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Review: Endometrial function in pregnancy establishment in cattle

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ABSTRACT

The endometrium is fundamentally required for successful pregnancy in ruminants and species where the posthatching conceptus undergoes a protracted elongation and peri-implantation phase of pregnancy. Moreover, there are substantial waves of pregnancy loss during this pre- and peri-implantation period of pregnancy the precise source of which has not been clearly defined i.e., the maternal uterine contribution to this loss. Understanding the molecular interactions required for successful pregnancy in cattle will allow us to intervene to support pregnancy success during this vulnerable window. The endometrium contributes to most key developmental milestones of pregnancy establishment, including (1) contributing to the regulation of the oestrus cycle, (2) nourishing the preimplantation conceptus, (3) responding to the conceptus to create a more receptive microenvironment, (4) providing essential biophysical support, and (5) signalling and producing factors which affect the mother systemically. This review will summarise what we currently know about conceptus-maternal interactions as well as identify the gaps in our knowledge that could be filled with newer *in vitro* model approaches. These include the use of microfluidics, organ-on-a-chip devices, and bioinformatic approaches. This will help maximise food production efficiency (both meat and dairy) and decrease the environmental burden, while enhancing our understanding of the fundamental processes required for successful implantation in cattle.

Implications

Understanding how the embryo and the maternal environment communicate is critical as this is when most pregnancy loss occurs. Understanding these fundamental interactions is difficult as we are limited with what we can investigate in live animals. However, newer tools are being developed that will allow us to answer fundamental questions. These include how does implantation occur, how is this affected by the developmental potential or sex of the embryo. This will allow us to understand what happens when these processes go wrong and how we can intervene to sustainably enhance food production.

Introduction

This review summarises current knowledge surrounding conceptus-endometrial communication during the preimplantation period in cattle. It aims to identify limitations in current techniques and proposes novel techniques to investigate this period including maternal systemic effects. The preimplantation period is of special interest as the majority of pregnancy losses occur during this period in cattle (Diskin and Morris, 2008; Diskin et al., 2012). These losses reduce the efficiency of the meat and dairy production industries, which is both financially costly and worsening the industries' environmental impact. Therefore, reducing early pregnancy losses in cattle by working to better study the preimplantation period is of increasing importance.

The critical processes of establishing uterine receptivity to implantation and the maternal recognition of pregnancy are dependent upon communication to occur between the endometrial tissue and a sufficiently elongated competent conceptus (Forde et al., 2011c; Sánchez et al., 2019b), and maternal systemic effects via the endometrium (Oliveira et al., 2008). These signalling events have been studied using *in vivo* techniques, but it is difficult to untangle the complex signalling between tissues and often requires slaughter, large numbers of animals involved, and is very costly. Traditional *in vitro* systems can be used to investigate

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certain aspects of the conceptus-endometrial cross-talk but cannot fully recapitulate the elongated conceptus, the maternal circulation, or the endometrial tissue architecture. We therefore propose that work should focus on developing novel *in vivo*-like techniques to study this process *in vitro* and discuss the limitations and possibilities of that in this review.

Origin, development, and structure of the endometrial tissue

The endometrium, the inner lining of the uterus, is a mucosal, highly dynamic tissue with regenerative properties. The uterine endometrium plays a critical role in pregnancy success by providing an appropriate environment for an embryo to undergo specified developmental milestones including morphological changes and tissue differentiation. It provides nutritional support prior to development of the placenta, as well as biophysical support for implantation. The ability of the endometrium to support pregnancy success begins with its development in utero when the reproductive tract is undifferentiated. This process of reproductive tract specification is conserved in a number of mammals, albeit with species-specific differences (Kobayashi and Behringer, 2003). In the 1940s, Alfred Jost's innovative work laid the foundation of the principle that before sexual differentiation occurs, the embryo possesses both Müllerian and Wolffian ducts, which are the progenitors of the female and male reproductive tracts, respectively (Jost, 1947). The development of the female phenotype is a default process and includes the maintenance of the Müllerian duct which ultimately differentiates into the mature female reproductive tract (i.e. oviduct, uterus, cervix, upper part of vagina) (Zhao and Yao, 2019). With the presence of uterine glands observed at about day 250 of gestation (Atkinson et al., 1984), the presence of foetal testicular hormones is required for the masculinisation of the reproductive tract by driving the regression of the Müllerian ducts and retention of the Wolffian ducts (O'Shaughnessy and Fowler, 2011).

Although most of the female reproductive tract organs are developed and differentiated by the time of birth, the uterine morphogenesis is completed postnatally in rodents, large domestic animals, and likely humans (Filant and Spencer, 2014; Spencer et al., 2019). The mammalian uterus has a Müllerian (also known as paramesonephric) origin and consists of a central tubular epithelium surrounded by undifferentiated mesenchyme at birth (Cooke et al., 2013). The stromal cells are differentiated from the uterine mesenchymal cells, whereas the Müllerian duct epithelium differentiates into uterine luminal epithelium. The postnatal developmental events that are related to the endometrial layer are (i) the organisation and stratification of endometrial stroma, and (ii) adenogenesis (development of glands) (Cooke et al., 2013). Uterine glands develop as invaginations of the luminal epithelium, which progressively penetrates the stroma; a process which begins primarily, if not uniquely, after birth and is completed within the first 8 weeks in sheep (Wilson et al., 2017). This critical window of uterine gland development has been demonstrated in animal models including the pig whereby bioactive molecules present in maternal colostrum/milk drive uterine gland adenogenesis. Therefore, establishing the capability of the endometrium to support successful pregnancy begins in utero, with additional postnatal development of glands and important components of endometrial function (Gray et al., 2000 and 2001; Cooke et al., 2013).

In its differentiated status, the endometrium is a heterogeneous tissue composed mainly of luminal columnar epithelial, glandular epithelial, and fibroblast-like stromal cells interspersed with cycle-related changes in immune cells, as well as vasculature (Atkinson et al., 1984; Cobb and Watson, 1995). The endometrium of adult bovine contains caruncular regions that are oval thickenings of the uterine mucosa, organised in irregular rows that run the length of each uterine horn (Atkinson et al., 1984). These play a vital role in placentation, as they are the maternal sites of attachment with foetal membranes (Filant and Spencer, 2014). The appropriate function of the endometrium, for which these three cell types are highly responsible, is crucial for the implantation and establishment of pregnancy (Forde and Lonergan, 2012).

Overall body growth and reproductive tract development occur in an asynchronous manner. Endometrial morphology prior to puberty onset undergoes a wave of luminal epithelium height regression at 1–2 months of age (Hopper, 2021). In heifers, the uterus grows at a similar rate to the body until 6 months postpartum, but from that point, it grows more rapidly until puberty (Hopper, 2021). The variability in age at puberty (average 9–10 months) is affected by genetic, nutritional, and environmental factors (Kinder et al., 1995). Luminal epithelial cell height then enters a period of rapid acceleration at about 6 months of age (Hopper, 2021). It has also been demonstrated that the onset of puberty is positively correlated with increased endometrial thickness in Nellore heifers (Monteiro et al., 2013).

