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Understanding conceptus–maternal interactions: what tools do we need to develop?

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ABSTRACT

Communication between the maternal endometrium and developing embryo/conceptus is critical to support successful pregnancy to term. Studying the peri-implantation period of pregnancy is critical as this is when most pregnancy loss occurs in cattle. Our current understanding of these interactions is limited, due to the lack of appropriate *in vitro* models to assess these interactions. The endometrium is a complex and heterogeneous tissue that is regulated in a transcriptional and translational manner throughout the oestrous cycle. While there are *in vitro* models to study endometrial function, they are static and 2D in nature or explant models and are limited in how well they recapitulate the *in vivo* endometrium. Recent developments in organoid systems, microfluidic approaches, extracellular matrix biology, and *in silico* approaches provide a new opportunity to develop *in vitro* systems that better model the *in vivo* scenario. This will allow us to investigate in a more high-throughput manner the fundamental molecular interactions that are required for successful pregnancy in cattle.

Keywords: endometrium, gene expression, *in vitro* models, *in silico* models, interferon tau, pregnancy, progesterone, uterus.

Introduction

Pregnancy loss affects all mammalian species and occurs at critical developmental timepoints, particularly during early pregnancy. In cattle and other food-producing animals, reproduction needs to be as efficient as possible to decrease environmental impacts, increase food production to feed an ever-increasing global population, and to increase profit margins for the farmers. In beef cattle, 50% of pregnancies fail before day 30 (Reese *et al.* 2020), with that number estimated to be even higher in dairy cattle (Ealy and Seekford 2019). Furthermore, up to 60% of human pregnancies are lost in the early stages (before 12 weeks) (Larsen *et al.* 2013). Although many of these losses can be attributed to developmental failures with the pre-implantation embryo, one underexplored cause is thought to be errors in embryo–maternal bilateral communication (Sánchez *et al.* 2019). Therefore, it is essential to understand the critical embryo–maternal interactions that occur *in vivo* and how these contribute to pregnancy success or failure. Much of what we know about the fundamentals of embryo–maternal communication comes from *in vivo* studies (recently reviewed by Idelevich and Vilella 2020) as well as more static and traditional 2D culture systems. However, recent moves in certain countries including the UK (e.g. by NC3Rs) are looking to reduce the number of animals used for research (https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/277942/bis-14-589-working-to-reduce-the-use-of_animals-in-research.pdf). This, combined with developments in novel *in vitro* and *in silico* methods (reviewed below), allow for an opportunity to examine and explore conceptus–maternal interactions in a more high-throughput manner. The aim of this review will be to set into context what we know about the endometrium itself, its critical interactions with the developing embryo/conceptus, where the gaps in our knowledge are, and what developments will allow us to develop more high-throughput approaches to understand and mitigate pregnancy loss in food-producing animals and broader array of mammals.

Structure and function of the endometrium

The fundamental role of the endometrium is to support growth and development of the embryo/conceptus prior to development of functional cotyledonary placental structures (Spencer *et al.* 2016). This heterogeneous tissue that lines the mammalian uterus and is the first point of cellular contact for the conceptus provides nutrients via histotrophic secretions (termed uterine luminal fluid, ULF), establishes receptivity to implantation for an appropriately developed conceptus, and maintains pregnancy. The endometrium is composed of luminal epithelia (LE) and glandular epithelia (GE) cells, stromal/mesenchymal cells, immune cells and a blood supply, all of which support different functions of the endometrium (Fig. 1).

Factors driving conceptus elongation and embryonic nutritional cargo are secreted by LE and GE throughout elongation; during implantation, embryonic secretions to the glands induce specific gene expression changes, thought to be essential for successful pregnancy (Adhikari *et al.* 2022). In between these glandular areas are aglandular areas called caruncles – small protrusions on the surface of the endometrium (Chankeaw *et al.* 2021). Underlying this glandular region exists a supportive network of fibroblast-like stromal cells, holding vasculature, immune cells, and lymph vessels throughout an extracellular matrix (ECM) (Chankeaw *et al.* 2021).

Research has indicated that epithelial cell secretions include growth factors, hormones, cytokines, and exosome-encapsulated molecules that regulate uterine receptivity, stromal cell decidualisation (in species such as mouse and humans, which undergo this process) and blastocyst/conceptus implantation (Zhang *et al.* 2013; Filant and Spencer 2014; Cretoiu *et al.* 2016). The GE and LE provide nutritional sustenance to support the growth of the blastocyst prior to placental formation, during the elongation process through secretions into the histotroph in ruminants (Spencer 2014). This is evidenced by the fact that sheep blastocysts fail to elongate in sheep where uterine gland and luminal epithelia have been inhibited from developing (Gray *et al.* 2002; Spencer 2014).

