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The Equine Endometrial Cup Reaction: A Fetomaternal Signal of Significance

D. F. Antczak¹, Amanda M. de Mestre², Sandra Wilsher³, and W. R. Allen³

¹Baker Institute for Animal Health, College of Veterinary Medicine, Cornell University, Ithaca, New York 14853 USA; doug.antczak@cornell.edu

²Royal Veterinary College, London NW1 0TU, United Kingdom; ademestre@rvc.ac.uk

³Paul Mellon Laboratory, Newmarket, Suffolk CB8 9DE, United Kingdom; sandrawilsher@hotmail.co.uk; pml777@virginmedia.com

Abstract

A remarkable feature of equine pregnancy is the development of the invasive trophoblast of the chorionic girdle and its formation of the gonadotrophin-secreting endometrial cup cells in early gestation. The details of this process have been revealed only slowly over the past century, since the first description of the endometrial cups in 1912. This centennial presents an opportunity to review the characteristics of the cells and molecules involved in this early, critical phase of placentation in the mare. The invasiveness of the chorionic girdle trophoblast appears to represent an atavistic attribute more commonly associated with the hemochorial placentae of primates and rodents but not with the more recently derived epitheliochorial placentae of the odd-toed ungulates. The nature of and *raison d'être* for the strong fetal signals transmitted to the mare by the endometrial cup reaction, and her responses to these messages, are the subject of the present review.

Keywords

horse; pregnancy; placenta; trophoblast; development; immunology

EARLY PROMISE, FALSE STARTS AND CLOSING THE LOOP

Discovery of the Endometrial Cups and the Chorionic Girdle

One hundred years ago, German anatomist Wilhelm Schauder (Figure 1*a*) published a seminal paper of work he had carried out while undertaking his veterinary training at the University of Giessen. In it, he described the existence of unusual structures in the endometrium of pregnant mares (1). This is the first known description of the endometrial cups, which constitute a circle of raised, ulcer-like protuberances at the base of the gravid horn in the equine uterus in early pregnancy (Figure 2*a,b*). Each of the protuberances has a

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central depression filled with yellow exocrine secretion, which Schauder speculated might be an important component of fetal histotroph. His singular observation followed 15 years after Professor J. Cossar Ewart of Edinburgh observed a “whitish band nearly a quarter of an inch in width consisting of numerous delicate folds separated from each other by deep furrows, which helps to fix the horse embryo to the uterus” (Figure 1*b*) (2). Ewart’s “chorionic girdle” (Figure 2*c,d*), a discrete, thickened band of rapidly multiplying and specialized trophoblast cells that encircles the spherical equine conceptus between days 25 and 35 of gestation in the region between the enlarging allantois and regressing yolk sac, is now well known to be the progenitor of the gonadotrophin-secreting endometrial cups in the mare, although the link was not made until another 60 years had passed (3). During that period, the main secretory product of the endometrial cups, equine chorionic gonadotrophin (eCG), became the object of intense study and application in reproductive biology and agriculture.

Pieces of the Puzzle

Not until 31 years after Schauder’s original description were the endometrial cups identified as the source of the high concentrations of eCG in the blood of pregnant mares (4). Professor Harold Cole (Figure 1*c*) and his colleagues in Davis, California had made the startling discovery 13 years previously that small volumes of serum recovered from mares between 40 and 150 days of gestation, but not before or after these gestational ages, would stimulate marked ovarian and uterine enlargement and follicular growth when injected into sexually immature rats (5). A search for gonadotrophin production during pregnancy in large domestic animals had been stimulated by the discovery three years earlier of high concentrations of a gonadotrophin, initially termed Prolan B but now known more correctly as human chorionic gonadotrophin (hCG), in the blood and urine of pregnant women (6).

The discovery of high concentrations of gonadotrophin in the blood of pregnant mares initiated a decade of intense experimental activity by the Davis group. This research showed that gonadotrophic activity first became detectable in the mare’s serum between 37 and 41 days after mating. Levels rose steeply thereafter to an individually variable peak between 60 and 75 days and then declined again steadily until hormone activity disappeared completely between days 120 and 150 (5,7–9). The group examined the biological properties of eCG in laboratory rodents and farm animals (10–13), determined its long biological half-life in serum (14), purified it from mare serum to examine its chemical properties (15–18), and modified and improved the biological assays for its quantitative measurement in both serum and saline extracts (19,20).

A curious twist of fate occurred in 1934, when Cole’s research student, Hubert Catchpole, measured high concentrations of eCG in saline extracts of allantochorion recovered from mares between 60 and 100 days gestation (21). Swayed by Zondek’s (22,23) findings that the trophoblast was the source of hCG in women, the Davis group concluded that the trophoblast of the allantochorion was the likely source of the gonadotrophin in pregnant mare serum. They measured high concentrations of eCG in the saline extracts of allantochorion, particularly those areas covered with a yellow, glutinous exocrine secretion,

but they failed to determine the source of this secretion and paid no particular heed to Schauder's endometrial cups arranged in a circle in the gravid uterine horn (Figure 2*a,b*).