The complex endocrine events that culminate in the onset of reproductive capacity are based on the maturation of the hypothalamic-pituitary-ovarian-uterine axis and are thoroughly reviewed by Kinder et al. (1995). Increased luteinising hormone pulse frequency is a prerequisite for the onset of puberty in heifers, as it causes a cascade of downstream interactions leading to sexual maturation. Genome analysis has identified molecular pathways which regulate puberty initiation in cattle (reviewed by Fortes et al., 2016) and RNA sequencing identified the expression of the proenkephalin (***PENK***) gene is significantly higher in prepubertal compared to postpubertal heifers in tissues including the uterus and endometrium (Cánovas et al., 2014). As increased progesterone levels have been associated with elevated *PENK* expression in sheep (Taylor et al., 2007), it has been proposed that these progesterone surges, common in puberty-transitioning heifers, might be required to upregulate *PENK* expression and activate its pathways, which in turn regulate gonadotrophin-releasing hormone expression (Fortes et al., 2016). It is clear that *in utero* and postnatal development of the endometrium is critical for its function to support pregnancy success.

Response of the endometrium to hormonal cues in circulation

The oestrous cycle in cattle ranges between 18 and 24 days consisting of a long luteal phase (14–18 days), characterised by the presence of corpus luteum (**CL**), and a shorter follicular phase (4–6 days), with degeneration of the CL until ovulation (Forde et al., 2011b). These cyclical events are finely orchestrated by actions of hormones released from the hypothalamic-pituitary–gonadal axis, with the concentrations of oestradiol and progesterone (**P4**) exerting the greatest influence on the spatial and temporal changes that occur in the endometrium during the oestrus cycle. The endometrium plays a pivotal role in the complex hormonal interactions that regulate the oestrus cycle. It is the source of prostaglandin $F_{2\alpha}$ (**$PGF_{2\alpha}$**), which serves as a luteolytic hormone (Lamothe et al., 1977), leading to the structural and functional regression of the CL. The release of $PGF_{2\alpha}$ is pulsatile and oxytocin-dependent. In sheep, it has been demonstrated that the maintenance of the luminal and glandular epithelium integrity is required for $PGF_{2\alpha}$ production able to induce luteolysis (Gray et al., 2000). This finding implies the presence of oxytocin receptors in these cells, as well as a $PGF_{2\alpha}$ synthesis mechanism. Indeed, Jenner et al (1991) showed that there is an increase in the concentration of oxytocin receptors in the uterine tissue of cows around the time of luteolysis, whereas pregnant animals exhibited suppression of

the oxytocin receptors at the expected time of luteolysis (Vejlsted and Struve-Christensen, 1977). In the presence of pregnancy, the release of specific factors such as interferon-tau (**IFNT**) by the elongating embryo inhibits the transcription of the oxytocin receptor gene specifically in the endometrial luminal and glandular epithelium in sheep and cattle, leading to the maintenance of the CL and P4 production (Hansen et al., 2017).

After the onset of puberty (usually by 12 months in cattle), the hormonally controlled oestrus cycle begins. The bovine oestrus cycle can be split into two distinct stages, a follicular phase of 4–6 days followed by a luteal phase of 14–18 days (reviewed by Forde et al., 2011a, 2011b and 2011c). Briefly, during the follicular phase, P4 concentrations in maternal circulation are low, and luteinising hormone acts upon the follicle to cause ovulation thereby ending the follicular phase (Roche, 1996). The site of the ovulated follicle develops into a CL structure on the ovary, the presence of which determines the length of the luteal phase. After the CL regresses, the follicular phase begins and the follicle matures fully in readiness for ovulation, and the cycle repeats. Many other hormones produced by other maternal tissues are involved in regulating the bovine oestrus cycle which are not the focus of this review but have been well reviewed (Kojima, 2003; Forde et al., 2011b). The endometrium is exposed to these fluctuating maternal hormones via the blood supply and the endometrium is itself the source of the hormone $PGF_{2\alpha}$ which is responsible for CL regression and the continuation of the cycle (Hansel and Convey, 1983). A number of more recent studies have shown that manipulation of the size of the ovulated follicle and therefore the maternal hormonal profile alters the endometrial transcriptome (Sá Filho et al., 2017), endometrial amino acid transporter expression (França et al., 2017), uterine tissue phospholipid profile (Belaz et al., 2016), as well as specific pathways in the endometrium (Scolari et al., 2016; Oliveira et al., 2017). In addition, oestradiol concentrations alter the expression of selected pregnancy recognition transcripts in the endometrium as well as overall transcriptional profile of the endometrium, and uterine luminal fluid (**ULF**) composition (Northrop-Albrecht et al., 2022a and 2022b).

There is substantial evidence in the literature that higher concentrations of P4 in maternal circulation lead to enhanced conceptus development and pregnancy success (Lonergan and Sánchez, 2020). These changes are mediated by direct changes to the endometrial transcriptome (Forde et al., 2009, 2011a, and 2012; Forde and Lonergan, 2012) and composition of the ULF (Forde et al., 2014b; Simintiras et al., 2018; 2019a) rather than a direct effect on the embryo/conceptus itself (Clemente et al., 2009). Rising P4 concentrations during the luteal phase correlates with the rapid growth phase of the conceptus (known as elongation), the energy requirements of which are provided by the histotroph (Simintiras et al., 2019b). Low circulating concentrations of P4 during the luteal phase are linked with lower pregnancy success rates (Lonergan, 2011) and reduced elongation (Forde et al., 2011a). Therefore, maternal P4 concentration indirectly contributes to early pregnancy success via alterations to the composition of ULF. Spatial differences in exposure also result in differences in conceptus signalling response with differences observed between the ipsilateral and contralateral endometrial transcriptome (Sánchez et al., 2019a; Bagés-Arnal et al., 2020). In addition, increased exposure to P4 during the luteal phase also leads to spatial and temporal transcriptional changes within the endometrium, including loss of expression of the P4 receptor by day 13 in the endometrial luminal epithelial cells and in LE and surface glandular epithelium by day 16 (Spencer and Bazer, 1995). This is critical as loss of the P4 receptor from the luminal epithelium and superficial glands is a prerequisite to establish uterine receptivity to implantation in all mammalian species studied thus far and it is clear that concentrations of P4 in circulation modify the timing of establishing receptivity to implantation (Bazer et al., 2009; Okumu et al., 2010).

The endometrium supports conceptus development

The preimplantation bovine embryo enters the uterus by day 5 and undergoes morula compaction, blastocyst formation and subsequent hatching from the zona pellucida by day 13, followed by a period of rapid elongation to form a long thin filamentous conceptus, going from around 6 cm on day 16, to 20 cm by day 19 (Betteridge et al., 1980). This process occurs prior to placentation and so the conceptus relies solely on nourishment from the histotroph, as evidenced by the absence of endometrial glands in a sheep model resulting in a failure of the blastocyst to elongate (Gray et al., 2001). The bovine conceptus is particularly dependent upon the ULF due to the huge energy expenditure required during elongation and due to the protracted preimplantation stage lasting until around day 20 before placentomes begin to form (Brooks et al., 2014).

ULF contains secretions from the conceptus itself but is primarily composed of histotroph. Histotroph is a nutrient-dense fluid produced by the endometrium, particularly the endometrial glands, composed of enzymes (Simintiras et al., 2022), proteins (Forde et al., 2014a), amino acids (Forde et al., 2014b), growth factors (Keller et al., 1998), extracellular vesicles (**EVs**) (Nakamura et al., 2019), ions and other factors (Simintiras et al., 2019c). Conceptus elongation does not occur in the absence of glands in ewes (Gray et al., 2001), and preflushing the ULF prior to embryo transfer reduces pregnancy success in cattle (Martins et al., 2018), indicating that components of ULF are critical for conceptus elongation. Specifically, the conceptus relies upon the ULF as a source of glucose, adenosine monophosphate, and ATP as sources of energy, adenosine as a building block for DNA, RNA, and adenosine monophosphate, and hypoxanthine as another source of components for DNA and RNA synthesis (Simintiras et al., 2019b).