Pre-implantation embryo development

In cattle and other mammals, fertilisation occurs in the oviduct (or fallopian tube) and this is where the process of

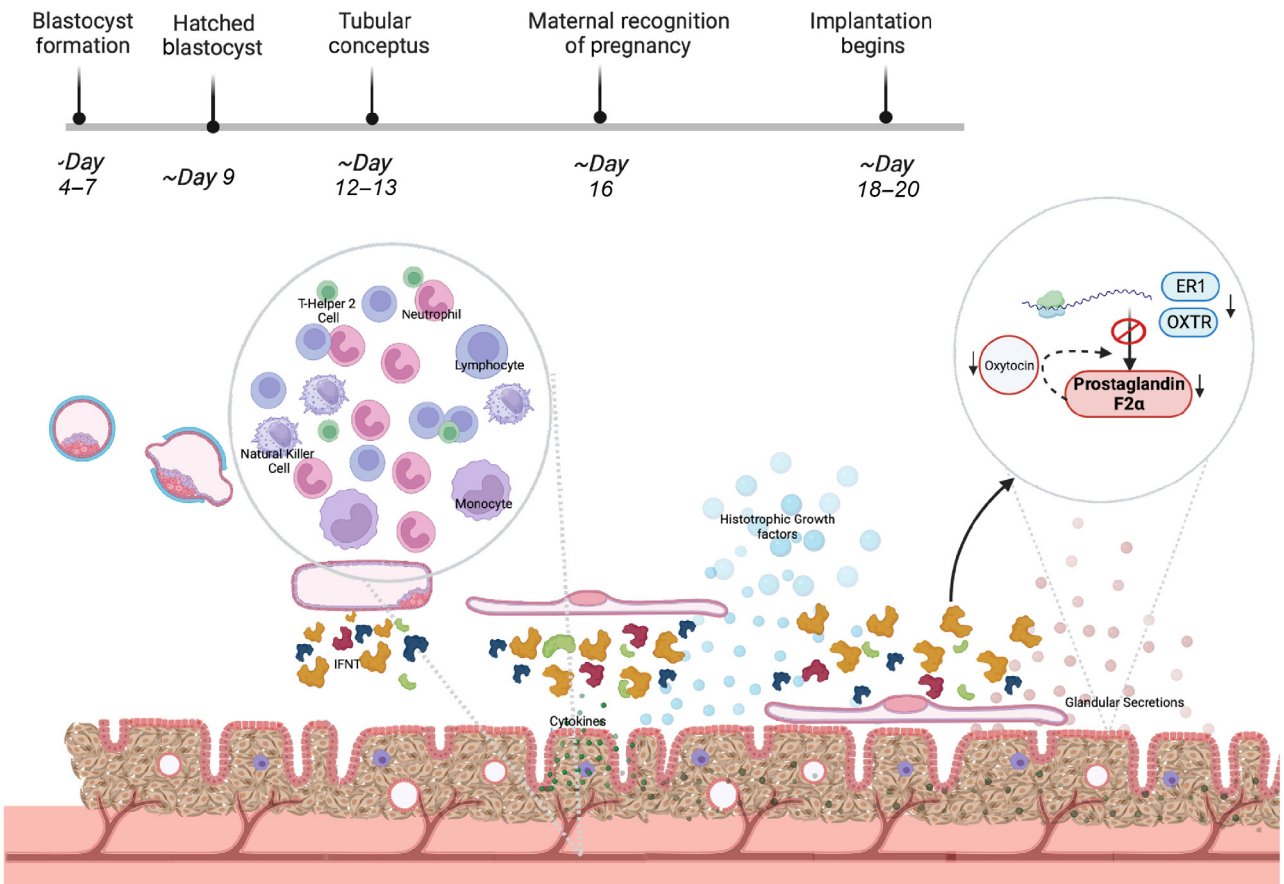

Fig. 1. Schematic diagram of the function of the bovine endometrium during the peri-implantation period of pregnancy. IFNT – interferon tau, ER1 – oestrogen receptor 1, OXTR – oxytocin receptor. Created with Biorender.com.

embryonic genome activation occurs. There is conservation in terms of the morphological steps that occur, e.g. development to the morula and transition to the blastocyst stage of development. This is key for pregnancy success as this is when differentiation between the inner cell mass (which goes on to form the fetus) and the outer cell mass (the trophectoderm that forms the extraembryonic membranes) occurs (Idelevich and Vilella 2020). The trophectoderm is not only the site of the first physical interaction between the conceptus and the endometrium but also produces the species-specific pregnancy recognition signal (detailed below). Bovine embryos don't depend on exposure to the maternal system until after blastocyst hatching, as evidenced by *in vitro* embryo culture and successful pregnancy after transfer at this point. In cattle, the blastocyst hatches from the zona pellucida around day 9, where the conceptus undergoes a period of rapid elongation into a filamentous conceptus (Spencer 2013), which begins implantation on day 16–20 after ovulation (Eozenou *et al.* 2020). This differs in humans, where the implantation processes of apposition occur immediately post-hatching (Kim and Kim 2017). The maternal endometrium provides nourishment for the conceptus prior to placenta formation via endometrial histotrophic secretions from endometrial glands (Forde and Lonergan 2012).

Successful implantation in all species relies on the maternal exchange of nutrition and sustenance. Ruminants characteristically have an extended period of conceptus development prior to implantation, called trophoblast elongation. This gives the conceptus a long thin appearance, beginning at a length of 2 mm and measuring at ~200 mm (20 cm) at the time of implantation (Brooks *et al.* 2014). At this point, the endometrium and uterine lumen are key to conceptus survival. During elongation, the conceptus is reliant on the histotrophic nutrition and growth factors provided mostly by the uterine glands (Forde and Lonergan 2012). This has not been successfully recapitulated *in vitro* and thus limits our understanding of pregnancy loss/success. It is therefore essential to explore the mechanisms by which uterine lumen and endometrial sensitivity are initiated and maintained. As bovine conceptus elongation is a maternally dependent process, it is currently extremely challenging to study the conceptus–maternal interaction *in vivo*. It is also necessary to develop new techniques and models accurately reflecting the start of this interaction (Lonergan and Fair 2014).