But Cole remained puzzled by the disappearance of eCG from mares' blood in midgestation, despite the continued function of the epitheliochorial equine placenta. Accordingly, he and Harold Goss reexamined some pregnant mare uteri and this time determined that saline extracts of the endometrial cups and the exocrine secretion accumulated on their luminal surface yielded levels of eCG activity that were orders of magnitude higher than equivalent extracts of normal endometrium or allantochorion. Because the cups had no physical connection to the overlying allantochorion, and because they degenerated and died by midpregnancy, whereas the allantochorion continued unchanged, Cole and colleagues concluded quite reasonably, but wrongly, that the cups were some form of localized, maternal decidual response in the endometrium, akin to the pregnancy decidualization of the endometrium in women and rodents (4).

The Davis group produced a detailed description of the gross and histological development and regression of the cups, from their first appearance in the endometrium at approximately day 40, through their period of maximum eCG secretion at approximately day 60–75, and continuing until their eventual degeneration and dehiscence from the endometrium during days 120–150 (24). They observed that the basal portions of the endometrial glands persisting within each cup became distended, and the accumulated exocrine secretion and their lining epithelium stained strongly with periodic acid Schiff acid-Schiff. (PAS), which indicated their likely secretion of glycoproteins. Purification studies had, by then, established that eCG was a high-molecular weight glycoprotein (11,15,18,25–28). Because the highest concentrations of eCG were measurable in the exocrine secretion accumulated on the luminal surface of the degenerating cups and adhered to the overlying allantochorion, they speculated, again wrongly as it turned out, that eCG was probably synthesized and secreted by the gland epithelium.

Increasing quantities of partially purified eCG were now being used in agriculture to stimulate follicular development and ovulation in noncycling sheep, cattle, and pigs and to cause superovulation in these and laboratory species for the purposes of embryo transfer (29). The hormone was plentiful and relatively easy to isolate from blood serum from pregnant mares, and it showed the unusual property of possessing both follicle stimulating hormone (FSH)-like and luteinizing hormone (LH)-like biological activities in a ratio of approximately 1.4:1 (30,31). However, the link between the chorionic girdle and the endometrial cups, and the true source of eCG, remained undetermined.

The Origin of the Endometrial Cups

The chorionic girdle Ewart (2) described (Figure 2*c*) was not mentioned again in the literature until van Niekerk (32) remarked upon its sudden disappearance from the surface of the conceptus after day 35 of gestation. W.R. Allen then looked more closely at Ewart's chorionic girdle, but he too perpetrated yet another major error of interpretation. Swayed by the earlier conclusions of Clegg et al. (24) and Amoroso (33) that the endometrial cups were a type of maternal decidual response of the endometrium to the presence of the conceptus, he concluded wrongly from histological evidence that the chorionic girdle must transmit a

local signal that induces mesenchymal cells in the endometrium to transform into the large binucleate endometrial cup cells. He also confirmed the earlier finding of strong PAS staining of the epithelium and lining in the distended glands within the cup, which thereby seemed to support a maternal source for eCG (34).

“There’s none so blind as those who will not see,” and when Dr. Bob Moor (Figure 1d) cultured small explants of equine chorionic girdle tissue *in vitro* at the Animal Research Station in Cambridge in the early 1970s, he finally closed the loop in the fragmented story of the origin of the endometrial cups. The explants grew vigorously and formed stable colonies of large, binucleate trophoblast cells (Figure 3a), which secreted high concentrations of eCG into the culture medium for >150 days (Figure 3b) (35). Moor’s demonstration of the ready ability of chorionic girdle cells, but not the normal trophoblast of the allantochorion, to secrete eCG *in vitro* in the absence of any endometrial involvement at last revealed the true picture. It stimulated a morphological study that demonstrated active invasion of the endometrium by the chorionic girdle during days 35–38 after ovulation, when the already binucleate girdle cells (36) push between, and sometimes straight through, the luminal epithelial cells before progressing down the lumenae of the endometrial glands by similarly dislodging the lining epithelium (Figure 4a) (37). Then, presumably by enzyme action, they break through the basement membrane of the glands and stream out into the surrounding endometrial stroma. Here, within only one to two days, and as though tripped by an inbuilt development switch, they suddenly become sessile, cease to divide, enlarge greatly, assume a rounded, epithelioid appearance, and begin to display the organelles associated with protein hormone secretion (Figure 4b) (3,37). Their enlargement causes them to pack tightly together in the endometrial stroma to form the bulk of each endometrial cup (Figure 4c) (38).

The gonadotrophin secreted by these fetal cup cells reaches the maternal bloodstream via large lymph sinuses that develop in the stroma beneath each cup (Figure 4c) (33,34). Here its LH-like component synergizes with 10–12-day waves of pituitary FSH (39,40) to stimulate the development of the secondary corpora lutea, which persist in the maternal ovaries between 40 and 150 days of gestation (41,42), and which are the source of the secondary rise in serum progesterone concentrations during this period of pregnancy (Figure 5) (38,43).

Thus, the revelations of the early 1970s in Cambridge corrected two fundamental misunderstandings. First, the equine endometrial cups are of fetal, not maternal, origin (35). Second, the invasive trophoblast cells of the chorionic girdle, not the epithelial cells lining the distended endometrial glands, secrete eCG, which thereby verifies the original conclusion of Catchpole and Lyons that the placenta produces this hormone (21).

