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Preimplantation development in ungulates: a ‘ménage à quatre’ scenario

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Abstract

In ungulates, early embryonic development differs dramatically from that of mice and humans and is characterized by an extended period of pre- and peri-implantation development *in utero*. After hatching from the zona pellucida, the ungulate blastocyst will stay free in the uterus for many days before implanting within the uterine wall. During this protracted peri-implantation period, an intimate dialog between the embryo and the uterus is established through a complex series of paracrine signals. The blastocyst elongates, leading to extreme growth of extra-embryonic tissues, and at the same time, the inner cell mass moves up into the trophoblast and evolves into the embryonic disc, which is directly exposed to molecules present in the uterine fluids. In the peri-implantation period, uterine glands secrete a wide range of molecules, including enzymes, growth factors, adhesion proteins, cytokines, hormones, and nutrients like amino and fatty acids, which are collectively referred to as histotroph. The identification, role, and effects of these secretions on the biology of the conceptus are still being described; however, the studies that have been conducted to date have demonstrated that histotroph is essential for embryonic development and serves a critical function during the pre- and peri implantation periods. Here, we present an overview of current knowledge on the molecular dialogue among embryonic, extraembryonic, and maternal tissues prior to implantation. Taken together, the body of work described here demonstrates the extent to which this dialog enables the coordination of the development of the conceptus with respect to the establishment of embryonic and extra-embryonic tissues as well as in preparation for implantation.

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Introduction

In most mammals, the embryo develops in a dedicated environment, the uterus, which serves both nutritive and protective functions. As a consequence, the main activity of the first period of embryonic development is the production of extraembryonic tissues which are essential for placentation and for the survival of the embryo *in utero*. Early mammalian embryogenesis has been extensively studied in rodents and primates, with the mouse as the main reference species of the last several decades. These studies have identified the key principles that govern early development including morphogenesis, fate commitment, and pluripotency (Artus & Hadjantonakis 2012). From fertilization to the blastocyst stage, a conserved succession of morphogenetic events is observed in all mammalian species and occurs at a similar rate, lasting from 4.5 days to 7–8 days. During this period, highly conserved developmental steps result in the differentiation and segregation of embryonic from extraembryonic tissues (although these tissues sometimes remain intermingled

within the same cell layer, as in marsupials). At the end of this process, the resulting blastocyst is composed of three distinct cell populations: (i) the epiblast, which is pluripotent and forms the embryo proper, and two sets of extraembryonic tissues that are necessary for specialized interactions with the maternal uterus, (ii) the trophoblast or trophoctoderm, an extra-embryonic layer that contributes to the fetal placenta, and (iii) the hypoblast or primitive endoderm, an extra-embryonic layer that gives rise to the visceral and parietal hypoblast (Acloque *et al.* 2012). At this point, different developmental strategies evolved in different mammalian species, leading either to implantation (as in mice or humans) or to an extended period of pre-implantation development frequently associated with extreme growth of extra-embryonic tissues and the initiation of gastrulation and morphogenesis (at the end of this process). This specific developmental window has been described in many ungulate species (Hue *et al.* 2012). During this period, the conceptus remains in contact with uterine fluids, through the external layers composed of the trophoblast and the epiblast.

To date, little is known of the complex molecular dialog that occurs between the maternal uterus and the first three main cell populations of the conceptus. This dialog and its molecular dynamics are key to understanding the coordination of numerous crucial developmental processes, including the elongation of extra-embryonic tissues, visceral hypoblast formation, epiblast priming, initiation of gastrulation, primordial germ cell formation, left–right symmetry specification, and finally conceptus implantation. The aim of this review is to summarize the current state of knowledge of this ‘ménage à quatre’. We also examine how pluripotency is controlled in some ungulate species and why it remains so complicated to reproduce *in vitro* this amazing *in vivo* process.

Preimplantation development in ungulates and early lineage specification

Timing and early morphogenesis of an ungulate conceptus

During its journey from the oviduct to the uterus, the embryo undergoes a series of cleavage and morphogenetic events whose dynamics and timing vary depending on the species in question. The first noticeable change in the embryo is a flattening of the blastomeres, giving it the appearance of a mulberry and thus the name morula. This process, referred to as compaction, results from an increase in intercellular adhesion and the acquisition of cell polarity. Compaction is also closely associated with the differentiation of the inner cell mass (ICM) from the trophectoderm (TE) in the mouse. In ungulates, this occurs around day 5, at the 16–32-cell stage in bovines (Van Soom *et al.* 1997), pigs (Reima *et al.* 1993), and sheep (Bindon 1971). The second major morphogenetic event is the formation of the blastocyst, characterized by a fluid-filled cavity known as the blastocoel. This process of cavitation occurs around day 6–7 in bovines (Van Soom *et al.* 1997) and day 7–8 in pigs (Reima *et al.* 1993) and sheep (Bindon 1971). The timing of these developmental processes is quite similar in all mammalian species – activation of the embryonic genome occurs between the 5-cell and the 16-cell stage – and the main molecular players and signaling pathways described in rodents and primates are also conserved in ungulates (Piliszek & Madeja 2018). The main differences that have been described among species so far mostly relate to variations in functional dynamics, which will be detailed later.

In most ungulates, after the blastocyst hatches from the zona pellucida, it remains free in the uterine tract for several days before implanting within the uterine wall. This protracted peri-implantation period distinguishes ungulate development from that of rodents and primates and is further characterized by the establishment of an intimate dialog between the embryo and the uterus through a complex series of paracrine signals (detailed

in ‘Maternal influence on conceptus development’ section). During this period, which varies widely among species, the spherical blastocyst elongates into a tubular and filamentous form. In pigs, this process starts on day 10 and transforms a sphere 0.5–1 mm in diameter into a filamentous blastocyst 1000 µm long by day 16. Interestingly, elongation of the blastocyst seems to be initially driven by cellular hypertrophy rather than cellular hyperplasia (Geisert *et al.* 1982, reviewed in Bazer & Johnson 2014). As Rauber’s layer disappears, the ICM moves up into the trophectoderm and evolves into an embryonic disc which eventually starts to gastrulate (Fléchon *et al.* 2004, van Leeuwen *et al.* 2015).

The terminology used to describe the different layers that make up an ungulate blastocyst can sometimes be misleading, mostly because either similar terms are assigned to inequivalent cell layers or different terms are used to describe the same cell layer. As emphasized in Fig. 1 and according to Pfeffer *et al.* (2017), we propose the following terminology. The early blastocyst is composed of the trophoblast (TB) and the inner cell mass (ICM). After hatching, the three main cell populations of the blastocyst are the trophoblast (TB), the epiblast (EPI), and the hypoblast (HYPO). At the spherical stage, two main territories can be observed: (1) the embryonic disc, which includes the EPI, the visceral hypoblast (VH), and the disappearing Rauber’s layer (the equivalent of the polar trophectoderm in mice), and (2) the extra-embryonic parietal layers which include TB (external layer) and the parietal hypoblast (PH, internal layer). At the ovoid stage, TB can be subdivided into two types: the adjacent mural trophoblast (AMTB), located in close contact with the EPI, and the mural trophoblast (MTB) overlying the PH. As development progresses, the EPI also starts to differentiate through the formation of the posterior marginal zone of the epiblast (PMZE) which delineates the antero-posterior axis. The VH can also be subdivided into two regions: the anterior visceral hypoblast (AVH) overlying the EPI and the VH underlying the PMZE. We will use this terminology for this review.

TB/ICM specification

The first specifications in cell lineage in the mouse integrate various inputs including cell positioning, cell polarity, mechanical tensions, and metabolic constraints (Kim *et al.* 2018, White *et al.* 2018). These signals seem to be integrated by the Hippo signaling pathway which in turn regulates the expression of key lineage transcription factors (TFs); these establish a stable gene regulatory network that controls cell lineage specification and subsequent cell fate determination (Nishioka *et al.* 2009). Cell polarization at the time of compaction is one of the key determinants and asymmetric cell division generates polar and apolar cells on the basis of inheritance of the apical domain (polar cell) (Johnson & Ziomek 1981). While apolar cells tend to be located inside the