In vitro model development

Decreased endometrial receptivity is one of the major contributing factors to a failed pregnancy within the first 3 weeks of gestation. Studying the development of an embryo within the maternal uterus and consequential endometrial development is not only extremely expensive but also technologically challenging to replicate *in vitro*. Ideally, an *in vitro* model would be able to recapitulate the transcriptomics and structural changes that occur in the epithelium during the peri-implantation period of pregnancy and the differentiation of stromal cells following exposure to steroids such as progesterone and oestradiol, as well as various signalling molecules and growth factors such as interferon tau (IFNT) and insulin-like growth factor (Donofrio *et al.* 2008; Adhikari *et al.* 2022). Moving forward to begin the formation of a suitable model, a detailed insight into each cell type, its individual function, and its interactions with surrounding cell types is required. We must also consider biophysical structure, multicellular interactions, and the flow of components and signals between the trophoblast and endometrium (Table 1).

Cellular maps of the endometrium

Most of our knowledge of endometrial function from *in vivo* studies has come from bulk RNA sequencing of endometrial tissues at different stages of pregnancy. Generating single-cell resolution maps of the endometrium and its response to cues important for early pregnancy and implantation would allow us to understand cell-specific changes that occur during successful pregnancy and allow us to map the *in vitro* systems to understand which are appropriate to answer specific functional questions of the endometrium. Single-cell sequences of other mammalian species, including humans (Lucas *et al.* 2020) and murines (Kirkwood *et al.* 2021), have resolved different subcellular phenotypes of the endometrium, but this technique has been used in a more limited way in bovines, with bovine placental single-cell sequencing only identifying different trophoblast cell lineages (Davenport *et al.* 2023). To develop effective *in vitro* systems, a better understanding of the single-cell resolution and the temporal changes of the endometrium in response to progesterone, oestradiol and IFNT is required.

Providing biophysical structure

One largely under investigated component of the endometrium is the contribution of the ECM to early pregnancy success. The ECM is the non-cellular component of any tissue, which supports the tissue in a variety of ways, including providing biophysical structure to the cells and supplying biochemical cues (Frantz *et al.* 2010). The ECM is mainly composed of proteins such as collagen (mostly secreted by fibroblasts; De Wever *et al.* 2008) elastin, fibronectin, some proteoglycans, water, and polysaccharides, although the exact composition is unique to each tissue and determines the properties of that tissue (Frantz *et al.* 2010). Cell adhesion and migration within the ECM is mediated by ECM receptors and cellular cytoskeletal adherence to the ECM (Schmidt and Friedl 2010). The 3D structure and properties of the ECM itself are controlled by enzymatic degradation (such as by metalloproteinases) or inhibitors of those enzymes (Cruz-Munoz and Khokha 2008). Changes to the ECM are known as ECM remodelling and have been implicated in disease states (Nallanthighal *et al.* 2019), tissue stiffness (Engler *et al.* 2006), and tissue morphology and structure (Chevalier *et al.* 2016).

Table 1. Advantages and limitations of different approaches to determine conceptus–maternal interactions in bovines.

Models	Advantages	Disadvantages
2D cultures	• Inexpensive • Quick to grow • Easy to maintain • Fast data • Easy to replicate experiments • Larger surface area for nutrient and gas exchange	• Lack of ECM • Not a representative micro-environment of host tissue • Do not encapsulate the natural 3D structure of tissue • Variable phenotype/genotype • Will not consider inter-species variation in data • Cell–cell contact is limited
Primary cultures	• Cultured directly from animal tissue • More accurate representation of the micro-environment *in vivo*. • Closer genetic makeup to cells *in vivo* • Responses to variable conditions prove more indicative of *in vivo* • Can be used for further 3D formation of organoids/spheroids and co-cultured systems • Could be used for patient-specific diagnosis/treatment of disease	• Cultures still grown as a monolayer, unless used for 3D culturing • Potential discrepancies in genotype between cells and tissue of the same species • Do not encapsulate the natural 3D structure of tissue • Cell–cell contact is limited • Isolation methods may cause cellular damage • Contamination of cell types in cultures • Cell growth is dependent on quality of tissue and isolation
3D cultures (Spheroids/ Organoids)	• Mimic the natural response of cells *in vivo* to variable conditions • Cells have a phenotype and genotype different to those in 2D models. • Dimensions differ to 2D cells, allowing more cell–cell contact • Larger surface area for receptors and substrates • Higher proportion of endo/Eeocytosis leads to a more accurately represented micro-environment of tissues *in vivo* • Self-forming; forming *in vivo*–like structures	• Take longer to establish a functional model • Expensive to maintain culturing media • Many growth factors involved unknown • No vascular network; therefore, no way of internal cells undergoing gas exchange or receiving nutrient transport
In silico	• No direct testing on experimental subjects required • Specific experimental conditions can be incorporated • Can be used to model complex physiological systems • Multiple parameters and conditions can be considered simultaneously • Can be combined with *in vivo* and *in vitro* studies to give greater insight	• Requires high quality 'omics' datasets to be available • Often requires validation using *in vivo* or *in vitro* studies

