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Regulation of Implantation and Establishment of Pregnancy in Mammals

Tribute to 45 Year Anniversary of Roger V. Short's "Maternal Recognition of Pregnancy"

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Chapter 1
Introduction

Rodney D. Geisert

Abstract Establishment and maintenance of pregnancy in a number of mammalian species depends upon a tightly regulated interaction between the semiallogeneic conceptus and the maternal uterine endometrium. The term "Maternal Recognition of Pregnancy" is attributed to Roger V. Short's paper titled "Implantation and the Maternal Recognition of Pregnancy" which was published in proceedings from the 1969 Symposium on Foetal Autonomy. Professor Short's landmark paper stimulated increased interest in elucidating how the conceptus signals its presence to assure maintenance of the corpus luteum beyond the normal length of the estrous or menstrual cycle to allow pregnancy to be established and maintained. Ten years following publication of Professor Short's paper, a Ciba Foundation Symposium entitled "Maternal Recognition of Pregnancy" brought together leading scientists to discuss the multiple mechanisms and pathways by which different viviparous species establish a successful pregnancy. The present volume on "Regulation of Implantation and Establishment of Pregnancy in Mammals" brings together current reviews from leading experts to address the diversity of mechanisms by which species establish and maintain pregnancy. Implantation in mice, dogs, pigs, cattle, sheep, horses, primates, humans and species in which embryonic diapause occurs are discussed. Reviews will provide current knowledge on the role of endometrial steroid receptors, adhesion factors, cytokines, interferons, steroids, prostaglandins, growth factors and immune cells involved with regulation of conceptus development.

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Establishment and maintenance of pregnancy in a number of mammalian species depends upon a tightly regulated interaction between the semiallogeneic conceptus and the maternal uterine endometrium. The term "Maternal Recognition of Pregnancy" is attributed to Roger V. Short's paper titled "Implantation and the Maternal Recognition of Pregnancy" which was published in proceedings from the 1969 Symposium on Foetal Autonomy (Short, 1969). Professor Short's landmark paper stimulated increased interest in elucidating how the conceptus signals its presence to assure maintenance of the corpus luteum beyond the normal length of the estrous or menstrual cycle to allow pregnancy to be established and maintained. To gain an historical perspective on maternal recognition of pregnancy, I recommend that every graduate student and young investigator involved with reproductive biology read his review paper. The following quote, taken from the introduction of Professor Short's paper, indicates that the establishment of pregnancy involves more than a simple biological pathway to "rescue" the corpus luteum from regressing during pregnancy and outlines the fundamental questions regarding pregnancy recognition signaling mechanisms which researchers today continue to investigate across a diverse variety of species.

> The maternal organism first becomes aware of the presence of an embryo in the uterus in diverse ways. In most mammals, this critical piece of information must be relayed to the mother at an early stage of gestation, and we will begin by considering in general terms both the nature of the message and the mode of its transmission. We shall then be in a position to investigate variations on the basic pattern, species by species.
>
> One of the first outward and visible signs that an embryo has made its presence felt in the uterus is when the corpus luteum of the cycle becomes transformed into a corpus luteum of pregnancy, and estrous or menstrual cycles cease to recur. Let us therefore examine this luteotropic action of the conceptus in a little more detail. Can the stimulus be initiated by the embryo before it has achieved an anatomical union with the endometrium? Is the stimulus itself mechanical in nature, giving rise to afferent neural stimuli to the hypothalamus, which in turn bring about the release of luteotropic hormone(s) from the anterior pituitary, or does the conceptus have a hormonal action, elaborating its own luteotropic substances? In those species in which the endometrium of the nonpregnant uterus seems to produce a luteolytic factor, how does the embryo act to neutralize this effect? These are some of the questions to which we must attempt to the find the answers. Furthermore, it may be a mistake to concentrate all our attention on luteal maintenance as the first premonition of a pregnancy; fundamental differences between the pregnant and nonpregnant animal may begin to become apparent soon after fertilization, and in a number of species, the lifespan and secretory activity of the corpus luteum is unaffected by pregnancy. Undoubtedly much still lies outside our comprehension in this most fascinating area of investigation (Short 1969).

Ten years following the publication of Professor Short's paper, a Ciba Foundation Symposium entitled "Maternal Recognition of Pregnancy" (Ciba Foundation Symposium, 1979) brought together leading scientists to discuss the multiple mechanisms and pathways by which different viviparous species establish a successful pregnancy. The diversity of mechanisms to establish and maintain luteal function alone are clearly evident from the following species variation: (1) in the bitch, the corpora lutea (CL) are maintained for the length of pregnancy whether or not mating occurs; (2) in mice and rats, a sterile mating extends the lifespan of the CL from 4 to 12 days through vaginal stimulation by the erect penal spines (Fig. 1.1) that

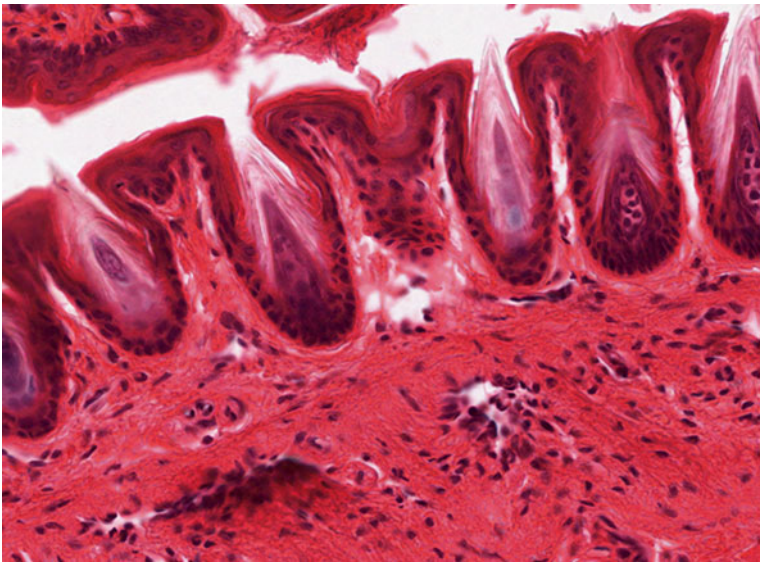


Fig. 1.1 Penile spines of the glans penis of rats stimulate the vagina of the female during mating to extend CL lifespan beyond a normal 4-day estrous cycle. If maternal recognition of pregnancy is considered extending CL function beyond the estrous cycle, the penile spines could be considered one of the earliest signaling mechanisms evoked even before fertilization. However, establishment of a pregnancy by viable blastocysts/conceptuses involves a more elaborate interaction between the maternal endometrium and implanting conceptus. Note that the short estrous cycle, mating-induced CL extension, and the ability to induce embryonic diapause place mice and rats among the more efficient and prolific species of mammals for reproduction

induce the diurnal release of prolactin; (3) in humans and subprimates, release of a conceptus-derived factor (chorionic gonadotrophin, CG) acts directly on CL to maintain function; and (4) the release of conceptus-derived factors indirectly inhibits the release or production of luteolytic pulses of prostaglandin F2α (luteolysin) from the endometrium. Of course, PGF2α had not been identified as a luteolytic hormone at the time of Short's paper. However, maternal recognition of pregnancy involves considerably more than extending luteal function. The attaching or implanting conceptus must stimulate adequate maternal blood flow to the placenta for transfer of oxygen and nutrients and induce the maternal endometrium to provide the spatiotemporal pattern of secretions and nutrient transport mechanisms necessary for continued development and survival of the conceptus throughout pregnancy while altering the maternal immune system to prevent rejection of the semiallogeneic conceptus.

Over the past few decades, technological advances in transcriptomics, proteomics, metabolomics, and glycomics along with the ability to selectively knockout genes of interest has greatly advanced our understanding of maternal-conceptus interactions that are essential for the establishment and maintenance of a successful

pregnancy. This knowledge provides a foundation from which to build research endeavors to help resolve infertility, embryonic loss, and recurrent abortion in humans, captive wild animals, and important farm species. The present volume on "Regulation of Implantation and Establishment of Pregnancy in Mammals" brings together current reviews from leading experts to address the diversity of mechanisms by which species establish and maintain pregnancy. Implantation in mice, dogs, pigs, cattle, sheep, horses, primates, humans, and species in which embryonic diapause occurs are discussed. Reviews will provide current knowledge on the role of endometrial steroid receptors, adhesion factors, cytokines, interferons, steroids, prostaglandins, growth factors, and immune cells involved with regulation of conceptus development. This knowledge provides a foundation for the development of strategies to resolve infertility, embryonic loss, and recurrent abortion in humans, captive wild animals, and important farm animal species in the future.

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Chapter 2
History of Maternal Recognition of Pregnancy

Fuller W. Bazer

Abstract The mechanism for signaling pregnancy recognition is highly variable among species, and the signaling molecule itself varies between estrogens in pigs to chorionic gonadotrophin in primates. This chapter provides insight into the menstrual cycle of women and estrous cycles of rodents, dog, cat, pigs, sheep, rabbits, and marsupials, as well as the hormones required for pregnancy recognition. Pregnancy recognition involves specific hormones such as prolactin in rodents or interferons in ruminants and estrogens in pigs that in their own way ensure the maintenance of the corpus luteum and its secretion of progesterone which is the hormone of pregnancy. However, these pregnancy recognition signals may also modify gene expression in a cell-specific and temporal manner to ensure the growth and development of the conceptus. This chapter provides some historical aspects of the development of understanding of mechanisms for the establishment and maintenance of pregnancy in several species of mammals.

2.1 Introduction

Professor Roger V. Short presented a historic paper with respect to pregnancy recognition at a Ciba Foundation Symposium in 1969 (Short 1969). Papers presented at that meeting and, in particular, the paper by Short (1969) were historic in introducing and clarifying terms like luteotrophic, luteolytic, and antiluteolytic, as well as concepts of uterine-independent ovarian cycles in primates and uterine-dependent ovarian cycles in subprimate species. An example of a luteotrophic signal was chorionic gonadotropin (CG) that acts directly on the corpus luteum (CL) to maintain or increase secretion of progesterone. The common luteolytic factor or hormone is recognized to be prostaglandin $F_{2\alpha}$ (PGF) which causes regression of the CL and cessation of secretion of progesterone. Antiluteolytic signals were not well known

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in 1969, but available evidence indicated that they either prevented actions of the luteolytic hormone PGF or abrogated a mechanism to prevent PGF from inducing luteolysis. We now recognize the most common pregnancy recognition signals in subprimate species as estrogens in pigs and interferon tau in ruminants. Short (1969) noted that a luteotrophic signal seemed unique to humans and other primates, but not pigs, sheep, or horses, and that species such as the red kangaroo do not appear to have a pregnancy recognition signal. Further, luteotrophic signals for pregnancy recognition, such as CG, stimulate the production of progesterone while the production of progesterone by the CL is merely sustained in species producing an antiluteolytic signal. There was also recognition that mechanisms for pregnancy recognition signaling were very diverse and that some species, such as roe deer, experience a long period of blastocyst diapause which makes defining the time of maternal recognition of pregnancy problematic.

If we now move ahead to the symposium on maternal recognition of pregnancy at the Ciba Foundation in London in May 1978, presentations by various scientists indicated solid advances in understanding pregnancy recognition signaling in several species including humans, laboratory animals, and livestock. Ross (1979) reported that lutectomy before the 7th week of pregnancy resulted in abortion in women and that abortion could be prevented by administering exogenous progesterone. It was also established that the trophectoderm secretes CG in humans and that declining concentrations of progesterone in serum of women could be prevented by injections of CG; therefore, Ross (1979) proposed that human CG is the pregnancy recognition signal which sustains the production of progesterone by CL in women who become pregnant. Flint et al. (1979) reported on the importance of estrogens secreted by the pig conceptuses between days 10 and 12 after the onset of estrus and suggested that estrogens act to reduce uterine secretion of PGF which was recognized as the luteolytic hormone in pigs. Flint et al. (1979) also reported (1) the lack of evidence for a pregnancy recognition signal in certain carnivores, e.g., dog, ferret, and marsupials; (2) the lack of transport of unfertilized ova from the oviduct into the uterus in the mare; (3) the requirement for progesterone and estrogen for implantation in rodents; and (4) systemic effects of estrogens on CL function in pigs based on studies by Kraeling et al. (1975). Poyser and Walker (1979) provided a summary of information regarding the luteolytic role of PGF in guinea pigs and evidence for an undefined antiluteolytic factor in that species, as well as evidence that PGF is the luteolytic hormone in uterine vein blood of sheep, pig, cow, horse, and rabbit. Finally, Short (1979) concluded that genetic anomalies in oocytes or embryos result in abnormal conceptuses that fail to signal pregnancy recognition.

2.2 Pregnancy Recognition Signaling

The endocrinology of recurring estrous/menstrual cycles and pregnancy in primates, ruminants, swine, horses, cats, dogs, and rodents and hormonal signaling for maternal recognition of pregnancy will be discussed briefly in subsequent sections of this

review. The estrous cycle of subprimate species is uterine dependent, because the uterus is the source of PGF, the luteolytic hormone responsible for functional and structural regression of ovarian CL in the absence of an appropriate maternal recognition of pregnancy signal. In primates, the menstrual cycle is uterine independent since luteolytic PGF derives from an intra-ovarian source and conceptus trophectoderm-derived CG acts directly on the CL as the pregnancy recognition signal to sustain or increase secretion of progesterone. In mice and rats, mating-induced pulses of prolactin from the maternal anterior pituitary gland and lactogenic hormones from the uterine decidua and placenta are luteotrophic for the formation and maintenance of CL, but luteolysis is dependent on actions of PGF in the event that mice or rats fail to establish pregnancy. Sterile mating or early loss of embryos results in a period of pseudopregnancy (12 days). In rodents, CL maintenance beyond day 12 of gestation is supported by lactogenic hormones from the uterine decidua and placenta that replace the maternal source of PRL after day 8 until term. During the peri-implantation period of pregnancy in subprimate species, maternal recognition of pregnancy signals from the conceptus are antiluteolytic as they modify uterine release of luteolytic PGF to prevent luteolysis; however, the antiluteolytic signals do not inhibit basal secretion of PGF.

2.3 Local Versus Systemic Pathways for PGF-Induced Luteolysis

Ginther (1976) described the role and nature of the utero-ovarian vasculature of ewe, cow, sow, mare, laboratory rodents, monkeys, dogs, and cats that determines in large part whether luteolytic effects of PGF are mediated locally or systemically. In sheep, for example, PGF must be transferred locally within the utero-ovarian vascular pedicle from uterine venous blood to ovarian artery in sufficient amounts to induce luteolysis. The local transfer of PGF for luteolysis is required because the ewe's lungs convert 99 % of the PGF to its inactive metabolite PGFM (15-keto, 13, 14 dihydro-PGF) which precludes systemic luteolytic effects of PGF (Davis et al. 1980).

In contrast to the ewe, the mare does not have a utero-ovarian vascular pedicle for local transfer of PGF from uterine venous drainage to ovarian artery; therefore, PGF acts systemically to induce luteolysis. A comparison of plasma clearance and half-life of PGF between heifers and mares revealed that for mares (1) plasma clearance was five times less, (2) maximum concentrations of PGF in plasma were five times greater, and (3) the distribution half-life and elimination half-life for PGF were three times longer (Shrestha et al. 2012). Those results indicate that the lungs of the heifer are significantly more efficient in converting PGF to PGFM.

The equine conceptus produces a factor that inhibits uterine release of luteolytic PGF (Sharp et al. 1989). In cycling mares, concentrations of PGF in uterine venous plasma and uterine flushings increase between days 14 and 16 when luteolysis occurs. In pregnant mares, the equine conceptus does not elongate, so it migrates between the two uterine horns exerting its antiluteolytic effect (Leith and Ginther 1984; Stout and

Allen 2001). The conceptus-derived antiluteolytic mechanism results in reduced amounts of PGF in uterine fluids and uterine venous plasma and PGFM in peripheral blood, and endometrial production of luteolytic PGF in response to endogenous and exogenous OXT is abrogated. Thus, the migrating conceptus antiluteolytic mechanism presumably reduces expression of endometrial OXTR or sensitivity of the uterus to OXT. Equine conceptuses produce estrogens and unique proteins such as interferon delta, between days 8 and 20 of gestation (Tayade et al. 2008), but those molecules are not known to be pregnancy recognition signals in mares.

For pigs, the estimated conversion of PGF to PGFM in one pass via the lungs is only 19 % (Davis et al. 1980), so there is likely a local luteolytic effect of PGF via the utero-ovarian vascular pedicle and a systemic effect of PGF as the luteolytic hormone. In unilaterally pregnant pigs, the CL ipsilateral to the nonpregnant uterine horn regress first and then the CL on the contralateral ovary regress (Flint et al. 1982). Therefore, it is critical that pig conceptuses elongate rapidly to expose the uterine endometrium to estrogens and prevent endocrine secretion of PGF. Polge et al. (1966) reported that four or more elongated conceptuses within the uteri of gilts are required to produce sufficient estrogens for the establishment of pregnancy.