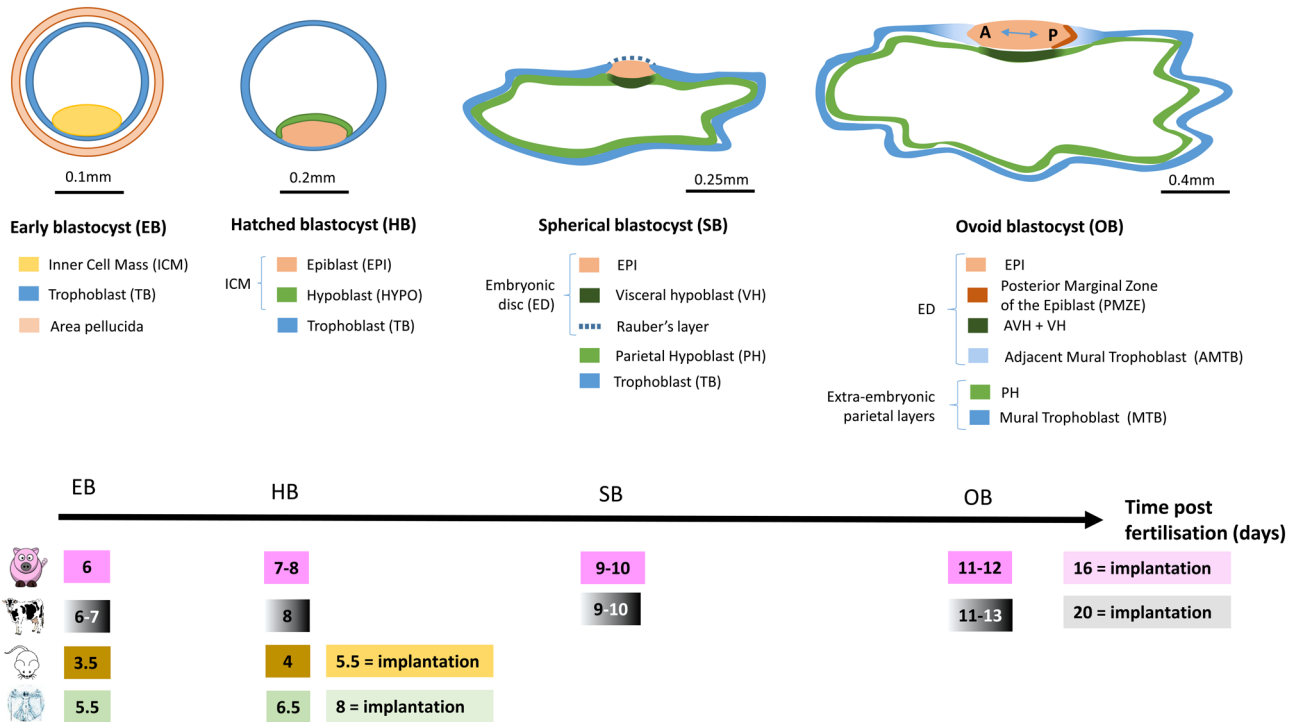


Figure 1 Schematic representation of blastocyst stages in pigs and bovines. The terminology used to identify the different cell layers and tissues is explained for each stage. The timeline depicts, for each species (pig, bovine, mouse, and human), the developmental time needed to reach a given blastocyst stage.

developing embryo, polar cells remain outside, facing the external environment. The apical domain sequesters some components of the Hippo signaling pathway, including AMOT, an activator of LATS kinases, so that the pathway is inactive in polar cells (Hirate *et al.* 2013, Leung & Zernicka-Goetz 2013). This absence of Hippo signaling activity leads to the nuclear localization of Yes-associated protein (YAP) which, by interacting with the transcription factor TEAD4, drives the expression of TB-specific genes. Conversely, the active Hippo pathway in apolar cells leads to the phosphorylation of YAP and its consequent cytoplasmic localization, which prevents its interaction with TEAD4 and eventually blocks the commitment of these cells toward the TB lineage.

In other mammals, the Hippo signaling pathway is likely to be a similarly major driver of TB/ICM specification. Comparable signaling machinery has been described in pigs (Emura *et al.* 2016, Liu *et al.* 2018) and bovines (Home *et al.* 2012, Ozawa *et al.* 2012, Sakurai *et al.* 2016). As in the mouse, ROCK inhibition in pig embryos promotes Hippo signaling activity, suppressing CDX2 (Caudal type Homeobox 2) expression, a key transcription factor for TB specification and maintenance (Kono *et al.* 2014, Liu *et al.* 2018). However, the reverse effect was reported in bovines, which suggests a certain degree of variability among species (Negrón-Pérez *et al.* 2018). Despite this difference, alterations in YAP or AMOT expression affect bovine blastocyst

formation and the number of CDX2-positive cells is negatively correlated with TEAD4 expression level (Sakurai *et al.* 2016, Negrón-Pérez *et al.* 2018). While the functional link between cell polarization, activity of the Hippo signaling pathway, and CDX2 expression is conserved between mice and pigs and, by extension, other mammals, the molecular kinetic for TB lineage determination differs among species from that described in the mouse (Liu *et al.* 2018).

In the mouse, the Hippo pathway regulates key TB-specific genes as *Cdx2*, *Gata2*, and *Gata3*, which encode transcription factors that are involved in setting up the TB genetic program (Ralston *et al.* 2010). In particular, CDX2 appears to be critical for TB maintenance rather than TB specification (Strumpf *et al.* 2005). Similarly, in pigs, CDX2 is necessary for TB cell proliferation and the maintenance of cell polarity (Bou *et al.* 2017), and in bovines, for TB maintenance at later embryonic stages (Berg *et al.* 2011, Goissis & Cibelli 2014, Sakurai *et al.* 2016).

GATA2 and GATA3 (GATA-binding proteins 2 and 3) are also involved in mouse TB maintenance (Ray *et al.* 2009, Ralston *et al.* 2010, Home *et al.* 2017). Interestingly, these GATA-TFs also regulate TB markers in the bovine trophoblast CT-1 cell line, which suggests a conserved role in TB biology. However, they also act on species-specific targets and this role is exemplified by their regulation of *Interferon tau* expression in bovines,

which is critical to pregnancy recognition in ruminants (Bai *et al.* 2011). However, to date, no analysis has been reported that assesses the function of GATA2 and GATA3 in the early embryo in ungulates. Additional TFs have also been identified as regulators of TB physiology by Pfeffer (2018) and Piliszek and Madeja (2018); these studies highlighted both common and species-specific TB regulators.

In addition to Hippo signaling, the specification of TB/ICM cell lineages appears to be regulated by multiple other pathways, including Wnt signaling. Indeed, at least two studies have examined the effect of modulation of Wnt signaling activity on early bovine embryonic development (Denicol *et al.* 2013, Madeja *et al.* 2015). Denicol *et al.* (2013) reported that activation of Wnt reduced numbers of TB and ICM cells, while treatment with DKK1, a Wnt inhibitor, had no major effect on blastocyst formation. In contrast, Madeja *et al.* (2015) showed that Wnt activation upregulated pluripotent markers at the expense of TB markers. In addition, Wnt signaling played a role in trophoblast stem cell maintenance *in vitro*, likely through the regulation of YAP/TAZ activity (Wang *et al.* 2019). This study suggested the presence of essential cross-talk between Wnt and Hippo signaling pathways. Interestingly, DKK1 was reported to regulate blastocyst elongation during the peri-implantation period (Tribulo *et al.* 2019). Taken together, these studies highlight the important role(s) of the Wnt pathway in TB cell biology, specifically in promoting self-renewal and cell differentiation.

Another pathway that seems to be involved in early ungulate embryonic development is JAK/STAT signaling. For example, STAT5 is expressed during early bovine embryogenesis (Flisikowski *et al.* 2015) and some STAT1 and STAT3 SNPs were found to be associated with improved embryonic survival (Khatib *et al.* 2009). Lastly, inhibition of JAK1/2 affects formation of the ICM but not the TB (Meng *et al.* 2015). Several questions still remain to be answered, including which upstream signal(s) regulate(s) JAK/STAT, which genes are targeted, and whether cross-talk exists with Hippo signaling.

An aspect of ungulate development that appears to be unique relates to the fate of the TB. At the hatched blastocyst stage, TB can be subdivided into two spatially distinct tissues: TB that covers the blastocoel cavity, and the polar trophoblast, also known as Rauber's layer (RL). In rodents and primates, the polar trophoblast contributes to the post-implantation development of the fetal part of the placenta; in ungulates, instead, this function is fulfilled by the mural trophoblast. Indeed, one characteristic of ungulate early embryonic development is the disappearance of the RL, occurring around days 9–11 in pigs (Sun *et al.* 2015), days 10–12 in horses (Enders *et al.* 1988), and day 14 in bovines (Maddox-Hyttel *et al.* 2003, van Leeuwen *et al.* 2015). After RL disintegration, the EPI is completely devoid of TB and thus directly exposed to uterine fluids. RL disappearance

is thought to occur via apoptosis rather than as the result of a difference in proliferative rate with the EPI (Enders *et al.* 1988, Maddox-Hyttel *et al.* 2003). The role of RL loss during early embryonic development is still under debate, but it has been linked to the formation of the anterior visceral hypoblast and the emergence of the primitive streak (van Leeuwen *et al.* 2015).

EPI/HYPO specification

The ICM eventually differentiates into two distinct cell lineages, EPI and HYPO. In the mouse, their formation results from a sequence of events that was originally studied through the dynamic expression of EPI- and HYPO-specific TFs (Artus & Chazaud 2014). NANOG (EPI) and GATA6 (HYPO) are initially expressed in all blastomeres at the morula stage (Plusa *et al.* 2008). Their expression is progressively restricted and eventually becomes mutually exclusive, so that ICM cells express only the EPI or the HYPO genetic program. By the mid-blastocyst stage, the ICM is a mixture of EPI and HYPO progenitors organized in a salt-and-pepper pattern (Chazaud *et al.* 2006). Once specified, EPI and HYPO cells reorganize so that HYPO cells lie in contact with the blastocoel cavity, encapsulating the EPI. This cell-sorting process is likely to involve cell adhesion, active migration, positioning information, epithelialization, and selective apoptosis of mispositioned cells (Gerbe *et al.* 2008, Plusa *et al.* 2008, Meilhac *et al.* 2009). Whether this succession of steps is conserved in other mammals remains to be clarified. However, based on expression studies, this pattern seems to be replicated in ungulates, although with different kinetics, as reported from studies of bovines (Khan *et al.* 2012, Kuijk *et al.* 2012), pigs (Ramos-Ibeas *et al.* 2019), and horses (Choi *et al.* 2015).