The ECM also contains factors which are required for tissue homeostasis, cell differentiation, and tissue morphogenesis. It acts as a reservoir for growth factors (such as transforming growth factor-b), which are released from the ECM to act upon neighbouring cells when the ECM is remodelled (Taipale and Keski-Oja 1997). ECM-resident matrix-bound extracellular vesicles (EVs) have also recently been identified (Huleihel *et al.* 2016). EVs can contain coding and non-coding RNA species and proteins, among other cellular components, which can influence target cells after uptake (Tkach and Théry 2016). EVs can be targeted to specific cell types by the presence of surface receptors which interact with those expressed on the target cell membrane (Tkach and Théry 2016). Matrix-bound EVs have been shown to influence the ECM directly through vesicle surface factors (Sanderson *et al.* 2019) and indirectly by fusing with cells (Sajeesh *et al.* 2020).

The correct morphology, functionality, and remodelling of the endometrium are critical for endometrial receptivity and subsequent implantation, and change drastically during the oestrous cycle in species such as humans (Strowitzki *et al.* 2006). Therefore, the dynamics and regulation of the ECM contribute to early pregnancy success (O'Connor *et al.* 2020). In cattle, the transcription of genes involved in ECM remodelling is altered during the oestrous cycle (Mitko *et al.* 2008), and similarly the abundance of collagen in the endometrium (Scolari *et al.* 2016). Authors also found that reduced ECM remodelling on day 6 is correlated with pregnancy success in cattle (Scolari *et al.* 2016). By studying the endometrium *in vitro* using novel techniques, we can better model the endometrium to gain a greater understanding of early pregnancy processes and how the ECM contributes to their success.

Until recently, very few *in vitro* models successfully considered and incorporated the endometrial ECM. Work by Díez *et al.* (2023) utilised a 3D polystyrene scaffold populated with bovine endometrial stromal cells which deposited ECM proteins *in vitro* (Díez *et al.* 2023). The resulting tissue-like structure supported by ECM allowed the formation of an epithelial monolayer, thereby recapitulating the basic 3D structure of the endometrium *in vitro* (Díez *et al.* 2023). Similarly, an electrospun scaffold has been used to culture bovine endometrial cells in which ECM was deposited, although culture was limited to a shorter timeframe than the polystyrene scaffold more recently reported (MacKintosh *et al.* 2013). These scaffolds require an artificial scaffold into which fibroblasts are seeded, following which they can then secrete ECM. The scaffolds themselves cannot be altered through remodelling.

Although these advances in 3D cell culture and ECM deposition are a leap forward in research addressing ECM in

in vitro culture systems, they still do not fully recapitulate *in vivo* endometrial ECM. A noteworthy method for addressing this utilises decellularisation of tissue to leave behind the natural ECM of that tissue. Cells can then be seeded or the remaining tissue ECM investigated. A recent example successfully used decellularisation of porcine endometria to produce an endometrial ECM 'hydrogel' which was then seeded with human primary endometrial cells for long-term culture (López-Martínez *et al.* 2021). Although the authors identified many points of optimisation for the technique, the method is an interesting example of utilising the *in vivo*–produced ECM *in vitro* to improve endometrial models. The ECM is a complex matrix which undergoes remodelling and contains EVs and other factors which can influence the cells of that tissue. The ECM deserves consideration when designing *in vitro* tissue models, especially when the composition and architecture of the tissue is critical for its function, such as in the endometrium.

Multicellular endometrial model approaches

Investigating tissue and cellular interactions and the functional understanding of tissues currently relies heavily on 2D *in vitro* models. Using static monolayers of cells in culture does not portray the intricacy of a 3D multicellular network and the interactions which take place in a physiologically accurate micro-environment. Three-dimensional cellular models are now increasingly popular *in vitro* models as they are able to replicate this multicellular network. Such models currently include organoid cultures and systems, scaffolds, various hydrogel-based assays, and microfluidic and organ-on-chip approaches.

Cell lines

Immortalised cell lines are often used as tools for preliminary data; however, they may not provide an accurate phenotype or genotype of the tissue of origin (Nallanthighal *et al.* 2019). Inter-species genetic variation cannot be accounted for with immortalised cultures; therefore, it is preferable to use primary cultures when possible (Li *et al.* 2016). Primary cells have allowed researchers to better observe cellular characteristics as they would exist *in vivo*. Isolated directly from host tissue samples taken via biopsies, resections, or post-mortem autopsies, these cell lines better represent both phenotypical and genotypical signatures as they exist within the host than immortalised secondary cultures. They prove particularly beneficial when observing factors within the cellular micro-environment, especially when co-cultured with systemically relevant primary cell lines in model systems incorporating microfluidic technology. A more representative micro-environment would produce responses more indicative of what occurs *in vivo* (Richter *et al.* 2021).