PHYSIOLOGY OF INVASIVE TROPHOBLAST IN THE HORSE

Molecular Mechanisms That Regulate Chorionic Girdle Development

Development of mature invasive trophoblast of the chorionic girdle requires three phases: an initial period of rapid proliferation, which is closely followed by differentiation of uninucleate trophoblast cells into eCG-expressing binucleate cells, and, finally, acquisition

of an invasive phenotype that permits entry of the cells through the luminal epithelium of the endometrium. These three sequential and overlapping events are completed within a window of approximately ten days. Several growth factors are expressed by the endometrium and avascular mesoderm that abut the chorionic girdle and are likely to act as extrinsic regulators of one or more of these three phases.

Epidermal growth factor (EGF) and transforming growth factor β (TGF β) expression are dramatically increased in the gland epithelium between days 30 and 40 of pregnancy, correlating with the acquisition of an invasive phenotype by trophoblast cells (44,45). *In vitro* experiments in a trophoblast cell line indicate EGF regulates the promoter activity of the alpha subunit of eCG acting via protein kinase C and mitogen-activated protein kinases pathways (46). Vascular endothelial growth factor (VEGF) is expressed by endometrial gland and luminal epithelia throughout the period of chorionic girdle development (47). Hepatocyte growth factor–scatter factor (HGF-SF) is expressed exclusively by allantoic mesenchyme and mesothelial cells that underlay the chorion and chorionic girdle, which led Stewart and colleagues (48,49) to propose that HGF-SF may act as the mitogenic stimulus for the rapid period of trophoblast proliferation observed between days 30 and 32 of gestation. Limited functional data exist that demonstrate how these growth factors influence trophoblast development, although mature invasive trophoblast cells express the receptors for EGF, VEGF, and HGF; namely, EGFR (44), c-met (48), and Flt and KDR (47), which indicates that the cells are capable of responding to these signals.

Several intrinsic factors likely also control the development of the chorionic girdle. Day-38 chorionic girdle trophoblast expresses VEGF and its receptors KDR and Flt-1, which suggests VEGF may act in an autocrine manner on mature chorionic girdle cells. The developing chorionic girdle expresses several transcription factors, including glial cells missing 1 (GCM1), heart and neural crest derivatives expressed transcript 1 (HAND1), distal-less homeobox 3 (DLX3), and estrogen-related receptor β (ERR β) (46,50). An interesting temporal expression pattern in the chorionic girdle was noted for the placental-specific transcription factor, GCM1. *GCM1* mRNA expression was minimally expressed in the chorionic girdle prior to day 30 of gestation but concentrations rose rapidly from that day with peak values in mature invasive trophoblast at day 34 of gestation (50). There is molecular evidence from *in vitro* studies that *GCM1* expression is restricted to terminally differentiated binucleate trophoblast, which suggests that it may function in the latter stages of development as a regulator of binucleate trophoblast differentiation, eCG expression, and/or trophoblast migration.

Transcriptome profiling using expression microarrays has been applied to compare gene expression between invasive (chorionic girdle) and noninvasive (chorion) trophoblast from day-33–34 horse conceptuses (51). Over 300 differentially expressed genes were detected in the comparison, and among the most dramatically upregulated transcripts in the chorionic girdle was the cytokine interleukin-22 (IL22). IL22 is produced by specialized T lymphocytes (e.g., Th17 and Th22 T helper cells), and it is an unusual cytokine in that its target tissue is not another type of lymphoid cell but, rather, epithelium in which it is thought to be a mediator of inflammation and tissue homeostasis at mucosal surfaces (52,53). The discovery of IL22 expression by equine chorionic girdle is the first description

of IL22 production by a nonlymphoid cell. Its function may be to hasten reepithelialization of the endometrial surface after chorionic girdle invasion.

A Brief Existence: Development and Death of the Endometrial Cups

Following invasion of the chorionic girdle during days 35–38 after ovulation, the endometrial cups first appear macroscopically as a series of pale, slightly raised plaques on the surface of the endometrium that are arranged in a circle around the conceptus at the base of the gravid uterine horn (Figure 2*b*) (38). Each cup measures 0.8–1.5 cm in width, depending upon the breadth of the progenitor chorionic girdle (38), and may range in length from small, isolated structures of only 1–2 cm to unbroken 20-cm ribbons of cup tissue. This variation stems from the degree of folding of the endometrium at the time of chorionic girdle invasion. The peak concentration of eCG in maternal blood and the total quantity of eCG secreted during early gestation are directly related to the total amount of endometrial cup tissue that develops, which, in turn, is governed by the amount of chorionic girdle tissue that invades the endometrium at approximately day 36 (38). eCG secretion typically reaches its peak levels within three weeks after its first detection in the mare's blood and then begins to decline with a slope that approximates the six-day half-life of the eCG molecule, becoming undetectable by day 120–150 (Figure 5).

Beginning at the time of chorionic girdle invasion of the endometrium, the endometrial cup trophoblast cells are the focus of an accumulation of maternal lymphocytes, largely CD4⁺ and CD8⁺ T cells (Figure 6*b*) (54). At first, these lymphocytes remain clustered in the endometrial stroma at the periphery of the cup (Figure 6*a*), but by day 60–70 they move into the main cup structure (Figure 6*c*). Over the next 30–40 days, the lymphocytes are joined by increasing numbers of neutrophils, macrophages, and eosinophils. Eventually, between days 100 and 140 in most mares, the whole necrotic cup and admixed, inspissated exocrine secretion are sloughed off the surface of the endometrium (Figure 6*d*) to leave a dense layer of leukocytes in the remaining stroma and an avillous scar on the surface of the overlying allantochorion (38).