2.4 Primates (*Homo sapiens* and *Macaca mulatta*)

2.4.1 The Menstrual Cycle

The mechanism(s) responsible for luteolysis in primates has not been clarified, but several possibilities have been suggested (see Stouffer and Hearn 1998). Estradiol (E2) may act directly on luteal cells of primates to initiate luteolysis or suppress secretion of luteinizing hormone (LH) required for luteal maintenance; however, evidence for this E2-mediated luteolytic pathway is controversial. Similarly, intra-ovarian PGF may act directly on the primate CL to initiate luteolysis in the absence of stimulatory effects of CG during pregnancy. Inhibitors of PGF synthesis do not extend CL lifespan, whereas intra-luteal infusion of PGF, but not other prostaglandins, causes premature luteolysis in monkeys. Therefore, PGF may be an intra-ovarian paracrine or autocrine hormone that acts in concert with other intra-ovarian peptides/proteins to regress CL in primates. Nevertheless, regression of the CL is clearly uterine independent in primates as hysterectomy does not alter cyclic ovarian function in either women or monkeys.

2.4.2 Pregnancy in Women and Other Primates

Maternal recognition of pregnancy signaling extends CL function throughout pregnancy or until the time of the luteal-placental shift when the production of progesterone by the placenta is adequate to support pregnancy in the absence of

a CL (see Fazleabas et al. 2004). CG alone appears to be the maternal recognition of pregnancy signal in nonhuman primates and humans. There are also effects of CG on uterine receptivity to implantation and pregnancy in primates (see Fazleabas et al. 2004). Although there is no evidence that either prolactin (PRL) or placental lactogen (CSH1) is luteotropic for primate CL, an excellent review of roles of those hormones with emphasis on CG, PRL, and CSH1 has been published (see Ben-Jonathan et al. 2008). Pregnancy recognition signaling, implantation, and uterine decidualization in primates are discussed in detail in Chap. 12.

2.5 Rodents (Rat, *Rattus norvegicus*; Mouse, *Mus musculus*)

Pregnancy recognition signaling appears to be similar for mice and rats. Deansely (1966) noted that the maintenance of adequate amounts of progesterone was required for pregnancy in rodents. Morphological changes in CL of pregnant mice were reported by Choudary and Greenwald (1969) to be associated with a shift from dependency on maternal prolactin, to day 6 of pregnancy, to LH on days 9 and 10 of pregnancy and then placental "luteotrophins" for the remainder of pregnancy which was consistent with other published results (Newton and Beck 1939; Cerruti and Lyons 1960). However, more recent reviews provide clarity to mechanisms for pregnancy recognition in rodents (see Soares et al. 2007; Ben-Jonathan et al. 2008).

2.5.1 Pseudopregnancy and Pregnancy

Extension of CL lifespan in rodents beyond day 12 requires the presence of viable conceptuses within the uterus. The establishment and maintenance of pregnancy requires two endocrine events. First, mating elicits diurnal and nocturnal surges of PRL from the maternal anterior pituitary that increase LHCGR on luteal cells for the formation of CL and suppress aldo-keto reductase family 1, member 1 (AKR1C1) activity in CL to prevent the conversion of progesterone to 20α-OH progesterone. The maintenance of pregnancy beyond day 12 in rodents is dependent on implantation, conceptus development, and production of lactogenic hormones by uterine decidua and placentae. Those lactogenic hormones replace PRL from the maternal anterior pituitary to maintain CL function for the production of progesterone through the remainder of gestation. The main luteotropic hormone of uterine decidual cells is PRL that maintains secretion of P4; however, various forms of lactogenic hormones produced by the placentae of rodents maintain secretion of progesterone by CL during pregnancy (see Soares et al. 2007; Ben-Jonathan et al. 2008). In-depth discussions of pregnancy in rodents can be found in Chaps. 2, 3, and 4.

2.6 Swine (*Sus domestica*)

2.6.1 The Estrous Cycle and Luteolysis

Gilts and sows are spontaneously ovulating, polyestrous, litter-bearing pigs that reach puberty between 4 and 9 months of age and have recurring estrous cycles of 18–21 days throughout the year (see Anderson 1993). Behavioral estrus lasts for 24–72 h and ovulation occurs 36–42 h after onset of estrus. Corpora lutea are well formed by day 4 or day 5 of the estrous cycle, and progesterone secretion increases from that time to maximum production between days 12 and 14 of the estrous cycle (Guthrie et al. 1972). Luteal regression begins on about day 15 in nonpregnant females, and concentrations of progesterone in plasma decline rapidly to basal levels (1 ng/ml or less) by day 17, leading to recurrent estrous cycles unless interrupted by pregnancy.

The pig uterine endometrium is the source of luteolytic PGF responsible for morphological regression of CL and cessation of progesterone secretion in the absence of pregnancy. Loeb (1923) noted that hysterectomy of guinea pigs during the luteal phase of the estrous cycle allowed prolonged CL maintenance and this was later demonstrated in pigs (Spies et al. 1958; Du Mesnil Du Buisson and Dauzier 1959). Anderson et al. (1969) found that bilateral hysterectomy in the early- to mid-luteal phase of the estrous cycle resulted in CL maintenance 114 or more days, and this was also true for gilts in which there was the absence of endometrial epithelia or congenital absence of the uterine endometrium (Anderson et al. 1969). PGF is luteolytic when injected into the uterine lumen or intramuscularly in swine on day 12 or later of the estrous cycle (see Bazer et al. 1982). Pig CL are refractory to PGF until day 12 which is consistent with reports that the CL are "autonomous" with respect to the lack of a need for pituitary support for the first 12 days of the estrous cycle (Du Mesnil Du Buisson and Dauzier 1959; Moeljono et al. 1977). Henderson and McNatty (1975) suggested that pig CL remain refractory to about day 12 when conformational changes within the luteal cell membrane facilitate PGF binding. The PGF was proposed to inactivate the adenyl cyclase system to inhibit progesterone secretion and activate lysosomal enzymes responsible for morphological regression of the CL. Guthrie and Rexroad (1981) reported that a single injection of hCG on day 12 of the cycle temporarily blocked luteolysis, but daily gonadotropin replacement therapy did not override luteolytic effects of PGF and prevent the return to estrus (Du Mesnil Du Buisson 1966). Endometrial extracts from days 13 to 17 of the estrous cycle and day 19 of pregnancy exert a luteolytic effect in unilaterally pregnant gilts having induced CL. and this effect is blocked by indomethacin that inhibits PTGS2 (Christenson and Day 1972; Patek and Watson 1976; Watson and Patek 1979). PGF is produced in vitro by pig endometria from days 8, 12, 14, 16, and 18 of the estrous cycle (Guthrie and Rexroad 1981), but the production of PGF was significantly greater on days 16 and 18 compared to days 8, 12, and 14. Later, concentrations of immunoreactive PGF in utero-ovarian vein plasma are greater and secreted in a pulsatile manner during the period of expected luteolysis

in nonpregnant gilts as compared with pregnant gilts (Gleeson et al. 1974; Killian et al. 1976; Moeljono et al. 1977; Frank et al. 1977, 1978). Thus, endometrial production of PGF is greater during the period of luteolysis, and exogenous PGF is luteolytic in gilts after day 12 of the estrous cycle.

Endocrine requirements for luteolysis in pigs are not well defined. Whereas OXT from CL and posterior pituitary act via uterine OXTR to elicit pulsatile release of PGF in ruminants, the CL of pigs contain little OXT and vasopressin. Thus, the role(s) of these neuropeptides in luteolysis in pigs is not known. Interestingly, the uterine endometrium is a source of OXY in pigs, and exogenous OXT decreases the inter-estrous interval in gilts when administered between days 10 and 16 post-estrus, but not when administered to ovary-intact hysterectomized gilts, suggesting that the effect of OXT is uterine dependent (Mirando et al. 1995). The endometrium of pigs contains receptors for OXT and lysine vasopressin but only responds to OXT with increased secretion of PGF, whereas both OXT and vasopressin stimulate inositol phosphate turnover indicative of stimulation of the protein kinase C and calcium-calmodulin kinase cell signaling pathways. OXT stimulates phospholipase C activity, and phosphatidylinositol hydrolysis increases intracellular concentrations of calcium and diacylglycerol which activate protein kinase C and calcium-calmodulin kinase to activate phospholipase A2 which generates arachidonic acid for the synthesis of PGF in pigs. Although concentrations of OXT increase in the peripheral circulation of pigs during luteolysis, OXT-induced increases in circulating concentrations of 13,14-dihydro-15-keto-PGF (PGFM), the inactive metabolite of PGF, are lower in pregnant than cyclic gilts or gilts induced into pseudopregnancy by injection of exogenous E2 from day 11 to day 15 post-estrus. However, prostaglandins are critical for the establishment of pregnancy in pigs as inhibition of PTGS2 results in pregnancy failure and concentrations of PGFM in the circulation of pregnant gilts increase beginning on day 12.

2.6.2 Pregnancy

After hatching from the zona pellucida, pig blastocysts expand and undergo a rapid morphological transition to spherical (10–15 mm diameter), tubular (15 mm by 50 mm), and filamentous (l mm by 100–200 mm) forms between days 10 and 12 of pregnancy and achieve a length of 800–1000 mm between days 12 and 15 of pregnancy (see Bazer 1992, 2013b). During this period of rapid elongation of the conceptus, the trophectoderm produces estrogens, as well as IFN gamma (IFNG) and IFN delta (IFND). The pregnancy recognition signal in pigs is estrogen with estradiol-17β (E2) being the dominant form produced by the conceptus between days 11 and 12 and then days 15–30 of pregnancy. Estrogen induces undefined cellular events that direct secretion of PGF away from the uterine vasculature and into the uterine lumen (exocrine secretion) where it is sequestered and metabolized to prevent luteolysis. In nonpregnant gilts, PGF is released from the uterine endometrium into the uterine venous drainage (endocrine secretion) to be transported

systemically to the CL to induce luteolysis. The "endocrine-exocrine theory of pregnancy recognition" in pigs was published in 1977 (Bazer and Thatcher 1977).

In a seminar in 1977, I discussed observed changes in the direction of uteroferrin release as being basolateral from uterine GE into the stromal tissue in cyclic gilts but release being into the uterine lumen of pregnant gilts (Chen et al. 1975). This led Professor Donald H. Barron to note that the uterine epithelia of pigs can secrete in both an endocrine and an exocrine manner. That comment was then extrapolated to results of our studies of PGF in blood from the uterine vein versus the uterine lumen, and the manuscript on the theory of maternal recognition of pregnancy in pigs was drafted the evening of that same day. The paper was accepted with little change and published in *Prostaglandins* (Bazer and Thatcher 1977). A detailed account of pregnancy recognition signaling in pigs is presented in Chap. 9.

2.7 Ruminants: Sheep, Cows, and Goats

2.7.1 Estrous Cycle and Luteolysis

The estrous cycle of ruminants is uterine dependent as the uterine endometrium is the source of luteolytic pulses of PGF. During diestrus, progesterone increases phospholipid stores and prostaglandin synthase 2 (PTGS2) in uterine epithelia. Arachidonic acid generated by phospholipase A2 is converted by PTGS2 to substrates for the synthesis of PGF during late diestrus. Exposure of the uterus to progesterone for 10–12 days results in downregulation of progesterone receptors (PGR) which allows an increase in expression of receptors for estrogens (ESR1) and oxytocin (OXTR) initially in uterine LE/sGE and then GE and stromal cells. Following upregulation of ESR1 and OXTR in uterine epithelia, E2 induces phospholipase A2 to mobilize arachidonic acid for the conversion to PGF and oxytocin (OXT) from the CL, and the posterior pituitary acts via OXTR to induce pulsatile release of luteolytic PGF that culminates in the regression of the CL. If ewes are hysterectomized during the luteal phase of the estrous cycle, the source of luteolytic PGF is absent and lifespan of the CL is prolonged to about 5 months which is similar to the duration of a normal pregnancy.

2.7.2 Pregnancy

Rowson and Moor (1967) reported that ovine conceptuses secrete a substance that extends CL lifespan. Transfer of a blastocyst into the ligated uterine horn contralateral to the ovary bearing the cyclic CL in ewes had no effect to extend the lifespan of CL; however, when the conceptus or a homogenate of conceptuses was introduced into the uterine horn ipsilateral to the CL, a functional CL was maintained.

Thus, the conceptus factor acted locally to prevent regression of the CL, and it was determined that the antiluteolytic factor was thermolabile, inactivated by proteases, and produced by conceptuses between days 12 and 21 of pregnancy (Rowson and Moor 1967; Moor 1968; Moor and Rowson 1966a, b).

The estrogen-dependent endocrine-exocrine theory of pregnancy recognition in pigs (Bazer and Thatcher 1977) biased our thinking toward that being a common mechanism for pregnancy recognition in sheep and cows. Accordingly, we studied steroid metabolism by uterine endometria and conceptuses from sheep and cows only to find no evidence for the secretion of significant amounts of estrogens by sheep or cow conceptuses during the period of pregnancy recognition (Eley et al. 1979). Therefore, we cultured sheep conceptuses in the presence of radiolabeled amino acids as we were doing for studies of proteins secreted by pig endometrial explants cultures (Basha et al. 1979). The ovine conceptus-conditioned culture medium was analyzed and found to contain significant amounts of a low molecular weight radiolabeled protein originally named Protein X and now known as interferon tau (IFNT) (Wilson et al. 1979). Low molecular weight radiolabeled proteins were also found to be secreted by cow conceptuses (Lewis et al. 1979). Protein X was introduced into the uterine lumen of cyclic ewes and found to increase the lifespan of the CL of ewes (Godkin et al. 1982, 1984). Protein X had a 14 kDa contaminating protein later determined to be galectin 15 (Gray et al. 2004). Protein X was renamed ovine trophoblast protein 1 (oTP1). During that same period, Martal et al. (1979) reported purification of "trophoblastin" that is equivalent to oTP1.

Dr. James Lauderdale, a colleague of Dr. Thatcher and I, obtained permission for Dr. Russell Anthony, a postdoctoral fellow with us, to work in the laboratories of Drs. K. R. Marotti and H. G. Polites at the Upjohn Company to clone the gene for oTP1. This effort was continued after Dr. Michael Roberts' laboratory moved to the University of Missouri. The gene for oTP1 was cloned and sequenced at the Upjohn Company to reveal that it is a type 1 interferon later designated interferon tau (IFNT) by the International Cytokine and Interferon Society (Imakawa et al. 1987; Roberts 1993). In the meantime, we used highly purified IFNT to demonstrate its potent antiviral, antiproliferative, and immunosuppressive activities and initial insight into its structural motif (Pontzer et al. 1988, 1990, 1991, 1994; Jarpe et al. 1994). We produced a synthetic gene for IFNT, expressed it in the *Pichia pastoris* yeast system, and showed that its biological activities were equivalent to those of native IFNT (Ott et al. 1991; VanHeeke et al. 1996). We also conducted experiments to unravel the mechanism whereby IFNT prevents luteal regression to allow the establishment of pregnancy in ewes (see Thatcher et al. 1989; Meyer et al. 1995; Newton et al. 1996; Bazer 2013a, b) using Dr. John McCracken's model of the "progesterone block" for the regulation of the estrous cycle in ewes (Schramm et al. 1983). Results supported our hypotheses relevant to mechanisms whereby IFNT acts to signal pregnancy recognition in sheep and other ruminant species. An in-depth discussion of pregnancy recognition signaling in ruminants is provided in Chap. 10.

2.8 Horse (*Equus ferus caballus*)

2.8.1 Estrous Cycle and Luteolysis

Mares are seasonally polyestrous, with the onset of cyclicity beginning in late winter in the Northern Hemisphere (see Irvine 1995). Ovarian activity is inhibited by decreasing day length (photoperiod) but often lags several months after the solstice. The effects of photoperiod appear to be regulated by melatonin from the pineal gland. Mares typically exhibit estrous cycles of 21–22 days, although variability in length of estrus and diestrus is common, particularly during transition into and out of the breeding season. Mares have the highest fertility between May and July; however, pregnancy lasts 11 months and the goal among breeders is for mares to foal near January 1 (the arbitrary birth date for all foals born in a given calendar year). Estrus lasts from 3 to 7 days and is accompanied by swelling and reddening of the vulva in response to estrogens from ovarian follicles. Ovulation occurs after the oocyte undergoes the first meiotic division, typically 24–48 h before the end of behavioral estrus. Ovulation is preceded by an increase and then decrease in concentrations of LH in serum over a period of up to 10 days. Multiple ovulations are not uncommon, but secondary ovulations typically occur within 48 h of the initial ovulation. Additional ovulations during the luteal phase may occur, but the physiological basis for them has not been defined. Secretion of FSH and LH increases in parallel, with the initial increase during late estrus/early diestrus and a second increase during mid-diestrus.