Bovine endometrial research regularly uses primary cells. These cells are routinely harvested from the bovine uterus, isolated, and cultured. Established protocols are followed for this process, as previously described in detail (Cronin *et al.* 2012). This allows the study of molecular properties, phenotypical characteristics, and cellular responses to treatments and conditions. These studies allow us to gain insight into cellular-specific properties, aiding our understanding of how the bovine endometrium aids implantation and gestation (Li *et al.* 2016; Tinning *et al.* 2020; Mathew *et al.* 2022). Previous studies have successfully isolated bovine endometrial epithelial cells and used these cells for further processing such as single-cell sequencing and immune factor investigation (Kelly *et al.* 2020; Chankeaw *et al.* 2021), and for further use in 3D co-cultured systems (Murillo and Muñoz 2021). Endometrial cells have also successfully been harvested from humans, pigs, sheep, horses, and rodents (Wang *et al.* 2000; De Clercq *et al.* 2017; Rink *et al.* 2017; Tavakol *et al.* 2018; Hu *et al.* 2022), with advances allowing the transformation of some primary cultures into immortalised-like cultures, able to maintain primary cell–like characteristics but for prolonged passages (Hu *et al.* 2022).

Although primary cultures prove beneficial and provide researchers with a platform for insight and data, there will still be differences in cellular characteristics depending on the tissue origin (Qadan *et al.* 2018), especially when taken from different animals. The lack of multicellular stimulation and therefore altering of cellular micro-environment will alter the molecular structure of cells (Lee *et al.* 2006). The process of endometrial and stromal cell isolation involves degradation of the endometrial structure as a whole, potentially causing cells to release damage associated with molecular patterns, altering downstream genetic profile and functioning of cells (Borges *et al.* 2012).

There can be concern over the methods of cell isolation used due to proteolytic and other digestive enzymes potentially altering cellular makeup. The next question which arises is the number of passages for which these cells will continue to represent cells *in vivo*. One way to combat some of these issues is using tissue explants, co-culturing, or 3D models of the various cell types involved in implantation. Furthermore, *in vitro* culturing often uses serum-based media with additional antibiotic components, which overtime likely influence cellular characteristics and morphology. This is evident by numerous studies displaying alterations in primary cell lines through serum starvation, e.g. shortening of microglial processes alongside increase in cell body size, cessation of proliferation in human endometrial mesenchymal cells and induction of autophagy in endometrial epithelial cells.

Endometrial explant models

Explant cultures are another form of primary tissue culture and involve the culturing of the tissue or organ of interest until tissue-specific progenitor cells migrate into the culturing vessel and can be maintained. Progenitor cells can then differentiate into the relevant organ-specific cells while being reliant on the growth factors and conditions they are exposed to. This method can only be maintained for 2 to

3 weeks and requires a delicate balance of culturing. This ensures all components of the media are a precise replica of the *in vivo* environment (Lee *et al.* 2006).

The continuation of cells alongside host tissue proves hugely advantageous for mimicking the cellular microenvironment (Bedzhov and Zernicka-Goetz 2014). Factors such as the ECM, vesicular secretions, growth factors, cytokines and cellular contents are functionally active within the tissue explant once it has been established, which allows for cellular growth and stability (Borges *et al.* 2012). The stem cells subjected to less biological stress when this method is utilised will retain more *in vivo*–like characteristics (Hendijani 2017).

Endometrial tissue explants have proved advantageous in exploring the conceptus–maternal relationship across species. They have been successfully cultured and used for downstream applications such as determining mechanisms of innate immunity/inflammation, and the influence of blastocyst presence to cellular molecular profile (Borges *et al.* 2012; Passaro *et al.* 2018; Sánchez *et al.* 2019). Explanted endometrial tissue taken from various stages of the oestrous cycle has also shown changes in expression across the cycle (Ault-Seay *et al.* 2022). The same tissues showed significant differences in gene expression in response to lipopolysaccharide treatment, indicative of a difference in immune response dependent on the stage of oestrous cycle the host is in, potentially resulting in changes to endometrial function and therefore pregnancy success (Ault-Seay *et al.* 2022). Other factors influencing immune cell populations and consequential responses may include: immunological stresses present in the mother (e.g. infection) and fluctuations in hormone levels.

In another study, bovine endometrial tissue was cultured alongside long and short length embryos, all 15 days old. The observations showed a difference in molecular patterns in endometrial cells, which were dependent on which length conceptus they had been exposed to (Sánchez *et al.* 2019). This study showed the importance of how this relationship can influence the genotype of both the endometrium and conceptus. While two conceptuses may be the same age, growth rate may differ and consequentially influence the endometrial transcriptome (Sánchez *et al.* 2019). In 2005, Tan *et al.* successfully developed and optimised the culture conditions of a co-culturing system using mouse endometrial tissue explants taken alongside blastocysts on day 4 of pregnancy. Tissue taken at this specific time allowed the group to encapsulate the short lived 'window of implantation' in mice, lasting only a few hours on this day, thus providing a step in the right direction for research into implantation (Tan *et al.* 2005).

3D culture models

2D immortalised cell cultures provide an ideal baseline for disease modelling, and the development of both primary isolated cultures and explant cultures provides a closer mimicking cellular profile to that of host tissue. However, recent biological models are turning to 3D cell cultures such as spheroids and organoids for an increasingly physiologically accurate representation of *in vivo* conditions.