On a superficial level the endometrial cup reaction appears to be a cell-mediated immune response to the semiallogeneic invasive trophoblast cells which results in the eventual destruction of the cup trophoblasts. Indeed, the lymphocyte accumulations are restricted to the area of the cups; and they do not normally occur along the allantochorion-endometrial border that makes up the interface of the placenta proper (54). Furthermore, the invasive trophoblast cells of the chorionic girdle and early endometrial cups express high levels of paternal major histocompatibility complex (MHC) class I antigens (55), whereas the allantochorion does not express these molecules (56).

However, after several decades of investigation, the immunological explanation for the lifespan and demise of the endometrial cups remains incomplete. For example, no extension of cup lifespan and no decrease in lymphocyte accumulations around the endometrial cups were observed in experimental MHC-compatible pregnancies compared with MHC-incompatible pregnancies (57). Furthermore, immunological sensitization of mares to the MHC antigens of the mating stallion, by skin allografting before establishment of pregnancy, did not reduce the lifespan of the endometrial cups or cause detectable changes

in the lymphocyte accumulations (58). The survival time and function of isolated chorionic girdle trophoblast after ectopic transplantation to fully allogeneic, nonpregnant recipient mares suggests that the lifespan of the endometrial cups may be determined primarily by factors intrinsic to the cup cells themselves (59). The invading chorionic girdle cells and early endometrial cup cells are clearly immunogenic. However, the role of the maternal immune response in endometrial cup cell death is still poorly understood.

Factors Influencing Endometrial Cup Development

Many factors have been shown to influence eCG concentrations in mares' blood, including mare size (8,60,61), mare parity (62), paternity of the conceptus (63), twin pregnancy (64), and conception at the first postpartum oestrus (65). More recent experiments, in which mare size, age, parity, and conceptus paternity were standardized, have also shown that both body condition and exercise have a significant influence on eCG concentrations in maternal blood (66). The eCG profiles of 61 Thoroughbred mares all mated to the same stallion showed peak eCG concentrations occurring between days 50 and 85 after ovulation (mean $\pm$ s.e. 62.4 ± 1.0 days) and ranging from as low as 14.5 to as high as 126 iu/ml (64.5 ± 3.7 iu/ml). eCG became undetectable in the circulation on day 134.1 ± 1.7 (range, 105–150 days), thereby taking a mean of 71.7 ± 1.4 days (range, 50–95 days) to reach baseline after the peak concentration (66). In this study, body condition score affected eCG levels, such that mares with high scores (fat) had lower circulating eCG levels than mares with low scores (thin). Finally, an effect of exercise was reported in this same investigation with non-exercised mares showing higher eCG levels from 60 days after ovulation than a group undergoing mild exercise (one hour of brisk walking/trotting twice daily).

The most profound effects on eCG levels are determined by fetal genotype. This was first observed by Wadslaw Bielanski and colleagues in Krakow, Poland, who measured much lower concentrations of eCG in the serum of mares when carrying hybrid mule (female horse $\times$ male donkey) fetuses than when carrying normal intraspecies horse fetuses (67), and it was later confirmed and extended by research in California (68) and Cambridge, United Kingdom (69). When eCG levels were measured in groups of mares (*Equus caballus*, $2n = 64$) and female donkeys (*Equus asinus*, $2n = 62$) carrying normal intraspecies pregnancies or interspecies mule (donkey-sire) or hinny (horse-sire) pregnancies, the striking outcome was that the level of eCG in maternal serum appeared to be determined by the genotype of the sire (Figure 7) (69). This was later shown to result from reproducible differences in the amount of endometrial cup tissue formed in the four types of pregnancy. Namely, large amounts of endometrial cup tissue in mares carrying horse and donkeys carrying hinny pregnancies versus much smaller endometrial cups in female donkeys carrying donkey or mares carrying mule pregnancies (70). The amount of cup tissue in the various types of pregnancies was, in turn, determined by the amount of chorionic girdle tissue that invaded the endometrium. Horse and hinny chorionic girdles were found to be wider and generally larger and better developed than donkey or mule girdles (Figure 8) (70).

This marked divergence in the breadth and eCG-secreting capacity of mule and hinny chorionic girdles could be explained by the action of paternally expressed, imprinted genes that influence the development of the chorionic girdle: namely, a broad and active chorionic

girdle when a stallion sires the offspring compared with a narrower and less-active girdle when a male donkey is the father (Figure 8). However, this putative imprinted influence can be affected by maternal uterine environment, as demonstrated convincingly by between-species embryo transfer. Placing a mule embryo, which would normally produce a narrow chorionic girdle and small endometrial cups in its genetic horse mother, into the uterus of a female donkey results in the development of a broad chorionic girdle and larger and more active endometrial cups, similar to those generated in a donkey carrying an interspecies hinny pregnancy (Figure 9) (71). The question of which growth factors or other endometrial products can exert such a profound influence on placental development prior to implantation in the equid mother remains a fascinating topic for future investigation.

