometrium: What is Required to Establish Uterine Receptivity to Implantation in Cattle ?

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**Abstract.** In cattle, the majority of pregnancy loss can be attributed to early embryonic loss which occurs prior maternal recognition of pregnancy on Day 16 (Day 0 = ovulation). During this time, carefully orchestrated spatio-temporal alterations in the transcriptomic profile of the endometrium are required to drive conceptus elongation, via secretions from the endometrium (termed histotroph) and establish uterine receptivity to implantation. The two main modulators of these processes are progesterone (P4) and the pregnancy recognition signal interferon tau (IFNT). Altered concentrations of P4 in circulation mediate its effects via the endometrium and have been associated with different rates of conceptus elongation in cattle. Transcriptomic analysis of the endometrium has shown that modulation of circulating P4 alters endometrial expression of genes that can contribute to histotroph composition, which is beneficial (when P4 is supplemented) or detrimental (when P4 is reduced) to the developing conceptus. In addition, down-regulation of the progesterone receptor, required to establish uterine receptivity, is altered in the endometrium of heifers with altered P4 concentrations. IFNT, a type 1 interferon, also significantly impacts on the endometrial transcriptome. It induces the expression of a large number of classical interferon stimulated genes as early as Day 15 of pregnancy. In summary, the successful establishment of pregnancy in cattle requires a sequence of key events to ensure appropriate maternally derived secretions, establish uterine receptivity to implantation as well as an adequate endometrial response to IFNT production.

**Key words:** Estrous cycle, Gene expression, Pregnancy, Progesterone

(J. Reprod. Dev. 58: 189–195, 2012)

In cattle, as in other ungulate ruminants, the implantation process is characterized by a protracted period of elongation of the hatched blastocyst followed by apposition, attachment and adhesion of the trophectoderm to the uterine luminal epithelium (LE) [1, 2]. Following successful fertilisation of an oocyte in the oviduct, the resulting embryo enters the uterus on approximately day 4 of pregnancy where it undergoes a number of cell divisions to form the morula which, after further differentiation, forms a blastocyst consisting of the inner cells mass (which will eventually give rise to the embryo/foetus) and an outer cell mass consisting of trophectoderm cells which ultimately give rise to the placenta. Up to the blastocyst stage of development, the embryo is relatively autonomous i.e. contact with the maternal uterine environment is not strictly required, as evidenced by the fact it is possible to culture embryos *in vitro* up to the blastocyst stage of development. However, once the embryo hatches from the zona pellucida (approximately day 9–10), significant morphological changes occur in the conceptus of domestic animals, including cattle, that differentiate the early pregnancy events from those of rodents, humans and non-human primates. The hatched blastocyst forms an ovoid-shaped conceptus between days 12–14 and the elongation process begins, whereby rapid proliferation of the conceptus trophectoderm cells occurs. During this period the conceptus is entirely reliant on maternally derived secretions, collectively termed histotroph, for survival [3].

The dependence of conceptus elongation on histotroph composition of the uterine lumen has been confirmed in an ovine model, whereby ablation of uterine glands peri-natally [4] resulted in the inability of the hatched blastocyst to elongate [5]. Moreover, in cattle, despite attempts to induce elongation *in vitro* [6, 7], hatched blastocysts fail to elongate in a morphologically normal way but will do so if transferred to synchronised recipients [8].

The uterine endometrium is a complex tissue, consisting of luminal epithelial cells (LE), superficial (sGE) and deep glandular (dGE) epithelial cells as well as fibroblast-like stromal cells (STR) and these different cell types, play key roles in driving the elongation process, via endometrial secretions. In addition both spatial and temporal alterations to the endometrial transcriptome are required to establish uterine receptivity to implantation, vital to which, is the loss of expression of the nuclear progesterone receptor from the luminal epithelium [9]. Indeed, these changes are critical to the likelihood of successful pregnancy occurring in cattle given that the pre-implantation period of pregnancy is when most embryonic loss occurs [10–13] and the coordinate actions of progesterone from the corpus luteum as well as conceptus derived Interferon Tau (IFNT) regulate these changes. In the present review, the use of large scale transcriptomic analysis to identify key alterations in the endometrial transcriptome of cattle will be discussed. In particular, emphasis on the effects of progesterone and pregnancy in driving conceptus elongation and establishing uterine receptivity to implantation will be discussed. A model of the spatial and temporal changes in gene and protein localisation that lead to the establishment of uterine receptivity in the bovine endometrium at distinct stages of early pregnancy will be presented.