P4 production begins about 24 h after ovulation and is maximal between days 6 and 18–20 post-estrus. Concentrations of P4 range from 4 to 8 ng/ml during diestrus but decline rapidly in the nonpregnant mare at the end of diestrus in response to uterine release of PGF and luteolysis. The uterine endometrium releases luteolytic PGF, but neither the pattern of release required for luteolysis nor endocrine regulation of uterine production of luteolytic PGF is clearly established. However, cervical stimulation results in the release of OXT via the Ferguson reflex and stimulates uterine secretion of PGF as does the administration of exogenous OXT. It is assumed that the combined effects of P4, E2, and OXT are responsible for the activation of the luteolytic mechanism in mares and CL of mares are responsive to luteolytic effects of PGF after day 5 post-ovulation (Sharp et al. 1989).

2.8.2 Pregnancy

The equine conceptus produces an unknown factor that inhibits uterine release of luteolytic PGF (see Sharp et al. 1989; Sharp 2000). In cycling mares, concentrations of PGF in uterine venous plasma and uterine flushings increase between days 14 and 16 when luteolysis occurs and concentrations of P4 in plasma decline. Receptors for PGF on luteal cells are abundant between day 14 of the estrous cycle and estrus

and day 18 of pregnancy. The equine conceptus migrates between the two uterine horns until fixation on day 18 of pregnancy to activate an antiluteolytic mechanism as amounts of PGF in uterine fluids and uterine venous plasma are reduced and the pattern of release of PGFM into blood is not pulsatile in pregnant mares. Further, the presence of the conceptus abrogates endometrial production of PGF in response to both cervical stimulation and exogenous OXT, indicating the absence of or a reduction in the expression of endometrial OXTR in pregnant mares. Equine conceptuses produce increasing amounts of E2 between days 8 and 20 of gestation; however, attempts to prolong CL lifespan in mares by injections of E2 have yielded variable results. The equine conceptus also secretes proteins of 400, 65, and 50 kDa between days 12 and 14 of pregnancy (Sharp 2000), as well as IFND (Cochet et al. 2009), but their roles in pregnancy recognition are not known. Pregnancy recognition signaling in the mare is discussed in detail in Chap. 11.

2.9 Rabbits (*Oryctolagus cuniculus*)

2.9.1 Ovarian Cycle and Estrus

The rabbit doe is polyestrous from puberty at 3–5 months to the end of her reproductive life at 12–36 months and reproduces throughout the year (see Miller and Pawlak 1994; Rameriz and Beyer 1994). After puberty, mature follicles persist in the ovary for 7–10 days before undergoing atresia and being replaced by another wave of follicles that secrete high levels of both E2 and inhibin. E2 stimulates sexual receptivity and inhibin suppresses additional follicular growth by blocking FSH secretion. During this period of growth and atresia of follicles, does are receptive to mating and exhibit lordosis when mounted by does or bucks. The rabbit doe is an induced ovulator and ovulates in response to mating or stimulation of the perineal or vaginal area. The ovulatory response to mating is followed by the formation of CL and a 16–18-day period of pseudopregnancy analogous to diestrus or pregnancy which lasts for about 31 days.

Mating of the doe elicits a neural input into the hypothalamus for the secretion of GnRH and a subsequent ovulatory surge of LH and ovulation 9–12 h post-coitum (pc). The ovulatory surge of LH increases cholesterol mobilization and production of 20α-OH P4 by ovarian interstitial cells within 10–60 min pc that peaks at 4–6 h and returns to below basal levels by 9–12 h pc. Concentrations of LH and FSH are highest at 1–2 and 2–3 h pc, respectively, and both return to pre-mating levels within 6–12 h pc. Circulating concentrations of both LH and FSH are low throughout pregnancy except for a second peak of FSH on days 1–2 of pregnancy. Concentrations of testosterone and E2 in plasma increase about threefold between 90 and 120 min pc and return to below basal levels by 12 h pc. At mating, concentrations of PRL in plasma decrease transiently, return to basal levels in about 30 min and increase again 3–4 days pc, and remain elevated for up to two-thirds of gestation. In addition to its role in lactation, PRL stimulates steroidogenesis in the rabbit ovary. Concentrations

of OXY in blood increase in response to mating, parturition, and lactation. There is a second release of OXY about 5 h pc, and it may stimulate uterine and oviductal contractions responsible for sperm transport.

Regression of the CL occurs at the end of pregnancy or pseudopregnancy in response to PGF produced by the ovary and/or endometrium or in response to exogenous PGF. The uterus synthesizes prostaglandins; however, hysterectomy delays but does not completely block luteolysis in pseudopregnant does. The CL also synthesizes PGF, PGE2, and 6-keto-prostaglandin F1α, suggesting intra-ovarian mechanisms for luteolysis. The increase in uterine PGFs may initiate luteal regression, because levels of P4 in serum decline prior to increases in PGFs. Purified PGE reductase from the CL of pseudopregnant rabbits possesses both PGE-9-keto-reductase and 20α-HSD activity, suggesting that both P4 and PGE2 are substrates and that PG production and steroid metabolism are tightly linked in the luteolytic cascade in rabbits. The CL from pseudopregnant does become responsive to exogenous PGF around day 12, whereas the CL of pregnant does are responsive to luteolytic effects of PGF as early as day 7 but unresponsive by day 15. Thus, the conceptus affects the CL prior to maternal recognition of pregnancy on day 12 and renders the CL more resistant to PGF after maternal recognition of pregnancy (Marcinkiewicz et al. 1992). The basis for the altered sensitivity of rabbit CL to PGF is not known. There is also participation of immune cells in luteolysis in rabbits (Nariai et al. 1995).

2.9.2 Pregnancy

The rabbit has a duplex uterus, each with a cervix, so the male deposits semen into the anterior vagina so that sperm can be transported into each uterine horn to fertilize ova which are ovulated from each ovary approximately 10 h pc (Browning et al. 1980). Fertilization occurs at the ampullary-isthmic junction of the oviduct 1–2 h post-ovulation, and embryos enter the uterus on day 3 and blastocysts undergo implantation on day 7. Rabbits have a hemochorial placenta with discoid villous distribution. The placenta is not a source of P4, so the CL are required for the production of P4 sufficient to maintain pregnancy to term. For pseudopregnant and pregnant does, P4 increases from 1 to 2 ng/ml on day 2 pc to 12–20 ng/ml between days 6 and 8 pc. Between days 8 and 10 pc, P4 profiles of pregnant and pseudopregnant does diverge as concentrations decline rapidly to days 16 and 18 of pseudopregnancy, whereas concentrations of P4 are maintained until 3–4 days prior to parturition between days 28 and 36 post-coitum in pregnant does. Circulating levels of P4 and E2 are not different between pregnant and pseudopregnant does until after implantation (Browning et al. 1980).

Maternal recognition of pregnancy in rabbits occurs between days 10 and 12 pc in response to E2 and an unidentified placental luteotropin (see Keyes et al. 1994). Luteal cells contain LH receptors; however, LH does not stimulate P4 production in vivo. Rather, estrogen exerts its luteotropic effect by uncoupling P4 production from cyclic AMP (cAMP). If E2 is withdrawn from does, exogenous CG stimulates

luteal cAMP and both CG and cAMP stimulate P4 production. The luteotropic effect of the placenta does not result from increased concentrations or affinity of luteal ESR1 for E2. Rabbit placentae secrete immunoreactive GnRH-like activity which acts locally on the uterus, but not directly on luteal cells (Nowak and Bahr 1987). A putative 6–8 kDa placental luteotrophic factor also has been reported to enhance P4 production by cultured luteal cells alone or in conjunction with E2 (Gadsby 1989). Further, a 12–14 kDa rabbit placental luteotropin that is acidic and trypsin and heat sensitive also has been reported (Marcinkiewiz et al. 1992; Marcinkiewicz and Bahr 1993). This factor stimulated the production of P4 by luteal explants in the presence of E2; however, 200 μg/ml of conceptus protein was necessary to achieve a modest increase in P4 production. Rabbit placental giant cells contain immunoreactive CG and cytotrophoblast cells contain immunoreactive CSH1/PRL. However, the effects of CSH1 and PRL on luteal cells are not known (Grunder et al. 1994). It is known that circulating levels of E2 increase, P4 levels decrease, and PRL levels increase about 2 days prepartum. PRL influences nest-building behavior but mainly affects lactogenesis and milk production, whereas E2 and P4 are only temporally associated with nest building (Negatu and McNitt 2002). As for other species, E2 and P4 also affect mammogenesis and lactogenesis.

2.10 Domestic Cats (*Felis catus*)

2.10.1 Estrous Cycle

The domestic cat is a seasonally polyestrous, induced ovulator with onset of cyclicity occurring as early as 4 months and as late as 21 months of age depending on breed, photoperiod, and level of nutrition (Tsutsui and Stabenfeldt 1993). Cats are long-day breeders, so litters are generally born in the spring and summer months in the Northern Hemisphere, but cats exhibit sexual activity throughout the year in the tropics. In the absence of mating, estrus lasts 2–10 days (average 7 days) with females vocalizing and exhibiting "treading" with hind legs, rubbing against objects, and accepting males for mating. Interestrous periods of 3–14 days (average 10 days) separate periods of estrus and are characterized by low circulating concentrations of E2 and non-receptivity to mating. With growth of ovarian follicles, there is increased secretion of E2 over a 2–3-day period prior to estrus with circulating levels of 40–100 pg/ml at estrus. Queens ovulate mature follicles in response to mating-induced activation of a neuroendocrine reflex which releases GnRH and an ovulatory surge of LH as early as 5 min pc that peaks at 20 min and returns to basal levels by 60 min. However, cats sometimes ovulate in the absence of cervical stimulation and physical contact with other cats. The ovulatory surge of LH varies in amplitude (10–100 ng/ml) and duration (1–24 h) based on the number of copulations. Multiple matings on successive days of estrus ensure ovulation in all queens, but less than 50 % of queens in estrus ovulate after a single copulation (see Wildt et al. 1981).

The CL of domestic cats are resistant to exogenous PGF even late in gestation. However, the production of PGF by the fetal-placental unit and endometrium increases during the last half of pregnancy, reaches a plateau around day 45, and increases sharply just before parturition. The administration of exogenous PGF to cats after day 40 of gestation induces abortion, but the mechanism is not known (Tsutsui and Stabenfeldt 1993).

2.10.2 Pregnancy

Cats have a bipartite uterus and the male deposits semen in the anterior vagina at ejaculation (see Tsutsui and Stabenfeldt 1993). Ovulation occurs 25–50 h pc and frequent matings reduce the time to ovulation. Fertilization takes place in the oviduct up to 48 h after ovulation, and embryos enter the uterus at the blastocyst stage 4–6 days post-ovulation. Blastocysts hatch from the zona pellucida on day 11, and implantation occurs on days 12–13 of pregnancy. The cat has an endotheliochorial-type placenta with zonary villous distribution. Following mating, concentrations of P4 in plasma increase to 15–90 ng/ml between days 10 and 40 of pregnancy and days 13–30 of pseudopregnancy. Pseudopregnancy typically lasts 40 days, whereas the length of gestation averages 63–65 days but ranges from 56 to 71 days. By day 30, circulating levels of P4 are higher in pregnant than pseudopregnant queens. Evidence for a specific pregnancy recognition signal in cats has not been reported. However, major proteins secreted by feline conceptuses between days 10 and 25 of pregnancy have been reported, and there was no evidence for secretion of an interferon based on the lack of antiviral activity of the proteins (Thatcher et al. 1991).

The placenta does not produce sufficient P4 to maintain pregnancy as ovariectomy on day 45 results in a rapid decline in circulating P4 and abortion within 6–9 days (Verstegen et al. 1993). PRL levels increase during the last trimester of gestation to peak values at parturition (5–10 ng/ml) and remain elevated during lactation in response to suckling stimulus. PRL is considered an important luteotropin in late gestation. RLX, produced by the fetal-placental unit, increases to 5–10 ng/ml plasma during the second half of gestation and, acting in concert with P4, maintains a quiescent uterus but later facilitates parturition by softening the connective tissues of the pelvis. Following parturition, queens experience anestrus during lactation and resume cycling 2–3 weeks after weaning kittens. Brown (2006) provides a comprehensive review of domestic and nondomestic felids regarding differences in the type of ovulation (spontaneous versus induced), steroid metabolism, seasonal effects on reproduction, adrenal responses to husbandry practices, and ovarian responses to exogenous gonadotrophins and steroids, as well as variations in circulating concentrations of various hormones during of pregnancy.

2.11 Domestic Dog (*Canis lupus familiaris*)

2.11.1 Ovarian Cycle and Estrus

The bitch is monestrous and spontaneously ovulates once or twice per year from 7 to 12 months of age to reproductive senescence (Concannon 1993). Follicles grow and secrete E2 and inhibin at the end of anestrus in association with a transient increase in LH and a slight reduction in FSH just prior to proestrus. Ovarian inhibin suppresses FSH secretion during proestrus as E2 increases to 50–100 pg/ml. Proestrus lasts about 7 days but ranges from 3 days to 3 weeks and is characterized by bloody discharge from the vagina, and the vaginal and perineal areas increase in size and turgidity. An LH surge at the onset of estrus is accompanied by a decrease in circulating levels of E2, increasing concentrations of P4, and increased sexual receptivity. The GnRH-induced LH surge lasts 2–3 days, and concentrations of FSH peak shortly after the LH surge and return to basal levels in 1–2 days. Bitches ovulate about 48 h after the LH surge. Luteolysis is protracted in the bitch as concentrations of P4 decrease gradually at the end of the luteal phase and remain at less than 1 ng/ml until the next follicular phase. However, luteolysis occurs rapidly immediately postpartum due to the absence of effects of LH and PRL. Frequent administration of exogenous PGF is luteolytic in the bitch, but hysterectomy does not prolong CL lifespan which suggests that luteolysis is uterine independent (Concannon and McCann 1989). The length of the luteal phase is similar in pseudopregnant and pregnant bitches, and concentrations of P4 are maximum between days 10 and 40 post-LH surge and then decline to term at 64–66 days of gestation (Concannon 1993), which suggests that there is not a specific pregnancy recognition signal from conceptuses in bitches.

2.11.2 Pregnancy

Oocytes are at the germinal vesicle stage when ovulated, reach metaphase II in the oviduct, and are fertilizable for 2–3 days as spermatozoa are viable in the female reproductive tract for 6–7 days. Fertilization occurs four days after the LH surge, morulae/blastocysts enter the uterus on days 9–10, and implantation occurs on days 16–18 after the LH surge. Blastocysts enter the uterus on day 10 where they are free floating until hatching, and implantation occurs around day 16 (Concannon and McCann 1989). The dog has an endotheliochorial placenta with a zonary villous distribution. The CL are the primary source of progesterone as both ovariectomy and hypophysectomy at any stage of pregnancy result in abortion. Since the CL of pregnancy and pseudopregnancy have similar lifespans, a pregnancy recognition signal does not seem to be required for CL maintenance during pregnancy. A

comprehensive review of the endocrinology of pregnancy in the bitch indicates that much is yet to be learned about reproduction in dogs (Verstegen-Onclin and Verstegen 2008). Current knowledge of pregnancy in the dogs is presented in Chap 11.

2.12 Marsupials

2.12.1 Pregnant Versus Nonpregnant Marsupials

An extensive review of reproduction in marsupials was published by Tyndale-Biscoe (1984). In that chapter, it is noted that Hill and O'Donoghue (1913) did not consider that differences existed between nonpregnant and pregnant marsupials; however, it is also noted that Owen (1834) noted that the thickness of the endometrium of the gravid uterus was greater than for the nongravid uterus. Renfree (1972) and Renfree and Tyndale-Biscoe (1973) later confirmed and extended the observations of Owen (1834).

2.12.2 Pregnant Marsupials

A recent review by Renfree (2010) and information provided in Chap 11 provide clear evidence that there is maternal recognition of pregnancy in response to the presence of the trophoblast in the gravid uterine horn.

2.13 Summary

As suggested by many, reproduction is essential for the maintenance of a species and, therefore, nature has been very liberal in exploring means to establish and maintain pregnancy. This review has attempted to capture aspects of scientific discovery of mechanisms for pregnancy recognition signaling by reproductive scientists during the late nineteenth century with a few observations, the major breakthroughs in the twentieth century in understanding pregnancy recognition signaling. No doubt that information and new discoveries will facilitate researchers seeking to increase reproductive efficiencies in animal agriculture, improve reproductive health in women, and seek to development methods for fertility control that are acceptable by various peoples across our world. Other researchers will continue research to unravel fine details of the mysteries of the reproductive systems of various species of animals to fully understand mechanisms that underlie successful development and function of the reproductive system.

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Chapter 3
The Role of Steroid Hormone Receptors in the Establishment of Pregnancy in Rodents

Nyssa R. Adams and Francesco J. DeMayo

Abstract The ovarian hormones, estrogen and progesterone, and their receptors, the estrogen receptor (ER) and progesterone receptor (PR), orchestrate the complex sequence of events required for uterine receptivity and the establishment of pregnancy. The actions of ER, PR, and other steroid hormone receptors (SHRs) direct the uterus through the processes of implantation and decidualization. Due to the ethical concerns of studying pregnancy in humans, genetically engineered rodent models have facilitated many of the discoveries that have elucidated the molecular events directing early pregnancy. This chapter will cover the conserved structure and function of the SHRs. ER and PR will be highlighted for their pivotal roles in uterine receptivity, implantation, and decidualization. The dynamic regulation of ER and PR expression and activity throughout the estrous cycle and early pregnancy, and the importance of SHRs in coordinating paracrine signaling between the endometrial compartments will also be explored. Finally, the roles of androgen receptor (AR) and glucocorticoid receptor (GR) in the establishment of pregnancy will be discussed.