In the mouse, the specification of EPI and HYPO cells is accompanied by a progressive loss of plasticity (Chazaud *et al.* 2006, Grabarek *et al.* 2012). Interestingly, HYPO formation is associated with the sequential expression of different markers, which is assumed to represent the activation of a lineage-specific genetic program. These markers are, successively, GATA6 (8-cell), PDGFR α (16-cell), SOX17 (32-cell), GATA4 (64-cell), and SOX7 (sorted PrE cells) (Plusa *et al.* 2008, Artus *et al.* 2011). The role of these factors remains to be determined in other mammals. In this situation, cell fate specification of a bi-potential ICM cell can be viewed as a process in which one genetic program is shut down while the other is maintained. This mechanism must be tightly regulated temporally and perhaps spatially and requires the regulation of transcriptional activity as well as of protein stability (Bessonard *et al.* 2017).

In mammals, EPI/HYPO formation seems to be strictly regulated by receptor tyrosine kinase (RTK) signaling. In rodents in particular, EPI/HYPO specification is controlled by fibroblast growth factor (FGF) signaling,

and this process has been largely characterized through gain- and loss-of-function experiments (Chazaud *et al.* 2006, Nichols *et al.* 2009, Yamanaka *et al.* 2010, Kang *et al.* 2013, 2017, Krawchuk *et al.* 2013, Molotkov *et al.* 2017). There is abundant evidence, however, to suggest that the role of FGF signaling in EPI/HYPO cell-fate decisions is not confined to rodents. In bovines, the inhibition of specific FGF signal transducers like the mitogen-activated protein kinase (MEK) and FGF receptor (FGFR) has different outputs. Indeed, inhibition of MEK but not FGFR biases cell-fate decisions toward EPI, while the addition of FGF4 promotes development into HYPO (Kuijk *et al.* 2012). Similar observations were reported in ovine blastocysts (Moradi *et al.* 2015). However, in humans, MEK inhibition does not affect EPI/HYPO specification (Kuijk *et al.* 2012, Roode *et al.* 2012). These observations suggest that (i) other input signals regulate MEK activity and (ii) one or multiple additional downstream effectors control the EPI/HYPO genetic program. For example, platelet-derived growth factor (PDGF) signaling is critical in regulating HYPO cell survival through the PI3K-mTOR pathway in mice (Artus *et al.* 2010, Bessonard *et al.* 2019).

Functional interactions at work between the three main blastocyst tissues during elongation

The extraembryonic and embryonic tissues that make up the blastocyst are frequently defined according to their future fate, by their eventual contributions to the placenta and extraembryonic membranes or to the embryo proper. However, much experimental evidence, old and new, suggests that these tissues also have functional interactions during preimplantation development, in particular to harmonize and synchronize their reciprocal development and growth. It is likely that these interactions are necessary for the proper development of each of the three main tissues that make up the blastocyst.

Experimental evidence from trophoblastic vesicles

To our knowledge, only a few studies have demonstrated the importance of interactions between extraembryonic and embryonic tissues for conceptus elongation and survival in ungulates. The earliest studies were carried out using novel (at the time) cellular tools, the trophoblastic vesicles (TV) developed by Gardner and Johnson (1972). TVs consist of fragments of blastocysts that are devoid of EPI cells. Depending on the embryonic stage from which they are derived, TVs can be composed either of TB cells only or both TB and parietal hypoblast (PH) cells. TVs can be cultured *in vitro* and transferred into synchronized recipient females. In a pioneering study, Surani and Barton (1977) delayed implantation by

transferring murine blastocysts or TVs into progesterone-treated ovariectomized females. In that environment, the embryos remained in a period of quiescence that resembled diapause. Following estradiol injection, both blastocysts and TVs were able to resume implantation, indicating that implantation does not require the presence of an ICM. Interestingly, while the number of cells increased in quiescent embryos from 60 to 120, this number remained unchanged in TVs, an indication that paracrine signals from the ICM regulate TB cell proliferation. Likewise, co-culture of single bovine blastocysts with TVs improved their development *in vitro*, further evidence of the existence of paracrine signals between the TB and ICM (Camous *et al.* 1984, Pool *et al.* 1988, Mori *et al.* 2012).

In the ovine model, TVs can be maintained *in vitro* for up to 20 days, but do not proliferate or elongate (Fléchon *et al.* 1986). However, they do elongate when transferred into recipient females, but to a lesser extent than control embryos. These data demonstrate that the embryonic disc is not necessary for TB elongation but suggest again that paracrine signals and direct interactions between the embryonic disc and extra-embryonic parietal tissues may be important for the survival, coordinated growth, and morphogenesis of the conceptus during elongation (see also Hue *et al.* 2007).

In elongating pig embryos, several paracrine signals have been identified between the EPI and TB, and involve both the FGF and BMP signaling pathways (Valdez Magaña *et al.* 2014). EPI cells express FGF4, which acts through FGFR2 on adjacent mural TB cells to activate MAPK phosphorylation. In parallel, BMP4 is produced by the extraembryonic mesoderm and HYPO cells and interacts with BMPR2 expressed in TB cells. These molecular dialogs between embryonic and extraembryonic tissues appear to be evolutionarily conserved, as they play important roles also in early embryogenesis in the mouse (Goldin & Papaioannou 2003, Graham *et al.* 2014, Kurowski *et al.* 2019). However, several aspects still remain to be clarified in ungulates, including (i) the precise origin of the paracrine signals required during elongation, (ii) the mechanisms of molecule delivery, and (iii) the effects of these signals on their target cells.

The use of trophoblastic vesicles may help to answer these questions if future studies can take into consideration their cell composition. Indeed, in previous studies, TVs derived either from equine (Ball *et al.* 1989) or ovine blastocysts (Fléchon *et al.* 1986) were composed of numerous cell types that have not been fully characterized. But another complementary strategy could be the use of medium- and high-throughput transcriptomic technologies, which have already shed some light on various aspects of ungulate preimplantation development.

Evidence from early transcriptomic studies: differences between developmental stages

An indispensable tool in elucidating differences among developmental stages has been the use of transcriptomic profiling, which has helped to identify important cellular processes triggered in TB and HYPO during elongation.

In sheep and cattle, comparisons of gene expression between ovoid, tubular, and early filamentous stages confirmed that a key molecular transition occurs between the ovoid and tubular stages with the initiation of elongation (Cammass *et al.* 2005, Degrelle *et al.* 2005). By integrating multiple independent transcriptomic studies of different stages of elongation (Ushizawa *et al.* 2004, Hue *et al.* 2007, Mamo *et al.* 2011), researchers were able to identify key differentially expressed genes (DEGs) whose functions are linked to cell proliferation, cellular growth and differentiation and connective tissue formation (Hue *et al.* 2012). Interestingly, one recurring function of trophoblast DEGs is an association with lipid metabolism. This particularity of ungulate blastocysts, which has not been reported from mice or humans, seems to be linked to the fact that the elongation process in ungulates is coupled with a long preimplantation period. During this time, the conceptus relies only on its own resources and uterine histotroph to meet its significant resource demands for cell growth and cell proliferation. For example, for bovine blastocysts, the wet weight of the conceptus can increase by a factor of 20 between gestational days 16 and 19 (Lewis *et al.* 1982). A recent transcriptomic comparison between ovoid, tubular, and filamentous bovine blastocysts clearly highlighted important changes in lipid metabolism and fatty acid biosynthesis during elongation, together with alterations in arachidonic acid metabolism and prostaglandin synthesis and transport (Ribeiro *et al.* 2016a). This confirms the importance of lipids as a key element during early embryogenesis for many aspects of cell biology, including cell growth and phospholipid membrane synthesis, energy production, and intercellular signaling (see review by Ribeiro *et al.* 2016b). Similar observations have been made in ovine or porcine conceptuses undergoing elongation (Charpigny *et al.* 1997, Blomberg *et al.* 2006, Waclawik *et al.* 2013, Brooks *et al.* 2015). In ruminants, the regulatory factor PPAR γ (peroxisome proliferator-activated receptor gamma) has proven to be an integral aspect of this process: it plays a significant role in governing prostaglandin synthesis and lipid metabolism and is required for conceptus elongation (Brooks *et al.* 2015, Ribeiro *et al.* 2016b) and likely for TB differentiation as well (Degrelle *et al.* 2011). A recent gene expression analysis presented evidence for a similar function for PPAR γ in the extra-embryonic layers in pigs (Blitek & Szymanska 2019).

An important limitation of these studies is that they compare gene expression profiles acquired from whole

embryos. This provides functional information on the molecular dynamics among developmental stages but does not clarify precisely the specific functions and connections between the cell populations that make up the conceptus. One way to better answer this question would be to isolate each subpopulation and analyze its transcriptome profile independently or, better yet, to characterize the blastocyst at the single-cell level during the preimplantation period.