3D models of cell culture are most often grown using acellular scaffolds of either a biological or synthetic nature. They can be anchorage dependent (scaffold based), formed in biological or synthetic hydrogels, or independent (non-scaffold based) systems such as spheroids, formed using the hanging drop, low attachment plates or gels, formation through agitation, and magnetic levitation (Langhans 2018). One study successfully modelled formation of spheroidal cultures of trophoblasts, fibroblasts and epithelia. Through this they observed a decrease in the expression of polarisation proteins ezrin and CK18 in the presence of trophoblast spheroids, indicative of the depolarisation of epithelial lining at implantation (Haeger *et al.* 2015).

Another study developed a model of implantation in bovines using 3D trophoblast spheroids applied onto endometrial epithelial cells, aiming to investigate factors affecting attachment of trophoblast cells to uterine epithelium (Sakurai *et al.* 2012). Looking at the expression of IFNT in both these components, the authors attempted to mimic implantation by applying CT-1 trophoblast spheroids to endometrial epithelial cells, with the addition of concentrated uterine fluid from pregnant ewes. The attachment rate, alongside the expression of various other factors, was observed and quantified. Using this model they were able to conclude that IFNT showed a decreased expression in CT-1 trophoblast spheroids when applied onto endometrial epithelial cells at attachment, while no changes in IFNT expression were observed in the CT-1 spheroids with the absence of epithelial cells, proving that IFNT is a good indicator of successful attachment (Sakurai *et al.* 2012).

Although 3D cultures are a preferable model, they still lack the presence of vascular and supportive networks for waste removal and transport, similar to what is found *in vivo*. While a monolayer of cells can retrieve the nutrition and perform the gas exchange necessary, cells within the centre of a 3D spheroid or organoid will not have the same exposure to nutrients and gases as those on the surface. This is beneficial when studying tumour biology; however, it does not replicate tissue systems *in vivo*, where a vast network of exchange and communication would exist between cells (including immune cells), via the actions of cytokines, various microRNAs, and nutritional factors.

Mimicking physiological flow

While the advances in cell culture models have been beneficial, they still do not mimic the physiological systemic flow under which all biological structures function. Advances in bioengineering have introduced intricate microfluidic models (Pun *et al.* 2021), which allow a two-way exchange of relevant nutritional material and waste removal alongside sufficient gas exchange. These microfluidic approaches allow us to mimic physiological flow in *in vitro* cell culture systems. The devices are made of a translucent plastic, polymer, or

glass, and include a variable number of hollow microchannels within which cells grow. Under pump systems, channels conduct the flow of reagents, with volumes as small as 0.001 μL being required (Whitesides 2006)

Singular or multiple cell lines can be grown/layered in unison or under flow to recreate tissue-specific structures (Pun *et al.* 2021). Research conducted into complex systems such as neurological, cardiovascular, endometrial, and oncological physiologies are benefitting from microfluidic models ranging in complexity, allowing the recreation of such networks with the correct multicellular composition in a non-static state, and therefore a more physiologically realistic micro-environment (Nolan *et al.* 2023).

Within the study of uterine biology, hormonal fluctuations that occur throughout the oestrous cycle and their influence on endometrial function cannot be ignored. Recreation of endometrial biology encompassing the network of reproductive hormones has been successfully attempted, mimicking the menstrual cycle of humans using microfluidics modelling. These models have been used to better mimic the hormonal fluctuations that the reproductive tract experiences *in vivo*. The synergistic relationship between embryonic development and endometrial structure and function cannot be accurately represented using static *in vitro* models.

A recent study (Ahn *et al.* 2021) included a vascularised multicellular endometrium with constitutive endothelial cells, stromal fibroblasts and finally epithelial cells that were all grown under flow. Oestradiol and progesterone were incorporated into culture media in varying concentrations and combinations depending on menstrual cycle phase. Once established, the authors applied embryo-sized microbeads coated with either insulin-like growth factor binding protein 1 or heparin-binding EGF-like growth factor, both of which are known to be released by the embryo (Ahn *et al.* 2021). A successful model was indicated in doing so; researchers observed a marked increase in epithelial lining thickness and achieved successful attachment of the embryo, developing a model of implantation (Ahn *et al.* 2021).

De Bem *et al.* (2021) utilised microfluidic technology to replicate the bovine endometrial structure using primary isolated endometrial cells. By altering the flow with glucose and insulin-like growth factor 1 (IGF1) throughout the model, they were able to explore any concentration-dependent transcription changes to the endometrium and any resultant downstream proteomic changes within the surrounding media. Both glucose and IGF1 concentrations in circulation *in vivo* are associated with embryo development and are affected at times of metabolic stress, e.g. in dairy cows during negative energy balance (Forde *et al.* 2016; De Bem *et al.* 2021). Both factors could therefore could play a vital role in early pregnancy failure in bovines. Both endometrial and stromal cells responded to these factors with numerous changes in the protein secretome (De Bem *et al.* 2021).

Xiao *et al.* (2017) developed a unique microfluidic model capable of combining five different tissue structures: ovarian, fallopian, uterine, cervical, and hepatic (to determine tissue stability outside the reproductive system). Each of these tissue explants were incorporated into a microfluidic model with an individual recirculation of media. A second circulating system allowed for recirculation of media within and around the whole model. An individual follicular model was also incorporated, containing murine ovarian follicles, with sustained human chorionic gonadotrophin (hCG). Through the sustenance of hCG and development of these follicles within this complex system, the group observed identical hormonal fluctuations as seen in the human menstrual cycle (Xiao *et al.* 2017). This final model is an example of how microfluidic or organ-on-a-chip devices can be developed to interconnect various physiological systems and utilise cell-to-cell communication to produce an *in vitro*–like micro-environment.