Received: December 11, 2011

Accepted: December 20, 2011

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## Pregnancy Changes

In cattle, the conceptus trophoctoderm must secrete sufficient quantities of IFNT by Day 16 [14, 15], in order to inhibit the production of luteolytic pulses of prostaglandin F2 $\alpha$  by the endometrium [12, 13, 16] and allow pregnancy recognition to occur. Prior to the advent of large-scale gene expression analysis, knowledge on the effect of pregnancy and/or IFNT on the bovine endometrium *in vivo*, was dependent on a candidate gene approach with particular emphasis on the fact that IFNT is a Type I IFN. Testing this hypothesis revealed that pregnancy and IFNT induced the production of uncharacterised proteins by the endometrium [17]. Some of these proteins and genes induced by pregnancy/IFNT were later revealed to be classical Type I IFN stimulated genes (ISGs) 2',5'-oligoadenylate synthetase 1, 40/46 kDa; *OAS1* [18, 19], ISG15 ubiquitin-like modifier; *ISG15* [20, 21], Chemokine (C-X-C motif) ligand 5; *CXCL5* [22], interferon induced transmembrane protein 3; *IFITM3*, interferon induced transmembrane protein 1; *IFITM1* [23], myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse) MX1, myxovirus (influenza virus) resistance 2 (mouse); MX2 proteins [24] and ubiquitin-like modifier activating enzyme 7; *UBE1L* [25] as well as proteins involved in the luteolytic mechanism (oxytocin receptor; OXTR, estrogen receptor alpha; ESR, nuclear progesterone receptor; PGR: [26], prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase); PTGS2 [27], and cytokines (granulocyte-macrophage colony-stimulating-factor; GMCSF: [27]).

With the widespread availability of large-scale transcriptomic analysis, the molecular mechanisms required to establish uterine receptivity to implantation and pregnancy recognition are beginning to be elucidated. The initial *in vivo* analysis of the effect of the conceptus on endometrial gene expression was carried out using subtracted cDNA libraries [28] and a model of monozygotic twins to reduce genetic variation. The focus of this study was on Day 18 of pregnancy/ non-pregnant heifers and of the 87 differentially expressed genes (DEGs) identified, almost half of these were classical type I IFN-stimulated genes (ISGs). Despite this large induction of Type I IFN genes, this technology allowed for the identification of genes that are involved in cell adhesion (connective tissue growth factor; *CTGF*, glycosylphosphatidylinositol specific phospholipase D1; *GPLD1*, milk fat globule-EGF factor 8 protein; *MFG8*) as well as genes involved in endometrial remodeling (matrix metalloproteinase 19; *MMP19*, tissue inhibitor of metalloproteinase 2; *TIMP2*) that were not previously known to be involved in pregnancy recognition/implantation in the endometrium of cattle. The second model utilised suppressive subtractive hybridisation technology also but used *in vivo* derived embryos [29]. Additional transcripts were identified in this study (109) as differentially regulated in endometria of pregnant compared to cyclic heifers on Day 18. Again, a large proportion of these genes are recognised as Type I IFN responsive genes; however, additional genes involved in the roles of cell adhesion, endometrial remodelling and modulation of the maternal immune response to pregnancy were identified (Agrin; *AGRN*, complement component 1, r subcomponent; *C1R*, complement component 4A (Rodgers blood group); *C4A*, CD81 molecule; *CD81*, *claudin 4 CLDN4*, *claudin 10 CLDN10*, meprin