Evidence from dissection-based transcriptomic studies: compartmentalization of layer functions

Using dissection-based strategies multiple studies have recently performed transcriptomic analyses of equine (Iqbal *et al.* 2014), pig (Bernardo *et al.* 2018), and bovine embryos (Hosseini *et al.* 2015, Zhao *et al.* 2016, Pfeiffer *et al.* 2017, Bernardo *et al.* 2018). These have provided new insights into the signaling pathways that are potentially active in the blastocysts of these three species. Specifically, Iqbal *et al.* (2014) compared the transcriptome of ICM and TB using bisected D7 equine blastocysts, while Bernardo *et al.* (2018) characterized the transcriptomes of the EPI from three mammalian species (mouse, cattle, pigs) at three equivalent stages: ICM, early epiblast, and late epiblast. Hosseini *et al.* (2015) characterized ICM and TB isolated from *in vivo*-produced D7.5 expanded bovine blastocysts, while Zhao *et al.* (2016) used immunopurified TB and ICM cells from *in vitro*-produced D7 bovine blastocyst and Pfeiffer *et al.* (2017) compared the transcriptomes of the late epiblast and the embryonic disc (including early epiblast and the underlying hypoblast) of bovine embryos.

The latter studies used the mouse model as a reference for identifying developmental stages and tissues. Taken together, these studies enable us for the first time to (1) identify shared and species-specific molecular players that govern pluripotency at these three stages and (2) highlight the differences between mice and ungulates with respect to pluripotent tissues. By looking at orthologous genes shared among the different species, Bernardo *et al.* (2018) first observed that the mouse EPI (regardless of the stage under examination) is quite divergent from that of pigs or cattle, which are more similar to each other transcriptionally. A similar observation was made by Hosseini *et al.* (2015) in a comparison of the transcriptomic profile of bovine ICM to that of humans and mice. In addition, Bernardo *et al.* observed that the three species' ICM profiles were distinct from those of early and late epiblasts, but differences between ICM and EPI were much more pronounced in mice than in ungulates. These results suggest two intriguing hypotheses: that the current definition of naive and primed states of pluripotency could merely represent a particularity of rodents or that naive pluripotency could be a very labile and transient state in ungulates. Both

studies also confirmed the molecular similarity between early and late epiblast, supporting the existence of a stable and unique primed-like pluripotent state that is maintained during elongation in equine, pig, and cattle embryos. Indeed, most of the molecular players known to regulate primed pluripotency in the EPI of mammalian embryos are expressed in the embryonic disc of pig and bovine embryos.

NODAL signaling, which is thought to sustain primed pluripotency in humans and mice (Vallier *et al.* 2005, Brons *et al.* 2007), is active in both bovine and pig blastocysts (Blomberg *et al.* 2008, Alberio *et al.* 2010, Hosseini *et al.* 2015). The studies highlighted above confirmed the expression of *GDF3* in the ICM of horse embryos (Iqbal *et al.* 2014) and the strong expression of *NODAL* and *GDF3*, together with their receptors and transducers, in the bovine embryonic disc (EPI+HYPO) (Pfeffer *et al.* 2017) and in pig EPI cells (Bernardo *et al.* 2018). Instead, genes necessary for the JAK/STAT3 pathway, which has been described as a key feature of naive pluripotency (Hall *et al.* 2009, van Oosten *et al.* 2012), are downregulated between the ICM and early epiblast stages (Eckert & Niemann 1998, Alberio *et al.* 2010, de Ruijter-Villani *et al.* 2015, Pfeffer *et al.* 2017, Bernardo *et al.* 2018). The same was also observed for WNT signaling, which is known to maintain naive pluripotency by inducing the nuclear translocation of TCF3 and is apparently inactive in the EPI of pig and bovine blastocysts (Pfeffer *et al.* 2017, Bogliotti *et al.* 2018) but active in bovine TB (Zhao *et al.* 2016).

Interestingly, BMP signaling is also active in EPI of pig, equine and bovine embryos. In mice, BMP signaling has been shown to support the maintenance of pluripotency through the activation of *Id1* and *Id3* (Ying *et al.* 2003). In ungulates, *BMP4* is expressed in EPI at all stages, but with notable variability. *BMP2* is also strongly expressed in the ICM of equine blastocysts (Iqbal *et al.* 2014) and in the embryonic disc at the ovoid stage in pigs (Valdez Magaña *et al.* 2014) and cattle (Pfeffer *et al.* 2017). These results suggest a synergistic action of these two molecules to regulate the balance between pluripotency and differentiation of embryonic cells in mice and ungulates. Altogether, although many questions remain, the work that has been done to date confirms the biological importance of NODAL and BMP signaling pathways in the primed epiblast in mammals.

Looking beyond the embryonic disc, Pfeffer *et al.* (2017) tried to analyze the flow of molecular information between embryonic and extra-embryonic tissues by dissecting the four main tissues of the bovine ovoid blastocyst (embryonic stage 5 at D14 post fertilization, Van Leeuwen *et al.* 2015) and comparing their transcriptomic profiles. Pathway analysis confirmed the functional proximity of HYPO and EPI within the embryonic disc, while the parietal hypoblast (PH) was less similar and occupied an intermediate position. The transcriptome of the TB, instead, was quite unique,

demonstrating specific enrichment for biological functions linked with steroid biosynthesis. This was consistent with an earlier study of equine blastocysts, in which genes that were overexpressed in TB with respect to other tissues were specifically associated with biological processes related to lipid biosynthesis, ion transport, and Golgi vesicle transport (Iqbal *et al.* 2014). By performing a detailed analysis of the genes encoding signaling molecules and their respective receptors, Pfeffer *et al.* (2017) confirmed that the four tissues should be theoretically able to transactivate each of the signaling pathways analyzed. Indeed, while the bovine TB expressed only a few signaling molecules (including *FGF2*, *PDGFA*, and vascular-endothelial growth factor *VEGFA/VEGFB*), molecules secreted by either the underlying HYPO (*FGF10*, *Indian Hedgehog*, *Insulin-like growth factor IGF2*, *Angiopoietin*, or *WNT11*) or the adjacent embryonic disc (*NODAL*, *GDF3*, or *BMPs*) may also act on TB through a paracrine action (Fig. 2).

Unfortunately, the challenges inherent in performing microdissection – namely, the limited amount of biological material obtained and possible contamination with adjacent tissues – have limited our ability to produce an accurate, dynamic molecular atlas of the developing blastocyst.

Evidence from single-cell studies

Recent developments in single-cell transcriptomics have opened new dimensions through the resolution of spatial cellular heterogeneity and the ability to capture cellular changes over time. Single-cell transcriptomes from whole embryos and tissues could help to identify cell–cell interactions, such as those between fetal and maternal cells during placentation in humans (Vento-Tormo *et al.* 2018) or to clarify cell lineages and inter-cellular signaling (Deng *et al.* 2014, Petropoulos *et al.* 2016, Mohammed *et al.* 2017, Rivron *et al.* 2018, Stirparo *et al.* 2018). Indeed, by combining blastoid culture and single-cell transcriptomic studies, Rivron *et al.* (2018) were able to describe the molecular exchange between TB, HYPO and EPI of mouse blastocysts. They showed that paracrine signals emitted by the EPI, including IL11, FGF4, BMP4, and NODAL together with autocrine signals such as IL11, WNT6, and WNT7B, are necessary for TB development and *in utero* implantation.

To date, only a few single-cell gene expression studies have been performed on ungulate species, and only on pig and bovine blastocysts (Negrón-Pérez *et al.* 2017, Wei *et al.* 2017, 2018, Ramos-Ibeas *et al.* 2019).

In ruminants, two independent groups recently reported single-cell qPCR analyses on *in vitro*-produced blastocysts (Negrón-Pérez *et al.* 2017, Wei *et al.* 2017). Both studies used a set of genes known to be linked in mammals with early embryonic development (cell fate, pluripotency, signaling pathways). They isolated, respectively, 96 and 67 cells from the morula stage to

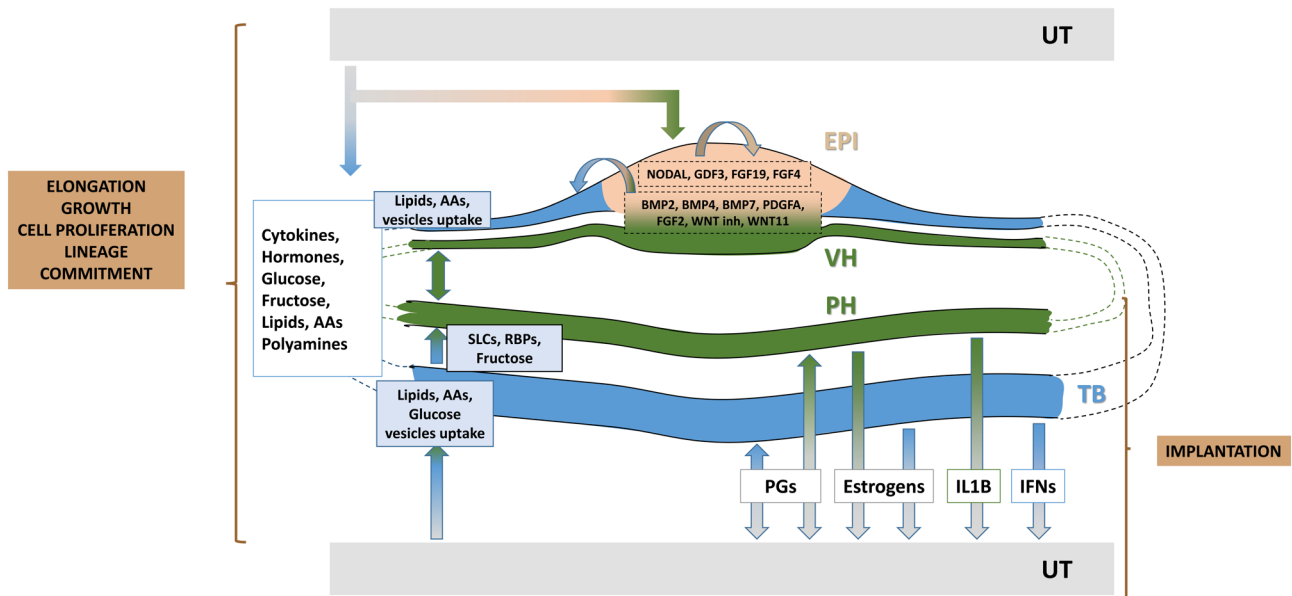


Figure 2 Representation of molecular flows among different embryonic, extra-embryonic, and maternal tissues. AAs, amino acids; EPI, epiblast; IFNs, interferons; PGs, prostaglandins; PH, parietal hypoblast; RBP, retinol-binding protein; TB, trophoblast; SLC, solute carrier transporters; UT, uterus; VH, visceral hypoblast.

