

# Chapter 9

## Pregnancy Recognition and Implantation of the Conceptus in the Mare

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**Abstract** Few, if any, biological processes are as diverse among domestic species as establishment of early pregnancy, in particular maternal recognition of pregnancy. Following fertilization and initial development in the mare oviduct, selective transport of the embryo through the uterotubal junction driven by embryo-derived PGE2 occurs. Upon arrival in the uterus, an acellular glycoprotein capsule is formed that covers the embryo, blastocyst, and conceptus (embryo and associated extraembryonic membranes) between the second and third weeks of pregnancy. Between Days 9 and 15/16 of pregnancy, the conceptus undergoes an extended phase of mobility. Conceptus mobility is driven by conceptus-derived PGF2 $\alpha$  and PGE2 that stimulate uterine contractions which in turn propel migration of the conceptus within the uterine lumen. Cessation of conceptus mobility is referred to as fixation and appears to be attributable to increasing size of the conceptus, preferential thickening of the endometrium near the mesometrial attachment referred to as encroachment, and a reduction in sialic acid content of the capsule. During maternal recognition of pregnancy, endometrial PGF2 $\alpha$  release is attenuated, a consequence of reduced expression of key enzymes involved in prostaglandin production. Oxytocin responsiveness is altered during early pregnancy, and reduced expression of the oxytocin receptor appears to be regulated at the posttranscriptional level rather than the transcriptional level. Prostaglandin release is attenuated temporarily only during early pregnancy; during the third week of pregnancy, the endometrium resumes the ability to secrete PGF2 $\alpha$ . The equine conceptus initiates steroidogenesis as early as Day 6 and synthesizes estrogens, androgens, and progesterone. Estrogens are metabolized locally, presumably regulating their bioavailability and actions. Results of experiments attempting to prove that conceptus-derived estrogens are responsible for extension of corpus luteum function have been inconclusive. By the fourth week of pregnancy, the chorionic girdle becomes visible on the trophoblast. Subsequent invasion of chorionic girdle cells leads to formation of

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R.D. Geisert, F.W. Bazer (eds.), *Regulation of Implantation and Establishment of Pregnancy in Mammals: Tribute to 45 Year Anniversary of Roger V. Short's "Maternal Recognition of Pregnancy"*, Advances in Anatomy, Embryology and Cell Biology 216, DOI 10.1007/978-3-319-15856-3\_9

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endometrial cups which secrete equine chorionic gonadotropin. Equine chorionic gonadotropin has luteinizing hormone functions in the mare, causing luteinization of follicles resulting in the formation of secondary corpora lutea essential to production of progesterone and maintenance of pregnancy.

## 9.1 Introduction

Few, if any, biological processes are as diverse among domestic species as establishment of pregnancy. The mare has several unique features, including selective transport of embryos through the uterotubal junction, an extended phase of conceptus mobility throughout the two uterine horns, and an acellular glycoprotein capsule covering the conceptus between the second and third weeks of pregnancy. Furthermore, the horse is the mammal with the longest known preimplantation phase of pregnancy; implantation does not occur until Day 40 (Allen and Stewart 2001). Of particular interest are events leading to prolongation of luteal lifespan, collectively termed “maternal recognition of pregnancy” (Short 1969). The maternal recognition of pregnancy factor secreted by the conceptus to signal its presence to the maternal system varies among species. These processes are best understood in domestic ruminants and other ungulates, such as the pig; interferon tau has been identified as a conceptus-derived paracrine factor exhibiting antiluteolytic properties in ruminants, whereas conceptus-derived estrogens are the primary pregnancy recognition signal in pigs (Bazer et al. 1997; Geisert et al. 1990). Although the horse is one of the few domestic species in which the conceptus-derived pregnancy recognition signal has not been definitively identified, equids appear to be distinct from ruminants and pigs in the signal(s) used for maternal recognition of pregnancy. Contrasting with the bovine conceptus, equine conceptuses express interferons at negligible levels only; interferons delta 1 and 2 and interferon alpha 1 are expressed at low levels, but only after the critical time of maternal recognition of pregnancy only (Budik et al. 2010; Cochet et al. 2009; Klein 2015). Similar to the porcine conceptus, the equine conceptus synthesizes large amounts of estrogen during the critical time of maternal recognition of pregnancy; unlike in the pig though, a role for conceptus-derived estrogens in maternal recognition of pregnancy remains to be proven (Vanderwall et al. 1994; Woodley et al. 1979). The signal/signals released by the equine conceptus appear to have a molecular weight between 1 and 6 kDa, and its ability to reduce release of prostaglandin F2alpha by endometrial explants in culture is eliminated through proteinase K and charcoal stripping (Ababneh et al. 2000; Sharp et al. 1989).

The earliest evidence for systemic recognition of the conceptus in mares is the presence of an immunosuppressive protein termed “early pregnancy factor” which can be detected (rosette inhibition test) in serum as early as 2 days after ovulation (Ohnuma et al. 2000; Takagi et al. 1998). This early pregnancy factor has been characterized as an extracellular form of heat shock protein 10 (Cavanagh 1996); Day-25 conceptuses have higher transcript abundance than Day-8 blastocysts and protein expression was localized to trophoblast cells (Hatzel et al. 2014).

## 9.2 Selective Transport of Equine Embryos

Following fertilization and initial development of the equine embryo in the oviduct, there is selective transport of equine embryos through the uterotubal junction. In that regard, unfertilized oocytes are retained in the oviduct (Betteridge and Mitchell 1972, 1974; Flood et al. 1979), whereas embryos are transported through the oviduct and pass the uterotubal junction to reach the uterine lumen 6.0–6.5 days after fertilization (Battut et al. 1997). This selective transport is a unique feature among domestic animals. Coinciding with transport through the oviduct, the equine embryo secretes considerable amounts of prostaglandin E2 (PGE2; Weber et al. 1991b), and intraoviductal application of PGE2 not only hastens oviductal transport of embryos but also results in recovery of unfertilized oocytes from the uterine lumen (Weber et al. 1991a). Taken together with the observation that the equine oviduct contains receptors for PGE2 (Weber et al. 1992), it appears that the equine conceptus facilitates its own transport and passage through the uterotubal junction via secretion of PGE2. Furthermore, laparoscopic application of PGE2 on the serosal surface of the oviduct in mares has been reported to overcome unexplained infertility (Arnold and Love 2013).