A, beta; *MEPIB*, met proto-oncogene (hepatocyte growth factor receptor); *MET*, lectin, galactoside-binding, soluble, 9; *GALS9*, lectin, galactoside-binding, soluble, 3 binding protein; *LGALS3BP*, serpin peptidase inhibitor, clade G (C1 inhibitor), member 1; *SERPING1*, transglutaminase 2 (C polypeptide, protein-glutamine-gamma-glutamyltransferase; *TGM2*). Using an oligonucleotide microarray, Mansouri-Attia *et al.* [30] identified similar DEGs in response to pregnancy at a later time-point (Day 20). However, uniquely to this study the intercaruncular and caruncular regions of the endometrium were analysed separately. This resulted in the identification of 446 DEGs in the caruncular and 1,295 in the intercaruncular region. The fact that more genes were altered in the intercaruncular compared to caruncular region is not surprising, given that the intercaruncular region is suffused with glandular epithelial cells and in sheep, a number of non-classical ISGs are induced by IFNT during pregnancy recognition in these cells [31].

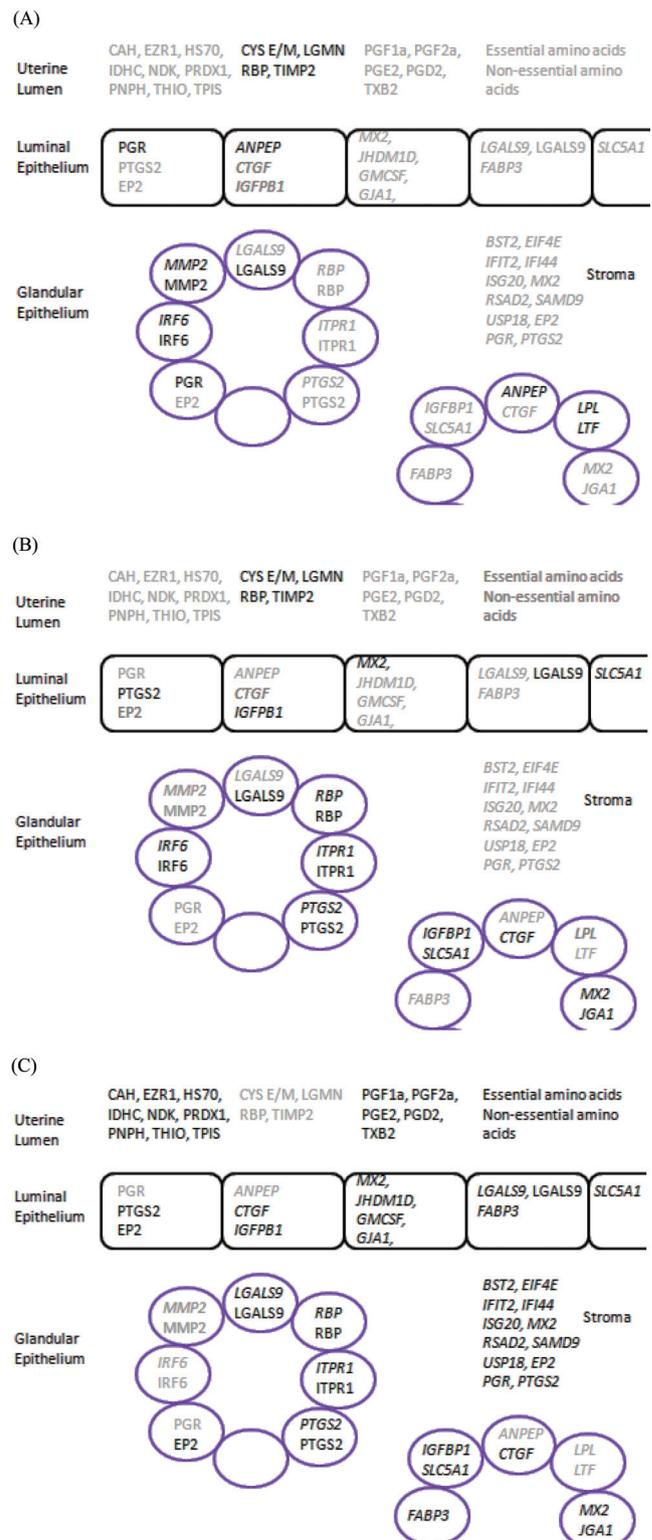
These studies were focused on the endometrial response to the conceptus after pregnancy recognition had occurred. Our group has recently published how the presence of the embryo affects endometrial gene expression at distinct developmental stages of early pregnancy compared to non-inseminated cyclic controls. Using the Affymetrix microarray platform, no differences were detectable between pregnant and cyclic heifers on Day 5 (8- to 16-cell stage embryo), Day 7 (morula/early blastocyst stage of development) or on Day 13 (ovoid conceptus and initiation of elongation) [32]; the only detectable differences were on Day 16. This suggests that the transcriptomic alterations that occur in the endometrium as time from estrous to the luteal phase progresses, occur in a similar manner irrespective of whether an embryo/conceptus is present. In addition, the majority of these genes are also involved in the classical Type I IFN response and are comparable to those identified by Walker *et al.