the expanded blastocyst. From these datasets, they were able to discriminate among the three main cellular populations of the blastocyst. While the bovine EPI expressed known markers of core pluripotency from mice and humans (*NANOG*, *SOX2*, *POU5F1*, *NR5A2*), most of the prototypical hypoblast markers were not hypoblast-specific in bovine blastocysts and were also expressed either in TB cells (*GATA6*), in EPI (*GSC* and *HNF4A*), or in both (*FN1*). Markers that were indeed largely hypoblast specific included *SOX17* and *GATA4*, together with *PDGFRA*. Bovine TB cells specifically expressed or overexpressed TB-specific transcription factors known from humans and mice (*CDX2*, *GATA3*, *GRHL1*, *GRHL2*, *MSX2*, *TFAP2A*) and genes that control cell–cell interactions (*KRT8*, *PECAM1*, *DAB2*, *ATP12A*). While these two studies helped to identify blastomere-specific gene expression and early lineage specification in bovine blastocysts, the numbers of genes and cells analyzed (a total of 17 cells for epiblast, 51 for hypoblast, and 95 for TE) were too limited to go deeper into the molecular interactions among these three tissues.

In pigs, two studies also addressed similar questions, one with single-cell qPCR on *in vitro*-produced pig blastocysts (Wei *et al.* 2018) and the other with mRNA-seq sequencing on a panel of cells isolated from *in vivo*-derived pig conceptuses at several developmental stages (morula, early blastocyst, late blastocyst (hatched), and spherical blastocyst; Ramos-Ibeas *et al.* 2019). The first interesting comparison to be made from these two publications is the difference between blastocysts produced *in vitro* and those produced *in vivo*. Among the blastocysts produced *in vitro*, there was no clear

transcriptomic segregation between HYPO and EPI even at the late blastocyst stages; instead, segregation was quite evident between hypoblast and epiblast in late blastocysts produced *in vivo*. These results provide evidence that *in vitro*-produced blastocysts 8 days after fertilization are not as mature as those developed *in vivo* and that culture conditions seem to be inadequate at this stage.

The two studies confirmed the presence of specific markers for TB (*GATA2*, *GATA3*, *DAB2*, *CDX2*) and EPI (*POU5F1*, *SOX2*, *NANOG*, *KLF4*) that are conserved among mammals in early blastocysts. In addition, Ramos-Ibeas *et al.* reported specific markers for the pig hypoblast in hatched and spherical blastocysts (*PDGFRA*, *GATA4*, *GATA6*, *COL4A1*, *NID2*, *RSPO3*). The genes *POU5F1*, *KLF4*, *GATA6*, and *PDGFRA* were also expressed in the morula and ICM of early blastocysts, which supports the shared origin of HYPO and EPI cells from the ICM. This latter study also investigated the signaling pathways active in the different cell layers and their importance for cell commitment to different lineages. To do this, they first performed differential gene expression analysis between cell groups to identify DEGs associated with known signaling pathways. Then, they cultured pig embryos with inhibitors of these previously identified candidate pathways. Using this two-step approach, they observed a switch in the dependence on JAK/STAT, PI3K, and TGF β pathways for the maintenance of pluripotency in epiblast cells. While JAK/STAT and PI3K seem to be necessary at the morula and early blastocyst stages, NODAL signaling is preponderant later in the EPI. PI3K also seems to be important for TB development in early

blastocysts. Unlike reports from cattle, these authors did not observe a particular effect of WNT inhibition on these cells but they did confirm the dependence on MAPK signaling in the ICM for the formation of HYPO (see also 'EPI/HYPO specification' section).

This study did not pay particular attention to peroxisome proliferator-activated receptor (PPAR) signaling, even if the DEG analysis clearly highlighted its activity in both the hypoblast and epiblast of hatched and spherical blastocysts (Ramos-Ibeas *et al.* 2019). PPARs are nuclear receptor proteins that are able to dimerize with RXR to control gene expression; they are particularly relevant because their main ligands are free fatty acids and eicosanoids, of which the latter group is produced by the oxidation of arachidonic acid or other polyunsaturated fatty acids and includes prostaglandins and leukotriens. The importance of prostaglandins during elongation has been extensively documented in ungulates (Dorniak *et al.* 2011, Brooks *et al.* 2015). Together with gene expression studies in cattle (Ribeiro *et al.* 2016, Pfeffer *et al.* 2017), this work confirms the importance of the embryonic and parietal extra-embryonic tissues (HYPO and TB) for the synthesis of PPAR ligands from fatty acids present in the uterine fluids.

Taken together, all the studies discussed here highlight the interdependence that exists among the tissues of the conceptus (TB, EPI, HYPO) to ensure its development and survival. Importantly, these studies also suggest that these tissues, through the production and secretion of many different molecules (Table 1), interact strongly with the maternal uterine environment (ovary and endometrium), constituting an inseparable 'ménage à quatre'.

Maternal influence on conceptus development

Evidence of maternal effects on conceptus development

Current limits of embryo culture

Efforts to optimize *in vitro* embryo production and reduce differences between *in vitro*- and *in vivo*-produced embryos in many different species (mouse, human, rabbit, cattle, pig, sheep) have led to a deep appreciation for the extent of the maternal influence on conceptus development. While the quality of oocytes and the conditions of *in vitro* maturation or fertilization undoubtedly matter (Smith *et al.* 2009, Leroy *et al.* 2015), the maternal influence on the developmental phase that precedes implantation and placental formation is unmistakable and appears to derive mostly from the molecular crosstalk at work within the maternal tract. This effect arises first in the oviduct (from one cell to early blastocyst stage, especially in horses due to a longer stay therein, Smits *et al.* 2016), and then moves into the uterus (from early blastocyst to implantation).

For decades, efforts have been made to establish *in vitro* systems that mimic the oviduct environment to produce blastocysts from different species (Smits *et al.* 2012, Fowler *et al.* 2018, Hamdi *et al.* 2018). Instead, *in vitro* development of older stages has only succeeded in human and mice through cultures in enriched media (Hsu 1973) or, recently, the establishment of embryoids/gastruloids (Govindasamy *et al.* 2019). In livestock species, embryonic discs have been successfully cultured for a few days with no effect on gastrulation patterns but extra-embryonic tissues have failed to elongate *in vitro* despite their good *in vitro* survival in most species (Hochereau-de Reviers & Perreau 1993, Wianny *et al.* 1997, Valdez Magaña *et al.* 2014, Stankova *et al.* 2015, see also 'Experimental evidence from trophoblastic vesicles' section).

Diapause: when the maternal environment controls the onset of blastocyst development

Scientific interest began to focus on the *in vivo* maternal influence on embryonic tissues following reports that mouse embryonic stem (ES) cells were more easily derived from females in diapause (Evans & Kaufman 1981, Kaufman *et al.* 1983), a state of embryonic dormancy controlled by signals from the uterus (Renfree & Fenelon 2017). In ungulates, the roe deer is the only species in which naturally occurring embryonic diapause has been studied. During this diapause, which lasts for 5 months, the TB and the EPI exhibit a minimum proliferation rate, do not differentiate, and display unique structural features including a lack of mitochondria, ribosomes, Golgi apparatus, and endoplasmic reticulum (Aitken *et al.* 1975). In parallel, the endometrium also presents specific and dynamic ultrastructures, indicating that endometrial glands also play a major role in diapause induction and the subsequent reactivation of embryonic development (Aitken *et al.* 1975). Recently, characterizations of the proteome of uterine fluids at different times during and after diapause highlighted the importance of polyamine biogenesis and degradation during roe deer diapause and the importance of the regulation of cell–cell adhesion during this process (van der Weijden *et al.* 2019). Hormonal regulation is also associated with exit from diapause in roe deer, but this remains poorly characterized; data from the existing literature are contradictory regarding levels of estrogen, progesterone, and prolactin during and after diapause (Aitken 1974, Lambert *et al.* 2001, Korzekwa *et al.* 2019, van der Weijden *et al.* 2019).

