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Review article

Embryo–maternal communication during the first 4 weeks of equine pregnancy

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ABSTRACT

The first month of equine pregnancy covers a period of rapid growth and development, during which the single-cell zygote metamorphoses into an embryo with a functional circulation and precursors of many important organs, enclosed within extraembryonic membranes responsible for nutrient uptake and gaseous exchange. After exiting the oviduct, the conceptus must influence uterine physiology to ensure adequate nutrition and preparation for implantation, while continued development results in the chorioallantois superseding the yolk sac as the primary interface for maternal interaction and exchange. Throughout the first month, pregnancy maintenance depends absolutely on progesterone secreted by the primary corpus luteum. However, although extension of luteal life span via maternal recognition of pregnancy is clearly essential, it is still not known how the horse conceptus signals its presence. On the other hand, our understanding of how luteolytic prostaglandin $F_{2\alpha}$ release from the endometrium is averted has improved, and we are increasingly aware of the biological and practical significance of various events characteristic of early horse pregnancy, such as selective oviductal transport, the formation and dissolution of the blastocyst capsule, and prolonged intrauterine conceptus migration. It is also increasingly clear that embryo–maternal dialog during the first month is essential not only to conceptus survival but also has more profound and long-lasting implications. In this latter respect, it is now accepted that the maternal environment (e.g., metabolic or health status) may epigenetically alter gene expression capacity of the developing embryo and thereby permanently influence the health of the resulting foal right through adulthood.

1. Introduction

The last 30 years have witnessed numerous breakthroughs in our understanding of the pathogenesis of, and diagnostic and therapeutic approaches to, a range of conditions that previously compromised fertility in horses. There have also been significant advances, including improved success rates, in assisted reproductive technologies, such as embryo and oocyte transfer as well as intracytoplasmic sperm injection (ICSI) and somatic cell nuclear transfer for *in vitro* embryo production [1,2]. The advances in general reproductive medicine and management have resulted in marked improvements in per-cycle pregnancy rates in intensively managed horses [3]. Indeed, recent surveys have reported mean per-cycle pregnancy rates of more than 60% in well-managed naturally mated thoroughbred mares [4] and exceeding 45% in commercial warm-blood mares artificially inseminated with frozen-thawed semen [5]. Despite these developments, the incidence of early pregnancy loss has changed little, if at all [3], and early pregnancy loss remains a significant source of economic loss to the breeding industry.

The incidence of detected pregnancy loss between initial pregnancy detection at around Day 15 and eventual

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foaling varies between about 5% and 15% in young fertile mares but can reach much higher incidences (>20%) in mares older than 18-years [3,6], and after suboptimally synchronized embryo transfer (ET) [7] or transfer of *in vitro* produced (IVP) embryos [8]. Approximately 60% of all pregnancy losses occur in the first 6 to 7 weeks of the 11-month gestation [9], a bias toward pregnancy loss early in gestation that is not surprising if one considers that embryos with gross intrinsic defects, such as numerical chromosome abnormalities [10], are most likely to fail during early development. Moreover, the horse embryo undergoes a series of profound developmental changes during the first 7 weeks in transforming itself from a ball of undifferentiated cells into a fetus with precursors of all the organs required for extrauterine life. During this same period, the developing embryo must communicate with its dam to extend the period of luteal progesterone production beyond that of normal diestrus and to secure a qualitatively and quantitatively adequate supply of histotrophic nutrients during an unusually long period (40–45 days) before the development of a stable, definitive chorioallantoic placenta [11]. In this respect, uterine receptivity for, and modification to allow, implantation is critical to survival of the embryo, and the way in which the embryo and its dam communicate to bring about coordinated changes in maternal, endometrial, and embryonic physiology is vital to the successful maintenance of pregnancy. It is also increasingly evident that the conditions that the developing embryo experiences during very early gestation can, via epigenetic modification, fundamentally influence the health and susceptibility to disease of the resulting foal up to and including adult life. This review focuses on some of the critical events that characterize equine embryo–maternal signaling during the first 4 weeks of gestation.