Achieving a multicellular system in bovine reproductive physiology would allow for more accurate predictions and observations of which maternal factors influence embryonic development, and vice versa, in order to accurately predict factors which may be affecting implantation failure and therefore pregnancy loss in early pregnancy.

Investigating the conceptus side

While there has been progress made in modelling the *in vitro* endometrium, in order to understand pregnancy loss we must also consider models of trophoblast (TE) cells and how they interact with *in vitro*–produced endometria. Previous models have used TE cells (BT-1 cell line, Awad *et al.* 2020; CT-1 cell line, Talbot *et al.* 2000). More recently, bovine trophoblast stem cells (TSCs) have been isolated from blastocyst cultures (Wang *et al.* 2023). These cells can be maintained undifferentiated in long-term cultures and they can be induced to produce differentiated TE cells in ~5 days, as determined by the expression of trophoblast markers PTGS2, placental lactogen and IFNT. When injected into NOD-SCID mice, these cells differentiate in mature TE cells, evidenced by the presence of binucleated cells and expression of MMP2, normally detected in the trophoblast–endometrial interphase. These assays demonstrate the potential of these TSCs; however, more work is needed to demonstrate their ability to recapitulate the dynamic changes of TE cells during the complex process of TE elongation and show how these are related to the interaction with the maternal environment. Indeed, a recent transcriptomic study of bovine conceptuses between days 12 and 18 identified novel subpopulations of TE cells that may emerge during the transition from ovoid to tubular stage (Scatolin *et al.* 2023). Importantly, this study also identified possible signalling interactions between the epiblast, the TE and hypoblast during this critical period, highlighting the need to develop systems that include all the components of the conceptus in order to study the molecular

interactions defining the embryo–maternal communication required for successful pregnancy establishment. A step towards achieving this objective was reported recently with the generation of bovine blastoids by using stem cells (Pinzón-Arteaga *et al.* 2023). TSCs were aggregated with expanded potential bovine stem cells (EPSC) and cultured under conditions favouring the differentiation of the hypoblast lineage from epiblast cells, while forming a blastocyst. Remarkably, these blastoids produce IFNT in recipient animals similar to levels seen in control embryos; however, later pregnancies were not reported. These blastoids need to be studied in more detail to determine their functionality, thus future experiments combining blastoids and 2D endometrial cultures will be instrumental in establishing the functional interactions between maternal–fetal interphase during the critical stage of embryo elongation and implantation.

In silico approaches

Using *in silico* and computational modelling approaches allows multicellular networks of a physiological or pharmacological system to be investigated. Computational approaches mean specific experimental conditions can be incorporated into models without being tested directly on experimental subjects. While most often used in pharmacologically based assays, *in silico* approaches are now being introduced to other complex physiological systems such as cardiovascular and endometrial biology (Colquitt *et al.* 2011). A major strength of *in silico* approaches is that multiple parameters (conditions) can be considered simultaneously, unlike *in vivo* or *in vitro* approaches. *In silico* approaches can also be combined with *in vitro* and *in vivo* studies to gain detailed mechanistic and functional insights. *In silico* studies are already helping researchers understand various cell states at different points throughout the reproductive cycle, with the multi-omic analysis of different stages of the cycle/developmental competency of embryos as well as validation of new *in vitro* model systems (recently reviewed in three review papers from the International Ruminant Reproduction conference; Huang *et al.* 2021; Yiğit *et al.* 2022; Cheredath *et al.* 2023). In recent years, the quality, quantity, and taxonomic breadth of available gene sequences and full genomes has increased dramatically. This has facilitated greater insight into the adaptive and evolutionary history of genomic elements associated with variation in reproductive phenotypes. Such approaches can inform and target subsequent *in vitro* and *in vivo* analyses by highlighting which regulatory elements and/or genes may contribute to reproductive pathologies, or reproductive strategies and phenotypes.

Many significant insights into pregnancy in placental mammals have been gained from these comparative evolutionary biology approaches. For example, seminal work by Lynch *et al.* (2015) set out to determine the evolution of uterine gene expression across tetrapods by characterising transcribed genes in the endometria of eutheria (dog, cow, horse, pig, and armadillo), marsupial (opossum) and monotreme (platypus), and combining these data with similar datasets from the pregnant uteri of other tetrapod species (frog, lizard, and chicken). They discovered thousands of genes evolved endometrial expression on the lineage leading to mammals, with roles in communication and immune tolerance. On the same lineage, thousands of *cis*-regulatory hormone-responsive elements emerged that were co-opted from ancient transposable elements, suggesting a major rewiring and functional innovation on this stem mammal lineage required to establish the regulatory networks needed for pregnancy success in mammals. Recently, taking a similar comparative transcriptomic approach with endometrium samples from several mammal species, genes have been identified that are hypothesised to be involved in determining human unique pregnancy-related traits (Mika *et al.* 2021, 2022), some of which, are heavily implicated in pregnancy complications. More broadly, these studies strongly implicate changes in uterine gene expression with several evolutionary innovations during the origin of eutherian pregnancy. Indeed, reconstructing the ancestral transcriptome of 23 species shows how the maternal gene expression profiles are correlated with the degree of placental invasion, suggesting maternal control on placental invasiveness (Mika *et al.* 2022).