Natural diapause serves as a clear indicator of the importance of the maternal environment in controlling the timing of embryonic development before implantation. This observation has also been confirmed with experimental data. Diapause has been induced by (i) the transfer of embryos from a non-diapausing species to the uterus of a diapausing species

Table 1 Biological molecules known to be produced by the different embryonic, extra-embryonic and maternal tissues in different ungulate species.

	Growth factors and cytokines	Hormones	Amino acids	Fatty acids	Prostaglandins	Extracellular vesicles nucleic acids	Receptors	Carbohydrates
Embryonic Disc								
Epiblast	BMP4 ^[p,c] , PDGFA ^[p,c] , FGF2 ^[c,h] , WNT ^[inh] , WNT11 ^[p,c] , NODAL ^[p,c] , GDF3 ^[p,c,h] , FGF19 ^[p] , FGF4 ^[p,c,h]		Polyamines ^[p,s]	PPARs ^[p,s,c]		Proteins, Lipids, miRNAs ^[p,s,c,h]	FGFRs ^[p,c,h] , PDGFRA ^[p,c,h] , BMPRS ^[p,c,h] , TGF β ^[p,c,s] , HGFR ^[c] , IGF ^[c] , VEGFR ^[c] , WNTRS ^[c]	
Hypoblast	BMP4 ^[p,c] , PDGFA ^[p,c] , FGF2 ^[c,h] , WNT ^[inh] , WNT11 ^[p,c] , BMP2 ^[p,c,h] , BMP7 ^[p,c]	E ₂ ^[p]	Polyamines ^[p,s]	PPARs ^[p,s,c]		Proteins, Lipids, miRNAs ^[p,s,c,h]	FGFRs ^[p,c] , PDGFRA ^[p,c,h] , HGFR ^[c] , IGF ^[c] , VEGFR ^[c] , WNTRS ^[c]	
Parietal extra-embryonic layers								
Hypoblast	IH ^[c] , IGFs ^[c,h] , WNT11 ^[c] , FGF10 ^[c] , FGF2 ^[c]	E ₂ ^[p,c,h] , Placental Lactogene ^[s]	Polyamines ^[p,s]	PPARs ^[p,s,c] , SLCs ^[p,s,c,h] , Cortisol ^[s] , Arachidonate ^[p,s,c] , RBP ₅ ^[p,s,c,h]	LPA ^[p,s,c] , LPARs ^[p,s,c] , PGE ₂ ^[p,s,c,h] , PGF _{2α^[p,s,c], PG^[p]}	Proteins, Lipids, miRNAs ^[p,s,c,h]	FGFR ^[p,c]	Fructose ^[p,c,s]
Trophoblast	VEGF ^[c] , FGF2 ^[c] , PDGFA ^[c] , IFN- τ ^[c,s] , IFN- γ ^[p] , IFN- δ ^[p] , IL1B2 ^[p] , TNF α ^[h]	E ₂ ^[p,c,h] , Placental Lactogene ^[s]	Polyamines ^[p,s]	PPARs ^[p,s,c] , SLCs ^[p,s,c,h] , Cortisol ^[s] , Arachidonate ^[p,s,c] , RBP ₅ ^[p,s,c,h]	LPA ^[p,s,c] , LPARs ^[p,s,c] , PGE ₂ ^[p,s,c,h] , PGF _{2α^[p,s,c], PG^[p]}	Proteins, Lipids, miRNAs ^[p,s,c,h]	FGFR ^[p,c] , BMPR ^[p,c] , PTGIR ^[p,s] , PTGIR ^[p,s] , LPAR ^[p,s,c] , TGF β ^[p,c,s]	Fructose ^[p,c,s]
Endometrium								
Uterine glands	FGF7 ^[p,s] , FGF10 ^[s] , EGF ^[p] , TGF β ^[p] , IL6 ^[p] , FGF2 ^[p] , IGF ^[p,s] , WNTs ^[s,c] , GH ^[s] , CSF2 ^[p,c,s] , HGF ^[s] , SPPI ^[p,s]		AAs ^[p,c,s,h] , SLCs ^[p,c,s] , Polyamines ^[s]	RBP ₅ ^[p,s,c,h] , Retinol ^[p,s,c] , SLCs ^[p,s,c,h] , Arachidonate ^[p,s,c]	LPA ^[p,s,c] , LPARs ^[p,s,c] , PGE ₂ ^[p,s,c] , PGF _{2α^[p,s,c,h]}	Proteins, Lipids, miRNAs ^[p,s,c,h]	IL1R ^[p,s,c] , LPAR ^[p,s,c] , IFN-R ^[p,s,c] , FGFR ^[p,s,c] , ESR β ^[p,s,c] , PGR ^[p,c,s,h] , HGFR ^[s] , TGF β ^[p,s,c] , PTGIR ^[p,s]	Glucose, fructose, mannitol/ sorbitol ^[p,c,h,s]
Blood	IGF1 ^[p,c,h,s]	P4 ^[p,c,h,s]	NEFA					Glucose, fructose, mannitol/ sorbitol ^[p,c,h,s]

c, cattle; p, pig; h, horse; s, sheep.

(ferret embryo to mink uterus; sheep embryo to mouse uterus) or (ii) the addition of uterine luminal fluids (ULF) from a diapausing species to a culture of blastocysts from a non-diapausing species (mouse ULF to rabbit embryos; wallaby ULF to non-diapausing mouse embryos) (Ptak *et al.* 2012, Fenelon *et al.* 2017). Reactivation of diapaused embryos is inducible as well through the use of ULF, co-culture with uterine cells (mink), or transfer to a reactivated uterus. So far, the uterine signals that reactivate development are understood better than those that induce diapause (Renfree & Fenelon 2017), but the control of polyamine synthesis seems to be a key conserved factor for the induction and maintenance of and exit from diapause (Lefevre *et al.* 2011, Fenelon *et al.* 2017, van der Weijden *et al.* 2019). However, the maternal factors that control diapause and embryonic receptivity to these signals might not be universally identical: pig embryos transferred to diapaused rat uteri degenerated, whereas sheep embryos transferred to diapaused mouse uteri did not, and furthermore, some were even able to elongate when transferred back to sheep uteri (Ptak *et al.* 2012, Geisert *et al.* 2017).

Experimental evidence in vivo and ex vivo

So far, the maternal influence on extra-embryonic tissues *in vivo* has been described through its effects on the regulation of elongation processes in ungulates or on capsule formation in equids. Blastocysts as well as trophoblastic vesicles do not elongate *in vitro* but do so *in vivo* once transferred to the uteri of synchronized recipients (Heyman *et al.* 1984, Fléchon *et al.* 1986). When there is a reduced density of glands in the uterus, or no glands at all (such as in the sheep uterine gland knockout model UGKO), elongation is reduced or abolished (Gray *et al.* 2001, Spencer & Gray 2006). Scientific efforts have thus focused primarily on uterine secretions, but a few studies have examined physical constraints on embryo elongation. Cultures of bovine and porcine embryos using capillaries or hydrogels demonstrate only limited elongation *in vitro* (Vejlsted *et al.* 2006, Hue *et al.* 2007, Miles *et al.* 2017) and equine embryos, which do not elongate but instead expand into spherical vesicles, are protected by a capsule that needs the uterus to form and can withstand myometrial forces (Quinn *et al.* 2007, Stout 2016). Beside the role of physical forces, another open question is whether uterine secretions act directly or indirectly on extra-embryonic tissues. In pigs, priming from mesoderm cells is needed through BMP signaling before trophoblast cells can respond to specific uterine signals like FGF signaling (Valdez Magaña *et al.* 2014). Nonetheless, recent work has indicated that uterine secretions have many more functions beyond those involved in developmental progress, including the coordination of on-time implantation (mouse) or stromal decidualization (Kelleher *et al.* 2018, 2019).

Interplay between maternal and conceptus tissues to drive late blastocyst development

During pregnancy, maternal uterine secretions (or histotroph) contain a variety of proteins, amino acids, carbohydrates, lipids, ions, and extracellular vesicles, which are produced by the uterine glandular epithelium (GE) and the luminal epithelium (LE) or transported from the serum to the uterine cavity by the luminal epithelium. Developing conceptuses, instead, produce signals for MRP (maternal recognition of pregnancy), stimulate endometrial receptivity, and secrete proteins, lipids, and extracellular vesicles that contribute to the ULF. Together, the multitude of factors involved in communication between maternal tissues and those of the conceptus contribute to create an interplay of formidable temporal and spatial complexity. Below, we focus on the secretions from both sets of tissues and their consequences for embryonic and extra-embryonic development.

Uterine secretions

Studies of the metabolome and proteome of uterine fluids have been reported for several species, including cattle, horses, pigs, and sheep (Li *et al.* 2007, Forde *et al.* 2014a,b, Bastos *et al.* 2019, reviewed in Spencer *et al.* 2019). In addition, transcriptomic studies have revealed specific secretory functions for the epithelial and stromal components of the uterus (Bauersachs & Wolf 2012, Zeng *et al.* 2018). These secretions result from the action of regulatory pathways on the endometrium that initiate with priming of ovarian origin (estrogens then progesterone). Additional stimulations, such as those from the conceptus, can also modulate the composition of these secretions (Groebner *et al.* 2011, Gibson *et al.* 2017), together with other environmental factors like the physiological status (Satterfield *et al.* 2010, Forde *et al.* 2014a, Beyer *et al.* 2019) and the diet of the mother (Chartrand *et al.* 2003, Giller *et al.* 2018, Crouse *et al.* 2019).