2. Embryo–maternal communication during oviductal development

The equine embryo remains in the oviduct for an unusually long time, 6 to 7 days [12], during which the embryonic genome is activated [13] and development up to the late morula or early blastocyst stage occurs. That the oviduct is more than a passive bystander to this early development is indicated by studies demonstrating that IVP horse blastocysts lag considerably behind their *in vivo* counterparts in terms of cell number and developmental stage [14], contain a higher proportion of apoptotic cells [14,15], and are more prone to early embryonic death after transfer to a recipient mare [8]. Indeed, because 2- to 4-cell embryos transferred into the oviduct of either a recipient mare [16] or a sheep [14] after fertilization by ICSI develop more rapidly and are more likely to reach the blastocyst stage than embryos maintained *in vitro* after ICSI, it is clear that undefined aspects of the oviductal environment are required to optimally support or stimulate early post-cleavage development. Although it is also probable that the developing embryo induces changes in oviduct epithelial cell gene expression [17], these appear to have few, if any, lasting effects on a mare's subsequent ability to support pregnancy as witnessed by the high initial and ongoing pregnancy rates after conventional ET to well-synchronized recipient mares [18]. On the other hand, the early horse embryo has been reported to signal its presence beyond the confines of the oviduct via early pregnancy factor (EPF), which has been reported to be detectable in the serum of pregnant mares from Day 2 after ovulation via the rosette inhibition test [19]. Although the ultimate relevance of EPF to the establishment or maintenance of pregnancy is unclear, a reliable EPF assay would be a useful asset for studies into the scale and timing of embryonic death, for investigating unexplained infertility, and for improving the efficiency of ET programs. Unfortunately, field trials of an early conception factor test that claimed to detect EPF in mare's serum were disappointing, with a high incidence of false positives and limited ability to discriminate between pregnancy and nonpregnancy [20].

The end of the oviductal phase in the horse is characterized by an unusual form of embryo–maternal communication, namely selective oviductal transport. In the mare, only developing embryos reliably descend into the uterus, whereas unfertilized oocytes remain trapped in the ampulla of the oviduct [21,22]. Weber et al. [23,24] demonstrated that the oviduct's ability to discriminate between developing embryos and unfertilized oocytes depends on embryonic secretion of prostaglandin E_2 (PGE_2) from Day 4 to 5 after fertilization. The very early embryo is unable to exit the oviduct because tonic contraction of the isthmic circular smooth muscle results in the ampullary–isthmic junction acting as a closed sphincter. However, PGE_2 secreted by the developing morula [23] induces the isthmic circular smooth muscle to relax, resulting in dilation of the isthmic sphincter and rapid passage of the embryo through into the uterus [25]. Failure of oviductal transport of viable embryos (i.e., ectopic [tubal] pregnancy) has not been reported as a clinical complication in mares.

3. The blastocyst capsule

Another enigmatic feature of equine pregnancy that requires, and almost certainly plays a role in, early embryo–maternal communication is the acellular blastocyst capsule, which forms between the trophectoderm and zona pellucida soon after the horse embryo enters the uterus, and coincident with blastocyst formation [26]. Although the mucin-like glycoproteins that initially make up the capsule are secreted by the trophectoderm [27,28], they are unable to coalesce into a confluent structure *in vitro* [14]. The idea that capsule formation requires an endometrial contribution has been strengthened by the report of augmented capsular glycoprotein production by IVP embryos exposed to uterocalin [29], a progesterone-dependent endometrial protein known to associate with or contribute to the capsule of uterine embryos [30]. On the other hand, it is not clear whether the endometrium contributes structural components to the capsule or simply provides an environment that supports or induces cross-linking and agglomeration of the trophectoderm-derived glycoproteins.

Soon after capsule coalescence is completed *in vivo*, the zona pellucida is shed to leave the Day 7 blastocyst completely enclosed within its new tertiary embryo coat. The capsule continues to increase in thickness until