The composition of ULF is not only sensitive to steroid hormone concentrations in the mother but is also reflective of the maternal metabolic environment. The transcriptome of the endometrium (Bauersachs et al., 2017), the transcriptome of the conceptus (Forde et al., 2017), and composition of ULF (Bauersachs et al., 2017) are all altered by maternal metabolic status. More recent work has shown that disruption specifically to ULF composition directly impacts upon pregnancy success (Martins et al., 2018). ULF composition changes throughout the oestrus cycle according to circulating P4 concentration, including metabolites (Simintiras et al., 2019c) and certain amino acids (Mullen et al., 2012). Therefore, conceptus elongation is dependent upon the ULF composition which is itself dependent upon the endometrium being primed by circulating maternal hormones. Any factor (such as RNA, protein or hormone) secreted into the ULF is vulnerable to degradation and exposure to changes such as pH (which ranges from 6.85 to 7.35 in cattle (López-Albors et al., 2021)). In addition to classically secreted molecules, an additional way in which different components can be transported into the ULF is via EV-mediated transport. EVs are membrane-bound vesicles which are produced from a tissue of origin and have a variety of contents (DNA, coding and non-coding RNA species, proteins, lipids) which can then be released into the recipient tissue (O'Neil et al., 2020). EVs act as communication devices which can transfer material between cells and tissues as their contents are protected within the lipid bilayer (Doyle and Wang, 2019). Recent evidence also demonstrates that the packaging of contents into EVs is selective, and their uptake is specifically targeted to certain tissues through receptor-receptor interactions (Kwok et al., 2021). EVs have been characterised in the ULF of cattle (Kusama et al., 2018) and sheep

(Burns et al., 2014), and secreted by human endometrial cells in culture (Ng et al., 2013). Burns et al (2016 and 2018) showed that it was primarily epithelial glands that produced endometrial EVs in sheep, EV secretion increased from days 10 to 14 in cyclic (corresponding to the start of conceptus elongation) and also in P4-treated sheep, and that those EVs are taken up by the conceptus. Therefore, EVs provide a route of communication from the endometrium that protects their cargo and delivers them to the conceptus- potentially driving elongation and development.

The endometrium responds to signals from the conceptus

Up to around day 7 postovulation, the embryo and endometrium can act independently of each other i.e. the embryo does not need to interact with the endometrium, nor the endometrium interact with the embryo to drive successful morphological changes in the embryo or to provide a suitable environment in which implantation can occur. This is evidenced by the production of developmentally competent embryos *in vitro* and successful pregnancy outcomes following embryo transfer. As mentioned above, specific stages of development i.e., conceptus elongation, require endometrial exposure and uterine lumen biophysical support to form a conceptus. The converse is also true, and for a pregnancy to establish and continue the endometrium requires exposure to a competent conceptus by day 16 at the latest (Betteridge et al., 1980). If no conceptus or a conceptus that is not developmentally competent is present by day 16, the CL undergoes luteolysis and the animal remains cyclic. During the rapid elongation phase, the lengthening conceptus produces increasing amounts of IFNT into the ULF. IFNT is the pregnancy recognition signal unique to ruminant species first discovered in cattle in 1979 (Lewis et al., 1979; Wilson et al., 1979). The discovery and role of IFNT in cattle have been extensively reviewed in far greater detail than the scope of this review (Hansen et al., 2017). IFNT binds with the interferon receptor expressed on the apical endometrial epithelial cell membrane ultimately inhibiting the signalling cascade induced by $PGF_{2\alpha}$ when IFNT is present in sufficient quantities resulting in blocking luteolysis of the CL on day 16 (Hansen et al., 2017). This process is also known as the 'maternal recognition of pregnancy' or 'pregnancy recognition' and is an entirely conceptus-initiated process which is essential for pregnancy establishment in cattle.

Endometrial exposure to an embryo can occur as late as day 16 in cattle to produce a successful pregnancy (Betteridge et al., 1978), indicating that after day 16, conceptus modulation of the endometrium is essential for pregnancy progression. However, studies have demonstrated that the oviduct transcriptome responds as early as day 3 following multiple embryo transfer (Maillo et al., 2015) and that the embryo alters the composition of the ULF as early as day 7 (Sponchiado et al., 2019).

Aside from downregulating the oxytocin receptor in the endometrium (leading to maternal recognition of pregnancy), IFNT also acts as a type I interferon, stimulating the expression of interferon-stimulated genes (**ISGs**) in the endometrium (Fig. 1) (Chaney et al., 2021). Some classical ISGs include *ISG15*, *MX1*, *MX2*, *STAT1*, and *OAS1*. IFNT has also been shown to alter the expression of other genes, termed 'non-classical ISGs', including *LGALS15* in sheep and *SLC2A1* in cattle. Non-classical ISG expression is usually limited to the luminal endometrial and superficial glandular endometrial cells (Spencer et al., 2008). Classical ISGs are usually related to immune response regulation whereas non-classical ISGs are usually related to modifying the endometrium to support the developing conceptus (e.g., histotroph composition). The role of IFNT as the pregnancy recognition signal has been well described, but over the last few decades, efforts have also focussed on identifying other factors the conceptus produces during the critical peri-implantation period and what actions these factors may have. Bauersachs et al (2012) demonstrated a far greater transcriptomic effect of the conceptus upon the bovine endometrium than treating the endometrium with endogenous IFNT alone *in vivo*. Although some of this effect could be attributed to biophysical interactions that occur between conceptus and endometrium, their work indicates that the endometrium is responding to signals from the preimplantation conceptus other than IFNT. Recent work has utilised a range of techniques to identify these other factors secreted by the conceptus and their role in modulating the endometrium and the other roles of IFNT besides acting as the pregnancy recognition signal (Fig. 1) (Forde et al., 2011c; 2015).

The original studies on pregnancy recognition in cattle identified that the conceptus produces proteins other than IFNT; however, it is only recently with high-throughput proteomics approaches that we can identify what these other proteins are. For example, candidate proteins which are specifically conceptus-derived and secreted into the ULF which may therefore act upon the endometrium have been identified via mass spectrometry (Forde et al., 2015). Forde *et al* identified 30 novel conceptus-derived proteins in cattle using proteomic analysis of both ULF and day 16 conceptus-conditioned medium, including macrophage-capping protein (**CAPG**) and protein disulphide isomerase precursor protein. Many of the identified proteins were associated with GO terms for extracellular region, space, or vesicular exosome, indicating that they may be involved in communication from the conceptus to the endometrium by means of EV passage (Forde et al., 2015). Evidence from sheep has demonstrated the ability of the conceptus to secrete EVs which are subsequently taken up by the endometrium (Burns et al., 2016). Another study in sheep identified that ovine EVs in the ULF on day 17 of pregnancy altered the expression of inflammation regulatory-associated genes in bovine endometrium epithelium *in vitro* (Nakamura et al., 2020). The novel conceptus-derived proteins identified by Forde et al (2015) are also capable of altering the endometrial transcriptome *in vitro*. CAPG has been demonstrated to alter both classical and non-classical ISGs *in vitro*, indicating its role may support IFNT in enhancing receptivity of the endometrium to the conceptus (Tinning et al., 2020). CAPG has also been found in ovine ULF EVs (Nakamura et al., 2016) and to be highly expressed in the ovine conceptus compared to the endometrium (Nakamura et al., 2020). We know a substantial amount about the mechanisms by which IFNT induces the maternal recognition of pregnancy process, but less about the role additional factors play in receptivity (Fig. 1). By developing *in vitro* approaches to model these interactions, we will be able to build up a comprehensive picture of implantation in cattle and what defects may be contributing to pregnancy loss at this critical time.