## 9.3 Prostaglandin F2 $\alpha$ Release Is Attenuated during Maternal Recognition of Pregnancy in the Mare

Mares have an estrous cycle of 21 days and, as in other large animal domestic species, prostaglandin F2 $\alpha$  (PGF2 $\alpha$ ) is the endogenous luteolysin (Allen and Rowson 1973; Douglas and Ginther 1972). In the absence of a conceptus, luteal regression is initiated approximately 14 days after ovulation, as evident by high PGF2 $\alpha$  concentrations in uterine flushings (Stout and Allen 2002), endometrial tissue (Vernon et al. 1981), and uterine vein blood (Douglas and Ginther 1976). A recent study elucidated an auto-amplification system of prostaglandin F2 $\alpha$  production in the equine endometrium. Mares given a synthetic analog of prostaglandin F2 $\alpha$  during the mid-luteal phase responded with a two-phase increase in circulating concentrations of PGFM (13,14-dihydro-15-keto-PGF2 $\alpha$ , the main metabolite of PGF2 $\alpha$ ); the first peak within 45 min, attributed to administration of exogenous PGF2 $\alpha$ , and the second peak at 16 h, with concentrations of PGFM remaining elevated for 56 h. The second increase in PGFM was likely due to PGF2 $\alpha$  production by the endometrium triggered by exogenous PGF2 $\alpha$ . In vitro studies using endometrial explant cultures and cultured epithelial and stromal cells confirmed that exposure to PGF2 $\alpha$  increased PTGS2 expression and stimulated release of PGF2 $\alpha$  (Okuda et al. 2014).

A hallmark of maternal recognition of pregnancy in the mare is reduced endometrial secretion of PGF2 $\alpha$  in the presence of a conceptus during the expected time of luteolysis. In this regard, concentrations of PGF2 $\alpha$  in the blood from uterine veins are lower in pregnant than nonpregnant mares on Days 10 and 14 after ovulation

(Douglas and Ginther 1976). Likewise, PGF2 $\alpha$  is undetectable in uterine flushings collected from the non-gravid horns of pregnant mares before Day 18 after ovulation (Berglund et al. 1982; Stout and Allen 2002). Co-incubation of endometrium with embryos *in vitro* reduces the amount of PGF2 $\alpha$  released by the endometrium, reflecting the inhibitory effect of the conceptus on prostaglandin synthesis and/or release (Berglund et al. 1982; Ealy et al. 2010; Watson and Sertich 1989).

Oxytocin plays a central role in the release of PGF2 $\alpha$  from the endometrium at the time of luteolysis. There is accumulating evidence that oxytocin responsiveness is altered during early pregnancy in the mare. In nonpregnant mares, endogenous oxytocin, provoked through repeated transcervical endometrial biopsies on Days 12 and 14 after ovulation, induces an increase in circulating concentrations of PGFM, whereas pregnant mares lack a corresponding increase in PGFM following cervical stimulation (Sharp et al. 1997). Furthermore, exogenous oxytocin provokes release of PGFM in nonpregnant mares, whereas this response is attenuated in pregnant mares (Goff et al. 1987; Starbuck et al. 1998). Nonpregnant mares respond to exogenous oxytocin with the greatest increase in circulating concentrations of PGFM around the time of luteolysis, *i.e.*, between Days 13 and 16 after ovulation, whereas pregnant mares show no increase in circulating PGFM concentrations in response to exogenous oxytocin given around the time of expected luteolysis (Goff et al. 1987; Starbuck et al. 1998). In line with the differential response to oxytocin depending on pregnancy status, endometrial oxytocin receptor concentrations differ between pregnant and nonpregnant mares around the time of luteolysis; concentrations of oxytocin receptors increase in nonpregnant mares, whereas there is no corresponding increase in pregnant mares (Sharp et al. 1997; Starbuck et al. 1998). There are indications that expression of oxytocin receptors is regulated at the posttranscriptional level, rather than the transcriptional level, during maternal recognition of pregnancy in the mare; transcript levels remain unchanged throughout early pregnancy (de Ruijter-Villani et al. 2014; Klein et al. 2010), whereas protein levels are decreased (de Ruijter-Villani et al. 2014; Sharp et al. 1997; Starbuck et al. 1998). In addition to reduced expression of oxytocin receptors, their function is altered during early pregnancy, manifested as lower affinity of the receptor for binding oxytocin in pregnant versus nonpregnant mares (Sharp et al. 1997). Further supporting evidence for involvement of oxytocin in regulation of luteolysis and its alteration during maternal recognition of pregnancy comes from the observation that repeated administration of oxytocin prolongs luteal function in mares (Stout et al. 1999; Vanderwall et al. 1994, 2007). Oxytocin receptor transcript levels remain unaltered in mares displaying prolonged luteal function following repeated oxytocin exposure, whereas prostaglandin-endoperoxide synthase 2 expression is reduced (Keith et al. 2013).

In ruminants, the corpus luteum and the posterior pituitary are sources of oxytocin that stimulate luteolytic pulses of PGF2 $\alpha$  from the endometrium at the end of diestrus (Flint and Sheldrick 1986). Unlike in ruminants, the corpus luteum of the mare does not contain oxytocin (Stevenson et al. 1991; Stock et al. 1995), whereas the endometrium expresses oxytocin (Bae and Watson 2003; Behrendt-Adam et al. 1999). Oxytocin-neurophysin I transcript abundance is highest during estrus and is negatively correlated with circulating concentrations of progesterone in pregnant

and nonpregnant mares (Behrendt-Adam et al. 1999). Within the endometrium, oxytocin mRNA and protein localize to luminal epithelial cells and superficial glandular epithelial cells, in addition to being secreted into the uterine lumen (Bae and Watson 2003). Recently, leucyl-cystinyl aminopeptidase (LNPEP), an enzyme that cleaves oxytocin, has been reconsidered for its potential role in regulating levels of oxytocin expression during maternal recognition of pregnancy. Albeit more work is needed, it appears that oxytocinase levels in serum are below the detection limit during diestrus, whereas the highest concentrations of oxytocinase in serum are during early pregnancy (Diel de Amorim et al. 2014).