approximately Day 11 and develops a bilaminar appearance that suggests a second (possibly uterine) source of contributory glycoproteins [30,31]. From around Day 18, the capsule begins to attenuate such that it becomes discontinuous between Days 20 [11] and 23 [31] and has disintegrated altogether by Day 30 [11]. It is not known how capsular degeneration occurs, but it is likely that endometrial enzymes play a contributory role. As will be discussed later, induction of luteolysis shortly before the time of conceptus fixation (Day 16) interferes with both the stage-dependent desialylation of capsular glycoproteins [32] and stepwise degradation of capsule-associated proteins [33]. Although the exact functions of the capsule are not known, its presence at the interface between trophectoderm and endometrium means that it must play some role in mediating embryo–maternal interaction. Moreover, because embryos from which a fully formed capsule was removed by micromanipulation did not develop into visible pregnancies after ET [34], the capsule is clearly essential to conceptus survival *in utero*. With regard to its probable functions, the capsule is physically robust and elastic. These properties are thought to enable the capsule to both help maintain the spherical shape of the early conceptus and to provide mechanical protection during the Day 7 to 16 mobile phase when the delicate vesicle is squeezed around the uterine lumen by myometrial contractions [35]. In addition, the high proportion of negatively charged sialic acid residues on the glycoproteins is thought to confer antiadhesive properties that further facilitate conceptus migration [27]; indeed, the end of the conceptus mobile phase is associated with widespread desialylation of these glycoproteins [36]. As will be discussed later, the capsule may in this way play an indirect role in embryonic signaling for maternal recognition of pregnancy, that is, by facilitating migration. More intriguingly, the capsule appears to play a direct role in embryo–maternal dialog by acting as a mailbox [37] for temporary storage, modification, or transfer of endometrial proteins involved in nutrient transport (e.g., uterocalin [38,39]) or associated with the cessation of conceptus migration and imminent capsule degradation (e.g., beta-2 microglobulin, soluble phospholipase A2 [33,40]). Similarly, the capsule may act as a repository for trophectodermal proteins that may influence endometrial function or act on the conceptus itself, for example, insulin–like growth factor binding protein 3 which has been proposed to stimulate conceptus development either directly or indirectly by trafficking insulin–like growth factor 1 [41].

4. Maternal recognition of pregnancy

One of the most obvious examples of embryo–maternal signaling during the early intrauterine period is maternal recognition of pregnancy (MRP), during which the developing conceptus sends a biochemical signal to its dam to ensure that she undergoes the physiological changes necessary to maintain a uterine environment conducive to continued embryonic growth and survival [42]. Because the primary determinant of an adequate environment is the continued supply of progesterone, MRP is generally taken to refer specifically to the conceptus-initiated events that prolong the life span and secretory activity of the primary corpus luteum. MRP in the mare involves an absolute suppression of the endometrium's ability to release its luteolytic hormone, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), in response to oxytocin produced either by the hypothalamic pituitary axis [43] or the endometrium itself [44] during Days 10 to 16 after ovulation [45]. To ensure delivery of the presumed conceptus antiluteolytic signal to enough endometrium to adequately suppress $PGF_{2\alpha}$ release and thereby avert luteolysis, the spherical equine conceptus migrates throughout the entire uterine lumen during the Day 10 to 16 period when luteolysis would otherwise be initiated. Indeed, when McDowell et al. [46] surgically restricted horse conceptuses to a single uterine horn, luteolysis was not blocked. Conceptus vesicle migration itself appears to be driven by myometrial contractions induced, somewhat paradoxically, by prostaglandins produced either directly by the conceptus or locally in the uterine wall in response to other conceptus signals [35].

One of the reasons why it has, to date, not been possible to identify the conceptus factor responsible for inducing MRP in the mare may be that the process itself, as in other species, is multiphasic. It is however now established that the initial absolute suppression of endometrial $PGF_{2\alpha}$ secretion involves a conceptus-directed downregulation of endometrial prostaglandin endoperoxide synthase 2 (PTGS2: [47,48]) accompanied by a post-transcriptional inhibition of endometrial oxytocin receptor (OXTR) expression [49] and, thereby, oxytocin binding capacity and responsiveness [50]. After the cessation of conceptus mobility at around Day 16, however, the endometrium belatedly develops the ability to secrete $PGF_{2\alpha}$ in response to oxytocin [50,51] presumably as a result of contemporaneous upregulations in PTGS2 and OXTR expression [49]. Prevention of luteolysis beyond conceptus fixation must therefore depend on either the depletion of the source of oxytocin or on the disabling of another element of the luteolytic pathway. In this latter respect, expression of the endometrial $PGF_{2\alpha}$ receptor, presumed to be integral to establishing the local endometrial feedback ($PGF_{2\alpha}$OXT) and feedforward ($PGF_{2\alpha}PGF_{2\alpha}$) loops needed to generate the large pulses of $PGF_{2\alpha}$ release required to trigger luteal demise, is downregulated through to at least Day 21 of pregnancy [49], which may explain why inadvertent luteolysis is not common in the postfixation period despite abundant PTGS2 and OXTR.