Sex, paternal, and developmental competency influences on these interactions

Given the precise temporal and spatial interactions required for successful pregnancy, a major question has been around the response of the endometrium to different types of embryos/conceptuses. Seminal studies involving cattle from 2009 were the first to identify in any species the biosensor capability of the endometrium (Bauersachs et al., 2009; Mansouri-Attia et al., 2009). Evidence from two separate groups determined there was a difference in the transcriptional response of the bovine endometrium during the peri-implantation period of pregnancy to embryos with different developmental competencies i.e., *in vivo* (high quality), *in vitro* (intermediate quality), and somatic cell nuclear transfer clones (low quality) (Fig. 1) (Bauersachs et al.,

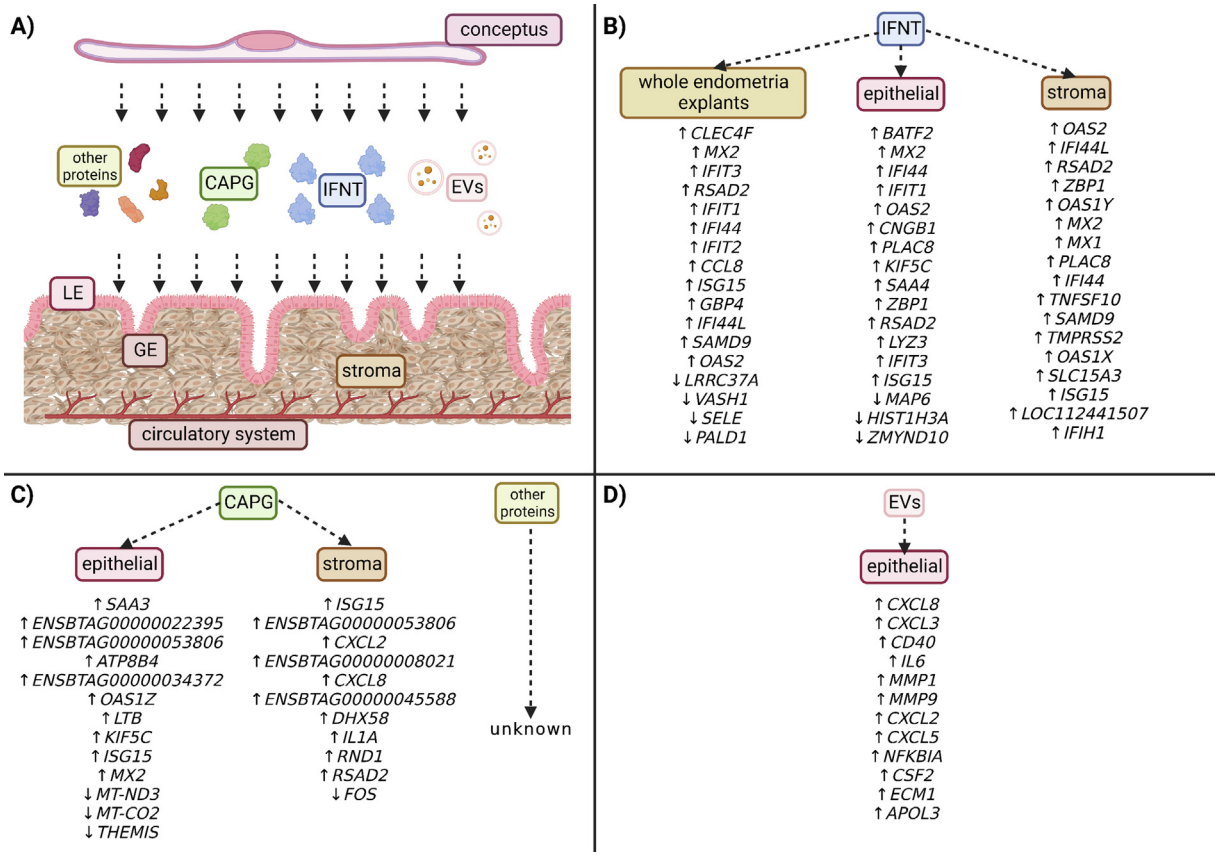

Fig. 1. Effect of bovine conceptus-derived proteins or extracellular vesicles (EVs) on the bovine endometrial transcriptome. (A) Recent work has demonstrated that the bovine conceptus secretes macrophage-capping protein (CAPG) and 29 other proteins (Forde et al., 2015), and that alongside uterine luminal fluid (ULF) extracellular vesicles (EVs) from pregnancy (Nakamura et al., 2019), they may act upon the endometrium to elicit a response, which is composed primarily of stromal, glandular epithelial (GE), and luminal epithelial (LE) cells. (B) RNA sequencing identified response to interferon-tau (IFNT) in bovine whole endometrial explants (Mathew et al., 2019), epithelial, and stromal cells (Chaney et al., 2021) *in vitro*. (C) Work by Forde et al. (2015) demonstrated that the day 16 bovine conceptus also secreted 30 other proteins, but the role of these in pregnancy establishment is as yet unknown. One of the 30 proteins, CAPG, alters the expression of a range of genes in bovine endometrial epithelial and stromal cells *in vitro*, some of which are also altered by IFNT (Tinning et al., 2020). (D) Recent data demonstrated that EVs isolated from bovine ULF during pregnancy can induce a transcriptional response in the bovine endometrium *in vitro* (Nakamura et al., 2019). The studies presented in (C) and (D) both found the response to CAPG or pregnant ULF-derived EVs to modulate the inflammatory response, despite controlling for the effect of IFNT. All data presented represent the most differentially expressed genes identified (up- or down-regulated). Created with Biorender.com.

2009; Mansouri-Attia et al., 2009). In addition to this endometrial response, there were also changes to ULF amino acid composition (Groebner et al., 2011). This hypothesis was further supported from studies in humans where the differences in decidualising stromal cells could sense the development competency of embryos and prevented energy expenditure supporting those (aneuploidy embryos) that would not develop a successful pregnancy (Macklon and Brosens, 2014). How this developmental sensing of the endometrium is mediated remains unknown but in cattle, at least it does not appear to be due to differences in IFNT production by the conceptuses.

There is substantial sexual dimorphism in the transcriptional landscape of bovine embryos and conceptuses. Differences have been observed as early as blastocyst stages of development (Bermejo-Alvarez et al., 2010) with more divergence in the transcriptional profile by the peri-implantation stage of development (Bermejo-Alvarez et al., 2011; Forde et al., 2016). Although no endometrial response to these were observed *in vivo*, studies have identified differences *in vitro* (Fig. 2) (Gómez et al., 2018; Mathew et al., 2019).