* [33] on Day 17 of pregnancy. Comparison of these DEGs with previous studies during the peri-implantation period of pregnancy revealed that 27 are differentially expressed on Day 16 as part of the early endometrial response to the conceptus (aconitase 2, mitochondrial; *ACO2*, beta-2-microglobulin; *B2M*, *XIAP associated factor 1*; *BIRC4BP*, *C4A*, *C14orf10*, *CD81*, *C1R*, *CLDN4*, *eukaryotic translation initiation factor 4E*; *EIF4E*, epithelial stromal interaction 1 (breast); *EPST11*, interferon, alpha-inducible protein 6; *IFI6*, GABA(A) receptor-associated protein like 1; *GABARAPL1*, *ISG15*, interferon regulatory factor 9; *IRF9*, RNA helicase LGP2; *LGP2*, *LGALS9*, *LGALS3BP*, *MX2*, *MX1*, *OAS1*, proteasome (prosome, macropain) inhibitor subunit 1 (PI31); *PSMF1*, receptor (chemosensory) transporter protein 4; *RTP4*, signal transducer and activator of transcription 1, 91kDa; *STAT1*, *SCOTIN*, *UBE1L* and tryptophanyl-tRNA synthetase; *WARS* [32]) and may represent early endometrial markers of a viable pre-implantation conceptus. More recently, Bauersachs *et al.* [34] used Gene Set Enrichment Analysis which revealed a strong correlation between those genes induced by pregnancy Day 16 [32], 17 [33], 18 [28, 29] and 20 [30] with their reported data on Day 15 and 18 of pregnancy [34]. This demonstrates that despite the use of different animals models, sample processing protocols, transcriptomic platforms and data analysis methods, there is a clear conceptus-induced gene expression signature during the peri-implantation period of pregnancy in cattle.