FETAL-MATERNAL SIGNALING AND PLACENTAL EVOLUTION

Two aspects of the biology of invasive trophoblast in the mare demonstrate strong signaling of the mother by the fetal-placental unit: the actions of eCG, the primary secreted product of the endometrial cup trophoblast cells, and the sensitization of the maternal immune system by the MHC antigens expressed by the chorionic girdle trophoblast cells.

The Molecular Physiology of Equine Chorionic Gonadotrophin

The original studies of Cole et al. (42) and others (41,72) drew attention to the close temporal relationship between the commencement of eCG secretion and the first of what becomes a succession of secondary ovulations and/or luteinizations in the maternal ovaries during the life span of the endometrial cups. Initially, it was assumed that the dual FSH-like and LH-like biological activities of eCG (31) stimulated both follicular growth and ovulation/luteinization of these secondary follicles to augment progesterone production by the primary corpus luteum until the allantochorion became sufficiently well established to take over progestagen production from the maternal ovaries entirely, from around day 100 of gestation onward (43,72). However, Evans & Irvine (39) and Urwin & Allen (40) both demonstrated that continued secretion of FSH by the pituitary gland in 10–12-day waves, just as if the mare was still cycling, is responsible for this follicular growth in pregnancy, whereas only the LH-like activity of eCG acts to mature and ovulate/luteinize the dominant accessory follicle in each wave. Terqui & Palmer (73) showed a pronounced rise in maternal serum estrogen concentrations that coincides with the onset of eCG secretion at day 37–40, and Daels and colleagues (74) subsequently demonstrated that the primary and secondary corpora lutea, not the enlarging ovarian follicles, secrete these additional estrogens in response to the LH-like property of the eCG.

This luteinizing action of eCG on follicles in the ovaries of the dam can be viewed from an evolutionary perspective as molecular instruction from fetus to mother, intended to benefit the fetoplacental unit (75). From such a viewpoint, eCG is more like an unusual type of pheromone than a traditional hormone. This perspective leads to potentially interesting outcomes in the case of interspecies (e.g., mule and hinny) and extraspecies (e.g. donkey-in-horse) pregnancies, in which the eCG produced by the endometrial cups can have different ratios of FSH:LH activity, depending upon the genotype of the conceptus.

The FSH:LH ratio of eCG is strongly influenced by fetal genotype; in normal intraspecies horse pregnancy the ratio is approximately 1.4, but it drops to as low as 0.1 in female donkeys carrying intraspecies donkey fetuses. In mares carrying hybrid mule fetuses, and in donkeys carrying reciprocal hybrid hinny fetuses, the FSH:LH ratio is midway between those of the horse and donkey extremes at 0.7-0.8. (Figure 10) (76). Radioreceptor assay studies also demonstrated a much lower binding affinity of eCG, but not pituitary FSH and LH, for gonadal FSH and LH receptors in the horse compared to binding with the equivalent receptors in other animal species (77). This helped to explain why the ovaries of the mare do not become hyperstimulated during the period of eCG production in each successive pregnancy.

Chorionic gonadotrophins have been identified only in species from two orders of mammals, the Perissodactyla (odd-toed ungulates) and primates. Humans and horses have taken very different paths to the production of their respective chorionic gonadotrophins. In the former the hCG beta chain is encoded in a gene cluster that arose in the primate lineage by duplication from the ancestral *hLH beta* gene (78–80). The hCG beta chain also has a 29–amino acid carboxyterminal extension which is not present on the hLH beta chain (81). In the horse and other equids, there was no duplication of the *eLH* gene. Instead, equids use their *eLH beta* gene to encode the eCG beta chain, and, thus, equine LH and eCG beta chains have the same amino acid structure, although they are glycosylated differently (82,83). Surprisingly, the horse *eCG beta* gene has a 3' extension of approximately the same length as that in the human, and this longer gene is also transcribed to produce equine LH (84–86). This curious example of convergent evolution between humans and equids can be extended to other aspects of placental form and function.

It is now considered likely that the earliest form of placentation in mammals was the invasive hemochorial type, which includes the placentae of rodents and primates (87), and the epitheliochorial placenta that is characteristic of equids and suids is a more recently derived type (88). At the level of gross anatomy, the horse and human placentae are distinctly different. However, at the level of cell type and function there are remarkable similarities. For example, the trophoblast cells that produce chorionic gonadotrophins in both humans and horses are multinucleate. The human syncytiotrophoblast is the sole source of hCG, and only the binucleate trophoblast of the endometrial cups produces eCG (89). Once again this seems to be a case of convergent evolution.

The evolutionary origin of the invasive trophoblast component of equids remains a mystery. If indeed the epitheliochorial placenta of the horse is a derived form, the minor invasive component of the chorionic girdle might be a relic retained from a more invasive ancestral type of placenta. However, the invasive trophoblast of equids, with its similarities to the invasive human extravillous trophoblast, might have developed independently in equids and would thus be yet another example of convergent evolution (89).