Male factor effects

One additional phenomenon that can influence the interactions between the conceptus and the endometrium is prior exposure to male factor(s). There is a substantial body of evidence in a variety of species to show that exposure to various components of the male ejaculate modifies the endometrial environment and tolerises the maternal environment and immune response to support successful pregnancy and prevent rejection of the conceptus allograft (reviewed in detail by Schjenken and Robertson, 2020). In cattle, there is more limited evidence of these interactions (summarised in Fig. 2). For example, conceptuses recovered from endometria exposed to seminal plasma were longer compared to controls with some modifications to the endometrial transcriptome (Mateo-Otero et al., 2020; Recuero et al., 2020) and seminal plasma itself has a detrimental effect on endometrial RNA integrity (Fernandez-Fuertes et al., 2019). Further, RNA sequencing of endometrial samples collected 24 h postmating with a vasectomised bull were not different to controls (Recuero et al., 2020) and minimal changes in those mated to intact bulls indicating that there are likely species-specific effects on the endometrium in response to male factor(s). Not only does the male factor alter the endometrium but the fertility status of the male can influence the endometrial response to conceptuses produced from sperm of different fertility indexes (O'Callaghan et al., 2022). Blastocysts produced *in vitro* using sperm from high- and low-fertile males were transferred into synchronised recipients (O'Callaghan et al., 2022). Sequencing of endometrial explants isolated at slaughter on Day 15 determined conceptuses produced from high-fertility males elicited a different response (overrepresented immune sig-

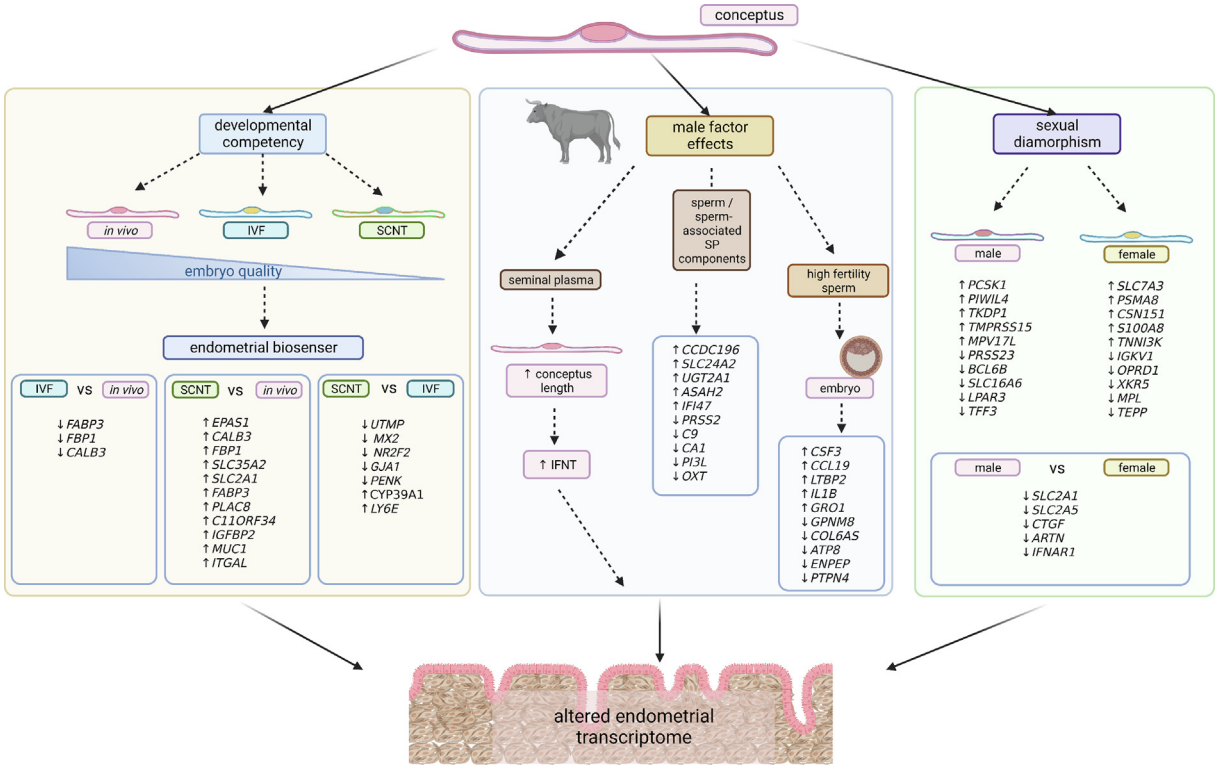

Fig. 2. Factors affecting endometrial response to conceptus developmental competency of the bovine conceptus result in altered endometrial response to the conceptus as the endometrium can act as a biosensor of embryo quality. Exposure to somatic cell nuclear transfer (SCNT) conceptus alters the endometrial transcriptome *in vivo* on pregnancy day 18 compared to *in vitro* fertilisation (IVF)-produced conceptuses (Bauersachs et al., 2009). IVF- or SCNT-produced conceptuses affect the endometrial transcriptome *in vivo* on day 18 of pregnancy compared to *in vivo* (artificial insemination)-produced conceptuses (Mansouri-Attia et al., 2009). These studies have described the effect of conceptus quality upon the endometrium *in vivo* but have limitations which could be overcome by using novel *in vitro* techniques to better understand the mechanisms behind endometrial developmental sensing. **Male factor effects** can also affect the endometrium. Priming the endometrium with seminal plasma (SP) results in longer filamentous conceptus formation (Mateo-Otero et al., 2020) which is associated with increased interferon-tau (IFNT) production (Bertolini et al., 2002), and exposure to sperm or sperm-associated components (but not seminal plasma alone) altered expression of genes in the endometrium (Recuero et al., 2020). Embryos produced from sperm from high-fertility males induced an altered endometrial transcriptional response to that from low-fertility males (O'Callaghan et al., 2022). **Sexual dimorphism** between conceptuses leads to altered expression profiles in endometrial explants *in vitro* after 6 hour exposure to a day 15 conceptus in cattle compared to controls (Mathew et al., 2019), and in endometrial epithelial cells *in vitro* exposed to day 8 male or female embryos for 48 hours compared to controls (Gómez et al., 2018). All these factors influence the endometrium *in vivo*, complicating *in vivo* studies investigating early pregnancy in cattle, therefore, novel techniques are required to better understand subtle differences other factors have upon the endometrium. Created with Biorender.com.

nalling pathways) to those produced from low-fertility males (overrepresentation of pathways associated with cell cycle and proteolysis) (O'Callaghan et al., 2022). The stimulation of IFNT in these explants was also modified.

The mechanism by which these competency and sex-specific responses are induced in the endometrium remains unknown, however, it is likely that these may be mediated by differences in proteomics and/or non-coding RNAs that are secreted from the conceptus in a traditional manner similar to IFNT or indeed packaged as different EV cargo. For example, we have shown that there are differences in the EV cargo from conceptuses with different development competencies (De Bem et al., 2021a; Malo Estepa et al., 2021; Imakawa et al., 2022). Development of novel tools to investigate these mechanisms of action will be required to fully understand the role of EVs in the biosensor capability of the endometrium.

The endometrium provides biophysical support

In cattle and other ruminant species, the blastocyst stage embryo hatches and rapidly elongates to form a filamentous conceptus which can span both uterine horns by day 24 (Bazer et al., 2011). The uterine structure and the endometrium are essential for conceptus elongation as they do not elongate *in vitro* (Brandão et al., 2004) or in the absence of endometrial glands and diminished luminal epithelium development (Gray et al., 2000 and 2001, and 2002). Conceptus elongation has not yet been successfully recapitulated *in vitro*, although attempts have been made using agar/agarose gel tunnels and medium adapted to mimic oviductal fluid (Vajta et al., 2004; Brandão et al., 2004). The culture system had limited success, only achieving 12 mm conceptus length by day 16 (compared to 60 mm *in vivo* (Brooks et al., 2014)). More recent efforts involved using an alginate 3D system which successfully allowed for extended *in vitro* culture (up to 32 days) and attachment but did not mimic elongation, instead producing spherical structures (Zhao et al., 2015). To date, no successful extended *in vitro* culture system has mimicked the uterine/endometrial environment to produce successfully elongated conceptuses, indicating the uterine architecture and endometrium are essential for bovine conceptus development.