3.1 Introduction

Pregnancy is a complex process, requiring the intricate coordination of implantation, decidualization, placentation, and parturition. Following fertilization, blastocysts are transported to the uterus via the oviducts. If the epithelium of the uterine endometrium is receptive, competent blastocysts are able to implant and invade into

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the surrounding endometrium. In the mouse, embryo implantation induces the endometrial stromal cells underlying the implantation site to undergo the process of decidualization, or differentiation into decidual cells; in humans, this process occurs independently of embryo implantation (reviewed in Cha et al. 2012). Decidual cells serve many functions during early pregnancy, as they support the developing embryo, regulate trophoblast invasion, enhance vascularization, and modulate maternal immunity. Successful decidualization sets the stage for placentation, in which a robust placental vascular network develops to support the developing embryo until parturition. Implantation, decidualization, and placentation are tightly regulated, and defects in any of these early processes can result in adverse pregnancy outcomes or infertility (Cha et al. 2012). The complex coordination of these sequential, interrelated processes is achieved through the activities of steroid hormone receptors (SHRs), notably estrogen receptor (ER) and progesterone receptor (PR). This chapter will detail the conserved structure and function of the SHRs, and the role of these crucial signaling molecules in the establishment of pregnancy in rodents. ER and PR, which mediate the activities of the ovarian hormones, estrogen and progesterone, will be highlighted for their critical roles in uterine receptivity and the establishment of pregnancy. Additionally, the roles of the androgen receptor (AR) and glucocorticoid receptor (GR) in the establishment of pregnancy will be discussed. Throughout the chapter, the activities of the SHRs will be illustrated by findings obtained from genetically engineered mouse models, which have facilitated essential insights into the biology of reproduction.

3.2 The Structure and Function of Steroid Hormone Receptors

Nuclear receptors (NRs) are crucial effectors of signaling networks, directing changes in gene transcription in response to changing conditions. The NR superfamily includes 48 proteins that regulate gene transcription to direct critical cellular processes, including survival, proliferation, and differentiation (reviewed in Tata 2002). Many of the NRs are activated by the binding of ligand molecules, while others are considered "orphan" receptors with no identified ligand regulating their transcriptional activity. The estrogen receptor-like subfamily of NRs (NR3) houses the steroid hormone-binding receptors, including ER, PR, AR, and GR. These SHRs function as transcription factors, effecting changes in gene transcription in response to extracellular signals that are communicated via hormone ligands.

Steroid hormones are secreted by endocrine organs to coordinate complex developmental and physiological processes. These molecules circulate through the blood to reach their target organs, where the lipophilic nature of their steroid rings allows them to cross the cell membrane via simple diffusion. Because diffusion is a nonspecific process, the cellular response to these hormones is directed by the specific expression of SHRs. In the absence of ligand, SHRs reside in the cytoplasm, bound to a complex of chaperone proteins (Smith 1993). These chaperones hold the recep-

tor in an inactive state, primed to bind ligand (Picard et al. 1990). Upon ligand binding, SHRs undergo a conformational change that triggers release from the chaperone complex and favors receptor dimerization. Dimerized hormone-receptor complexes translocate to the nucleus, where they bind to DNA and direct the recruitment of transcriptional coactivators, corepressors, and the transcriptional machinery to modulate the expression of target genes (reviewed in Shibata et al. 1997).

The SHRs share a common structure, composed of an amino-terminal DNA-binding domain (DBD), a carboxy-terminal ligand-binding domain (LBD), and a flexible hinge region that bridges these modular domains (Fig. 3.1) (reviewed in Beato and Klug 2000). The LBD is highly conserved among SHRs, forming a pocket for hormone binding near the receptor's carboxy-terminus (Wurtz et al. 1996). The LBD is also responsible for SHR interactions with the chaperone protein, heat shock protein 90 (HSP90) (Ricketson et al. 2007). Upon ligand binding, a conformational change is induced, facilitating release of the SHR from the large chaperone complex. The ligand-bound conformation also favors SHR dimerization and DNA binding (Kumar and Chambon 1988). Additionally, the LBD contains a nuclear localization signal (NLS) that targets SHRs to the nucleus (Guiochon-Mantel et al. 1989). This NLS is ligand-dependent, enhancing the translocation of ligand-bound SHRs to the nucleus.

The flexible hinge region bridges the DBD and LBD and serves several functions, as it can affect DNA-binding and dimerization activities (Daniel et al. 2010). The flexible hinge region contains an additional, constitutively active NLS (Guiochon-Mantel et al. 1989). Further, this region houses protein-protein interaction domains that, along with the LBD, are responsible for SHR interactions with chaperone proteins.

The DBD is highly conserved among SHRs, encoding two zinc fingers that are responsible for sequence recognition and DNA binding (Green et al. 1988). The DBD targets dimerized SHRs to a specific nucleotide sequence, called a hormone response element (HRE). The nucleotide sequence of an HRE is palindromic; each SHR monomer recognizes one-half site of the HRE (Luisi et al. 1991). HREs are

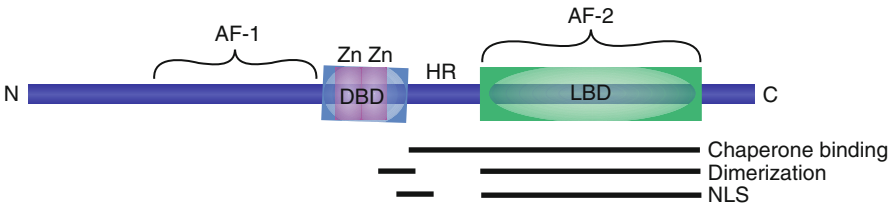


Fig. 3.1 The conserved structure of SHRs. Receptor isoforms vary by the length of their amino-terminus. The DNA-binding domain (*DBD*) is highly conserved between SHRs and encodes two zinc fingers (*Zn*). The ligand-binding domain (*LBD*) forms a pocket for the binding of steroid hormone ligands. The hinge region (*HR*) bridges the DBD and LBD. The constitutive transactivation domain (*AF-1*) is encoded by the amino-terminal amino acids, while the ligand-dependent transactivation domain (*AF-2*) is contained within the LBD. The regions responsible for binding chaperone proteins, receptor dimerization, and nuclear localization (*NLS*) are shown

enriched in the promoter regions of target genes, allowing SHRs to recruit the transcriptional machinery and coregulator proteins to alter transcriptional activity. Although each SHR has its own unique HRE, the SHRs are often able to bind to the response elements of other receptors.

SHRs regulate transcriptional activity through two transactivation domains, which directly interact with transcription factors and coactivators to regulate the initiation of transcription at target loci. The first transactivation domain, referred to as activation function 1 (AF-1), is located at the amino-terminus, near the DBD (reviewed in Lavery and McEwan 2005). AF-1 is constitutively activating, promoting the initiation of transcription in a ligand-independent fashion. An important exception is the AF-1 domain of human PR, which encodes an inhibitory domain, resulting in constitutive inhibition of transcriptional activity (Giangrande et al. 1997). The second transactivation domain (AF-2) is ligand inducible, directing enhanced transcriptional activation in response to ligand binding.

In addition to sharing a conserved structure, SHR activity can be regulated by common mechanisms. First, the activity of SHRs is modulated by subcellular localization. SHRs must translocate to the nucleus in order to alter transcription, a process that is directed by the NLSs encoded by each SHR. SHRs are also regulated through differential promoter usage and alternative splicing, resulting in the production of distinct receptor isoforms (Hollenberg et al. 1985; Conneely et al. 1989; Kastner et al. 1990; Encio and Detera-Wadleigh 1991; Mosselman et al. 1996; Wilson and McPhaul 1996). Further regulation of SHR activity can be achieved through posttranslational modifications. Notably, all of the SHRs are phosphoproteins, subject to phosphorylation and dephosphorylation events that modulate their activity (reviewed in Weigel and Moore 2007). Finally, SHR-driven changes in transcription are influenced by the action of transcriptional coregulators. Notably, steroid receptor coactivator (SRC) family proteins are critical coactivators of ER, PR, and GR, interacting with the AF-2 domains of these SHRs in a ligand-dependent fashion (reviewed in Lonard and O'Malley 2012).

3.3 The Establishment of Pregnancy in Rodents

The establishment of pregnancy is an intricate process requiring the coordination of multiple, interdependent steps. The processes of ovulation, fertilization, implantation, decidualization, placentation, and parturition must be coordinated across both time and space. The ovarian hormones, estrogen and progesterone, and their cognate SHRs, ER and PR, are responsible for the orchestration of these events and are absolutely essential for the successful establishment of pregnancy (reviewed in Vasquez and DeMayo 2013). Due to their pivotal importance in directing female reproduction, expression of ER and PR in the uterus is highly specific and dynamic,

subject to compartment-specific regulation that changes rapidly during early pregnancy (Tibbetts et al. 1998; Tan et al. 1999).

The uterus is composed of two main compartments: the myometrium and the endometrium. The myometrium is located externally, consisting of an outer, longitudinal muscle layer and an inner, circular muscle layer. The endometrium forms the inner layer of the uterus and is further subdivided into an epithelial compartment, composed of luminal and glandular epithelia, and a subepithelial stromal compartment. The luminal epithelium is the site of blastocyst attachment and implantation. In mice, attachment occurs at the antimesometrial surface of the uterine lumen. The secretory products of the endometrial glands are critical for successful attachment and implantation (Jeong et al. 2010). In rodents, blastocyst attachment triggers the stromal cells underlying the implantation site to differentiate into specialized decidual cells in a process termed decidualization. Decidual cells support the developing embryo, regulate trophoblast invasion, and modulate maternal immunity. Importantly, implantation and decidualization set the stage for healthy pregnancy, and defects in these processes can result in negative outcomes at later time points (reviewed in Cha et al. 2012).

In the mouse, female reproductive function is regulated by the estrous cycle, which lasts 4–5 days. The murine estrous cycle consists of four phases: proestrous, estrous, metestrous, and diestrous. The proestrous phase is marked by estrogen production and the accumulation of mature follicles in the ovaries. Ovulation occurs in the estrous phase, during which females are most receptive to mating. Following fertilization, day 1 of pregnancy (the time of vaginal plug) is marked by proliferation of the endometrial epithelium under the influence of preovulatory estrogen. This parallels the proliferative phase of the menstrual cycle. During days 2 and 3, corresponding to the early secretory phase of the menstrual cycle, the endometrium prepares for implantation under the influence of progesterone, which is produced by the corpus luteum. In the mouse, the maintenance of the corpus luteum and progesterone secretion requires cervical stimulation from mating. ER-driven epithelial proliferation ceases due to declining levels of estrogen and the antagonistic action of progesterone. Expression of PR in the endometrial epithelium spikes, resulting in downregulation of ER target genes. By day 4, however, epithelial PR levels drop, resulting in loss of PR signaling in this compartment despite high circulating levels of progesterone. At the same time, a preimplantation surge in estrogen induces the expression of genes that are critical to uterine receptivity, including leukemia inhibitory factor (LIF) (Finn and Martin 1974; McCormack and Greenwald 1974; Hewitt et al. 2012). This marks the beginning of the window of receptivity. The murine uterus is receptive to blastocyst implantation on day 4 of pregnancy. Blastocyst implantation triggers the differentiation of stromal cells, and decidualization spreads throughout the stroma surrounding the implantation chamber. In the absence of blastocyst implantation, the uterus becomes nonreceptive by day 5. If pregnancy is not achieved, the mouse enters the diestrous phase, characterized by involution of the corpus luteum and resorption of the endometrium.

3.4 Estrogen Receptor (ER)

ER (*Esr*) exists as two distinct isoforms, ERα (*Esr1*) and ERβ (*Esr2*), which are transcribed from distinct genes (Mosselman et al. 1996). These isoforms can form homo- and heterodimers to regulate transcriptional activity (Pettersson et al. 1997). ERα, the first ER isoform to be discovered, is well characterized. Isoform-specific ablation of ER in mice has shown that ERα regulates uterine function during reproduction. *Esr1* knockout (αERKO) mice are infertile due to defects in ovarian function and signaling along the hypothalamic-pituitary axis (Couse and Korach 1999). In contrast, *Esr2* knockout (βERKO) mice exhibit a mild subfertile phenotype, producing fewer and smaller litters than wild-type mice (Krege et al. 1998; Dupont et al. 2000). This subfertile phenotype is due to loss of the luteinizing hormone (LH) surge, resulting from loss of *Esr2* expression in the ovary (Jayes et al. 2014).

The ERα cistrome in the murine uterus has been characterized by chromatin immunoprecipitation followed by deep sequencing (ChIP-seq) (Hewitt et al. 2012). ERα was found to bind 5,814 sites basally, in vehicle-treated control uteri, and 17,240 sites following 1-hour estrogen treatment. Overlaying the ERα cistrome with gene expression data revealed that ERα binding is enriched near the transcription start site (TSS) (<10 kb) of genes that are positively regulated by estrogen. In contrast, genes that are downregulated by estrogen treatment show binding of ERα more distally.

ERα expression is tightly regulated in the reproductive organs of both humans and mice. ERα is expressed in all compartments of the uterus, the androgen-producing theca cells of the ovary, and the mammary glands. ERα is also expressed in the hypothalamic and pituitary glands. In the endometrium, ERα expression varies during the estrous cycle and early pregnancy (Tibbetts et al. 1998; Tan et al. 1999). ERα is strongly expressed in the glandular epithelium of the murine uterus and moderately expressed throughout the luminal epithelium and stroma. On day 1 of pregnancy, epithelial ERα expression is critical to mediate estrogen-dependent proliferation. ERα can still be detected in the epithelium and stroma at day 3 of pregnancy, immediately prior to the window of receptivity. By day 4, however, ERα expression has been lost from the luminal epithelium and stroma and is mostly limited to the mesometrial pole of the implantation chamber. This expression pattern reflects the loss of ERα in the decidualizing stromal cells that surround the implantation chamber. Importantly, strong ERα expression is maintained in the glandular epithelium of the uterus throughout days 3 and 4. During this period, the induction of ER target genes in the uterine glands is critical to prepare the uterus for embryo implantation. The expression of ERα is also tightly regulated during the human menstrual cycle. ERα expression is highest in the endometrial stroma and glands during the proliferative phase of the menstrual cycle. ERα levels decrease during the secretory phase of the menstrual cycle, as progesterone signaling becomes predominant.

3.4.1 *ER Signaling Drives Proliferation of the Endometrial Epithelium*

Estrogen regulates proliferation in a biphasic manner, directing both early and late proliferative events in the endometrium. Estrogen signaling stimulates a number of changes in the proliferating endometrium, including the transcription of cell cycle genes, DNA synthesis, epithelial mitosis, hyperemia, stromal edema, and the infiltration of immune cells. Estrogen-driven proliferation is mediated by paracrine signaling involving fibroblast growth factor (FGF) ligands. ER signaling induces FGF expression in stromal cells (Fujimoto et al. 1997; Tsai et al. 2002). Stromal FGF ligands activate epithelial FGF receptors (FGFRs), which drive the proliferative response by activating the downstream extracellular signal-regulated kinase (ERK1/2) or phosphoinositide 3-kinase (PI3K) pathways. Estrogen-dependent proliferation is also driven by insulin-like growth factor 1 (IGF1) (Zhu and Pollard 2007). IGF1, an ER target in the endometrium, is required for reproduction; deletion of *Igf1* results in infertility in both male and female mice (Baker et al. 1996). The proliferative effect of estrogen is conserved in humans, as endometrial proliferation is dramatically enhanced as estrogen increases during the proliferative phase of the menstrual cycle (Ferenczy et al. 1979) (Fig. 3.2).