Global transcriptomic analysis of the endometrial response to pregnancy has not only been used to unravel the molecular events surrounding the process of pregnancy recognition, but can reflect the quality of the conceptus present. As early as Day 18 of pregnancy the response of the intercaruncular endometrium to an IVF-produced conceptus is different to that elicited by a cloned conceptus [35], while this differential response is magnified by Day 20 of pregnancy, in particular, in the caruncular region of the endometrium [36]. Genes implicated in this differential response include those involved in the classical IFNT response of the endometrium (*MX2*, bone marrow stromal cell antigen 2; *BST2*, *IFIT1* [35]; radical S-adenosyl methionine domain containing 2; *RSAD2*, *OASI*[36]) as well as those involved in the establishment of uterine receptivity (fatty acid binding protein 3, muscle and heart (mammary-derived growth inhibitor); *FABP3* [37], serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 14; *SERPINA14*, *CA2* [38]) in both cattle and sheep.

What is clear from these studies is that the predominant effect of the conceptus on the endometrium is to elicit a classical Type I IFN response during the peri-implantation period. This is unsurprising given that both IFNT and IFNA interact with the same receptors in the bovine endometrium [39]. However, it is likely that additional cross talk between the conceptus and the endometrium does occur. Recent studies using RNA sequencing of the bovine embryo/conceptus at distinct stages of development identified that a large number of transcripts are specific to the conceptus only [40] and may reveal novel insights into what additional factors the conceptus produces, other than IFNT, that may modulate the uterine endometrium to establish uterine receptivity; similar to the effects of prostaglandin identified in the sheep endometrium [41]. Indeed, recent data from Bauersachs *et al.* [34] has begun to address this question by comparing gene expression changes due to IFNA, with those induced by the conceptus. One could speculate that the expression of genes induced by the presence of the conceptus and that are not just a result of a non-specific type I IFN response will help unravel additional conceptus-derived factors and the conceptus-maternal cross talk that is required for establishing uterine receptivity to implantation (Fig. 1).

### Cycle Related Changes

The carefully orchestrated temporal changes that occur throughout the estrous cycle in the uterine endometrium have been described by a number of studies [42, 43]. Indeed, correspondence analysis of the factor that contributes most to alterations in endometrial gene expression throughout the luteal phase of the estrous cycle indicates that it is stage of the cycle that contributes most to the overall transcriptional changes that occur [37, 43]. Interestingly, these changes coincide with the time during which the majority of embryonic loss occurs in cattle i.e. prior to pregnancy recognition [12, 13]. The fact that little detectable differences can be determined between pregnant and cyclic endometria prior to Day 15 [33, 34], means that alterations to cyclic endometria associated with different models of pregnancy outcome, can be exploited. One of the key factors that can modulate these temporal changes is circulating concentrations of progesterone. Retrospective studies



**Fig. 1.** Schematic representation of the temporal changes required in the bovine endometrium to establish uterine receptivity to implantation during (A) zona enclosed blastocyst stage, (B) elongation of the conceptus and (C) pregnancy recognition and peri-implantation period. Gene expression is given in italics and protein abundance is given in normal font. Data compiled from references [20, 27, 34, 35, 49, 64, 65, 67, 74–77].

in both beef and dairy cattle have identified a correlation between concentrations of progesterone up to Day 7 following insemination and subsequent pregnancy rates [44, 45]. Other studies in both sheep and cattle have shown that progesterone supplementation increases the length of the conceptus [46] and IFNT output, increasing the likelihood that pregnancy recognition will occur and pregnancy will be maintained.

There is substantial literature available in sheep on the effect of systemic progesterone on endometrial gene expression as well as the coordinate induction by progesterone and further stimulation by IFNT of endometrial gene expression [47]; however, considerably less evidence is available for progesterone effects on endometrial gene expression in cattle. Prior to the availability of large scale transcriptional analysis, the candidate gene approach was used to examine the expression pattern of genes that could be involved in mediating conceptus elongation e.g. those that encoded for growth factors/ cytokines or those involved in the progression of the estrous cycle. The expression of insulin-like growth factor 2; *IGFII*, insulin-like growth factor binding protein 2, 36 kDa; *IGFBP2* [48], prostaglandin E receptor 2 (subtype EP2), 53 kDa; *PTGER2* [49], vascular endothelial growth factor receptor 2; *VEGFR2* [50] in the endometrium, increases during the elongation stage of conceptus development while the expression of *ESR1* [51], matrix metalloproteinase 2 (gelatinase A, 72 kDa type IV collagenase; *MMP2* [52], and *VEGF* [50] declines. Treatment with exogenous progesterone increased the expression of uterine serpins [53] as well as *PGR*, *ESR1* and retinol binding protein 4, plasma; *RBP4* [54]. These data indicate that as progesterone concentrations increase, either as the luteal phase of the estrous cycle progresses, or with exogenous progesterone supplementation, the expression of genes and proteins in the endometrium is altered.