Posthatching elongation driven by endometrial secretions is a prerequisite for successful implantation and placenta formation. The cotyledonary nature of the bovine placenta is characterised by the presence of approximately 80 cotyledons by day 70 (Assis Neto et al., 2009). These clumps of villous trophoblast cells biophysically interact with the caruncles on the endometrium by microvillous interdigitation to produce a structure known as a placentome. Placentomes begin to form from day 20 of pregnancy in cattle and are functionally established by days 40–50 (Peter,

2013). These placentomes are the locations of the blood vessels, and the sites where foetal:maternal placental exchange will occur (Haeger et al., 2016). Placentomes form creating a synepitheliochorial interhemal barrier (Pfarrer et al., 2003; Carter and Enders, 2004), where the trophoblast cells do not invade past the maternal basement membrane in cattle. Two types of trophoblast cells are present: columnar trophoblast cells, and trophoblast giant cells (**TGCs**) (also known as trophoblast binucleate or multinuclear cells). The columnar trophoblast cells comprise the foetal contribution to the area of foetal maternal contact (Klisch et al., 1999). Bovine TGCs migrate into the endometrial epithelium, which modify the endometrium by fusing with uterine epithelial cells to produce a temporary syncytium, eventually being replaced by day 40 by uterine epithelial cells in cattle (Carter, 2020). Postimplantation in cattle, trinuclear hybrid cells are present which are one binucleate TGC fused with one endometrial epithelial cell, in contrast to other species such as ovine implantation, in which there is fusion of cells resulting in 25 nuclei (Wooding, 1992). Some extracellular matrix protein changes in the endometrium occur during attachment processes. Both collagen IV and laminin demonstrate altered localisation during the early stages of implantation, with both initially distributed most abundantly around the basement membrane of the epithelium. Expression of these proteins increase in areas deeper into the stromal layer (MacIntyre et al., 2002). It is thought that TGCs may migrate along a laminin matrix (Pfarrer et al., 2003). Integrin subunits α_1, α_3, and α_6 are present in the uterine epithelium, with α_1 distribution increasing throughout the stromal cells with progression of the pregnancy (MacIntyre et al., 2002). It is hypothesised that changing patterns of expression may be in response to the TGCs fusing with the epithelium. Therefore, biophysical interactions of the conceptus trophectoderm with the uterine endometrium, even in bovine species which are superficial in terms of implantation, are necessary to support successful pregnancy.

The endometrium signals back to the mother systemically

Pregnancy recognition signalling in domestic ruminants is considered a local phenomenon involving the production of IFNT by the trophoblast cells of the conceptus that acts as a paracrine signal directly on the uterus to alter gene expression (Spencer et al., 2004). In cattle, the anti-luteolytic effects of IFNT inhibit oxytocin receptor synthesis and prolong the lifespan of the ovarian CL, an essential event for further implantation and placentation (Bazer et al., 2018).

Overall, IFNT can locally modify the expression of more than 750 ISGs in the maternal endometrium (Forde et al., 2011c). Nonetheless, intrauterine infusion of IFNT revealed that their effects are not only local in the endometrium. IFNT can be released by the uterine vein and enter the maternal circulation with effects in extra uterine tissues (Oliveira et al., 2008). Therefore, ISG expression during early pregnancy in ruminants occurs in other tissues such as the CL (Chen et al., 2006; Oliveira et al., 2008; Bott et al., 2010), liver (Ruhmann et al., 2017), and cervix (Kunii et al., 2018). Additionally, since IFNT is transported to these different tissues and cells through the blood circulation, it also induces an increase in the abundance of ISGs in circulating peripheral blood cells (Kizaki et al., 2013; Hansen et al., 2017; Melo et al., 2020).

The bovine conceptus produces highest concentrations of IFNT by about week 3 of pregnancy but concentrations in circulation remain low impeding the use of IFNT as a biomarker of pregnancy (Hansen et al., 2017). The traditional pregnancy diagnosis made by transrectal ultrasound around 30 days postinsemination is a highly accurate method, although the diagnosis is still performed later than the ideal period, defined as before return to oestrus. Chemical methods to diagnose early-stage pregnancy in cattle, such as the production of pregnancy-associated glycoproteins in the circulation, have been extensively explored after day 24 of gestation (Silva et al., 2007; Ricci et al., 2015; Pohler et al., 2016), however, they are not an accurate method in previous stages of pregnancy although there has been some success in heifers on days 18–20 (Green et al., 2010b).

In addition to its anti-luteolytic effects, IFNT is also an immunosuppressive cytokine, known to have antiproliferative action on lymphocytes. In general, during an inflammatory process, polymorphonuclear neutrophils are the first cells recruited to inflammatory sites, providing cytokines and proteolytic enzymes (Talukder et al., 2019). Since IFNT induces ISGs in the endometrium and can go to other tissues via the circulation, detecting the ISG levels in peripheral blood may be used as a good marker of early pregnancy. The idea of using peripheral blood ISGs as a pregnancy marker was postulated years ago (Hansen and Rueda, 1991), but there are still no reliable blood biomarker candidates that are useful in determining pregnancy status before the expected time of oestrus return.

Assessment of ISGs in peripheral blood mononuclear cells (**PBMCs**) using microarray revealed that many hundreds of genes were up- and down-regulated in bovine PBMC in response to pregnancy (Hansen et al., 2010). Previous works have evaluated the expression of specific ISGs, including the interferon-stimulated protein 15 kDa, myxovirus-resistance proteins 1 and 2, and 2′–5′-oligoadenylate synthetase, in peripheral blood leukocytes as an alternative to diagnose early pregnancy during the peri-implantation period in dairy cows (Han et al., 2006; Gifford et al., 2007; Green et al., 2010b; 2010a; Shirasuna et al., 2012). In general, all the ISGs mentioned above have activities related to modulated immunity. For example, the ubiquitin-like protein interferon-stimulated protein 15 kDa is one of the most strongly and rapidly induced, and it can directly inhibit viral replication and modulate immunity. Interferon-stimulated protein 15 kDa is a member of the ubiquitin family, which includes ubiquitin and ubiquitin-like modifiers. They are involved in the regulation of several cellular activities, including protein stability, intracellular trafficking, cell cycle control and immune modulation (Perng and Lenschow, 2018). *MX1* and *MX2* ISGs were originally identified as factors conferring resistance to lethal influenza A virus infections in mice and that encode the interferon-inducible guanine triphosphatases (Zav'yalov, 2019). The 2′–5′ oligoadenylate synthetases are a family of antiviral proteins. As is typical for antiviral genes, the transcription of the 2′–5′ oligoadenylate synthetase genes are induced by both virus infection and interferon stimulation (Kristiansen et al., 2010). Bovine 2′–5′ oligoadenylate synthetase genes include *OAS1* and *OAS2* (Perelygin et al., 2006).