Immune Signaling of the Mare

A second aspect of fetus-to-mother signaling in equine pregnancy involves the strong humoral immune responses to the developing equine conceptus (89). Within 10–20 days after formation of the endometrial cups, virtually all maiden mares carrying MHC-

incompatible fetuses develop high titers of antipaternal lymphocytotoxic antibodies directed against the foreign paternal MHC class I antigens (90,91). The sources of this antigenic stimulation of the dam are the invasive chorionic girdle tissue and the early endometrial cup trophoblast cells, which express high levels of paternal and maternal MHC class I, but not MHC class II, molecules for the brief period between days 30 and 50 after ovulation (55,56,92,93). The terminally differentiated endometrial cup cells lose their expression of MHC class I antigens during the 10-day period after their formation, in a process that appears to be intrinsic to the trophoblast cells themselves, because the same MHC downregulation occurs in cell cultures of mature chorionic girdle trophoblast cells (94).

This strategy for selective expression of MHC antigens on invasive trophoblast appears, at first consideration, to be counterintuitive. In the various species that have been studied for MHC antigen expression in the placenta, most trophoblast cell populations downregulate expression of both MHC class I and MHC class II molecules (95). Why express only MHC class I molecules, and why only on the most invasive trophoblast cells? For many years, the horse seemed an outlier with respect to its MHC class I molecule expression in the placenta. However, recent work in mice and humans has revealed a very similar pattern: selective expression of MHC class I antigens on invasive trophoblast cells (96). This expression of MHC class I molecules on invasive trophoblast therefore appears to be another characteristic shared between the highly diverged epitheliochorial placenta of equids and the more ancient hemochorial placentae of rodents and primates.

There are no reports of pathological consequences of maternal antifetal MHC antibody responses during pregnancy in any species, and recent research on so-called regulatory T cells (Tregs) in pregnancy suggests that the spatially and temporally limited expression of MHC class I antigens by equine invasive trophoblast may play an important role in inducing maternal tolerance to the equine conceptus (97,98). Indeed, the local environment around the developing endometrial cups is rich in TGF β (45), which has been shown to be critical for induction of Tregs through the action of the FOXP3 transcription factor (99). There is also evidence for enrichment of FOXP3-expressing T cells around the endometrial cups (100). Finally, in equine pregnancy there is a dramatic manifestation of T cell tolerance in the systemic reduction in the capacity of circulating T cells from pregnant mares to develop into cytotoxic killer T cells (101,102). The strong antibody responses to the equine conceptus, coupled with dampening of cytotoxic T cell responses in the circulation, have led to formation of a hypothesis of split immunological tolerance to trophoblast (100). This hypothesis can explain many of the observed immunological phenomena in equine pregnancy. The cytotoxic activity of the striking maternal T cell accumulations around the endometrial cups described earlier may be kept in check by small populations of regulatory T cells induced by the MHC class I molecules expressed on the invading trophoblast cells of the chorionic girdle.

The Donkey-in-Horse Model of Pregnancy Failure

From the research summarized above, it is unclear whether the maternal lymphocytes surrounding the endometrial cups can have destructive immunological properties. A unique experimental system of extra- (cross) species embryo transfer has shed some light on this

question. Extraspecies transfer of donkey embryos into the uteri of horse mares results in development of a very small chorionic girdle (Figure 11), which fails completely to invade the endometrium of the surrogate mare at day 35–38 of gestation. Despite the resulting absence of endometrial cup development and eCG secretion, the conceptus continues to develop normally to approximately day 60–65, but without any attachment and interdigitation of the allantochorion and endometrium from day 40 onward, in some 70% of these pregnancies (Figure 12*a,b*). The resulting starvation of the growing fetus leads to its death at approximately day 75 (Figure 12*c*) and abortion of the degenerating conceptus between days 80 and 95 (Figure 12*e*), in conjunction with a vigorous maternal lymphocytic reaction against the unattached xenogeneic donkey allantochorion (Figure 12*d,f*) (103). In mares carrying donkey fetuses this lack of placentation and the resulting fetal death, cannot be prevented by the administration of either large quantities of partially purified eCG or the synthetic progestagen, allyl trenbolone, or a combination of both, to mimic endogenous eCG production and secondary luteal development (103). Curiously, the remaining 30% of donkey-in-horse pregnancies do manage to achieve placentation, albeit later and more slowly than in normal gestation, and the pregnancies proceed to term with the birth of donkey foals that range from mature and healthy to small and immature or dysmature (104). Transfer of second or third consecutive donkey embryos to mares that had or had not aborted previous donkey fetuses revealed a puzzling mixture of genetics and immunological memory. Namely, recipient mares that implanted and carried a first donkey pregnancy did so again successfully when second donkey embryos were transferred to them, whereas recipients that aborted their first extraspecies donkey fetus absorbed again at successively earlier stages of gestation when subsequent donkey embryos were transferred to them. Thus, an as-yet-unidentified genetic component seems to make the mare able, or unable, to implant and gestate a xenogeneic donkey embryo (104). The donkey-in-horse pregnancy model provides some of the strongest evidence in any species for destructive maternal antifetal immune responses, including the cardinal attribute of immunological memory.