Using a combination of transcriptomic data, morphological analyses, evolutionary and comparative genomics, the narrative around implantation has transformed in recent years from that of conflict between a 'passive maternal side' and an 'aggressive embryo side' to a model where cooperative inflammation and the role of endometrial receptivity in pregnancy are centre stage (Mika *et al.* 2022). The process of attachment in implantation is derived from an attachment reaction in the ancestral therian mammal (model = opossum), which leads to parturition (Mika *et al.* 2021). However, in eutheria a key shift to a non-inflammatory phase of pregnancy permits the extended period of placentation (Chavan *et al.* 2021). Comparative uterine transcriptomics revealed that this transition to a non-inflammatory phase of pregnancy in eutheria is underpinned by suppression of interleukin-17A (IL17A) from T helper cells by decidual stromal cells (Chavan *et al.* 2021)

The use of *in silico* approaches with high-quality genomes also helps to elucidate the role of non-coding regulatory elements of the genome. The human chromosome 19 microRNA cluster (C19MC) was identified by Bentwich *et al.* (2005) using computational methods, and then validated through in multiple tissues using *in vitro* methods. C19MC has since been shown to be expressed exclusively in the placenta during pregnancy and, once expressed, is trafficked to the maternal circulation via extracellular vesicles (Donker *et al.* 2012; Chang *et al.* 2017; Morales-Prieto *et al.* 2020). Furthermore, variations in expression of this cluster in humans have been associated with multiple pregnancy-related pathologies, including pre-eclampsia (Hromadnikova *et al.* 2013, 2017).

Importantly, variation in evolutionary rates of proteins doesn't always correlate with functional shift as has been shown by comparative analysis (*in silico* and *in vitro*) of a sperm protein (CatSper). Despite differences in evolutionary history of the CatSper gene between marsupial and eutherian mammals, much of the function of the CatSper protein is conserved (Hwang *et al.* 2021).

Taylor *et al.* (2023) explored the pattern of microRNA presence and absence in the genomes of 10 therian mammals, representing a range of implantation methods, and identified a 'core toolkit' of 13 mammal-exclusive microRNAs present in all species therein. Many of these core toolkit microRNAs were implicated in placental function and pregnancy-related pathologies. Using computational predictions of the targets of these microRNAs, they found that genes predicted to be under positive selection (indicative of protein functional shift) on the ancestral eutherian mammal lineage are preferentially targeted by these microRNAs. *In vitro* experiments then demonstrated that expression of these microRNAs was affected by early pregnancy molecules. Furthermore, dysfunction of these microRNAs and their targets contributes to endometrial-derived recurrent pregnancy loss (Hume *et al.* 2023).

To better predict the factors determining success in implantation and pregnancy, many recent studies have used artificial intelligence (AI) and machine-learning (ML) methods such as random forest and neural networks (Uyar *et al.* 2015; VerMilyea *et al.* 2020; Siristatidis *et al.* 2021; Li *et al.* 2022). Typically, these approaches take a selection of maternal or conceptus phenotypes and assess the extent to which these factors predict developmental outcomes. Data is randomly subset into a training set, which is used to develop a predictive model, which is subsequently validated on the remaining data. These models are then run until their predictive power reaches an optimal value. ML approaches have been taken to better understand the factors underlying success in IVF implantation (Larsen *et al.* 2013; Luo *et al.* 2017; Akbulut *et al.* 2018; Miao and Miao 2018; Araya *et al.* 2021).

ML approaches have recently become popular as a method of analysing large, complex omics-based datasets. Rabaglino *et al.* (2023) combined Bayesian logistic regression and neural network models with bovine transcriptomic data to identify eight genes whose expression was predictive of conceptus competence. In addition, the development of ML approaches, such as in Christmas *et al.* (2023), have led to finding enhancer regions associated with phenotypic differences in mammals, with many of the most highly conserved regions associated with embryonic development. Novel ML approaches and applications such as these are set to be increasingly important in understanding the role of non-protein-coding regions of the genome, which are typically more difficult to determine. These two studies highlight the potential for ML in better predicting developmental outcomes, as they have been leveraged to handle complex datasets across different scales, identifying potential portions of the transcriptome and genome, respectively, which may be essential in healthy fetal development. As ML approaches become more robust and reliable, it is likely that they will become an increasingly useful tool in the effort to better understand pregnancy-associated risk factors and conceptus–maternal interactions, and to reduce the need for *in vivo* and *in vitro* studies.

Outlook and future goals

Investigating conceptus–maternal interactions is critical if we are to overcome pregnancy loss in cattle. Most of what we know comes from bulk RNA sequencing and the scientific community would benefit from single-cell sequencing to fine map components of the endometrium and how they respond to signals during the peri-implantation period of pregnancy. This would allow the *in vitro* model development to map to what is known *in vivo*. Creation of representative models to study implantation would allow us to physically and mathematically recreate *in vivo* scenarios *in vitro* in a system that is currently difficult to study.

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