In pregnant cows, there is an increase of *ISG15* mRNA levels in peripheral blood leukocytes from day 15 to day 32 of gestation, with maximal levels on day 20. This higher amount of *ISG* on day 20 can be attributed to the greatest production of IFNT by the elongated conceptus (Hansen et al., 2017). Furthermore, it has already been shown that the presence of multiple embryos in the uterus produced an increased level of *ISG15* and *OAS1* in peripheral blood immune cells, when comparing pregnant and non-pregnant cows in early stages of development (Day 7) (Talukder et al., 2019). The strategy of transferring multiple embryos into the uterus of a cow is a good alternative for measuring the effects of a specific stimulus with expected variation based on trophectoderm production. However, this does not mimic the physiology condition of a mono-ovulatory species, such as in cattle.

An alternative approach used for pregnancy detection in dairy cows was to collect samples of peripheral blood by serial days and to assess associated P4 and *ISG15* levels (Han et al., 2006). Furthermore, an interesting feature is that apparently, there is an

effect caused by the greater ISG response in primiparous cows when compared to multiparous cows. On day 18, the ISG level was almost undetectable in the pregnant multiparous cows. The reason for this difference is not fully understood; however, it is possible that embryo size may differ between primiparous and multiparous cows with consequences in the total capacity of IFNT production by the embryo (Green et al., 2010b). The larger size of multiparous cows may be another possibility to explain the lower ISG response in blood leukocytes, as the systemic concentrations of IFNT would be diluted (Green et al., 2010b).

In a study with beef cows, ISG mRNA expression in PBMCs progressively increased from day 15, peaked at day 20, and decreased thereafter to day 22 (Pugliesi et al., 2014). Hence, the abundance of ISG in pregnant cows when compared to non-pregnant was higher at day 18 than at day 15 for all ISGs studied (*OAS1*, *ISG15*, *MX1*, *MX2*) (Pugliesi et al., 2014). More recently, this group examined the expression of these four ISGs (*OAS1*, *ISG15*, *MX1*, *MX2*) in PBMCs and polymorphonuclear leukocytes before, during, and after pregnancy recognition (Days 15–18) as previous work suggested that polymorphonuclear leukocyte cells may respond earlier to the IFNT stimulation compared to PBMCs (Perng and Lenschow, 2018). Greater abundance of *ISG15*, *OAS1*, *MX1* and *MX2* was observed in PBMCs in the immediate postpregnancy recognition period in those animals confirmed pregnant but, polymorphonuclear leukocytes do not respond earlier to the IFNT (Melo et al., 2020). In summary, the evaluation of ISGs in the blood of cattle seems to be a possible indicator of early pregnancy in ruminants. Although the use of ISG expression profiles in the maternal blood to predict early pregnancy in cattle have been extensively explored and show promise, the results are still inconstant and a quite controversial. Thus, improvements in the methodology to obtain more reliable results and the production of PCR machines which are simple to use on the farm and cost-effective for the end user are still needed. An alternative that has been shown to be more accurate is the association of ISG levels with other parameters, such as the dosage of P4 and/or pregnancy-associated glycoproteins.

Methods to studying these critical interactions

A major barrier to investigating the molecules that are required for successful conceptus-maternal interactions is it is difficult to untangle which are conceptus-derived, which are endometrial-derived, and which are endometrial-derived in response to the conceptus. Moreover, understanding which molecules are required for specific stages of pregnancy (e.g., IFNT is critical for pregnancy recognition), and which enhance or facilitate receptivity to implantation more broadly (e.g., the role of CAPG), is difficult to identify. A substantial barrier to these approaches has been the inability to recapitulate *in vitro*, conceptus elongation. Previous studies have used agar gels as well as glass capillaries (Vajta et al., 2004; Brandão et al., 2004; Alexopoulos et al., 2005) but these produced artificially elongated conceptuses that were not morphologically normal. More recent attempts used agarose gel culture systems with a different medium composition but resulted in limited survival of embryos to Day 15 with no or limited cells positive for *SOX2* expression (a marker of epiblast cells) observed postculture, indicating these are not morphologically or developmentally normal embryos (Ramos-Ibeas et al., 2020). It is clear from these studies, as well as uterine gland knock out studies mentioned above, that spatial and temporal-specific components of ULF as well as biophysical support from the endometrium are required to support posthatching elongation *in vitro*.

Typically, *in vitro* investigations of the endometrium utilise immortalised endometrial cells for ease of culture, or primary cells isolated from bovine endometrial tissue (Tinning et al., 2020). These cells are then typically seeded onto a 2D plastic surface, grown in a single cell type monolayer, and cultured in large volumes of a suitable medium. Therefore, all aspects of the environment of those cells cultured *in vitro* are different to their '*in vivo*' environment. Although these methods have provided invaluable information regarding early pregnancy and the endometrium in many species, moving forward efforts are being made to utilise novel techniques which better recapitulates the *in vivo* environment of the endometrium and resulting ULF (Fitzgerald et al., 2021).

Several newer approaches to provide the maternal endometrial component to *in vitro* elongation had been to use novel 3D culture approaches. One of the more well-developed systems are "microfluidics", or "organ-on-a-chip systems" or "microphysiological systems" (Marx et al., 2020) and have been applied to model reproductive processes (Young and Huh, 2021). These fluidic perfusion systems automatically move medium through a culture system, dynamically replenishing the medium, which is reflective of *in vivo* physiology and thereby promotes healthier *in vitro* cultures. They also have the ability to incorporate multiple cell types which allows communication between cell types, better mimicking *in vivo* conditions. Furthermore, shear stresses that are necessary for the physiological behaviour of certain cell types are also introduced. A human microfluidic reproductive system has recapitulated the entire menstrual cycle on a microscale (Xiao et al., 2017) as part of a full platform, called "EVATAR", where multiple modules recreate ovary, fallopian tube, uterus, cervix, and liver. While a similar hardware system could be adapted to host bovine cells to recreate the specific hormonal patterns involved in the bovine oestrus cycle or in early pregnancy phases in the future, simpler, single microfluidic devices already allow to observe cell type-specific behaviours and functions in the endometrium. Use of the bovine oviduct-on-a-chip systems (Ferraz et al., 2018) have demonstrated hormone responsiveness. Recent co-culturing of two cellular components in individually perfused chambers for example, demonstrate it is possible to recreate the endometrial perivascular stroma and to observe the biological responses to endocrine signalling, mimicking the natural oestrogen and P4 changes (Gnecco et al., 2017). Recent work used primary bovine endometrial stromal and epithelial cells cultured together in a 3D microfluidic system to investigate the effect of insulin and glucose on their transcriptome (De Bem et al., 2021b). Microfluidic bovine endometrium-on-a-chip systems could be used to better study the endometrium *in vitro* and its response to circulating maternal hormones.