Interestingly, attenuation of endometrial release of PGF $2\alpha$  seems to be a temporary event during maternal recognition of pregnancy in the mare; during the third week of pregnancy, the endometrium resumes the capability to secrete PGF $2\alpha$ . Although uterine flushings of pregnant mares contain no detectable amounts of PGF $2\alpha$  on Days 12, 14, and 16 after ovulation, the abundance of PGF $2\alpha$  in uterine flushings from pregnant mares starts to increase on Day 18 of pregnancy, peaks on Day 20, and decreases thereafter to negligible levels by Day 30 (Stout and Allen 2002). Likewise, by Day 18 of pregnancy, the uterus has regained the ability to respond to oxytocin with an increase in circulating concentrations of PGFM, although this is apparently not associated with an increase in expression of oxytocin receptors in the endometrium (Starbuck et al. 1998). Resumption of PGF $2\alpha$  production during early pregnancy is puzzling, given that the mare relies on luteal progesterone production throughout the first trimester of gestation. One way for the corpus luteum to escape the luteolytic action of PGF $2\alpha$  would be to reduce binding sites for PGF $2\alpha$ . During early pregnancy, binding capacity of the corpus luteum for PGF $2\alpha$  is high until Day 18. However, as of Day 20 of pregnancy-binding capacity of the CL for PGF $2\alpha$  begins to decline (Vernon et al. 1979). The hypothesis that reduced affinity of the corpus luteum to bind PGF $2\alpha$  prevents luteolysis after the second week of pregnancy has not been tested.

## 9.4 Regulation of the Pathway for Prostaglandin Synthesis

Enzymes involved in prostaglandin production are regulated with respect to level of expression throughout the estrous cycle and during early pregnancy. The first step in prostaglandin production is release of arachidonic acid from membrane phospholipids through the action of phospholipase A2 (PLA2). PLA2 has several isoforms that differ with regard to substrate specificity, dependence on calcium, and lipid modification: (1) cytosolic PLA2 (cPLA2), (2) calcium-dependent secretory PLA2 (sPLA2), and (3) calcium-independent intracellular PLA2 (iPLA2) (Chakraborti 2003). Phospholipase A2 activity is highest in equine endometria on Day 14 after ovulation (compared to estrus and Days 3 and 8 after ovulation), whereas the presence of a conceptus decreases activity at Day 14 of pregnancy (Ababneh and Troedsson 2013a). PLA2 kinetics in endometrial tissue obtained from pregnant mares indicates the presence of an unidentified competitive inhibitor of PLA2. Since

uteroglobin is a known inhibitor of PLA2, uteroglobin may function as the inhibitor of PLA2 during pregnancy (Ababneh and Troedsson 2013a). However, uteroglobin expression decreases during pregnancy (Hayes et al. 2012), necessitating more investigations to clarify the identity of this inhibitor. Endometrial expression of cytosolic PLA2 is reduced in the presence of a conceptus, despite uncertainty regarding timing and progesterone dependency. There is decreased expression of cytosolic PLA2 in pregnant mares although concentrations of progesterone are high on Day 14 and low on Day 18 (Ozel et al. 2014). Conversely, Ababneh and coworkers reported attenuated expression of cytosolic PLA2 at Day 15 of pregnancy, but only if circulating concentrations of progesterone were low. Nonpregnant mares with high circulating concentration progesterone had similar expression of PLA2 as pregnant mares on Day 15 after ovulation (Ababneh et al. 2011). Treating ovariectomized mares with estrogen and/or progesterone revealed that cytosolic PLA2 transcript expression is inversely correlated to concentrations of progesterone, whereas estrogen alone has no effect on its mRNA abundance (Ababneh and Troedsson 2013b). Taken together, expression of cytosolic PLA2 is attenuated during early pregnancy, consistent with reduced production of PGF2 $\alpha$  by the endometrium. Likewise, endometrial expression of secretory PLA2 is decreased during early pregnancy, both at the transcript (Ozel et al. 2014) and protein levels (Hayes et al. 2012). Expression of *PLA2* mRNA is inversely correlated with concentrations of progesterone, whereas estrogen alone has no effect on *PLA2* mRNA abundance in ovariectomized mares treated with estrogen and progesterone (Ababneh and Troedsson 2013b). The contribution of secretory PLA2 to endometrial production of PGF2 $\alpha$  that causes luteolysis is unknown. Secretory PLA2 functions as an innate immune protein, exerting antibacterial properties and enhancing the removal of cell debris (Beers et al. 2002; Birts et al. 2008), perhaps by contributing to functions of cells that form the innate immune defense system to maintain a normal uterine environment during estrus. Although endometrial expression of calcium-independent intracellular PLA2 is attenuated on Day 14 after ovulation in pregnant mares, its expression is upregulated on Day 22 after ovulation (Ozel et al. 2014). Increased expression of calcium-independent intracellular PLA2 during the third week of pregnancy may explain resumption of secretion of PGF2 $\alpha$  at that stage of pregnancy. Estrogen and progesterone concentrations do not affect expression of intracellular PLA2 transcript abundance in endometria of mares (Ababneh and Troedsson 2013b).

Following its liberation from membrane phospholipids, arachidonic acid is converted to prostaglandin-endoperoxide H<sub>2</sub> (PGH<sub>2</sub>) through the actions of prostaglandin-endoperoxide synthase 1 and 2 (PTGS1 and PTGS2). Expression of PTGS2 in endometria of nonpregnant mares is highest during the expected time of luteolysis, whereas expression is significantly reduced on corresponding days of pregnancy (Atli et al. 2010; Boerboom et al. 2004; de Ruijter-Villani et al. 2014; Ealy et al. 2010). Using endometrial explant cultures, Ealy and coworkers (2010) demonstrated that conceptus' secretions downregulate expression of *PTGS2* and reduce the amount of PGF2 $\alpha$  released by endometrial explants, indicating that altered expression of *PTGS2* is a central mechanism of maternal recognition of pregnancy in mares. However, *PTGS1* expression was unaltered around the time of