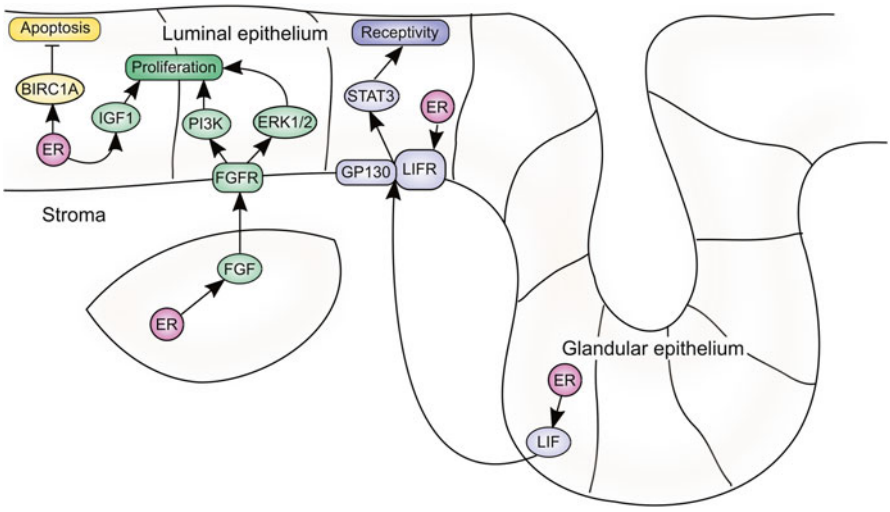


Fig. 3.2 ER signaling regulates endometrial proliferation and receptivity. Stromal ER drives proliferation of the endometrial epithelium via a paracrine signaling network. ER induces FGF expression in stromal cells, which act on FGFR expressed on the surface of epithelial cells. FGFR activates downstream signaling via PI3K and ERK1/2 to drive proliferation of epithelial cells. ER also induces IGF1 to further enhance proliferation. In addition, epithelial ER induces the expression of antiapoptotic targets, including BIRC1A. Uterine receptivity is mediated by ER-induced LIF signaling. ER induces expression of both LIF and its receptor, LIFR. LIF produced by the glandular epithelium signals to LIFR and the co-receptor GP130, which are expressed in the luminal epithelium. LIFR/GP130 activation results in activation of STAT3 and transition of the luminal epithelium to the receptive state

ERα-driven proliferation is a notable example demonstrating the importance of compartmental crosstalk in the endometrium. The proliferative response to estrogen is one of many endometrial functions that are coordinated by paracrine signaling between the epithelium and stroma. A reasonable hypothesis would be that estrogen stimulates ERα expressed in epithelial cells to drive proliferation in this same compartment. However, the use of mouse models has demonstrated that it is stromal ERα which is necessary and sufficient to drive proliferation of the endometrial epithelium. Indeed, in neonatal mice, the endometrial epithelium proliferates in response to circulating estrogen, despite the absence of ERα expression in this compartment (Bigsby and Cunha 1986). Instead, ERα is expressed in the stromal compartment and activates epithelial proliferation in a paracrine manner (Bigsby and Cunha 1986). Similar results have been obtained from tissue-grafting experiments, in which wild-type and αERKO endometrial tissues have been surgically recombined within the kidney capsule of mice (Cooke et al. 1997). Notably, estrogen treatment cannot stimulate proliferation in reconstructions of wild-type, ERα-expressing epithelium with αERKO stroma. However, grafts of αERKO epithelium with wild-type stroma exhibit a normal proliferative response to estrogen treatment, demonstrating that stromal ERα is necessary and sufficient to induce estrogen-driven proliferation of the epithelial compartment. Compartment-specific deletion of ERα from the endometrial epithelium using *Wnt7a*^{Cre} (UTEpiαERKO) further corroborates the role of epithelial-stromal crosstalk in estrogen-driven proliferation (Winuthayanon et al. 2010). UTEpiαERKO mice are infertile due to implantation failure and exhibit dysfunctional expression of estrogen target genes in the endometrial epithelium. Interestingly, the epithelium retains the ability to proliferate in response to estrogen, despite the absence of ERα expression in the epithelial compartment. Following estrogen treatment, epithelial cells exhibit DNA synthesis and mitogen expression, suggesting that these responses are directed by ERα in the endometrial stroma. While estrogen-dependent proliferation occurs normally in UTEpiαERKO epithelium, these mice display increased epithelial apoptosis. The induction of apoptosis in UTEpiαERKO epithelium is likely due to loss of expression of antiapoptotic ER targets, such as BIRC1A (Yin et al. 2008). This increase in epithelial apoptosis, along with the dysregulation of ER target genes that mediate uterine receptivity, may contribute to the infertile phenotype seen in these mice.

3.4.2 The ER Target LIF Mediates Uterine Receptivity

In mice, a preimplantation estrogen surge is required to prepare the uterus for receptivity (Finn and Martin 1974; McCormack and Greenwald 1974). This estrogen surge occurs 24–48 h prior to implantation and results in the induction of ER target genes that prime the uterus for blastocyst implantation. LIF, a secreted cytokine in the interleukin-6 family, is one of the critical mediators of uterine receptivity. LIF is produced in the uterine glands in response to the preimplantation estrogen surge, and functions as a ligand for the LIF receptor (LIFR), which is expressed in the luminal epithelium (Aghajanova 2004). Binding of LIF to LIFR and the co-receptor,

glycoprotein 130 (GP130), results in activation of the signal transducer and activator of transcription 3 (STAT3) signaling pathway in target cells (Ernst et al. 2001). Both *Lif* and *Lifr* are direct targets of ERα (Hewitt et al. 2012). LIF production is first evident in the glandular epithelium at day 3.5 of pregnancy and can later be detected in the endometrial stroma surrounding the site of blastocyst attachment (Stewart et al. 1992). Interestingly, LIFR and GP130 are also expressed on the blastocyst, suggesting a role for this ER signaling pathway in coordinating events between the mother and embryo. LIF is essential for uterine receptivity, as uterine deletion of *Lif* results in infertility due to implantation failure and defective decidualization (Chen et al. 2000). Notably, blastocyst implantation can be rescued by intraperitoneal injection of LIF (Chen et al. 2000). Implantation failure also occurs with deletion of the downstream mediators of the LIF pathway, GP130 (*Il6st*) or *Stat3* (Lee et al. 2013; Pawar et al. 2013; Sun et al. 2013). In humans, a similar surge in circulating estrogen levels occurs during the mid-secretory phase of the menstrual cycle. LIF expression increases at the time of implantation, suggesting that this pathway may play a role in uterine receptivity in humans (Dey et al. 2004). Notably, endometrial samples from women with unexplained infertility and multiple implantation failures exhibit decreased expression of LIF during the mid-secretory phase compared to healthy controls (Wu et al. 2013). However, clear evidence that ER activity is required for receptivity in humans and primates remains elusive.

3.5 Progesterone Receptor (PR)

Progesterone, the hormone of pregnancy, is absolutely required for the establishment and maintenance of pregnancy (reviewed in Wetendorf and DeMayo 2014). In most species, including rodents, PR maintains the conserved structures and functionalities common to the SHRs. The DBD targets dimerized PR to the progesterone response element (PRE), which consists of the palindromic sequence, AGAACAnnnTGTTCT (Ham et al. 1988). Interestingly, PR is able to bind promoters and modulate the transcription of genes that contain only half of the PRE sequence or no PRE at all (Rubel et al. 2012). A crucial exception to the conserved SHR structure and function occurs in human PR. The AF-1 transactivation domain, which constitutively activates transcription in most SHRs, has inhibitory activity in human PR (Giangrande et al. 1997). This results in constitutive repression of transcription of PR target genes. The AF-2 domain of human PR retains ligand-dependent transactivating activity and activates transcription of PR target genes in response to hormone binding.

3.5.1 PR Exists as Multiple Isoforms with Distinct Functions

Similar to ER, which exists as the differentially regulated ERα and ERβ isoforms, PR occurs as two isoforms, PR-A and PR-B. Unlike ERα and ERβ, which are encoded by distinct genes, the PR isoforms arise from differential promoter usage at the *Pgr*

gene (Conneely et al. 1989; Kastner et al. 1990). The longer isoform, PR-B, utilizes an upstream promoter. The amino-terminus of the PR-B isoform encodes an additional 164 amino acids, which form a third activation domain, termed AF-3 (Sartorius et al. 1994). This structural difference results in the strikingly different functions of the human PR-A and PR-B isoforms. Human PR-A suppresses transcription by recruiting the corepressor, nuclear corepressor 2 (NCOR2), to the loci of target genes (Giangrande et al. 2000). In contrast, PR-B functions primarily as a transcriptional activator (Vegeto et al. 1993). The AF-3 domain of PR-B is responsible for this dramatic functional difference. In the absence of an AF-3 domain, the activity of the human PR-A isoform is driven by the AF-1 domain, which is constitutively repressive. In the PR-B isoform, however, the inclusion of the AF-3 domain results in inhibition of the repressive activity of the AF-1 domain. Interestingly, the PR-B isoform is constitutively phosphorylated at Ser294 of the AF-1 domain. This phosphorylation is specific to the PR-B isoform and is thought to be responsible for the inhibition of the AF-1 domain's repressive activity (Clemm et al. 2000). The interplay between these PR isoforms likely has functional significance, as both PR-A and PR-B are expressed in the human endometrium and can from homo- or heterodimers. Unfortunately, the contrasting functions of the human PR-A and PR-B isoforms are not conserved in all species (Giangrande et al. 1997). This critical difference must be accounted for when translating discoveries from mouse models to human patients and may explain some of the species-dependent discrepancies in PR signaling.

In addition to the well-characterized PR-A and PR-B isoforms, the existence of a third isoform, PR-C, has been postulated (Wei and Miner 1994). The PR-C isoform, once thought to be an artifact, is now known to be transcribed from a TSS downstream from the PR-A TSS, resulting in the production of a 60 kDa protein. The truncated PR-C isoform cannot bind DNA, as it lacks the conserved SHR DBD (Wei et al. 1997). However, PR-C retains the ability to bind progesterone and undergo receptor dimerization, suggesting that it may inhibit progesterone signaling by ligand sequestration. PR-C expression is upregulated in human myometrium during parturition and may contribute to the functional withdrawal of progesterone during the induction of labor (Condon et al. 2006). However, many questions regarding the expression and activity of PR-C have yet to be answered.

3.5.2 PR Is Required for Multiple Reproductive Functions

The use of knockout mouse models has demonstrated that PR is absolutely essential for multiple reproductive functions. Complete knockout of both PR isoforms (PRKO) results in a spectrum of reproductive abnormalities in female mice (Lydon et al. 1995). PRKO mice are completely infertile due to defects in both ovarian and uterine functions. These mice display defective postnatal uterine development, ovulation, and decidualization. In addition, PRKO mice exhibit abnormalities in mating behavior and mammary gland development.

3.5.2.1 The PR-A Isoform Is Necessary and Sufficient for the Establishment of Pregnancy

Specific deletion of the PR-A (PRAKO) and PR-B (PRBKO) isoforms has demonstrated that PR-A, and not PR-B, is required for the establishment of pregnancy in mice. The PRAKO phenotype resembles that of PRKO mice, with complete infertility due to ovarian and uterine defects (Mulac-Jericevic et al. 2000). Intriguingly, PRAKO mice demonstrate increased epithelial proliferation in the endometrium in response to progesterone treatment. This phenotype is in striking contrast to wild-type uterine biology, in which progesterone treatment inhibits estrogen-driven epithelial proliferation. Notably, this proliferative phenotype requires PR-B expression, suggesting that PR-B has pro-proliferative activity that is normally inhibited by PR-A. Interestingly, this suggests that the murine PR-A and PR-B isoforms may exercise antagonizing inhibitory/activating functions, similar to the human PR isoforms. This underscores the importance of PR-A in repressing proliferation of the endometrial epithelium, as it may inhibit both ER-driven and PR-B-driven proliferative activity.

In stark contrast to the infertile PRAKO phenotype, PRBKO mice display normal fertility with no apparent defect in ovarian or uterine function (Mulac-Jericevic et al. 2003). Rather, loss of the PR-B isoform results in abnormal development of the mammary glands, with reduced ductal side branching and limited mammary gland maturation during pregnancy. This recapitulates the mammary gland defects observed in PRKO mice and further supports a role for PR-B in epithelial proliferation. While the murine PR isoforms cannot recapitulate the intricate interactions of the human PR-A and PR-B isoforms, these mouse models have nonetheless demonstrated that the PR-A and PR-B isoforms perform distinct functions during reproduction in rodents.

3.5.3 PR Coregulators and Chaperones Play Important Roles in Early Pregnancy

PR modulates transcription through the recruitment of transcription factors and specific coregulator proteins. Coregulators, either coactivators or corepressors, are specifically recruited by PR in an isoform-dependent manner. The human PR-A isoform exerts its repressive function by recruiting the corepressor, NCOR2 (Giangrande et al. 2000). PR-B, however, recruits steroid receptor coactivator-1 (SRC-1, *Ncoa1*) to activate transcription at target genes. Knockout mouse models have clearly demonstrated that SHR coregulators play important roles during early pregnancy. *Ncoa1* knockout mice are fertile, although they exhibit a decreased decidual response (Xu et al. 1998). Uterine ablation of steroid receptor coactivator-2 (SRC-2, *Ncoa2*) using Pgr^{Cre} results in infertility due to implantation failure and reduced decidualization (Mukherjee et al. 2006). Of note, crossing these mice to *Ncoa1* knockout mice

causes complete failure of decidualization, indicating that the coactivators SRC-1 and SRC-2 both play a role in the decidualization of stromal cells.

Chaperone proteins are also essential for optimal PR signaling; these proteins maintain cytosolic PR in an inactive state, prepared to bind ligand. One such chaperone, FK506 binding protein 4 (FKBP52, *Fkbp4*), forms part of the PR chaperone complex and enhances PR signaling (Barent et al. 1998). Complete ablation of *Fkbp4* results in infertility due to implantation failure (Tranguch et al. 2005). Importantly, less progesterone ligand is bound to PR in these mice. Expression of PR target genes is decreased, while ER targets are abnormally upregulated, highlighting the importance of FKBP52 in PR signaling. Interestingly, treatment with excess progesterone can rescue implantation in *Fkbp4* knockout mice in a background-dependent manner (Traguch et al. 2007). These findings highlight the importance of chaperone proteins in facilitating optimal PR signaling and bring to light the potentially confounding effects of genetic background.

3.5.4 PR in the Uterus

The roles of PR in the uterus have been further delineated by investigating the signaling pathways that lie downstream of progesterone signaling. These studies have demonstrated that progesterone signaling is absolutely critical during both implantation and decidualization and that PR orchestrates complex intracellular and paracrine signaling networks within the uterus. During implantation, PR activity regulates epithelial proliferation and modulates the activity of ER to establish receptivity. PR also induces signaling molecules in the endometrial epithelium that regulate subepithelial stromal cells to promote the decidual reaction that will take place following embryo attachment. The dynamic expression of PR in the epithelial and stromal compartments highlights the importance of progesterone signaling during both implantation and decidualization. Epithelial PR expression is high in the days preceding implantation, underscoring its importance in the establishment of uterine receptivity. PR expression in the epithelium declines at the time of attachment and instead increases in the endometrial stroma, where PR signaling will play a critical role in the decidualization of stromal cells that surround the implantation site (Fig. 3.3).

Identification of the signaling events that lie downstream of PR has illuminated some of the critical roles of PR during both implantation and decidualization. One essential function of endometrial PR is the induction of Indian hedgehog (IHH) during the pre-receptive phase. In the epithelium, PR stimulates expression of the target gene, *Ihh* (Takamoto et al. 2002). IHH from the endometrial epithelium acts on its receptors, Patched and Smoothened, which are expressed on the surface of endometrial stromal cells. This paracrine signaling pathway drives the proliferation and differentiation of stromal cells in a PR-dependent manner. As a result, the induction of IHH in the epithelium is critical to both embryo implantation and stromal cell decidualization. Indeed, uterine ablation of *Ihh* using PR^{Cre} ($PR^{Cre/+}Ihh^{f/f}$) results in infertility, with defects in decidualization in addition to implantation fail-

ure (Lee et al. 2006). In subepithelial stromal cells, IHH signaling induces the production of chicken ovalbumin upstream promoter transcription factor II (COUP-TFII, *Nr2f2*), an orphan member of the NR superfamily (Lee et al. 2006). Although COUP-TFII is expressed exclusively in the stromal compartment, it performs functions that are critical to both implantation and decidualization (Takamoto et al. 2005). Indeed, deletion of *Nr2f2* in the murine uterus using Pgr^{Cre} ($PR^{Cre/+}$*COUP-TFII*$^{f/f}$) results in infertility with implantation failure and defective decidualization (Kurihara et al. 2007). This PR-induced signaling pathway is critical to the process of decidualization. COUP-TFII drives decidualization by coordinating the activity of several growth factor signaling pathways, including epidermal growth factor receptor (EGFR), bone morphogenetic protein (BMP), and wingless-type MMTV