Because the oxytocin responsiveness that underpins cyclical luteolysis develops around Day 10 after ovulation [45,52], it is assumed that conceptus signaling to achieve MRP also begins at this time. Frustratingly, the conceptus signal responsible for modulating PTGS2 and OTXR expression, and thereby endometrial $PGF_{2\alpha}$ release capacity, remains elusive [53]. Early studies focused on molecules proven to function as MRP signals in the other large domestic species, such as interferon-τ (ruminants) and estrogens (pig), but these were either not secreted by equine conceptuses (interferon-τ: [54]) or were secreted in large quantities but did not reliably extend corpus luteum life span (estrogens: [55]). Studies to investigate the size of the conceptus product that inhibits $PGF_{2\alpha}$ secretion by equine endometrium concluded that the equine MRP signal must

have a molecular weight of 1 to 6 kDa [56] or 3 to 10 kDa [57] but were unable to identify specific candidates. On the other hand, MRP stage equine conceptuses have been shown to secrete a variety of hormones, including PGE_2 [51], insulin–like growth factor 1 [58], and estrogens [59] that, although they may not be directly involved in inhibiting endometrial $PGF_{2\alpha}$ secretion, almost certainly play significant roles in processes essential to pregnancy maintenance, such as conceptus migration [35], increased uterine vascularity [60], and qualitative and quantitative alterations in the composition of the uterine secretions that make up histotroph [61]. More recently, transcriptomic studies have indicated that a significant number of the genes differentially regulated in the endometrium of pregnant compared with nonpregnant mares on Days 8 to 13.5 after ovulation are estrogen or PGE_2 regulated [62,63]. Although a parallel study to examine differences in the transcriptome of conceptuses over the MRP period indicated a number of molecules likely to play roles in embryo–maternal interaction [64], it did not unearth a putative antiluteolytic signal.

5. Preparation for implantation

Successful implantation requires a normally developing embryo, an appropriately primed uterus, and carefully coordinated communication between embryonic trophectoderm and maternal endometrium to further modify the endometrial surface to permit attachment and stimulate trophectoderm cells to proliferate, attach, and subsequently either invade into, or interdigitate, with the endometrium [65,66]. Initial attachment takes place during the so-called window of receptivity to implantation [67,68]; during this period, the surface glycocalyx of the endometrial luminal epithelium must undergo changes to enable it to play its role in conceptus adhesion. An important aspect of this endometrial preparation is a period of exposure to progesterone that results in a paradoxical downregulation of progesterone receptors in the luminal and glandular epithelial cells but, possibly crucially, not in the stromal cells [49,65]. To explain how progesterone can play a critical role during implantation despite downregulation of epithelial cell progesterone receptors, it has been postulated that, during this phase, progesterone primarily stimulates the stromal cells to produce progestamedins, which in turn have paracrine effects on the endometrial epithelium and trophectoderm [65]. The next step in the implantation process is intimate apposition of trophectoderm and endometrium, an event that cannot begin in the mare until the capsule begins to fall apart shortly before Day 23 of gestation [31]. The subsequent attachment of trophectoderm to endometrium requires removal of surface antiadhesive components that previously inhibited this process [65]; in most species, this primarily involves mucins in the glycocalyx over the endometrial luminal epithelium (e.g., mucin-1 [69]). However, in the mare, there is no reduction in mucin-1 expression during conceptus–endometrium attachment [70]; on the other hand, the equine conceptus is itself surrounded by a capsule composed of mucin-like glycoproteins with antiadhesive properties. As discussed previously, these capsular glycoproteins are desialylated at the time of conceptus fixation, which should reduce their antiadhesive properties [36,64]. Moreover, desialylation of capsular glycoproteins appears to be progesterone dependent because it fails if luteolysis is induced in mares shortly before fixation [32]. In summary, desialylation of capsular glycoproteins may be a mechanism by which the progesterone-primed endometrium helps promote trophectoderm–endometrium attachment in the mare. Whether the degradation of the capsule alone is sufficient to permit attachment is not known, but it seems more likely that progesterone, aided by conceptus factors including estrogens, further promotes and stabilizes trophectoderm–endometrium attachment by upregulating osteopontin and its receptors (CD44 and integrin $\alpha V\beta 3$: unpublished observations), along with other extracellular matrix components, integrins and integrin-binding matrix proteins, such as fibronectin and fibrinogen [71].