luteolysis (Atli et al. 2010; de Ruijter-Villani et al. 2014). PGH2 is metabolized to PGF2 $\alpha$  or PGE2 through the action of prostaglandin F synthase (PGFS) or prostaglandin E synthase (PGES). In addition, expression of *PGFS* and *PEGS* does not differ between nonpregnant and pregnant mares on Day 15 after ovulation (Atli et al. 2010; Boerboom et al. 2004), whereas on Day 14 after ovulation, expression of endometrial PGFS mRNA is higher in nonpregnant versus pregnant mares (Atli et al. 2010). The prostaglandin receptor F (PTGFR) mediates the action of PGF2 $\alpha$ , and its expression is attenuated in pregnant mares around the time of luteolysis (Atli et al. 2010; de Ruijter-Villani et al. 2014). Actions of PGE2 are mediated via four receptors; expression of prostaglandin receptor E2 (PTGER2) and PTGER4 is unaltered around the time of expected luteolysis (Atli et al. 2010). Coinciding with the ability of the endometrium to secrete PGF2 $\alpha$  during the third week of pregnancy, upregulation of expression of *PTGS1* occurs in pregnant mares between Days 18 and 21 compared to Days 14 and 15 of pregnancy (Atli et al. 2010). However, there are conflicting reports regarding expression of *PTGS2* during the third week of pregnancy. Atli and coworkers (2010) detected low expression of *PTGS2* between Days 18 and 22 of pregnancy, whereas de Ruijter-Villani (2014) reported increased expression of *PTGS2* on Day 21 of pregnancy. Expression of *PGFS* and *PGES* was unchanged on Day 22 of pregnancy (Atli et al. 2010). Expression of solute carrier organic anion transporter family, member 2A1 (*SLCO2A1*), also known as prostaglandin transporter, increases during the third week of pregnancy, with expression being significantly greater at Day 22 of pregnancy than at estrus or on Days 14, 15, or 18 of the estrous cycle or pregnancy. No effect of day or pregnancy status on expression of *SLCO2A1* at 14, 15, or 18 days after ovulation was reported for pregnant or nonpregnant mares (Atli et al. 2010). In cattle, *SLCO2A1* expression is downregulated at the time of maternal recognition of pregnancy (Banu et al. 2003). The increased expression of *SLCO2A1* during the third week of pregnancy in mares could contribute to the resumption of secretion of PGF2 $\alpha$  by endometria of mares.

In addition to altered expression of key enzymes for prostaglandin synthesis, endometrial cytosol contains an inhibitor of prostaglandin synthesis during early pregnancy in the mare (Watson 1991). In cattle, early pregnancy is likewise associated with an increase of an intracellular inhibitor of prostaglandin synthesis in the endometrium (Gross et al. 1988) which has been identified as linoleic acid (Thatcher et al. 1994). The nature of the inhibitor of prostaglandin synthesis in the mare is unknown.

Alternatively, arachidonic acid can be converted to leukotriene A4 (LTA4) through the action of arachidonate 5-lipoxygenase, which is then either converted to leukotriene B4 or cysteinyl LTC4 through the action of LTC4 synthase and LTA4 hydrolase, respectively. In ruminants, local production of leukotrienes in the corpus luteum contributes to regulation of luteal function, whereas intrauterine administration of an arachidonate 5-lipoxygenase inhibitor delays luteolysis (Cooke and Ahmad 1998; Korzekwa et al. 2010; Milvae et al. 1986). Endometrial expression of arachidonate 5-lipoxygenase, LTC4 synthase, and LTA4 hydrolase is attenuated during early pregnancy in the mare, suggesting that regulation of the lipoxygenase pathway contributes to maintenance of pregnancy (Guzeloglu et al. 2013).

Recently, the effect of cytokines on endometrial prostaglandin production has been assessed. Interleukin-1 alpha, interleukin-1 beta, and interleukin-6 stimulate the production of PGF2 $\alpha$  and PGE2 by epithelial and stromal cells *in vitro*, with estrogen and progesterone modulating the response (Szostek et al. 2014). Tumor necrosis factor alpha stimulates prostaglandin production by mixed epithelial and stromal cells *in vitro* obtained from mare during the follicular phase but not when cells were obtained from mares during the mid-luteal phase (Galvao et al. 2013). How these findings relate to events during early pregnancy in the mare is unknown to date.

## 9.5 Spatial and Temporal Regulation of Endometrial Receptors for Estrogen and Progesterone

The actions of estrogen and progesterone are mediated via their respective receptors, of which estrogen receptor alpha (ESR1) and progesterone receptor (PGR) have been most extensively studied in the context of early pregnancy. Steroid hormones regulate the expression of their own receptors in endometrial cells, with estrogen and progesterone displaying differing effects: estrogens enhance expression of both ESR1 and PGR, whereas progesterone downregulates the expression of both receptors (Spencer and Bazer 2002). In ruminants, particularly ovine, spatial and temporal regulations of endometrial receptors for estrogen and progesterone during the estrous cycle and early pregnancy are well documented. Expression for both receptors is highest during estrus; however, continued progesterone exposure during diestrus results in the loss of PGR and ESR1 expression. This loss is most notable in luminal and superficial glandular epithelium, while deep glandular epithelium and stromal cells retain their already low levels of expression. Cyclic loss of PGR expression permits the reappearance of ESR1 expression, which is closely followed by an increase in PGR expression (Spencer and Bazer 1995). During early pregnancy, ESR1 and PGR expression in luminal and shallow glandular epithelium remains low; yet, the deep glandular epithelium and stroma maintain expression of both receptors (Spencer and Bazer 1995). A pregnancy-dependent block in receptor expression is due to the actions of interferon tau, which blocks expression of ESR1 (Spencer et al. 1995). It has been postulated that progesterone acts on the PGR-positive stroma during pregnancy, which in turn produces paracrine factors that act upon the PGR negative luminal epithelium (Spencer and Bazer 1995). During early pregnancy in the mare, a similar pattern of temporal and spatial expression of PGR and ESR1 in the endometrium can be observed, as expression of both receptors is highest during estrus and declines under the influence of progesterone following ovulation (de Ruijter-Villani et al. 2014; Hartt et al. 2005; McDowell et al. 1999; Tomanelli et al. 1991; Watson et al. 1992). Similar to sheep, expression of these receptors is primarily downregulated in the luminal epithelium, whereas the deep glandular epithelium and stroma retain low expression levels (de Ruijter-Villani et al. 2014; Hartt et al. 2005). By Day 15 after ovulation, expression patterns differ dependent on pregnancy status of the mare. While endometrial expression levels of