Another approach to overcome the disadvantage of culturing cells on a flat 2D surface in a monolayer is the use of spheroids, organoids, and assembloids as 3D culture systems. Bovine endometrial spheroids co-cultured with endometrial epithelial and stromal cells spheroid structures survived for up to 10 days (Yamauchi et al., 2003). Spheroids are relatively simple, may or may not self-assemble or require a matrix to form. Bovine endometrial spheroids have also been used to investigate the effect of IFN and P4 treatment (Rahman et al., 2020). Organoids differ in that they are derived from stem or pluripotent cells, are self-assembling, and require an extra cellular matrix to form (Gunti et al., 2021). The use of organoid systems in other species have facilitated the testing of a variety of important functions of the endometrium including response to hormones, prolonged culture, and expansion (Turco et al., 2017; Fitzgerald et al., 2019), as well as the formation of endometrium assembloids – better recapitulating the *in vivo* embryo, *in vitro* (Rawlings et al., 2021a). Assembloids are formed by co-culturing organoids with other cell types, ultimately incorporating them into the organoid (thereby known as an assembloid (Rawlings et al., 2021b)). Both human and bovine assembloids have been generated from endometrial organoids co-cultured with endometrial stromal cells and have been shown

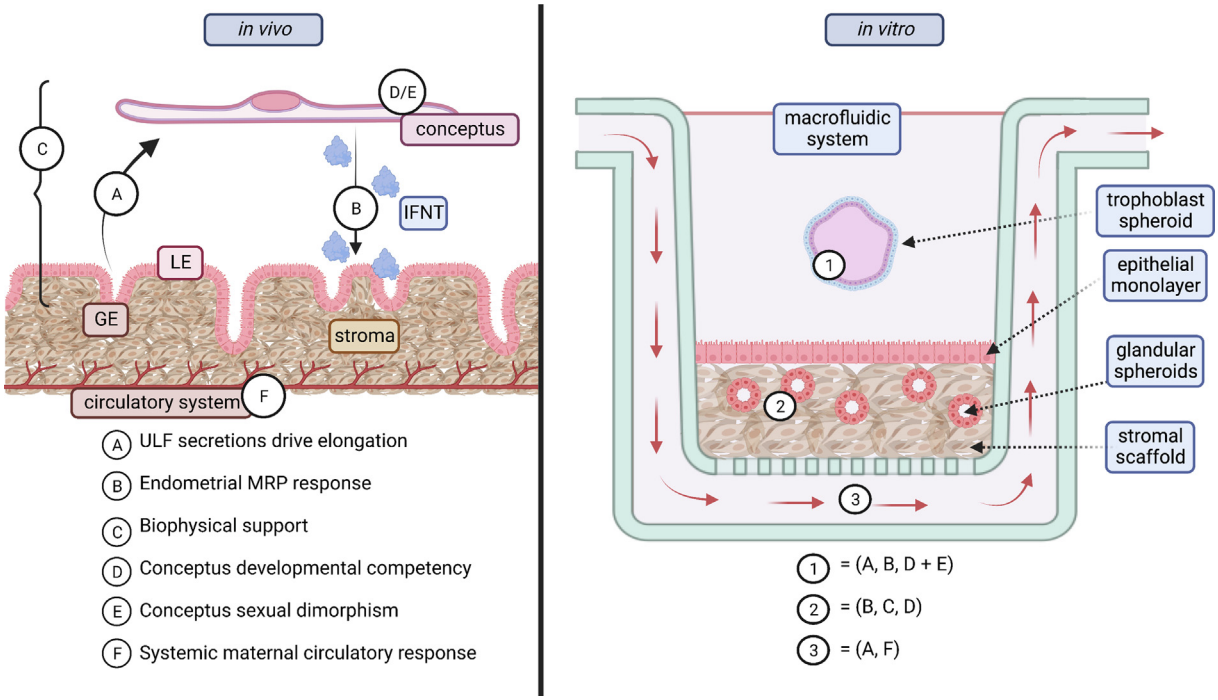

Fig. 3. Current limitations and outstanding questions from *in vivo* studies of conceptus-endometrial interactions and how novel *in vitro* systems could be utilised in understanding these interactions. We propose a novel macrofluidic system could be used to mimic early pregnancy in cattle, including systemic effects. Culture medium flowing through the system below the stromal scaffold acts as the maternal circulatory system and nourishes the endometrial tissue. The stromal scaffold contains seeded endometrial stromal cells, cultured glandular epithelial (GE) spheroids, and topped with a luminal epithelial (LE) monolayer. Within the isolated static well above the endometrial tissue, trophoblast spheroid(s) can be cultured within their own specific medium. CRISPR-Cas9 can be used to knock out the expression of specific genes of interest (GOI) to be able to study different aspects as described. (1) Knocking down a GOI in the trophoblast spheroid could be used to investigate the effect of expression of that gene on uterine luminal fluid (ULF) secretion, the maternal recognition of pregnancy (MRP), and the effect of conceptus developmental competency or sexual dimorphism upon endometrial/systemic response. (2) Knocking down a GOI in the endometrial tissue (stromal/glandular epithelial/luminal epithelial) can be used to determine the effect of that GOI upon the systemic response, conceptus development, or ULF secretion. (3) Altering the composition of the medium in the flow channel to mimic altering the systemic status of the mother can be used to assess the effect upon the endometrium, ULF composition, or conceptus development. Created with Biorender.com.

to respond to sex hormone treatment. Generating bovine organoids that can be built into assembled systems will facilitate our understanding of conceptus-maternal interactions by providing a high-throughput system to test these interactions. These 3D cell culture systems can be used to investigate the bovine endometrium and can be modulated to represent the stage of the oestrus cycle which is of interest.

An additional consideration is the use of extracellular scaffolds to allow for structural deposition of the extracellular matrix of the underlying stromal cells. Recent work has utilised a range of techniques to achieve a 3D cell culture system which may incorporate both stromal and epithelial endometrial cells in a range of species. Specifically in ruminants, recent work has achieved the culture of isolated endometrial glands in a 3D Matrigel system (Sugino et al., 2022). The 3D cultured glands differed to those cultured in 2D, with higher expression of secretory proteins in the 3D culture, while glands cultured in 2D did not respond to oestrogen or P4 treatment (Sugino et al., 2022). Another approach used in cattle has been the use of an electrospun material to create a 3D scaffold which can then be seeded with primary cells. A study published in 2015 found that culturing both stromal and epithelial cells on a 3D electrospun matrix resulted in a structure that resembled the *in vivo* endometrial structure, deposited extra cellular matrix, and responded to treatments (MacKintosh et al., 2015). Therefore, 3D culture may better support *in vitro* cultures to mimic *in vivo* conditions. In the last few years, many other techniques have been utilised, including collagen (Abbas et al., 2020) and alginate scaffolds (Stern-Tal et al., 2020), 3D bioprinting in humans (Ji et al., 2020), and the use of decellularised porcine endometrial extracellular matrix (López-Martínez et al., 2021), to support the culture of human endometrial cells in a 3D model. These techniques can be applied to the endometrium of cattle to investigate pregnancy establishment. A focus for the future should be production of micro- and macrofluidic modular 3D scaffold approaches that will allow us to test conceptus-maternal interactions required for successful pregnancy in cattle.

Concluding remarks

The full extent of the essential interactions between the conceptus and the uterine environment required to support successful pregnancy remains to be elucidated. We do have a wealth of evidence around conceptus-maternal interactions in cattle that is provided via *in vivo* slaughter experiments. However, the complex endometrial changes required for implantation success, including alterations to coding (mRNA/protein) and non-coding (micro RNA, long non-coding RNA etc) components of the endometrial landscape require further investigation. Untangling these interactions without understanding which are responses to the conceptus itself rather than endometrial-derived response to the conceptus is difficult. The lack of successful elongation *in vitro* is an additional limitation to progress. However, the key questions that remain around the required conceptus-maternal interactions for pregnancy success will be untangled as we progress towards more *in vivo*-like models *in vitro* (Fig. 3).

Ethics approval

Not applicable.

Data and model availability statement

No new datasets were created in this manuscript.

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Declaration of interest

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