Equine Invasive Trophoblast and the Initiation of Placental Attachment and Development

The mechanism involved in the prolonged (40-day) period between fertilization and implantation of the conceptus in equids has long puzzled reproductive physiologists. The blastocyst capsule, which develops soon after entry of the late morula/early blastocyst-stage embryo into the uterus on day 6 after ovulation (105), no doubt plays a major role in maintaining the spherical outline of the embryo while it grows over the next 16–18 days (106). But even after the capsule begins to disintegrate from day 21–23, to enable direct contact of the trophoblast layer of the allantochorion with the luminal epithelium of the endometrium, the conceptus remains essentially spherical for the next 15–20 days. Further, with the exception of the invasive chorionic girdle penetrating the endometrial barrier during days 35–38 to form the endometrial cups, the trophoblast shows no sign of any serious attempt to attach itself to the endometrium. Then suddenly, on or very close to day 40 and, hence, immediately after invasion of the chorionic girdle cells, a stable, microvillous attachment is achieved between trophoblast and luminal epithelium. This is followed quickly by the commencing growth of chorionic villi, which interdigitate with upward protrusions of endometrium to commence the feto-maternal interdigitation. This will increase in extent and complexity throughout the remainder of gestation to yield the diffuse

microcotyledonary epitheliochorial placenta that fills the entire uterus and encompasses an amazing 50+ meters² of microscopic feto-maternal contact at term in Thoroughbred mares (107).

Are the two major events, endometrial cup development and implantation/placentation, linked and interdependent? Temporally, this would seem to be the case, because the latter event commences hard on the heels of the former following a 20-day period during which direct and close physical contact has existed between the fetal and maternal epithelial layers but with no attempt at fusion and implantation. The failure of implantation and placentation to occur in the majority of donkey-in-horse pregnancies, following an apparent failure of the endometrial cup reaction to materialize, seems to support such a connection (104). Clearly, a century on, much more remains to be learned about the precise role of the endometrial cup reaction in the establishment and maintenance of equine pregnancy.

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Acronyms and Definitions

MHC	Major histocompatibility complex
Chorionic Girdle	invasive trophoblast cells of the horse
Allantochorion	noninvasive trophoblast cells of the horse
Endometrial cups	terminally differentiated, chorionic gonadotrophin-secreting trophoblast cells of the horse derived from the invasive chorionic girdle cells

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Summary Points

1. The chorionic girdle and endometrial cups of the equid placenta are invasive trophoblast cells that have no known counterparts in other species with epitheliochorial (diffuse) placentation.
2. Only primate and equine placentae secrete a chorionic gonadotrophin, but these functional states seem to have been achieved through convergent evolutionary pathways.
3. The basic cellular architecture and phenotypes of invasive and secretory trophoblast cells have been maintained in species as divergent as humans and horses.
4. The chorionic girdle and early endometrial cups display florid, but highly regulated, expression of antigenic paternal MHC class I molecules.
5. The maternal humoral and cellular immune responses to the conceptus in the mare are the most striking of any species yet described, but they do not appear to damage the fetus or placenta.
6. Equine pregnancy and/or equine trophoblast itself can induce a state of immune tolerance in the mare to her developing conceptus.

Future Issues

1. What factors initiate the rapid proliferation and differentiation of the chorionic girdle trophoblast cells at approximately day 30 of gestation?
2. What is the signal for terminal differentiation of chorionic girdle cells to a binucleate, migratory state?
3. What factors limit the invasion of the chorionic girdle cells into the superficial layer of the endometrium?
4. How does equine invasive trophoblast induce maternal immune tolerance?
5. What determines the lifespan of endometrial cup cells, and how are these cells destroyed in normal pregnancy?
6. Did the chorionic girdle arise by retention of an invasive phenotype from earlier placental forms, or did it arise de novo through a convergent evolutionary process?
7. Are the levels of eCG in horse, donkey, mule, and hinny pregnancies determined by the action of imprinted genes?
8. Is there a mechanistic link between chorionic girdle invasion and placental attachment to the endometrium in equine pregnancy?

Figure 1.

Pivotal figures in the endometrial cup saga. (a) Professor Wilhelm Schauder, Giessen, Germany (1); (b) Professor J. Cossar Ewart FRS, Edinburgh, England (2). (c) Professor Harold H. Cole, California, United States (4). (d) Dr. Robert M. Moor Fellow of the Royal Society, Cambridge, England (35).

Figure 2.

Endometrial cups and the progenitor tissue of the chorionic girdle. (a,b) Gross images of endometrial cups (arrows) in the uterus of a mare at day 43 of gestation. (b) The overlying allantochorion membrane has been removed. (c) Day-34 equine conceptus recovered by nonsurgical uterine lavage, showing prominent band of invasive trophoblast of the chorionic girdle (CG), allantochorion membrane (ALC), fetus (F), yolk sac (YS) beneath the chorion membrane (CH), and the bilaminar omphalopleure (BO). Conceptus diameter approximately 3.5 cm. (d) The CG of a day-34 conceptus viewed through a dissecting microscope at low magnification, showing the characteristic pattern of elongating folds of rapidly dividing cells. The girdle develops between the avascular CH on the left and the vascularizing ALC on the right. Scale bar = 1 mm.

(a)

(b)

Figure 3.

Chorionic girdle cells *in vitro*. (a) Photomicrograph of a monolayer culture of mature chorionic girdle cells grown *in vitro* for 18 days after recovery at day 34 of gestation. Note the large epithelioid appearance of the cells, their binucleate status, their characteristic cytoplasmic vesicles, and the cytoplasmic bridges between adjacent cells. Scale bar = 25 microns] (b) eCG profiles measured in four separate dishes of horse chorionic girdle cells maintained in monolayer culture for 180 days (35). Abbreviations: PMSG, pregnant mare serum gonadotrophin

Figure 4.