ESR1 and PGR are upregulated in nonpregnant mares, expression levels remain low in pregnant mares (de Ruijter-Villani et al. 2014; Hartt et al. 2005; McDowell et al. 1999). Low levels of expression are confined to the deep glandular epithelium and stroma, whereas the luminal epithelium remains receptor negative during early pregnancy (de Ruijter-Villani et al. 2014; Hartt et al. 2005; Wilsher et al. 2011). In all of the above-cited studies, receptor expression was localized to the nuclei of endometrial cells, whereas little to no cytoplasmic expression was observed. A recent study presents contrary results with respect to ESR1 expression during early pregnancy (Wilsher et al. 2011): minimal to moderate cytoplasmic expression of ESR1 was observed in luminal epithelium, in addition to more intense cytoplasmic expression in glandular epithelium throughout early pregnancy (Days 20–68). Only very occasional nuclear expression was observed in epithelial cells (Wilsher et al. 2011). The apparent discrepancy to reports that describe the absence of ESR1 expression in epithelial cells during early pregnancy may be the result of different antibodies being used. Subcellular staining patterns of ESR1 can depend on the primary antibody used for localization studies; for instance, a particular antibody can show a preference for cytoplasmic staining versus nuclear staining and vice versa (Schuler et al. 2002; Sierralta and Thole 1996).

It should be noted that during early pregnancy in the pig, luminal and glandular epithelial cells maintain ESR1 expression, albeit with immunoreactivity confined to nuclei and not the cytoplasm (Geisert et al. 1993; Knapczyk-Stwora et al. 2011). Similar to the equine conceptus, porcine conceptuses secrete significant amounts of estrogen during the time of MRP (Perry et al. 1973), which raises the likelihood of continued ESR1 expression by epithelial cells to be a unique feature to species in which conceptuses secrete large quantities of estrogen. It appears that ESR1 expression by luminal and glandular epithelial cells is retained during early pregnancy in the mare, but, in contrast to porcine, expression is retained in the cytoplasm, rather than in the nucleus. Even though the precise function of ESR1 found in cytoplasm is unknown, these receptors are likely to contribute to non-genomic actions of estrogen (Levin 2001, 2005). Continued expression of cytoplasmic ESR1 allows conceptus-derived estrogens to act upon epithelial cells and trigger events required for pregnancy maintenance in the mare. One could hypothesize that the different subcellular localizations of ESR1 in the epithelia of pregnant pigs and mares could explain the ability of conceptus-derived estrogens to block luteolysis in the pig (Geisert et al. 1990), whereas there is no clear evidence for the latter in mares.

## 9.6 Conceptus Mobility Is Essential to Maternal Recognition of Pregnancy in the Mare

Conceptus mobility is integral to maternal recognition of pregnancy in mares and can be observed as early as Day 9 after ovulation, with a marked increase in mobility on Day 10, and maximum mobility between Days 11 and 14. Restriction of conceptus mobility through experimental ligation of uterine horns results in a

decline in circulating concentrations of progesterone and return to estrus (McDowell et al. 1988). However, administration of exogenous progesterone to pregnant mares with experimentally ligated uterine horns prevents pregnancy loss, indicating that luteolysis is the cause of embryonic loss when conceptus mobility is restricted (McDowell et al. 1988). The period of maximum conceptus mobility coincides with the time of maternal recognition of pregnancy and the period of attenuated prostaglandin release by the endometrium. Conceptus mobility likely serves to distribute conceptus-derived factors over the entire surface of the endometrium. The utero-ovarian vein and the ovarian artery in the mare are clearly separate, resulting in a systemic pathway of luteolysis, i.e.,  $\text{PGF2}\alpha$  released by the endometrium reaches the ovaries via the systemic circulation (Ginther 1974). Consequently,  $\text{PGF2}\alpha$  production during maternal recognition of pregnancy has to be attenuated from the entire endometrium, explaining why conceptus mobility is integral to maintenance of pregnancy.

Coinciding with the end of conceptus migration, there is a marked decline in uterine contractility, indicating that uterine contractions are the driving force of conceptus mobility and that the embryo seems to be a direct stimulator thereof (Gastal et al. 1996). The independent migration of twin conceptuses is another indicator that the conceptus itself stimulates uterine contractility (Ginther 1985). De novo synthesis of prostaglandins is required for conceptus mobility, as administration of flunixin meglumine, a nonsteroidal anti-inflammatory drug, to pregnant mares on Days 12 and 14 after ovulation results in the immediate and marked reduction of conceptus migration (Stout and Allen 2001). Although it is generally accepted that prostaglandins stimulate uterine contractions that propel the conceptus within the uterine lumen, it does not define the site of prostaglandin production, i.e., conceptus versus endometrium. Given that the uterine lumen contains no prostaglandins during this time of pregnancy, whereas the conceptus secretes both  $\text{PGF2}\alpha$  and  $\text{PGE2}$  (Stout and Allen 2002), it is likely that the conceptus is the source of prostaglandins that stimulate uterine contractions. Alternatively, but less likely, localized release of endometrial  $\text{PGF2}\alpha$  could be caused by conceptus-derived estrogens (Stout and Allen 2001), since estrogens can stimulate secretion of  $\text{PGF2}\alpha$  by equine endometrium in vitro (Vernon et al. 1981) and in vivo (Goff et al. 1993).

Interestingly, flunixin meglumine does not reduce conceptus mobility on Day 10 of pregnancy (Stout and Allen 2001). However, Day-10 conceptuses produce much higher amounts of  $\text{PGF2}\alpha$  and  $\text{PGE2}$  per mg of tissue (Stout and Allen 2002) compared to later stages of conceptus development; therefore, flunixin meglumine concentrations may not have been sufficient to fully inhibit de novo prostaglandin synthesis. To date, no studies have addressed which of the two conceptus-derived prostaglandins,  $\text{PGF2}\alpha$  or  $\text{PGE2}$ , is the stimulator of uterine contractions. The addition of flunixin meglumine to Day-14 conceptuses in culture significantly reduced secretion of  $\text{PGE2}$ , but not  $\text{PGF2}\alpha$ , indicating that Day-14 conceptuses do not produce  $\text{PGF2}\alpha$  de novo. However, since de novo synthesis of prostaglandins is required for conceptus mobility on Day 14 (Stout and Allen 2001), it seems likely that  $\text{PGE2}$  is the conceptus-derived prostaglandin stimulating uterine contractility required for conceptus mobility. The actions of  $\text{PGE2}$  are mediated via four receptors, of which

PTGRE1 and PTGRE3 mediate smooth muscle contractility (Coleman et al. 1994). Unfortunately, expression of PTGRE1 and PTGRE3 in equine myometrium has not been reported. Everything considered, it appears that during the initial phase of conceptus mobility, conceptus-derived PGF $2\alpha$  and PGE2 contribute to conceptus mobility, whereas during the final phase of conceptus mobility, conceptus-derived PGE2 appears to be sole driver of uterine contractions. In pigs, estrogen-induced release of histamine from the endometrium has been suggested to contribute to conceptus migration (Pope et al. 1982). In mice, lysophosphatidic acid drives spacing of blastocysts, and in mice lacking the receptor for lysophosphatidic acid, blastocysts fail to distribute throughout the uterine lumen (Hama et al. 2007). Neither one of these concepts has been investigated in the mare.