The release of histotroph components in the ULF can occur either by direct secretion or in extracellular vesicles. Extracellular vesicles (originating from both LE and GE) contain a multitude of mRNAs, miRNAs, proteins, and lipids, as well as surface receptors/ligands, with a composition that varies based on cell type (Burns *et al.* 2014, 2016). The number and content of maternal extracellular vesicles are controlled with progesterone (Burns *et al.* 2018) but extracellular vesicles can also be produced by the conceptus itself and act either on conceptus cells, to facilitate implantation (Desrochers *et al.* 2016), or on endometrial cells (LE, GE; Kusuma *et al.* 2016, Nakamura *et al.* 2016).

Amino acids and carbohydrates are essential components for the development of early embryos (Booth *et al.* 2005, Thompson *et al.* 2016) and fetuses (Wu *et al.* 2008), as illustrated by the infertile phenotype of UGKO

sheep (Spencer *et al.* 2019). During early pregnancy in ewes, there is a marked increase in the abundance of most amino acids, including arginine (Arg), leucine (Leu), glutamine (Gln), glutamic acid (Glu), methionine (Met), serine (Ser), and histidine (His), as well as glucose, calcium, and sodium (Bazer *et al.* 2015), reflecting their importance for survival and growth of the conceptus. Interestingly, in addition to glucose, fructose is the most abundant hexose sugar in the uterine fluids of ungulate mammals, but its role in the growth and development of the conceptus remains imperfectly understood (Kim *et al.* 2012). The composition of amino acids and carbohydrates in ULF also reflects the expression levels of their respective transporters in endometrial and conceptus cells, as shown in studies of cattle and pigs (Forde *et al.* 2014b, Bazer *et al.* 2015, Steinhäuser *et al.* 2016).

As an example, concentrations of Arg and glucose in ULF of ewes increase significantly between D10 and D15 of pregnancy; these molecules are transported from maternal blood to uterine lumen through the activity of specialized transporters, SLC2A1 and SLC5A1 for glucose and SLC7A2B for arginine. Of these, the expression of *SLC5A1* is induced in the endometrium (LE, GE) by progesterone, while *SLC2A1* and *SLC5A11* are induced by P4 and IFN-tau (Gao *et al.* 2009a). Similarly, in pigs, luminal cells express AKR1B1 and SORD, two enzymes that are necessary to convert glucose into fructose, and endometrial cells also express the fructose transporter SLC2A8 during the peri-implantation period (Steinhäuser *et al.* 2016). Similar observations have been reported for other amino-acid transporters (Gao *et al.* 2009b,c, Satterfield *et al.* 2010, Bazer *et al.* 2015) and nutrient-sensing pathways (Gao *et al.* 2009d). As a result, the concentration of amino acids and carbohydrates varies in time in the ULF during early pregnancy and is dependent on the expression of transporters in the conceptus and/or uterus (Gao *et al.* 2009a, Forde *et al.* 2014a, Bazer *et al.* 2015, Gibson *et al.* 2018).

As a consequence of the increase in available amino acids, polyamines – a specific product of amino-acid biosynthesis – are also detected in significant quantities in ULF. Polyamine synthesis required Arg, Pro, and L-Ornithine together with the enzyme ornithine decarboxylase (ODC1), leading to the production of putrescine, spermidine or spermine (Lefevre *et al.* 2011). Polyamine levels in ULF are probably dynamically regulated by a balance between biosynthesis and catabolism and by transport between intra- and extra-cellular environments (Persson 2009). Indeed, fluctuations in polyamine levels during diapause in roe deer and mice have been linked with increased expression of ODC1 and SMS (spermine synthase) in the luminal stroma (Zhao *et al.* 2012, van der Weijden *et al.* 2019).

Histotroph also contains lipids and lipid mediators such as prostaglandins (PGs) or lysophosphatidic

acid (LPA). In ruminants, retinol and lipids have been detected in uterine fluids during diestrus, whereas studies of the conceptus at the onset of elongation have noted transporters and binding proteins for fatty acids (SLCs) or retinol (RBP4) (Ulbrich *et al.* 2009, Liszewska *et al.* 2012, Mullen *et al.* 2012). In pigs and horses, the histotroph also contains small-lipid-transport proteins such as RBP, uterocalin, and uteroglobin, with the last delivering lipids to the conceptus via the hydrophilic capsule glycan (Stallings-Mann *et al.* 1993, Suire *et al.* 2001, Quinn *et al.* 2007, Waclawik *et al.* 2013, Jeong *et al.* 2016). The active transfer of lipids from the endometrium to the conceptus was proposed decades ago by Boshier *et al.* (1987) but has only recently been supported by studies documenting extracellular vesicle formation and uptake (Mulcahy *et al.* 2014, Mathieu *et al.* 2019).

Conceptus secretions

Through its secretions, the conceptus contributes to the MRP (which mostly relies on the inhibition of luteolysis), but also prepares the intimate cellular dialogue between the TB and the endometrium that is necessary for implantation (Vento-Tormo *et al.* 2018, Biase *et al.* 2019). The production of prostaglandins by TB appears to be indispensable for this purpose in many ungulate species (Brooks *et al.* 2014), while other molecules appear to be more species specific, such as estrogens and IL1B2 in pigs (Ka *et al.* 2018) or IFN-tau in ruminants.

IFN-tau is produced by the ruminant conceptus and is known for its combined effects on the endometrium as well as on extra-uterine cells or tissues including PBMC, liver, and corpus luteum (Hansen *et al.* 2017, Imakawa *et al.* 2018, Passaro *et al.* 2018). In pregnant sheep, IFN-tau loss-of-function experiments using anti-sense oligonucleotides resulted in embryonic growth retardation and malformations (Brooks & Spencer 2015). Strikingly, loss of the type I IFN receptor has no effect on embryonic elongation (Brooks & Spencer 2015), which suggests that IFN-tau may act indirectly on conceptus elongation, potentially through its direct effects on endometrial cells.

Although IFN-tau has not been detected in non-ruminant ungulates, other mediators for the recognition of pregnancy have been identified in pigs. From the ovoid stage, the porcine blastocyst produces estrogens and IL1B2 (Perry *et al.* 1973, Spencer *et al.* 2004, Mathew *et al.* 2015) which act synergistically to drive endometrial functions and conceptus development. IL1B2 secreted by the conceptus is necessary for elongation and endogenous estrogen production and could help inhibit luteolysis by promoting estrogen production by endometrial cells (Mathew *et al.* 2015, Whyte *et al.* 2018). However, the direct effect of conceptus-derived estrogens remains unclear. CYP19A1-null pig embryos, which do not produce endogenous estrogen,

elongate and implant normally but fail to survive 30 days past elongation, supporting the hypothesis that conceptus-derived estrogens may instead be necessary after implantation for embryonic and fetal development (Meyer *et al.* 2019).

Effects of maternal secretions on blastocyst development

The conceptus requires histotroph for its development and utilizes specific molecular and cellular mechanisms to take up histotroph from the ULF. This is exemplified by the activation of the endosome-lysosome system in TB together with the increased expression of numerous transporters for amino acids, glucose, and fatty acids, as shown by numerous transcriptomic studies (see previous sections). Specific cellular structures can also contribute, such as multivesicular bodies, which arise from endocytosis and phagocytosis and can be observed in the mural trophoblast of mouse blastocysts just prior to implantation (Fu *et al.* 2014).

Variations in histotroph composition can also affect conceptus development and survival. Prior to implantation, the conceptus experiences exponential growth that is fueled by the increased levels of amino acids, glucose, and fructose in ULF (Bazer *et al.* 2015). TB, HYPO, and EPI cells express numerous transporters and enzymes that enable them to take up and metabolize these resources, but also to potentially secrete them to other tissues (extra-embryonic, embryonic, or maternal).

Amino acids such as Arg can regulate key signaling pathways, including the mTOR pathway which connects physiological and metabolic functions (Zhang *et al.* 2017). An increase in Arg and fructose in the ULF of pregnant gilts (between days 12 and 15 post-estrus) and ewes (between days 10 and 16 of pregnancy) is associated with an increase in cell migration and cell proliferation of primary trophoblastic cells *in vitro*, through their synergistic activation of the mTOR-RPS6K pathway (Kim *et al.* 2011, 2012, 2013). Interestingly, Arg also affects TB cell proliferation and motility through regulation of levels of nitric oxide (NO) and polyamines. Arg is converted into NO by nitric oxide synthase, and knockdown of this protein in ovine TB cells using morpholino antisense oligonucleotides delayed conceptus development and growth (Wang *et al.* 2014). Similarly, NO pathway inhibition in ovine TB cells reduced cell proliferation and migration, while its activation increased cell proliferation and migration (Wang *et al.* 2015). Arg can also be used as a substrate for polyamine biosynthesis, a process which requires the ornithine decarboxylase ODC1. Embryos of ODC1-knockout mice are not viable due to apoptotic cell loss in the ICM, but the phenotype can be rescued by the addition of polyamine (putrescine) in the drinking water (Pendeville *et al.* 2001). Similarly, supplementation of the culture medium with polyamines (spermine or

spermidine) increases the survival rate of pig or mouse blastocysts (Muzikova & Clark 1995, Cui & Kim 2005) and increases the proliferation and motility of ovine TB cells (Kim *et al.* 2011).