Formation of the endometrial cups. (a) Section of the chorionic girdle adhered to the surface of the endometrium at 37 days of gestation. The luminal epithelium has been obliterated, and the elongated binucleate trophoblast cells at the face of the girdle are about to penetrate the endometrial stroma, which already contains some attracted maternal lymphocytes. (b) High power field of mature, binucleate endometrial cup trophoblasts (arrow). (c) Low power section of an entire mature endometrial cup. The large binucleate cup cells are tightly packed in the endometrial stroma. The basal portions of the endometrial glands are becoming distended with accumulated exocrine secretions owing to ablation of the gland

outlets; note the large lymph sinuses beneath the cup (*arrow*). Scale bars = 45 microns (a), 30 microns (b), 175 microns (c).

Figure 5.

Progesterone profiles measured in the peripheral blood of two pony mares during the first 120 days of gestation, with diagrammatic profiles showing the period of eCG secretion superimposed. Note the regular increases in progesterone concentrations commencing at approximately day 38–40 with the formation of each successive secondary corpus luteum.

Figure 6.

The demise of the endometrial cups. (a) Low power view of an entire endometrial cup from day 60 of gestation. At this stage, large numbers of maternal lymphocytes have gathered around the edges of the cup. (b) High power field of maternal lymphocytes (T cells) surrounding a group of endometrial cup cells at day 60 of gestation. (c) Low power view of an entire endometrial cup from 75 days of gestation. The glands and lymphatics surrounding the trophoblast cells of the cup are distended, and lymphocytes have begun to move into the cup tissue. Many endometrial cup cells are degenerating. (d) Dead endometrial cup at 110 days of gestation, overlain by an equine chorionic gonadotrophin-rich mixture of degenerate cup cells and exocrine secretion released from the unblocked endometrial glands. Note the dense accumulation of leukocytes in the surrounding maternal stroma. Scale bars = 200 microns (a, c, d), 45 microns (b).

Figure 7.

The paternal genome determines equine chorionic gonadotrophin (eCG) levels in equid pregnancy. Comparison of eCG profiles measured at weekly intervals in the serum of (a) 30 pony mares carrying intraspecies horse conceptuses, (b) 11 mares carrying interspecies mule conceptuses, (c) 14 female donkeys carrying intraspecies donkey conceptuses, and (d) 6 female donkeys carrying interspecies hinny conceptuses (77).

Figure 8.

The size of the chorionic girdle (CG) determines the amount of endometrial cup tissue and the levels of equine chorionic gonadotrophin in maternal circulation. Intraspecies horse (*left*) and interspecies mule (*right*) conceptuses at 34 days of gestation showing the marked difference in width between their CGs.

Figure 9.

The maternal uterine environment can profoundly affect endometrial cup development and equine chorionic gonadotrophin (eCG) secretion levels. eCG profiles measured in the blood of a mare and a donkey recipient, each carrying a genetically identical demi-mule embryo created by splitting a mule embryo recovered from a donor mare on day 6 after ovulation (71).

Figure 10.

Profiles showing a pronounced effect of fetal genotype upon both the concentration of gonadotrophin in the serum (FSH and LH as measured by radioreceptor assay and the FSH:LH ratio of the gonadotrophin, between 30 and 90 days of gestation in the serum of: (a) a mare carrying a horse conceptus, (b) a donkey carrying a donkey conceptus, (c) a mare carrying a mule conceptus, and (d) a donkey carrying a hinny conceptus (76). [**Comment from Reviewer: It is unclear which response is the circles and which, the squares.**] Red lines (square symbols) = FSH:LH ratio; blue lines (circle symbols) = total gonadotrophin.

Figure 11.

Very low power sections showing the marked differences in width between the progenitor chorionic girdles of: (a) an intraspecies horse conceptus, (b) an intraspecies donkey conceptuses, (c) an interspecies mule conceptus, and (d) an extraspecies donkey-in-horse conceptus established by embryo transfer. Scale bar = 1.5 mm.

Figure 12.

Extraspecies donkey-in-horse pregnancies established by embryo transfer. (a) On day 65, the fetus is still viable, and the allantochorion is well vascularized, although the yellow coloration of the amniotic fluid indicates a level of fetal stress. (b) Histological section from a showing that the donkey allantochorion and horse endometrium lie closely opposed but without the expected attachment and interdigitation. (c) Distressed day-75 donkey-in-horse fetus showing contusion owing to hemolysis of fetal red blood cells; allantochorion is pale and poorly vascularized. (d) Histological section from c, showing appreciable quantities of exocrine secretion (histotroph) from the endometrial glands separating the fetal and maternal layers and significant numbers of leukocytes accumulated in the endometrial stroma. (e) Dead day-87 donkey-in-horse fetus; the amniotic fluid is heavily hemolysed and the allantochorion is pale, bloodless, and degenerating. (f) Histological section from e, with maternal leukocytes tracking through the luminal epithelium of the endometrium toward the degenerating allantochorion. Scale bar = 1 mm.