Cessation of conceptus mobility, referred to as “fixation,” occurs on average 15–16 days after ovulation (Ginther 1983b; Leith and Ginther 1984). During the last day of the mobile phase, the conceptus spends significantly more time in the uterine horn in which it will become fixed, with fixation occurring predominantly in the caudal segments of uterine horns (Silva and Ginther 2006). Interestingly, the site of future fixation can be predicted 1–4 days before fixation occurs, as endometrial thickness at the mesometrial aspect at the site of future fixation increases significantly (Silva and Ginther 2006). This noteworthy predictability reflects a conceptus-maternal interaction crucial to maternal recognition of pregnancy and implantation. Cessation of conceptus migration appears to be a multifactorial event. The diameter of the developing conceptus and time of fixation are negatively correlated (Gastal et al. 1996), indicating that physical impediments contribute to fixation, i.e., the conceptus becomes too large to move through the uterine lumen. An increase in uterine tone in conjunction with encroaching endometrial folds has been hypothesized to contribute to fixation (Ginther 1983a). Coinciding with cessation of conceptus mobility, the sialic acid content of the mucin-like capsular glycoproteins decreases, which has been suggested to be “a unique developmentally regulated mechanism for the control of embryo mobility” (Oriol et al. 1993b); the equine conceptus expresses NEU2, an enzyme also known as sialidase 2, which cleaves sialic acid from polysaccharide chains. Expression of *NEU2* increases from Days 8 to 16 of conceptus development, and conceptus-conditioned medium contains functional sialidase, whereas the endometrium was not identified as a major source of *NEU2*. Therefore, developmentally regulated expression of NEU2 provides a mechanism whereby the conceptus controls sialic acid content of its own capsule (Klein and Troedsson 2012).

## 9.7 The Embryonic Capsule of the Equine Conceptus

The equine conceptus is surrounded by an acellular glycoprotein capsule during the second and third week of gestation. It is composed of mucin-like glycoproteins; galactose, N-acetylglucosamine, sulfated sugars, and sialic acid represent the majority of the carbohydrates (Oriol et al. 1993a). Capsule formation starts as the embryo

enters the uterus, and it is surrounded by the zona pellucida (Betteridge et al. 1982). Within 24 h after entry into the uterus, the zona pellucida is shed and the hatched blastocyst is covered completely by the capsule (Flood et al. 1982). The trophoblast and not the endometrium is the source of capsular material, as evident through xenogeneic transplantation of trophoblast and endometrium into immunodeficient mice (Albihn et al. 2003). Indeed, transcriptional profiling of equine conceptuses revealed developmentally regulated expression of a sialic acid transporter and sialyltransferases (Klein and Troedsson 2011). By Day 22, the capsule disappears, but the responsible mechanism remains unknown (Oriol et al. 1993b). Nevertheless, shedding of the capsule allows first intimate contact between trophoblast and uterine luminal epithelium to initiate attachment and adhesion phases of implantation.

The capsule ensures that the equine conceptus maintains its spherical shape, a prerequisite to conceptus mobility. In addition, the capsule provides mechanical resilience, so the conceptus can withstand forces exerted on it during its mobility phase. Furthermore, the high sialic acid content of the capsule may confer anti-adhesive properties that facilitate conceptus mobility (Oriol et al. 1993b). Removal of the capsule between Days 6 and 7 after ovulation followed by transfer of the blastocyst to a synchronized recipient mare results in failure to establish pregnancy, indicating that the capsule is essential for pregnancy recognition and/or maintenance of pregnancy in the horse (Stout et al. 2005).

## 9.8 Estrogen Synthesis by the Equine Conceptus

Biosynthesis of steroid hormones such as estrogens by trophoblast/chorion is common among conceptuses of domestic animals. The equine conceptus initiates steroidogenesis as early as Day 6 and synthesizes estrogens, androgens, and progesterone in measurable quantities by Day 8 of development (Paulo and Tischner 1985). After Day 12, a large increase in estrogen content occurs in yolk sac and uterine luminal fluid (Zavy et al. 1984). The increased production of free and conjugated estrogens with increasing age of the conceptus is due to an increase in cell number and not due to an increase in estrogen production on a cellular level (Choi et al. 1997b). No increase in concentrations of free estrogens in the systemic circulation has been reported, indicating the conceptus is the sole source of estrogens at the conceptus-maternal interface during early pregnancy (Zavy et al. 1984).

By Days 18–20 of pregnancy, estrone is the main estrogen present in yolk sac fluid (Raeside et al. 2009). Local metabolism of estrogens plays a role in mediating the actions of estrogen and also contributes to regulating bioavailability as estrone is much weaker estrogen than estradiol (Zhu and Conney 1998). Estradiol is metabolized to estrone by extraembryonic tissues and to a lesser extent by the embryo proper. Estrone-to-estradiol conversion only occurs to a small extent in the wall of the bilaminar yolk sac. Both the embryo proper and extraembryonic tissues conjugate estrone and estradiol with sulfoconjugation dominating over glucuronidation (Raeside et al. 2009). The endometrium also contributes to conjugation of estrone and estradiol, with levels of conjugation being higher than trophoblast tissue

(Raeside et al. 2004). Taken together, it seems that the conceptus regulates the bio-availability of the massive amounts of estrogen synthesizes through conversion of estradiol to estrone and through extensive sulfoconjugation of both estrogen and estrone. Therefore, one must likely measure estrone sulfate in blood of mares to assess intrauterine production and metabolism of estrogens.