Different amino acids have been reported to affect epiblast development at different times of development: during the period of ICM/EPI proliferation (threonine) and during germ-layer commitment (glutamine, proline) (Moussaieff *et al.* 2015a). In human embryonic stem cells (ESCs), elevated glutamine levels are required to maintain high levels of the intracellular antioxidant glutathione, which protects the pluripotency factor OCT4 from cysteine oxidation. Depletion of glutamine leads to increased ROS levels and eventually to rapid degradation of OCT4 and consequent ESC differentiation (Marsboom *et al.* 2016). Similarly, in pig blastocysts, as pluripotent cells commit to the embryonic germ layers, glycolytic flux decreases and oxidative phosphorylation increases; together, these lead to the increased production of ROS, with the concomitant risk of DNA damage and increased degradation of pluripotency factors (Ramos-Ibeas *et al.* 2019). It is likely that elevated levels of glutamine protect epiblast cells from the effects of high ROS levels also in ungulates, which would be consistent with the report that glutamine and glycine levels peak on day 14 in the ULF of pregnant mares (Beyer *et al.* 2019).

In addition to their direct effects on cell metabolism and cell growth, deficiencies in amino acids can also impact the epigenome of the conceptus. These deficiencies can affect (1) the pool of available methyl groups used for DNA, RNA, and histone methylation (serine, methionine; Zglejc-Waszak *et al.* 2019), (2) the pool of acetyl-CoA and SAM used for histone acetylation (threonine; Moussaieff *et al.* 2015b), and (3) the pool of residues that are substrates for methylation and acetylation (arginine, lysine, and histidine residues; Bazer *et al.* 2015).

Lipid metabolism is also a determining factor in the growth and development of the conceptus (Barnwell *et al.* 2016, Ribeiro *et al.* 2016b). Transcriptomic studies have documented the intake and metabolism of lipids by the conceptus to produce prostaglandins and energy, and these seem to be key functions of parietal extra-embryonic layers (Ribeiro *et al.* 2016b, Pfeffer *et al.* 2017, Ramos-Ibeas *et al.* 2019). Lipids could also be essential for the viability of the conceptus by promoting pluripotency in EPI, based on reports that fatty acid synthesis and LPA-mediated signaling promote human and mouse pluripotency *in vitro* (Kime *et al.* 2016, Wang *et al.* 2017). Among well-known intracellular sensors for fatty acids and their derivatives, PPARs are key players during ungulate blastocyst development (see 'Evidence from single-cell studies' section), and PGs are precursors for or direct ligands of PPARs. Indeed, PG production by the conceptus plays a key role in regulating blastocyst growth (Stout 2016) and endometrium functions (Dorniak *et al.* 2012, Spencer *et al.* 2013, Kaczynski *et al.* 2016).

However, PGs are also secreted by the endometrium and regulate conceptus elongation (Dorniak *et al.* 2011) and luteolysis (Ford & Christenson 1991, Brooks *et al.* 2014). Altogether, the roles of lipid metabolism, PPAR signaling, and PG in the interplay between the conceptus and the uterus are clearly complex and as yet incompletely resolved (Ulbrich *et al.* 2009, Sandra *et al.* 2017).

In sum, the answer to the question of whether the histotroph determines blastocyst cell fate and differentiation in ungulate species remains elusive, even though metabolic fluxes (or switches) are clearly '*linked to cell identity just as gene expression, epigenetics, and morphology are*' (Cliff & Dalton 2017). In ungulates, further studies are still needed to explore the effects of nutrient composition on sensing and signaling between ULF and the ICM/epiblast, and comparisons involving diapause would be particularly illuminating (Boroviak *et al.* 2015).

Uterine nutrients as a potential trade-off in conceptus growth and development

As the uterus senses and drives conceptus development (Sandra *et al.* 2011), the embryo senses nutrient availability and responds to its environment, *in vitro* or *in vivo*, to compensate (Picone *et al.* 2011, Bertoldo *et al.* 2015, Fleming *et al.* 2015).

The importance of such a trade-off prior to embryonic implantation was described in mice, using pregnant females fed a low-protein diet (LPD) from fertilization to implantation. Surprisingly, the total pool of amino acids in blastocysts from LPD-fed and control mothers were similar, despite the decreased concentration of many free amino acids in the ULF of LPD-fed mothers and a reduction in mTORC1 signaling in embryonic cells (Eckert *et al.* 2012). These results clearly support the existence of compensatory mechanisms that rely on endocytosis or phagocytosis and that are stimulated in the TB and parietal HYPO (Bevilacqua *et al.* 2010, Sun *et al.* 2014, Fleming *et al.* 2015). Similar experiments using a low-fat/protein diet would be of particular interest, to determine whether similar compensatory mechanisms exist for lipid transport and intake from uterine fluids and to evaluate the long-term consequences.

Nowadays, it is clear that early nutrient deficiencies are detrimental to neonatal and/or adult health (Chavatte-Palmer *et al.* 2018, Duranthon & Chavatte-Palmer 2018). Still, much work remains to be done beyond straightforward characterizations of ULF composition in order to understand how the environment of the conceptus affects embryonic and extraembryonic tissues (proliferation/differentiation/pluripotency) across livestock species and in time – beyond the blastocyst stage and prior to placentation.

Concluding remarks

The molecular dialog between the conceptus and maternal tissues that takes place prior to implantation is slowly becoming better known. Rapid advances in the field of transcriptomics, proteomics, and metabolomics are certain to provide a clearer view of the molecular landscape behind the pre- and peri-implantation embryonic development of ungulates. In parallel, international initiatives such as the Functional Annotation of Animal Genomes project (FAANG, Giuffra *et al.* 2019) help in providing genomic tools and reference annotation maps for use in predicting the functioning of complex biological systems. Together with the recent revolution in single-cell-omics (Kelsey *et al.* 2017) and spatial transcriptomics (Rodrigues *et al.* 2019), we expect to soon have more precise maps of cell–cell interactions and cell–cell communications as well as progress in data integration and prediction. Paracrine signaling will however remain a complex issue which will have to be validated experimentally in order to decipher the extent to which cell–cell communications are involved in cell lineage specification.

This new knowledge will provide a solid framework for improved understanding of the biology of blastocyst development in ungulates. In particular, it could help in evaluating the contributions of epigenetic factors to embryogenesis during the preimplantation period. In mammals, rapid modifications of the epigenome occur before implantation and disturbance of this drastic reprogramming can also affect the developmental competence of ungulate embryos (Young & Beaujean 2004, Chung *et al.* 2017, Eckersley-Maslin *et al.* 2018, Duan *et al.* 2019). However, in ungulates, less is known about how epigenetic factors drive blastocyst formation and elongation, and the extent to which the maternal organism contributes to this type of regulation. Since the discovery that exosomes secreted by the endometrium contain epigenetic factors like miRNAs or lncRNAs (Burns *et al.* 2016, Gross *et al.* 2017), it is highly possible that this molecular dialog also acts at an epigenetic level. Indeed, miRNA levels are highly dynamic before and during elongation (Berg & Pfeffer 2018), and some have been associated with developmental competence in bovine preimplantation embryos (Lin *et al.* 2019).

Advances in this field could also be applied to provide molecular cues for the production of lines of true epiblast stem cells (EpiSCs), ESCs, extra-embryonic endoderm stem cells (XEN), and trophoblast stem cells (TS) for these species, and to improve culture conditions for embryos and organoids. The recent development of human TB and endometrium organoids (Turco *et al.* 2017, Turco *et al.* 2018) and mice embryoids (Rivron *et al.* 2018) are good examples of novel experimental approaches that could be adapted to ungulates.

Moreover, the possibility of obtaining elongating spherical and ovoid blastocysts from *in vitro*-produced

embryos or organoids, together with recent advances in genome editing technologies, would provide efficient tools for fine-scale dissection of the molecular mechanisms and signaling pathways that are relevant for elongation, implantation, maintenance of pluripotency, and early embryonic cell-fate commitment and differentiation in non-rodent species.

Such 3D cellular models are not only well suited for investigations of animal biology at the molecular level, but are also complementary to examinations of living farm animals: they enable us to reduce the complexity of traits through intermediate phenotypes, to perform medium- and large-scale studies, and to reduce the need for animal experimentation in agreement with the 3R (Replace, Reduce, Refine) guidelines. This virtuous circle, fed by these descriptive and functional approaches and whose success has been demonstrated for humans and mice, is now close to being accessible for livestock and ungulate species.

In addition to better understanding specific features of ungulate early embryonic development, these studies confirm the growing potential of ungulate species as alternative models to rodents for the investigation of biological mechanisms or pathologies that are not easily transposable from mice to humans. For example, recent works have highlighted interest in working with pig blastocysts to better understand the mechanisms that drive the induction of primordial germ cells in non-rodent mammals (Kobayashi *et al.* 2017) or in using the ovine model to study implantation failure in humans (Barry & Anthony 2008).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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Author contribution statement

J A, H A, and I H contributed equally to the conception and writing of this review.

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