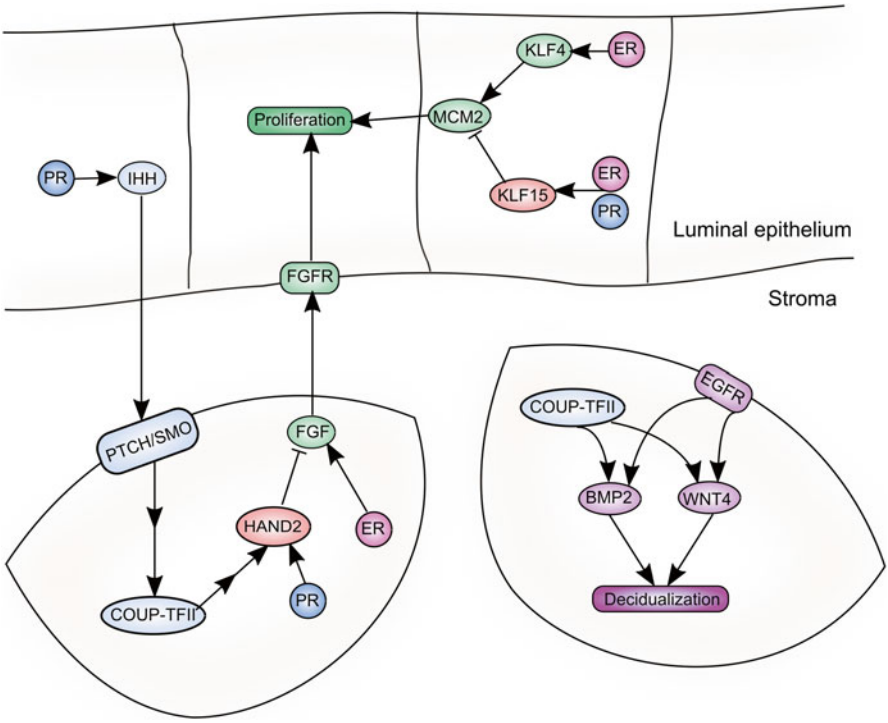


Fig. 3.3 PR regulates multiple signaling pathways in the endometrium and coordinates the functions of the endometrial epithelium and stroma via paracrine signaling networks. Epithelial PR induces expression of IHH, which acts on its receptors, Patched and Smoothened (*PTCH*/*SMO*), expressed on stromal cells. This results in induction of COUP-TFII in endometrial stromal cells. HAND2 is induced directly by PR, downstream of COUP-TFII signaling. HAND2 inhibits production of FGF ligands, thereby abrogating proliferative signaling to the epithelium. PR also inhibits epithelial proliferation directly, by favoring production of KLF15, resulting in downregulation of the proliferative target, MCM2. PR signaling also drives the decidualization of stromal cells. COUP-TFII, a downstream target of PR signaling, induces expression of BMP2 and WNT4. Induction of BMP2 and WNT4 also requires EGFR signaling. BMP2 and WNT4 are required for optimal PR signaling and decidualization of endometrial stromal cells

integration site family members (WNTs). Indeed, the signaling molecules BMP2 and WNT4 are induced downstream of EGFR signaling (Large et al. 2014). Further, uterine ablation of *Egfr* using PR^{Cre} ($PR^{Cre/+}Egfr^{f/f}$) results in defective decidualization, leading to infertility (Large et al. 2014). BMP2 is required for decidualization, and uterine ablation of *Bmp2* ($PR^{Cre/+}Bmp2^{f/f}$) results in inadequate decidualization and infertility (Lee et al. 2007). Interestingly, BMP2 expression appears to be crucial for optimal PR signaling in decidualizing stromal cells. BMP2 regulates the expression of FKBPs, and loss of BMP2 results in a decrease in activating phosphorylations of PR. Additionally, BMP2 regulates a multitude of targets in the stroma, including prostaglandin-endoperoxide synthase 1 (PTGS1) and the noncanonical WNT ligands, WNT4 and WNT6. WNT4 is itself required for the decidualization of endometrial stromal cells. Deletion of *Wnt4* from the murine uterus using PR^{Cre} ($PR^{Cre/+}Wnt4^{f/f}$) results in subfertility with impaired decidualization and implantation failure (Franco et al. 2011). Like $PR^{Cre/+}Bmp2^{f/f}$ mice, $PR^{Cre/+}Wnt4^{f/f}$ mice exhibit decreased responsiveness to progesterone, suggesting WNT4 signaling is also required for optimal PR signaling. Implantation failure, however, is unique to $PR^{Cre/+}Wnt4^{f/f}$ mice. Of note, $PR^{Cre/+}Wnt4^{f/f}$ mice exhibit a defect in uterine development that results in complete absence of uterine glands. Glandular secretions are critical to uterine receptivity, so this developmental phenotype may account for the implantation failure seen in $PR^{Cre/+}Wnt4^{f/f}$ mice.

3.5.5 PR Antagonizes Epithelial ER to Mediate Uterine Receptivity

Progesterone signaling has long been known to antagonize the activity of estrogen. The antagonistic activity of progesterone is of utmost importance in the uterus, where estrogen signaling must be abrogated for receptivity to be achieved. In the endometrium, progesterone mediates uterine receptivity by abrogating the expression of ER targets in the epithelium, as well as inhibiting estrogen-driven proliferation. The interplay between estrogen and progesterone signaling is highlighted by the fact that PR expression is directly regulated by ER. The *Pgr* promoter contains an estrogen response element (ERE), allowing ER to directly induce PR expression (Moutsatsou and Sekeris 2003). PR levels are also modified by progesterone, which represses PR expression through the same ERE in a negative feedback loop. During the menstrual cycle, endometrial PR expression is highest during the proliferative phase, consistent with ER-induced activation of *Pgr* transcription (Talbi et al. 2006).

One critical role of PR in preparing the uterus for implantation is the downregulation of estrogen-induced mucinous glycoproteins at the surface of the luminal epithelium. In the pre-receptive uterus, the endometrial lumen is lined by a mucous barrier consisting of highly glycosylated proteins, including the estrogen target, mucin 1 (MUC1) (reviewed in Carson et al. 2000). While MUC1 expression is critical to protect the endometrium from bacteria and other pathogens, it impedes embryo implantation and must be downregulated during the window of receptivity.

In mice, downregulation of MUC1 occurs on day 3 of pregnancy, following a surge in epithelial PR expression. Importantly, this downregulation is dependent upon the activity of the PR-A isoform (Brayman et al. 2006).

Abrogation of ER-dependent proliferation is another of the crucial functions of PR during days 2 and 3 of pregnancy. Progesterone signaling inhibits ER-driven proliferation by preventing translocation of cyclin D1 to the nucleus (Tong and Pollard 1999). This results in hyperphosphorylation of Rb and p107 and failure of cells to progress through G1/S checkpoint of the cell cycle. In endometrial epithelial cells, PR can directly inhibit proliferation by regulating minichromosome maintenance 2 (MCM2) (Ray and Pollard 2012). MCM2, a positive regulator of proliferation, is required for successful DNA replication during S phase of the cell cycle. Estradiol treatment induces MCM2 expression by enhancing the recruitment of ERα, Kruppel-like factor 4 (KLF4), and RNA polymerase II to the regulatory regions of the *Mcm2* gene. KLF4, a Zn finger transcription factor, is itself induced by ER. However, progesterone treatment prevents ER-dependent induction of KLF4. Instead, Kruppel-like factor 15 (KLF15) is induced by estrogen and progesterone co-treatment, resulting in recruitment of KLF15, rather than KLF4, to the *Mcm2* gene. This inhibits *Mcm2* promoter activity and expression, effectively halting DNA synthesis. These molecular mechanisms illustrate the importance of epithelial PR in directly antagonizing ER-driven proliferation within the epithelial compartment. The essential role of epithelial PR in ER antagonism has been further characterized by a genetically engineered mouse model, in which $Wnt7a^{Cre}$ was used to ablate PR specifically in the endometrial epithelium ($Wnt7a^{Cre}PR^{f/-}$) (Franco et al. 2012). $Wnt7a^{Cre}PR^{f/-}$ mice are infertile due to implantation failure and absent decidualization, highlighting the requirement for epithelial PR expression in both implantation and decidualization. Importantly, $Wnt7a^{Cre}PR^{f/-}$ mice exhibit excessive epithelial proliferation. Proliferation of the luminal epithelium continues into days 2 and 3 of pregnancy, and the proliferative ER targets, cyclin D1 and minichromosome maintenance 3 (MCM3), are aberrantly expressed at these later time points. These findings highlight the essential function of epithelial PR signaling in directly inhibiting estrogen-driven proliferation. However, stromal PR also plays a central role in the inhibition of ER-driven epithelial proliferation via a paracrine signaling pathway.

3.5.6 The Role of Stromal PR in ER Antagonism

The inhibition of estrogen signaling by PR is another illustrative example of the complex paracrine signaling that occurs between the epithelial and stromal compartments of the uterus. This paracrine signaling pathway is mediated by heart and neural crest derivatives expressed 2 (HAND2), a basic helix-loop-helix transcription factor. PR directly induces *HAND2* expression in human stromal cells (Mazur et al. 2015). In mice, HAND2 is expressed in the endometrial stroma at day 3 of pregnancy, consistent with PR-dependent induction (Huyen and Bany 2011). Importantly, uterine ablation of *Hand2* expression ($PR^{Cre/+}Hand2^{f/f}$) results in infertility (Li et al. 2011).

These mice exhibit implantation failure due to unopposed estrogen signaling in the endometrial epithelium. At the molecular level, HAND2 alters FGF signaling to abrogate estrogen signaling in a paracrine manner. HAND2 inhibits the production of several FGF ligands in stromal cells (Li et al. 2011). As a result, activation of FGFR in overlying epithelial cells ceases, and proliferative signaling is abrogated. The induction of HAND2 by PR in the stroma thus results in inhibition of estrogen signaling in the epithelium through this paracrine pathway. The importance of PR-driven compartmental crosstalk in the suppression of ER-induced epithelial proliferation is further supported by tissue-grafting experiments, in which wild-type and PRKO endometrial tissues were surgically recombined under the kidney capsule (Kurita et al. 1998). These studies demonstrated that stromal PR expression is necessary and sufficient to inhibit estrogen-driven proliferation of the endometrial epithelium. Estrogen-driven proliferation continued unabated, despite progesterone co-treatment, in reconstructions of wild-type epithelium with PRKO stroma. Grafts of PRKO epithelium with wild-type, PR-expressing stroma, however, showed suppression of epithelial proliferation in response to progesterone treatment. These findings are in direct disagreement with the phenotype of $Wnt7a^{Cre}PR^{f/-}$ mice, which demonstrate that epithelial PR is required for the inhibition of estrogen-induced proliferation in the pre-receptive uterus (Franco et al. 2012).

These discordant results underscore the need for better understanding of the complex signaling that takes place between the epithelial and stromal compartments. Importantly, these results indicate that both epithelial and stromal PRs play roles in the antagonism of ER activity at different time points during early pregnancy. PR expression changes dynamically during early pregnancy, suggesting that the relative importance of epithelial and stromal PR signaling varies temporally. Epithelial PR expression, which is high in the days preceding implantation, declines at the time of attachment. High expression of PR in the epithelium during the pre-receptive phase facilitates the direct inhibition of epithelial proliferation by epithelial PR. Indeed, the PR-dependent inhibition of cyclin D1 translocation and MCM2 expression can be seen in the pre-receptive uterus at days 2 and 3 of pregnancy. However, the loss of PR expression in the epithelial compartment at the time of attachment suggests a requirement for stromal PR in continued suppression of ER-driven proliferation. Indeed, stromal PR induces the production of HAND2 during decidualization, which facilitates inhibition of epithelial proliferation by a paracrine mechanism. Thus, PR may act directly within the epithelium, or indirectly via a paracrine mechanism, during different stages of early pregnancy.

3.6 Roles for Androgen Receptor (AR) in Early Pregnancy

AR, well known for its role in male reproduction, also performs important functions in female reproduction and pregnancy. AR binds to androgen ligands, most notably testosterone and dihydrotestosterone (DHT). Like ER and PR, AR is expressed as two unique isoforms. Similar to the PR isoforms, the AR isoforms are transcribed

from a single gene, with AR-A existing as an amino-terminally truncated form of AR-B (Wilson and McPhaul 1996). The AR-B isoform is expressed at higher levels than the AR-A isoform, and its activity is well characterized. Unfortunately, the action of AR-A is not well described. In humans, AR expression is detected in both the ovaries and the uterus (Cloke and Christian 2012). AR is expressed throughout the uterine epithelium and stroma, and is regulated during the different phases of the menstrual cycle. AR expression is highest in the stroma during the proliferative phase and then decreases during the secretory phase as stromal cells undergo decidualization (Horre et al. 1992). Indeed, AR regulates gene expression in decidualizing stromal cells. Addition of DHT during *in vitro* decidualization results in dose-dependent upregulation of the decidual marker, prolactin (PRL), by enhancing activation of the *PRL* promoter (Cloke et al. 2008). In vitro experiments have shown that AR regulates genes involved in cell motility, cytoskeletal organization, and regulation of the cell cycle in decidualizing stromal cells. In addition to its role in the differentiation of decidual cells, AR signaling may have antiproliferative functions that complement PR's ability to inhibit estrogen-driven proliferation. Androgens have been shown to inhibit cell growth and DNA synthesis in endometrial stromal cells, and knockdown of AR in stromal cells results in increased proliferation (Cloke et al. 2008). In mice, deletion of AR (ARKO) in females results in reduced fertility over time (Cloke and Christian 2012). This phenotype has previously been attributed to premature ovarian failure. However, ARKO females display additional abnormalities that suggest a role for AR signaling in the uterus during development and pregnancy. The uteri of ARKO females are small, with reduced uterine growth during the estrous cycle. Pregnancy-related phenotypes in ARKO females include reduced litter size, abnormal placental development, and placentomegaly. Although these phenotypes do not result in outright infertility, they underscore the need for further investigation of the role of AR signaling in uterine development and pregnancy.

3.7 Glucocorticoid Receptor (GR)

Finally, GR may perform important functions in the establishment and maintenance of pregnancy. GR, the first nuclear receptor to be cloned and sequenced, is well known for its diverse roles in homeostasis and stress responses. GR displays affinity for several different ligands, including dexamethasone, cortisol, corticosterone, mineralocorticoids, and even progesterone. GR is expressed as two isoforms, GRα and GRβ, which are produced through alternative splicing of the same gene (Hollenberg et al. 1985; Encio and Detera-Wadleigh 1991). GRα and GRβ are identical to amino acid 727, after which they diverge. GRα is slightly larger, at 777 amino acids, while GRβ is 742 amino acids in length. Intriguingly, the GRβ isoform does not bind hormone and is transcriptionally inactive. Unlike the other SHRs, GR exhibits markedly weak dimerization activity and monomeric GRα acts as a transcriptional repressor. Glucocorticoid signaling plays numerous, diverse roles

throughout development and adulthood, so it is unsurprising that GR is expressed in a multitude of tissues. Of interest, GR is expressed in the placenta throughout pregnancy. GR expression can also be detected in the uterine stroma in fibroblasts, endothelial cells, and lymphocytes (Bamberger et al. 2001). GR signaling may impede the process of decidualization. Glucocorticoids inhibit PRL production in differentiating endometrial stromal cells via upregulation of lipocortin-1 (Pihoker et al. 1991). GR signaling is also known to regulate prostaglandins, which play the critical function of increasing stromal vascular permeability during implantation and decidualization (Neulen et al. 1989).

3.8 Conclusions and Future Directions

Successful establishment and maintenance of pregnancy requires significant remodeling of the uterus; many of these changes are orchestrated by SHR signaling. In particular, steroid hormone signaling through ER and PR directs the endometrium through the dramatic changes that facilitate uterine receptivity, implantation, and decidualization. The expression and activity of these receptors change dynamically through the phases of the estrous cycle and early pregnancy. While we have uncovered many of the mechanisms that regulate the activities of ER and PR, many questions remain unanswered. In particular, the functional differences in the ER and PR isoforms, and the ways in which these isoforms interact, must be elucidated. The roles of the ERβ and PR-B isoforms in reproductive function, and the possible role of PR-B in promoting epithelial proliferation, merit further study. The roles of other SHRs, such as AR and GR, in early pregnancy also merit further exploration. Genetically engineered mice will continue to play a pivotal role in the investigation of pregnancy, leading to discoveries that can be translated into improved pregnancy outcomes for women around the globe.

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Chapter 4
Transmembrane Mucin Expression and Function in Embryo Implantation and Placentation

Pamela E. Constantinou, Micaela Morgado, and Daniel D. Carson

Abstract Transmembrane mucins (TMs) are extremely large, complex glycoproteins that line the apical surfaces of simple epithelia including those of the female reproductive tract. TMs provide a physical barrier consistent with their role as part of the innate immune system. This barrier function must be overcome in the context of embryo implantation to permit blastocyst attachment. Three major TMs have been identified in uterine epithelia of multiple species: MUC1, MUC4, and MUC16. MUC1 has been found in all species studied to date, whereas expression of MUC4 and MUC16 have been less well studied and may be species specific. The strategies for removing mucins to permit embryo attachment also vary in a species-specific way and include both hormonal suppression of TM gene expression and membrane clearance via cell surface proteases. Studies emerging from the cancer literature indicate that TMs can modulate a surprisingly wide variety of signal transduction processes. Furthermore, various cell surface proteins have been identified that bind either the oligosaccharide or protein motifs of TMs suggesting that these molecules may support cell attachment in some contexts, including trophoblast interactions with cells of the immune system. The intimate association of TMs at sites of embryo–maternal interaction and the varied functions these complex molecules can play make them key players in embryo implantation and placentation processes.