The conceptus expresses a number of enzymes involved in the synthesis and metabolism of steroid hormones. The rate-limiting step in the production of steroid hormones is the transfer of cholesterol within mitochondria to the inner mitochondrial membrane by steroidogenic acute regulatory protein (STAR), and equine conceptuses increase expression of STAR with advancing stage of development (Klein and Troedsson 2011). Similar to the expression pattern of STAR, cytochrome P450 cholesterol side-chain cleavage enzyme (P450SCC) transcript abundance increases with age of conceptus; P450SCC catalyzes the conversion of cholesterol to pregnenolone (Klein and Troedsson 2011). Then 3-beta-hydroxysteroid dehydrogenase (HSD3B1), a key enzyme in the production of progesterone, androgen, and estrogen, is expressed by cells of the trophoctoderm/chorion (Flood and Marrable 1975), and, like STAR and HSD3B1, expression increases with stage of conceptus development (Klein and Troedsson 2011). CYP19A1, commonly known as aromatase, catalyzes the last steps of estrogen biosynthesis and is expressed by trophoctoderm and extraembryonic endoderm just beneath the embryonic disk (Walters et al. 2000). Stage-specific expressions of 17 $\beta$ -hydroxysteroid dehydrogenases occur during equine conceptus development and are proposed to contribute to conversion of estrone to estradiol and vice versa (Klein and Troedsson 2011).

The estrogens synthesized by the equine conceptus have been explored in the search for the pregnancy recognition signal; however, experiments attempting to prove that embryo-derived estrogens are responsible for extension of corpus luteum function through systemic administration of estrogen to cycling mares have been inconclusive (Vanderwall et al. 1994; Woodley et al. 1979). The prolonged production of estrogens far past the time of maternal recognition of pregnancy suggests roles for estrogens and their metabolites in development of the conceptus well beyond the time of maternal recognition of pregnancy.

High concentrations of 19-norandrostenedione and its sulfoconjugate are present in yolk sac fluid, the biological significance of which is unclear (Raeside and Christie 2008). The high concentration of androgens is likely attributable to the expression of a blastocyst-specific isoform of aromatase that favors production of 19-nortestosterone over estradiol (Choi et al. 1997a).

## 9.9 Expression of Proteins Related to Uterine Receptivity to Implantation

Regulation of several endometrial proteins has been associated with endometrial receptivity to implantation across species. Mucin 1 (MUC1), a glycoprotein with an extensive extracellular domain, is expressed by epithelial cells and acts as an anti-adhesive molecule. In several species, there is a decrease in MUC1 expression by

uterine luminal epithelia at the time of implantation, either generalized (rodents and pigs) or localized at the site of implantation (rabbits) (Bowen et al. 1996; Hoffman et al. 1998; Johnson et al. 2001). Wilsher and coworkers demonstrated the presence of a MUC1 protein at the conceptus-maternal interface at varying stages of pregnancy (20–309 days after ovulation) in the mare and concluded that implantation and placentation in the mare occur despite persistence of expression of MUC1. Notwithstanding, this should be interpreted with care, as the antibody used likely recognized an isoform of MUC1, MUC1/Y, which has a smaller extracellular domain than MUC1, reflected by its smaller molecular weight (<58 kDa versus >120 kDa for full-length MUC1) (Levitin et al. 2005). Owing to the smaller extracellular domain, MUC1/Y does not confer anti-adhesive properties. Perhaps, expression of full-length MUC1 is downregulated at fixation/implantation in the mare, either through proteolytic cleavage of its extracellular domain (Parry et al. 2001) or by preferential transcription of the splice variant MUC1/Y (Obermair et al. 2001). However, expression of MUC1 in the nonpregnant mare has not been reported.

Secreted phosphoprotein 1 (SPP1), also known as osteopontin, is an extracellular matrix protein whose differential regulation of expression during early pregnancy has been implicated in uterine receptivity in humans, pigs, mice, and sheep (Johnson et al. 2014; Liu et al. 2013; Qu et al. 2008); in that regard, SPP1 is an extracellular matrix protein that mediates conceptus adhesion by bridging trophoblast and endometrial integrins. In the mare, there are limited studies characterizing expression and localization of SPP1 at the conceptus-maternal interface. No difference in expression of *SPP1* by the endometrium during early pregnancy and diestrus was reported for mares (Hitit et al. 2014). Furthermore, *SPP1* transcript abundance decreases markedly from Days 8 to 14 of conceptus development, which has been hypothesized to contribute to the prolonged preimplantation phase of conceptus development in the horse (Klein and Troedsson 2011).

Leukemia inhibitory factor (LIF) is an indispensable cytokine for murine implantation, as blastocysts fail to implant in LIF null mice (Stewart 1994). In the equine endometrium, there is one report that there is no significant upregulation of *LIF* mRNA expression within the first 22 days of pregnancy (Hitit et al. 2014), whereas another report indicated increases in expression of LIF mRNA between Days 14 and 21 of pregnancy (Villani et al. 2010). Therefore, the functional relevance of LIF expression at the conceptus-maternal interface in the mare remains to be determined. Macrophage migration inhibitory factor (MIF) is another cytokine implicated in reproductive processes at the fetal-maternal interface during pregnancy. In that regard, MIF is expressed by equine conceptuses and endometria, and it was postulated that “MIF is part of the molecular repertoire that contributes to normal endometrial function” (Klein and Troedsson 2013).

Fibroblast growth factor 2 (FGF2), along with the corresponding receptors FGFR1–4, is expressed at the conceptus-maternal interface in the mare (de Ruijter-Villani et al. 2013). Endometrial expression increases as early pregnancy advances, with FGF2 localizing to luminal and glandular epithelial cells of the endometrium.

Although the role of FGF2 during early pregnancy in the mare is unknown, its pregnancy-specific increase in expression suggests that it contributes to pregnancy maintenance, in particular during the fourth week of pregnancy.