That a precisely coordinated interaction between the progesterone-primed endometrium and the developing conceptus is critical to further development is highlighted by the delayed development observed when horse embryos are transferred asynchronously into the uterus of a recipient mare that ovulated more than 5 days after the donor [72]. This emphasizes the importance of a species-specific minimum period of progesterone priming if the endometrium is to adequately play its role in providing histotrophic nutrition by stage-specific adaptation of the expression of endometrial growth factors and nutrient transporters [65,66]. Indeed, recent transcriptomic studies have indicated the likely importance to conceptus development and survival of a range of cytokines, growth factors, and corresponding receptors that are upregulated in either the trophectoderm, endometrium, or both in the third and fourth weeks of pregnancy [64,71], under the combined influences of luteal progesterone and local trophectodermal hormones, including estrogens and PGE_2. There also appear to be roles for molecules known to be instrumental to implantation and conceptus survival in other species, such as leukemia inhibitory factor and its receptor (interleukin-6 signal transducer [73]), and various members, receptors, and binding factors from the fibroblast growth factor family [64,74]. What is not known is how the endometrial surface is specifically modified to play its contrasting roles in the two distinct implantation reactions seen during early equine pregnancy, namely invasion of the highly proliferative chorionic girdle cells and interdigitation of the remaining noninvasive chorion cells [11]. On the other hand, deficiencies in endometrial receptivity and/or aberrations in the embryo–maternal signaling processes required for implantation are undoubtedly significant contributors to the relatively high incidence of embryonic loss in the period between conceptus fixation (Day 16) and the onset of definitive placenta formation (Days 40–45) [9].

6. Developmental programming

To date, research into early conceptus–maternal interaction in the mare has concentrated on the possible consequences for pregnancy maintenance and loss; however, epidemiological studies in man and experimental studies in

animals have also identified the preimplantation period as a critical window in which the developing embryo responds to the maternal environment by permanently modifying its functional genome [75]. Indeed, preimplantation epigenetic modification is increasingly recognized as a central element of embryonic adaptation to predicted postnatal conditions, with genes permanently switched on or off (e.g., by methylation or histone modification) in response to environmental cues. This period of sensitivity appears to be concentrated into very early development (e.g., up to the blastocyst stage in mice and sheep [75]) and, besides its potential developmental advantages, represents a window during which the embryonic genome is exquisitely sensitive to disadvantageous developmental programming in response to adverse maternal metabolic status, health, or exposure to environmental toxins or pharmaceuticals. To date, studies to examine the epigenetic effects of maternal environment in horses have been limited to the transfer of embryos between large and small breeds, for example, thoroughbred, saddlebred, or draft horses and (Welsh) ponies, which have demonstrated that gestational environment can have marked effects not only on birth weight and initial postnatal growth but also on neonatal adrenal and pancreatic functions [76–79] and adult size. At what stage the equine embryo or fetus is (most) susceptible to developmental programming and how environmental conditions are communicated to the embryo have not yet been examined. On the other hand, the potential for maternal status during the peri-implantation period to induce irreversible epigenetic modifications and thereby preprogram health and disease susceptibility of resulting progeny is an area of early maternal–conceptus interaction that may ultimately be of equal or even greater relevance to the equine industry than pregnancy loss [80].

7. Conclusions

During the first month of pregnancy, the equine conceptus develops from a single-cell zygote to an embryo with a functional circulation and precursors of many of the organs required for postnatal life. During this period, embryo–maternal signaling plays critical roles in both the establishment and maintenance of pregnancy via chains of events resulting in luteal maintenance and ensuring adequate nutrient provision and preparation for implantation. In addition, it is likely that the environment that the developing conceptus experiences during the preimplantation period influences its epigenetic constitution in a fashion that could profoundly influence postnatal health and disease susceptibility.

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