### Progesterone Effects on the Endometrium

Considerable evidence in the literature has demonstrated that elevated concentrations of circulating progesterone in the immediate post-conception period are associated with advanced conceptus elongation [46, 55, 56], increased IFNT production [57, 58] and higher pregnancy rates in cattle and sheep [59–61] with a converse effect on pregnancy rates in dairy cows when progesterone concentrations are low in circulation [10]. Our group has recently begun to address the effect of how the endometrium responds to manipulation of progesterone concentrations *in vivo* and have been using the working hypothesis that when progesterone is altered in circulation, the endometrial transcriptome is changed, which impacts on the composition of the histotroph. This alters the elongation process of the conceptus resulting in different IFNT output which may affect pregnancy recognition. To address this hypothesis we have generated two *in vivo* models. Firstly we caused an early increase in progesterone concentrations by insertion of an intravaginal progesterone device on Day 3 of the cycle/pregnancy [56]. Secondly we have generated a model of induced low progesterone in circulation where output from the corpus luteum is consistently lower throughout the estrous cycle [62]. Manipulation of progesterone using both of these models has clear impacts on conceptus elongation [43, 56] and most interestingly, if the uterine

environment is primed to elevate/delay progesterone prior to Day 7, embryos transferred into these recipients either benefit (in the case of high progesterone) or are compromised (when progesterone output is low) with respect to elongation [8, 43]. Indeed, available evidence supports the notion that these progesterone mediated effects on elongation is via indirect alterations to the endometrial transcriptome [37, 43], rather than direct effects on the embryo itself [8, 63].

As there were clear impacts on the elongation of the conceptus in both of these models, the focus of our subsequent studies was on gene expression changes in the endometrium that could possibly contribute to the composition of the histotroph i.e. those genes that encode for secreted proteins or are transporters. In a normal progesterone environment i.e. no external progesterone manipulation, between Day 7 (when the embryo is enclosed in the zona pellucida) and Day 13 (when the hatched blastocyst begins to elongate) the expression of a large number of genes that may contribute to the histotroph are temporally modulated [32] including anlyl (membrane) aminopeptidase; *ANPEP*, chromogranin A (parathyroid secretory protein 1); *CHGA*, *CLDN4*, cystatin E/M; *CST6*, connective tissue growth factor; *CTGF*, *EEDA*, *FABP3*, *IGFBP1*, lipoprotein lipase; *LPL*, *lactotransferrin*; *LTF*, macrophage migration inhibitory factor (glycosylation-inhibiting factor); *MIF*, neuromedin B; *NMB*, *PTGS2*, solute carrier family 2 (facilitated glucose/fructose transporter; *SLC2A5*. The expression of these genes are also modulated co-ordinately with the presence (on Day 7) or absence (Day 13) of the nuclear progesterone receptor from the LE and GE [64]. In the model where progesterone is increased early and conceptus elongation is promoted there was a clear advance in the normal temporal changes that occur in the endometrium [37] including increases in the duration of expression of a number of selected genes (*ANPEP*, *CHGA*, *CTGF*, diacylglycerol O-acyltransferase 2; *GAT2*, dikkopf homolog 1 (*Xenopus laevis*); *DKK1*, *FABP3*, *LPL*, *LTF*, *NMB*, *SLC2A5*, solute carrier family 5 (sodium/glucose cotransporter), member 1; *SLC5A1*). Moreover, expression of the progesterone receptor was decreased in the LE and SG earlier [64]. In contrast, in the model of delayed post-ovulatory increase in progesterone and impaired conceptus elongation, the expression of these genes was also modulated as well as the progesterone receptor expression being maintained for longer in the LE and SG [43]. What is clear from these studies is that altered concentrations of progesterone in circulation affect the down-regulation of the progesterone receptor which is required to establish uterine receptivity to implantation. The large scale transcriptional analysis of the endometrium from these models has allowed us to identify genes that are turned on which may promote conceptus elongation. However, further study is required to identify if the protein products of these genes are secreted into the histotroph, as well as determine if these molecules directly drive proliferation of the conceptus trophectoderm.