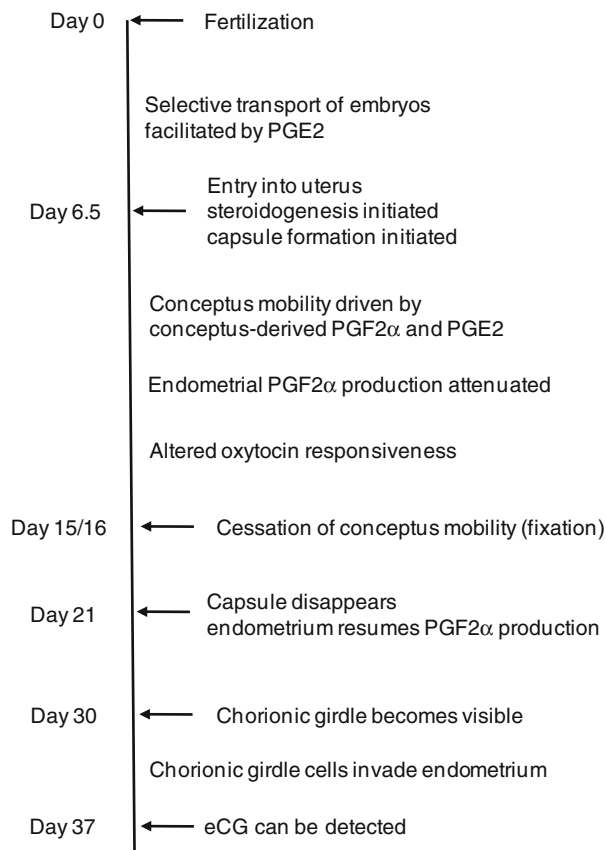
During the extended pre-attachment period, the conceptus relies on nutrients delivered through protein-rich uterine secretions termed histotroph (Zavy et al. 1982). The predominant protein of uterine histotroph in mares is uterocalin (P19), a member of the lipocalin family of proteins which transport small hydrophobic molecules (Flower et al. 1993). Large amounts of P19 are present in uterine flushings during the first 23 days of pregnancy (Stewart et al. 1995), and *P19* is the most abundant endometrial transcript at Day 16 of pregnancy (Klein 2015). Within the endometrium, expression of P19 mRNA and protein is restricted to the luminal and glandular epithelia (Crossett et al. 1996, 1998). Endogenous and exogenous progesterone stimulate P19 expression; however, disappearance of expression of P19 in the third week of pregnancy suggests that factors other than progesterone regulate its expression. The capsule of the developing conceptus contains large amount of P19 protein although there is the absence or low expression of *P19* mRNA, indicating that the capsule takes up or binds P19 from uterine secretions (Crossett et al. 1998). Equine uterocalin binds fatty acids and retinol; therefore, it seems likely that its main function is to deliver nutrients to the developing conceptus (Suire et al. 2001). Exposure of in vitro-produced equine embryos to P19 improves capsule formation, indicating a likely function of P19 (Smits et al. 2012).

## 9.10 The Endometrial Cup Reaction

By the fourth week of pregnancy, a distinct, temporary structure appears on the trophoblast at the interface between the expanding allantochorion and the regressing yolk sac, the chorionic girdle. Following a phase of rapid proliferation and transformation into binucleate cells, the cells of the chorionic girdle gain an invasive phenotype and penetrate through the luminal endometrial epithelium, resulting in the disappearance of the chorionic girdle from the surface of the conceptus (Enders and Liu 1991). Soon thereafter, the endometrial cups become visible as slightly raised pale white plaques on the endometrial surface (Yamauchi 1975). The horse is unique among domestic animals in the production of a chorionic gonadotropin (eCG, equine chorionic gonadotropin), which is the result of the endometrial cup reaction. eCG can be detected as early as Days 37–41, peaks between Days 60 and 75, after which time it starts to decline and disappears by Days 120–150 of gestation (Evans et al. 1933). eCG displays a remarkably long biological half-life (Catchpole et al. 1935), and its LH function induces luteinization of theca and granulosa cells of follicles resulting in the development of secondary corpora lutea which can be found between Days 40 and 150 of gestation (Amoroso et al. 1948; Cole et al. 1931). Formation of these secondary corpora lutea is essential to maintenance of pregnancy; they are the sole source of progesterone as progesterone production by

the primary CL wanes until onset of production of progestins by the chorion (Squires and Ginther 1975).

Invasion of chorionic girdle cells provokes an immune reaction evident through the accumulation of lymphocytes around the endometrial cups (Grünig et al. 1995). Invasive chorionic girdle cells express high levels of paternal major histocompatibility complex class I (MHC I) antigens (Donaldson et al. 1990), and an immunological mechanism leading to the temporary lifespan of the endometrial cups has been suspected for a long time. This hypothesis had to be revised, however, given that MHC-compatible pregnancies (Antczak et al. 1982) and prior immunological sensitization of mares to paternal MHC antigens (Adams et al. 2007) do not alter lymphocyte accumulation or lifespan of the endometrial cups. It seems that a mechanism intrinsic to the cells of endometrial cups determines their life cycle (Fig. 9.1).



**Fig. 9.1** Timeline of events during early pregnancy in the mare



## 9.11 Conclusions

Indirect conclusions can be drawn only about the time frame during which maternal recognition of pregnancy occurs. The conceptus has to be recognized before Day 14 after ovulation, as this is the time that luteolysis is initiated in the mare (Ginther et al. 2011). Although expression of oxytocin receptors is similar for nonpregnant and pregnant mares on Days 10 and 12 after ovulation, expression of oxytocin receptors is less for pregnant than cyclic mares on Day 14 after ovulation (de Ruijter-Villani et al. 2014; Sharp et al. 1997; Starbuck et al. 1998). Continuous infusions of oxytocin from 8 days after ovulation prolong luteal phase, whereas continuous infusions of oxytocin from 10 days after ovulation initiate luteolysis (Stout et al. 1999), indicating that maternal recognition of pregnancy is initiated by Day 10 post-ovulation. Wilsher and coworkers (2010) recently suggested reevaluation of the concept that maternal recognition of pregnancy occurs on or before Day 10. Surprisingly, all six asynchronous transfers of 10-day-old blastocysts into recipient mares on Day 12 of diestrus resulted in establishment of pregnancy and survival of the conceptus to the “heartbeat stage” of development of the embryo (end of observation period). Notwithstanding, various mechanisms lead to luteal maintenance when using repeated/continuous administration of oxytocin versus transfer of a blastocyst as in vitro factors secreted by the conceptus lead to a rapid (within 24 h) reduction in secretion of PGF2 $\alpha$  by endometrial explant cultures (Ealy et al. 2010). It seems likely that the mechanism leading to luteal maintenance via repeated or continuous treatments with oxytocin must be initiated well before maternal recognition of pregnancy occurs.

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