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4.1 Introduction

Embryo attachment to the uterine wall is a critical and highly regulated process in mammals. Embryo attachment is only allowed during a defined period of the estrous cycle, often called the "window of receptivity." While it was appreciated for many years that such a receptive window existed in many species, the molecular basis underlying receptivity remained unclear. Many investigations identified cell surface and extracellular matrix proteins capable of supporting embryo attachment *in vitro* with provocative correlations to their expression patterns *in vivo*, including certain integrins and heparan sulfate proteoglycan binding proteins [reviewed in Carson et al. (2000)]. Nonetheless, it also had long been recognized that the apical surface of uterine epithelia is covered with a thick glycocalyx that changes in complex ways during the cycle and in preparation for embryo attachment (Schlafke and Enders 1975). Yet, the identity of the molecules carrying these carbohydrates remained elusive. MUC1 was the first major molecule identified that was capable of carrying the large amount of oligosaccharides that could account for the major changes in carbohydrate composition at the uterine luminal surface. Subsequently, important functions of MUC1 and related glycoproteins were revealed as well as many aspects of the processes that control their expression. This review focuses on the role transmembrane mucins play in human implantation and placentation but discusses key, relevant findings in other species.

4.2 Implantation and Placentation

Following fertilization, the mammalian embryo, covered in a glycoprotein coat called the *zona pellucida*, passes through the oviduct to arrive at the apical surface of the uterine epithelium. Shortly after arrival in the uterus, the *zona pellucida* is lost and the embryo subsequently becomes attachment competent. Attachment competency includes expression of various adhesion-promoting proteins on the external surface of the trophectoderm surrounding the embryo. In parallel and coordinated by steroid hormone influences provided by *corpora lutea*, the uterus differentiates to a state in which it can support embryo attachment and implantation, the "receptive" state. This state is transient lasting for a period of hours to days in species like mice and humans, respectively (Cha and Dey 2014). If the embryo fails to attach during this period, the uterus converts to a refractory state where it will no longer support embryo attachment. When attachment occurs, the embryo may begin further development including formation of the placenta. In noninvasive species like pigs and sheep, placentation occurs essentially in the uterine lumen, while in invasive species like rodents and humans, the trophoblast penetrates the uterine epithelium and placentation occurs within the decidual tissue of the endometrium (Chavatte-Palmer and Guillomot 2007; Cha et al. 2012). In

humans, this process is particularly invasive with the trophoblast reaching the maternal arteries and displacing endothelial cells to establish contact with the maternal blood supply (Fisher 2004). Failure to reach the maternal arteries results in preeclampsia, a disease occurring in 3–5% of all human pregnancies with potentially life-threatening consequences to both the fetus and mother (Fisher 2004). The placenta is surrounded by a dense accumulation of maternal NK cells as well as regulatory T cells (Jennings et al. 1986; Alijotas-Reig et al. 2014). Thus, suppression of the potential maternal immune response to the allogeneic placenta is crucial.

4.3 Transmembrane Mucins (TMs): Expression and Functions

4.3.1 Structure

TMs are a subset of the family of high molecular weight, heavily glycosylated mucin glycoproteins. The hallmarks of mucins are their large size; the occurrence of multiple tandem repeat peptide motifs largely composed of serine, threonine, and proline; and the dense substitution of the tandem repeat motifs with O-linked oligosaccharides. Mucins may be either secreted or retained at the plasma membrane via membrane-spanning domains. The major TMs are MUC1, MUC4, and MUC16 (Fig. 4.1). Each is a type I membrane glycoprotein in which the vast majority of the structure is composed of the tandem repeat-containing ectodomain. The extremely large size and highly extended structures of the ectodomains account for many of the functions attributed to TMs. All three TMs undergo proteolytic cleavage and remain associated at the cell surface as heterodimers. Each has a single membrane-spanning sequence and a short cytoplasmic domain. Although still very much larger than conventional cell surface receptors, MUC1 is the smallest of these three TMs. MUC1's ectodomain is relatively simple being composed primarily of a series of tandem repeat motifs of 20–21 amino acids. Allelic polymorphism generates species of different sizes with the differences being in the number of tandem repeats (Gendler et al. 1990). Other forms of MUC1 lacking the cytoplasmic tail or ectodomain are generated by alternative splicing and are expressed by uterine tissues and uterine epithelial cell lines but usually constitute a minor amount of the total (Julian and Carson 2002; Hey et al. 2003). MUC1 has the largest cytoplasmic domain of the three TMs being approximately 70 amino acids in length. Consistent with this, MUC1's cytoplasmic tail has been implicated in more intracellular signaling processes than the other TMs (see below).

MUC4's ectodomain is substantially larger and more complex than that of MUC1. This structure is dominated by a large tandem repeat domain as well as a *nidogen*-like (NIDO) domain, an *a*dhesion-associated in *M*UC4 and *o*ther *p*rotein

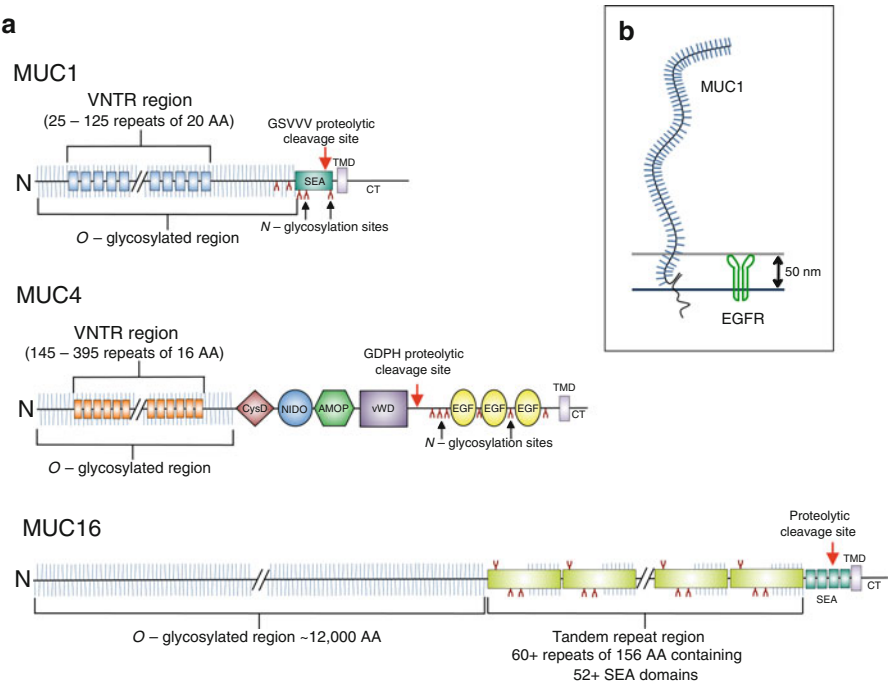


Fig. 4.1 Structural representations of TMs. (**a**) MUC1, MUC4, MUC16 (not drawn to scale). Abbreviations: *VNTR* variable number of tandem repeats, *SEA* sea urchin sperm protein–enterokinase–agrin, *TMD* transmembrane domain, *CT* cytoplasmic tail, *CysD* Cys-rich domain, *NIDO* nidogen homology sequence, *AMOP* adhesion-associated domain, *vWD* von Willebrand factor D domain, *EGF* epidermal growth factor-like regions. (**b**) Size comparison of MUC1, extending 200–500 nm from the cell surface, and epidermal growth factor receptor (*EGFR*), 50 nm from the cell surface (drawn to scale)

(AMOP) domain, *v*on *W*illebrand factor *D* (vWD) domain, and epidermal growth factor-like (EGF) domains. MUC4 splice variants also have been reported, although not in uterine tissues (Choudhury et al. 2000; Moniaux et al. 2000). MUC16 is the largest of the three and is the largest cell surface glycoprotein known. The ectodomain has a massive (12,000 amino acid) O-glycosylated domain, a tandem repeat domain consisting of more than 60 repeats of 156 amino acids, and *S*perm–*E*nterokinase–*A*grin (SEA) domains (O'Brien et al. 2001; Yin and Lloyd 2001; Gipson et al. 2014). Indirect evidence for the occurrence of MUC16 splice variants has been suggested [discussed in Haridas et al. (2014)]; however, mRNA splice variants have not been rigorously identified. Fragments of MUC16 released from the cell surface are the CA 125 antigens commonly used as a serum marker for certain cancers, notably ovarian and endometrial (Patsner and Yim 2013; Baser et al. 2014; Felder et al. 2014), as well as endometriosis (Spaczynski and Duleba 2003).

4.3.2 Functions

Not surprisingly, most of the functions attributed to TMs are accounted for by their massive ectodomains. Their heavy glycosylation makes them highly hygroscopic providing lubrication for mucosal surfaces. Their large size, high concentration at apical cell surfaces, extended structures, and generally antiadhesive nature make them excellent barrier molecules and a key part of the innate immune system (Hilkens et al. 1992; Voynow and Rubin 2009). In the context of embryo implantation, this barrier function is problematic since TMs are quite effective in inhibiting cell adhesion (Wesseling et al. 1995; Komatsu et al. 1997), including embryo attachment (DeSouza et al. 1999). Muc1, Muc4, and Muc16 (rodent nomenclature) are expressed by rodent uterine epithelia under most conditions (DeSouza et al. 1998; Idris and Carraway 1999, 2000; Wang et al. 2008). In rodents and certain other species, Muc1 is lost during the receptive phase providing access for embryo attachment to the uterine epithelium (DeSouza et al. 1998). Nonetheless, neither Muc1 nor Muc16 null mice display an implantation phenotype *in vivo* although Muc1 null uterine epithelia are constitutively "receptive" *in vitro* (DeSouza et al. 1999). In their roles as inhibitors of embryo attachment, loss of TMs would not be expected to inhibit implantation but rather would be expected to promote this process. Nonetheless, embryo transfer experiments have shown that uteri of Muc1 null mice are not chronically receptive but rather display the same window of receptivity as their wild-type counterparts (DeSouza et al. 1999). These observations led to the conclusion that factors in addition to loss of Muc1 expression are required to permit embryo attachment *in vivo*. These factors could include increased expression of various growth factors or expression of adhesion-promoting receptors; however, other TMs, e.g., Muc4 and Muc16, also may need to be lost during this process.

4.4 Control of TM Expression

4.4.1 Cytokines

TMs are almost exclusively expressed by simple epithelia throughout the body with low-level expression in some hematopoietic cells and activated T cells (Agrawal et al. 1998; Kruger et al. 2000). In most tissues, a substantial basal level of expression occurs; however, proinflammatory cytokines substantially elevate TM expression in many contexts, including epithelial cell lines derived from reproductive tract tissues (Lagow and Carson 2002; Thathiah et al. 2004; O'Connor et al. 2005; Dharmaraj et al. 2010; Kasimanickam et al. 2014; Chapela et al 2015; Morgado M et al. 2015, unpublished studies). These actions are mediated by the transcription factors *N*uclear *F*actor κ *B* (NFκB) and *S*ignal *T*ransducers and *A*ctivators of *T*ranscription (STATs) (Lagow and Carson 2002; Dharmaraj et al. 2010). Conversely, a class of transcriptional corepressors of cytokine actions, *p*rotein *i*nhibitors of

*a*ctivated *S*TATs (PIASs), suppresses *MUC1* gene expression in response to cytokines as well as progesterone (see below) in several cellular contexts and may serve as a feedback control on this system (Brayman et al. 2007). Cytokine responsiveness is likely to represent a system to elevate the barrier functions of epithelia when challenged by infection or irritants. Proinflammatory cytokine levels also change in dynamic ways in uterine tissues of various species during early stages of pregnancy and may drive TM expression (McMaster et al. 1992; Wessels et al. 2007; Bazer et al. 2009; Haider and Knofler 2009).

4.4.2 Steroid Hormones

Uterine TM expression is also strongly influenced by steroid hormones with estrogen elevating expression and progesterone antagonizing estrogen action, in this regard, in rodents (Surveyor et al. 1995; DeSouza et al. 1998; McNeer et al. 1998). While estrogen stimulates TM expression *in vivo,* estrogen receptor does not appear to directly regulate TM gene expression (Zhou et al. 1998; Brayman et al. 2006). Rather, it appears that estrogen effects on MUC1 expression are mediated via factors produced by other uterine cell types, e.g., stroma, in response to estrogen. In contrast, progesterone receptor directly binds to the MUC1 promoter region and regulates MUC1 gene expression in an isoform-specific fashion. Progesterone receptor B is a stimulator of MUC1 gene expression, whereas progesterone receptor A antagonizes the activity of the B isoform (Brayman et al. 2006). This isoform-specific response largely accounts for the apparently conflicting observations that progesterone inhibits TM expression in some species, e.g., rodents, while apparently elevating TM expression in other species, i.e., rabbits and humans (Hey et al. 1994; Hoffman et al. 1998).

4.4.3 PPARs and Trophoblastic Expression of MUC1

Studies in mice revealed two interesting features of Muc1 expression: (1) that another transcriptional coregulator, *P*eroxisome *P*roliferator-*A*ctivated *R*eceptor-γ (PPARγ), stimulates Muc1 expression and (2) Muc1 is expressed by placental trophoblast (Shalom-Barak et al. 2004). PPARγ is activated by various natural ligands including certain polyunsaturated fatty acids and prostaglandin J_2, as well as the synthetic thiazolidinediones, including rosiglitazone and pioglitazone. Investigation of PPARγ actions in human cell lines revealed an opposite response to that observed in mice, namely, inhibition with regard to both progesterone- (Wang et al. 2010) and EGF-simulated (Dharmaraj et al. 2013) MUC1 expression. The human MUC1 gene has a 21 bp insertion in the PPARγ-responsive region which appears to account for the differences in responsiveness between species. In addition to other actions, PPARγ and its agonists have anti-inflammatory actions (Kapadia et al. 2008). In this

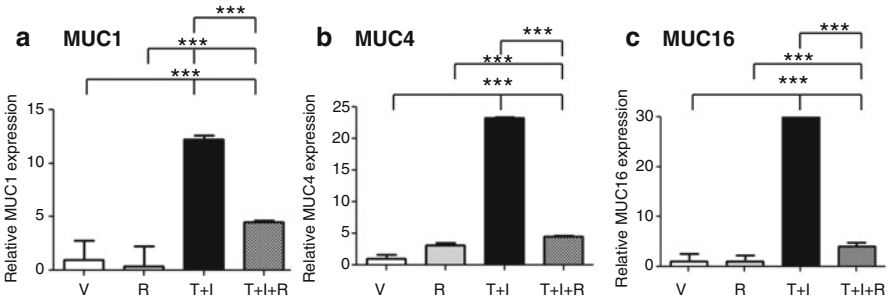


Fig. 4.2 Coordinate regulation of TMs by cytokines and rosiglitazone. MCF7 cells were incubated for 48 h with vehicle control (V), rosiglitazone (100 μM; R), TNFα (25 ng/ml) plus IFNγ (200 IU) (T+I), or TNFα plus IFNγ plus rosiglitazone (T+I+R). RNA was extracted from triplicate independent samples in each case for qRT-PCR analyses of (**a**) MUC1, (**b**) MUC4, and (**c**) MUC16 mRNA relative to that of β-actin. *** $p<0.001$ V vs. T+I and T+I+R, R vs. T+I and T+I+R

regard, PPARγ activators can inhibit cytokine-stimulated expression of all three TMs (Fig. 4.2). Therefore, it appears that TM expression can be regulated coordinately offering opportunities for broad therapeutic control. PPARγ activators have therapeutic value in placental dysfunction, including mitigation of symptoms associated with preeclampsia (McCarthy et al. 2011; Kadam et al. 2015). Whether this has relevance to reduction of trophoblast MUC1 expression is unclear.

Detection of Muc1 in murine placenta via cDNA microarray analyses was surprising since this tissue does not contain simple epithelia that would be the normal sites of Muc1 expression (Shalom-Barak et al. 2004). Nonetheless, several studies of human trophoblast and trophoblastic cell lines have confirmed that human trophoblasts not only express MUC1, but also that MUC1 is elevated in trophoblast of preeclamptic placentae and suppresses trophoblast invasion *in vitro* (Shyu et al. 2008, 2011). It is possible that the suppression of invasion is due to MUC1 physically inhibiting the interactions with the extracellular matrix through which trophoblast must invade; however, it also is possible that MUC1 interactions with specific cell surface receptors mediate aspects of this inhibitory response (see below).

4.4.4 Sheddases

In rabbits, MUC1 is removed locally at sites of embryo attachment *in vivo* (Hoffman et al. 1998). Human blastocysts also trigger clearing of MUC1 at their attachment sites on layers of human uterine epithelia cell lines *in vitro* (Meseguer et al. 2001). Thus, it appears that TM-removing activities exist that are either produced by blastocysts or activated in uterine epithelia by blastocysts. Conditioned media from human blastocysts do not stimulate MUC1 release by uterine epithelial cell lines (Thathiah A and Carson DD 2007, unpublished studies) implying that these

activities reside in the uterine epithelia themselves. Fragments of TMs lacking their cytoplasmic tails are released into serum in certain disease states (Spaczynski and Duleba 2003; Patsner and Yim 2013; Baser et al. 2014; Felder et al. 2014), as well as into female reproductive tract secretions (de Bruijn et al. 1986; Martinez et al. 1994; Andersch-Bjorkman et al. 2007). As mentioned above, mRNA slice variants can generate secreted forms of TMs lacking transmembrane and cytoplasmic domains; however, most of these fragments appear to be the result of ectodomain release or shedding from the cell surface via the action of cell surface proteases or "sheddases." Two sheddases that release MUC1 ectodomains have been identified in uterine epithelia, namely, TACE/ADAM17 and MT1-MMP (Ando and Kusano 1992; Thathiah et al. 2003). Sheddase activity can be controlled in various ways including through conversion from a proenzyme, transport to the cell surface from intracellular locales, and protein kinase C activation (Edwards et al. 2008). In addition, TNFα stimulates MUC1 shedding in uterine epithelial cell lines (Thathiah et al. 2004). In addition to the controls over TM gene expression mentioned above, TM shedding offers another point of intervention to control the levels of cell surface TMs either to enhance protective functions of the endometrium or to reduce TM expression to promote embryo attachment.