### Other Models of Uterine Receptivity

Microarray analysis has also been used in other models of fertility to categorise additional candidates' of uterine receptivity that are not directly regulated by progesterone or IFNT. Recent reports

by Salilew-Wondim *et al.* [65] used a unique model to analyse endometrial gene expression in the estrous cycle preceding embryo transfer and correlated these expression patterns to pregnancy outcome in the subsequent cycle. Relatively similar numbers of genes were up and down regulated in what was termed the receptive endometrium i.e. those resulting in successful pregnancy (612 and 514 respectively) on Day 7 with minimal DEGs identified on Day 14. What is particularly interesting regarding this study is the fact that progesterone concentrations are not repeatable between successive estrous cycles [66] indicating that additional factors are affecting the receptivity of the endometrium in these animals. The increased expression of adenosine A2b receptor; *ADORA2B*, *IRF6*, inositol 1,4,5-triphosphate receptor, type 1: *ITPR1* prostaglandin E receptor 4 (subtype EP4); *PTGER4*, *PTGS2* and TIMP metalloproteinase inhibitor 3; *TIMP3* with a coordinate decrease in angiotensin II receptor, type 1; *AGTR1*, *MMP2* and signal transducer and activator of transcription 5A; *STAT5A* seem to be indicative of a receptive uterine environment. Similar classes of genes were identified in another model of fertility where endometrial gene expression was compared between post partum dairy cows with mild (good pregnancy outcome) versus severe (poor pregnancy outcome) negative energy balance. The increased expression of alpha-2-HS-glycoprotein; *AHSG*, *MMPI*, *MMP3*, *MMP9* and *MMPI3* in cows with severe negative energy balance are possible markers of a compromised uterine environment [67], while increased secreted phosphoprotein 1; *SPP1* was a marker of reproductive superiority in heifers following an oxytocin challenge *in vivo* [68]. The use of large-scale transcriptional analysis in these and other studies will continue to allow the detection of an optimal versus a compromised uterine environment.

### Histotroph Composition

Modulation of endometrial gene expression in the ovine model has clearly demonstrated that these alterations can manifest in changes to the composition of the uterine histotroph [69–73]. One could hypothesise that this would hold true for cattle; however, there are very limited data available to test this hypothesis. The presence of both retinol and retinol binding protein (RBP) have been detected in uterine luminal fluid on Day 15 in cyclic animals [74] and the use of 2-D gel electrophoresis identified increased protein abundance of carbonic anhydrase, ezrin, heat shock protein 70, isocitrate dehydrogenase, nucleoside diphosphate kinase, peroxiredoxin 1, purine nucleoside phosphorylase, thioredoxin and triosephosphate isomerise with a coordinate decrease in cystatin E/M, legumain, RBP and tissue inhibitor of matrix metalloproteinase 2 [75]. In addition to these proteins, the concentration of 6-keto PGF1alpha, PEF2a, PGE2, PGD2 and TXB2 are higher in the uterine fluid from pregnant heifers on Day 15 and 18 compared to their cyclic counterparts [76] as well as increased concentrations of both essential and non-essential proteinogenic amino acids in the gravid uterine horn on Day 18 [77]. It is likely that these molecules play a role in driving the elongation process of the conceptus and contribute to successful pregnancy prior to implantation. A key area of research will be determining the regulation of these molecules by progesterone, IFNT and/or prostaglandins, how they function

to drive successful growth and development of the conceptus, as well as examining their composition in the uterine luminal fluid of *in vivo* models of compromised pregnancy outcome.

### Conclusions

In conclusion, the widespread availability of different transcriptomic platforms has significantly increased our understanding of what transcriptional events take place during the progression of the estrous cycle. In addition, the molecular mechanisms of pregnancy recognition and implantation as well as the role that circulating concentrations of progesterone and other models of a 'good' versus an inadequate uterine environment are beginning to be uncovered. Nonetheless, significant efforts must be made to follow up these studies with localisation of important genes and proteins as well as analysis of histotroph composition in the uterine lumen. This will allow us to truly understand what is required to drive conceptus elongation, establish uterine receptivity to implantation and promote successful pregnancy in cattle.

### Acknowledgments

Some of the authors' work referred in this review was supported by Science Foundation Ireland (07/SRC/B1156). NF is supported by a grant from Science Foundation Ireland (10/IN1/B3011).

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