4.5 Transmembrane Mucin Binding Proteins

Multiple proteins bind to TMs. This includes proteins that bind to oligosaccharide as well as protein motifs (Table 4.1). In the case of the former, these proteins often also can bind to other proteins or lipids carrying the same carbohydrate structures. Protein–oligosaccharide binding usually displays substantially lower affinity constants than protein–protein interactions, e.g., growth factor–growth factor receptor; however, TMs provide multiple binding sites for these proteins which can greatly increase the avidity of these interactions. In some of cases described below, binding may serve to aggregate or cross-link glycoproteins at the cell surface (galectins). In other cases, the interactions may support cell adhesion (mesothelin, selectins) or lead to intracellular signal transduction (Siglecs, β-catenin).

4.5.1 Galectins

Galectins are a family of small (14–39 kDa), soluble, β-galactoside binding proteins that occur both intracellularly and on the cell surface (Jeschke et al. 2013). Galectins do not have canonical signal sequences or transmembrane domains and reach the cell surface by nonclassical pathways. While the presence of β-galactose is required for galectin recognition, other aspects of oligosaccharide structure, e.g., sialylation, presence of lactosamine repeats, create differences in recognition by different galectins changing binding affinities up to 100-fold (Stowell et al. 2008; Zhuo et al.

Table 4.1 Transmembrane mucin binding proteins

Mucin binding proteins	Binding specificity	Biological activity	References
Galectins	*N*-acetyllactosamine (Galβ1-4GlcNAc) Lacto-*N*-biose (Galβ1-3GlcNAc) Increased affinity for poly-*N*-acetyllactosamine [Gal-1, Gal-3, Gal-7]	Increase barrier function of TM Promote anti-inflammatory effects [Gal-1, Gal-3] Involved with embryo implantation and placentation [Gal-1] Reepithelialization associated with wound repair [Gal-7]	Hirabayashi et al. (2002), Jeschke et al. (2013), Panjwani (2014)
Selectins	NeuAcα2-3Galβ1-4(Fucα1-3)GlcNAc (sLe^x) NeuAcα2-3Galβ1-4GlcNAcn1-3 [Galβ1-4(Fucα1-3)Glc-NAcβ1-3]$_2$[Galβ1-4GlcNAcβ1-3]$_2$Galβ1-4GlcβCer [E-Selectin] Core-2-based *O*-linked sLe^x on Thr residue on P-selectin glycoprotein ligand-1 near TyrSO(3) [P-selectin] NeuAcα2-3Galβ1-4(Fucα1-3)GlcNAc-6-SO_4 [L-selectin]	Potential to tether blastocysts to uterine epithelia	Hemmerich et al. (1995), Leppanen et al. (2000), Nimrichter et al. (2008)
Siglecs	Neu5Acα-6GalNacα [Siglec-6] Neu5Acα2-3Galβ1-4Glc [Siglecs-7,9] Neu5Acα2-6Galβ1-4Glc [Siglecs-7,9] NeuAcα2-8NeuAcα2-3Gal [Siglec 7]	Potential to restrict trophoblast invasion; potential to attenuate immune response to allogeneic placenta	Patel et al. (1999), Angata and Varki (2000), Zhang et al. (2000), Nicoll et al. (2003)
Mesothelin	N-linked glycosylation on MUC16	Supports MUC16-dependent cell adhesion and mesothelial colonization	Gubbels et al. (2006)

2008). Multiple galectins have been detected in human endometrium and placental tissues with a wider variety found in placental tissues (Jeschke et al. 2013).

Several galectins can bind to TMs, including MUC1, MUC4, and MUC16 (Bancalari et al. 1989; Seelenmeyer et al. 2003; Yu et al. 2007; Argueso et al. 2009; Senapati et al. 2011). Functional consequences of these interactions are suggested to include enhancement of the barrier function of TMs to activation of signal transduction cascades. A series of studies have implicated important functions for

galectins in embryo implantation and placentation, notably galectin-1 [Jeschke et al. (2013) for review]. In addition, elevated galectin-7 expression is associated with uterine repair after menstruation (Evans et al. 2014), miscarriage (Menkhorst et al. 2014b), and preeclampsia (Menkhorst et al. 2014a). Many of these observations relate to the proposed role for galectin-7 in reepithelialization associated with wound repair (Panjwani 2014).

4.5.2 Selectins

Selectins are a family of three cell adhesion-promoting transmembrane proteins (E-, L-, and P-) that bind complex oligosaccharide structures (Rosen and Bertozzi 1994). Originally discovered as mediators of lymphocyte homing responses, it since has been recognized that selectins are expressed in many other contexts, including by human blastocysts (Genbacev et al. 2003). In the endometrium, MUC1 carries selectin ligands throughout the cycle (Carson et al. 2006). Thus, it is possible that MUC1 and other selectin ligand-bearing TMs initially tether blastocysts to the epithelial cell surface at early stages of the implantation process. MUC16 also binds E- and L-selectin in pancreatic cancer cells (Chen et al. 2012); however, it is not clear if MUC4 or MUC16 carry selectin ligands in the uterus or placenta.

4.5.3 Siglecs

Siglecs (*s*ialic acid-binding *i*mmuno*g*lobulin-like *lec*tins) are a family of 14 mammalian cell surface proteins primarily expressed in cells of the immune system (Pillai et al. 2012; Macauley et al. 2014). Structurally, they are type I transmembrane glycoproteins with an oligosaccharide-binding ectodomain, a single transmembrane domain, and an intracellular domain that mediates signal transduction in most Siglecs. The intracellular domain usually contains either an ITIM (*i*mmunoreceptor *t*yrosine-based *i*nhibitory *m*otifs) or ITAM (*i*mmunoreceptor *t*yrosine-based *a*ctivation *m*otif) that functions to modulate protein phosphorylation-dependent signaling events. In the case of ITIM-containing Siglecs, ligand binding triggers phosphorylation of the ITIM creating a binding site for and activation of SHP phosphatases. Different Siglecs preferentially bind oligosaccharides with distinct structures, although all contain sialic acids. In humans, a unique ITIM-containing Siglec, Siglec-6, is expressed by trophoblast in a pattern complementary to the pattern of expression observed for its ligands (Brinkman-Van der Linden et al. 2007). Thus, Siglecs expressed by immune cells abundantly expressed in decidua as well as trophoblast Siglec-6 are likely to engage their complementary ligands in uteroplacental tissue. The identity of the molecules carrying Siglec ligands in uteroplacental tissue is unknown, but TMs may be among them. All three TMs can carry Siglec ligands in some contexts (Brinkman-Van der Linden and Varki 2000;

Swanson et al. 2007; Belisle et al. 2010; Tanida et al. 2013; Kiwamoto et al. 2015). Trophoblast TM binding to inhibitory Siglecs could potentially restrict trophoblast invasion as well as attenuate immune responses to the allogeneic placenta (Redzovic et al. 2013). Consistent with this notion, Muc1 null mice display greatly enhanced placental resorption (Croy et al. 1997). In addition, MUC16 binds to NK cells of pregnant women, presumably mediated by Siglec-9 on NK cells (Belisle et al. 2010; Tyler et al. 2012). With regard to trophoblast invasion, overexpression of MUC1 and Siglec-6 are associated with preeclampsia (Shyu et al. 2011); however, trophoblast TMs have not been formally demonstrated to carry Siglec-6 ligands.

4.5.4 Mesothelin

Mesothelin is a protein that recognizes the MUC16 ectodomain (Kaneko et al. 2009). Binding again is oligosaccharide dependent but requires N-linked, rather than O-linked, mucin-type oligosaccharides (Gubbels et al. 2006). Since mesothelin is retained at the cell surface via a glycosylphosphatidylinositol anchor binding does not result in any known signal transduction events. Rather, it is believed that mesothelin, primarily expressed by peritoneal mesothelial cells and certain cancers, supports MUC16-dependent cell adhesion and tissue colonization (Pastan and Hassan 2014). Mesothelin does not appear to be expressed by normal endometrium or placenta and is unlikely to play a role in embryo implantation or placentation.

4.5.5 TM Binding Signaling Proteins

The largest body of evidence for TM involvement in signal transduction processes relates to MUC1 [reviewed in Carson (2008), Kufe (2013)]. The MUC1 cytoplasmic domain is the largest of the TMs and appears to be able to support multiple interactions including phosphorylation, interaction with apoptosis modulators, and direct binding to β-catenin and several transcription factors. In almost all cases, these interactions have been demonstrated in cancer cells. Cancer cells not only lose apical restriction of TMs but also can accumulate substantial levels in intracellular locales which may account for many of these interactions (Hollingsworth and Swanson 2004; Bafna et al. 2010). None of these interactions have been demonstrated in normal endometrial or placental tissues or cells. Both MUC1 and MUC4 interact with members of the ERBB family and are suggested to promote responsiveness to EGF family members (Schroeder et al. 2001; Kozloski et al. 2010). ERBB family members are expressed by uterine epithelia, although interactions with MUC1 or MUC4 have not been demonstrated (Berchuck et al. 1989; McBean et al. 1997). It is possible that the apical restriction of TMs sequesters them from ERBBs under most circumstances. Both MUC1 and EGFR are expressed by trophoblast and could conceivably interact at these surfaces; however, MUC1:EGFR

interactions have not be demonstrated in trophoblast either. TMs also are implicated in promoting epithelial-to-mesenchymal transition (Comamala et al. 2011; Ponnusamy et al. 2013), although the relevance of this response to implantation is not clear.

4.6 Summary and Future Directions

TMs have emerged as major components of the apical surface of uterine epithelia. In addition to their general roles as hydrating agents and barriers to infection, TMs represent barriers to embryo attachment in the endometrium. In this regard, TMs contribute importantly to the creation of the non-receptive uterine state. Proinflammatory cytokines generally elevate TM expression in many contexts which is suggested to be part of an innate protective response. Different species have developed different strategies to remove TMs to permit blastocyst attachment. These strategies include hormonal downregulation of TM expression as well as local removal of TMs at blastocyst attachment sites through the action of cell surface proteases called sheddases. MUC1 also is expressed by trophoblast and elevated expression is associated with disease states, including preeclampsia. The discovery that TMs can carry ligands for and bind to various oligosaccharide-binding proteins found in the endometrium and placenta opens the possibility that TMs may play additional, adhesion-promoting roles in reproduction. These potential roles include promotion of certain cell–cell interactions as well as attenuation of the maternal immune response. Future work should focus on formally testing these exciting new roles for TMs as well as determining if control of TM expression and/or interference with TM–TM binding protein interactions can serve as points of therapeutic intervention in disease states and to enhance fertility.

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Chapter 5
Reflections on Rodent Implantation

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Abstract Embryo implantation is a complex process involving endocrine, paracrine, autocrine, and juxtacrine modulators that span cell–cell and cell–matrix interactions. The quality of implantation is predictive for pregnancy success. Earlier observational studies formed the basis for genetic and molecular approaches that ensued with emerging technological advances. However, the precise sequence and details of the molecular interactions involved have yet to be defined. This review reflects briefly on aspects of our current understanding of rodent implantation as a tribute to Roger Short's lifelong contributions to the field of reproductive physiology.

5.1 Introduction

Procreation is a cornerstone of evolutionary success. It helps to ensure the persistence of a species, while genetic diversification selects for traits optimal for survival of offspring. In eutherian mammals, the progression of pregnancy events is regulated by multiple checkpoints to ensure the successful birth of healthy young. Dysregulation at any critical stage of pregnancy can terminate the pregnancy or propagate adverse ripple effects throughout the remainder of pregnancy (Wang and Dey 2006; Cha et al. 2012).

Historically, implantation was dubbed "nidation" originating from the word "nidus," meaning a nest or a breeding place. Embryo implantation is the first intimate and cooperative physical and physiological interaction between the two genetically disparate epithelia derived from the embryo and maternal uterus. The prerequisites for embryo implantation include preimplantation embryo development

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to blastocyst stage, acquisition of its competency for implantation, uterine differentiation to the receptive state, reciprocal blastocyst–uterine dialogue, and the attachment reaction. Following implantation, stromal cell proliferate and differentiate to decidual cells (decidualization) along with matrix remodeling and angiogenesis. With the progression of pregnancy, the presumptive vascular system of the conceptus is brought into communication with the maternal circulation, establishing a functional placenta to direct pregnancy success.

Enders and Schlafke classified implantation into three stages: apposition, adhesion, and penetration (Enders and Schlafke 1967, 1969). During apposition, the embryonic trophectoderm becomes closely apposed to the uterine LE. This stage is then followed by intimate association of the trophectoderm and the luminal epithelium (LE) and is sufficiently intimate to resist dislocation of the blastocyst upon flushing of the lumen (adhesion). The penetration process is associated with the invasion through the LE by the trophectoderm. At this stage, stromal cells transform into decidual cells (decidualization) with extensive loss of the epithelial cells.

Implantation strategies vary between species and has been classified based on differing degrees of cell–cell interactions between the blastocyst and uterus. Bonnet classified implantation in diverse species into three principle categories: central, eccentric, and interstitial (Bonnet 1884). Central implantation is observed in mammals such as rabbits, ferrets, and some marsupials, in which blastocysts extensively expand prior to implantation to maximally interact with the uterine epithelium. In contrast, the blastocysts in mice, rats, and hamsters show only modest expansion and undergo eccentric implantation, and implantation chambers are formed by the evagination of the uterine epithelium. In many rodents, including mice and rats, implantation always occurs at the antimesometrial side of the uterus, opposite the entry site of blood vessels into the uterus, whereas implantation is mesometrial in bats. Finally, implantation is interstitial in guinea pigs, chimpanzees, and humans in which blastocysts embed into the subepithelial stroma.

Schlafke and Enders further classified implantation as intrusive, displacement, and fusion types based on the results of ultrastructural studies (Schlafke and Enders 1975). In intrusive types of implantation, as seen in humans and guinea pigs, trophoblast cells penetrate through the LE to reach the basal lamina. Displacement type of implantation occurs in rodents, in which the basal lamina lifts off the LE, facilitating trophoblast invasion into the subepithelial stromal bed. In contrast, rabbits exhibit fusion type of implantation in which trophoblast cells fuse with the LE by forming symplasma (trophoblastic knob).

Another type of implantation, dubbed noninvasive, is observed in large animals and marsupials, such as the pig, sheep, cow, horse, and wallaby (Renfree 1982). For example, in pigs with noninvasive implantation, the blastocyst remains in a free-floating state until day 12 at which point it elongates up to 100 mm in length primarily due to rapid growth of the extraembryonic tissue. This allows an efficient exchange of metabolites and nutrients between the uterus and conceptus until the attachment reaction and implantation occur.

5.2 Embryo Competency for Implantation

Embryonic migration through the oviduct towards the uterus and development of the embryo to the blastocyst stage are synchronized with the differentiation of the uterus to a state of receptivity for implantation (Psychoyos 1973). Loss of this synchrony results in defective implantation or implantation failure. In addition to appropriate development, the blastocyst must acquire molecular competency for implantation. Estrogen is essential for implantation in mice and rats (Psychoyos 1973; Ma et al. 2003; Yoshinaga 2013). Since the blastocyst does not respond to estrogen *in vitro* to acquire competency for implantation in a progesterone (P_4)-primed uterus (Paria et al. 1993), it was speculated that an estrogen metabolite could achieve blastocyst activation. Indeed, later studies found that catecholestrogens can activate the blastocyst *in vitro* and that an enzyme which generates catecholestrogens from primary estrogen is present in the uterus at the time of implantation (Paria et al. 1998), suggesting that estrogen has a dual role – while primary estrogen guides differentiating the uterus to the receptive state, its metabolite catecholestrogen can activate blastocysts for implantation.

Ovarian secretion of P_4 and estrogen are critical for implantation in mice and rats, but ovarian estrogen is not essential for implantation in several species such as pig, guinea pigs, rabbits, and hamsters. Since P_4 alone can support implantation in these species, a role for embryonic estrogen was speculated. In fact, later studies found that blastocysts in rabbits, pigs, and hamsters have the capacity to synthesize estrogens (Heap et al. 1981; Hoversland et al. 1982; Sholl et al. 1983). In contrast, the mouse embryo lacks the enzymatic machinery for estrogen synthesis (Stromstedt et al. 1996). It remains unknown whether embryonic estrogen plays a role in human implantation. In this respect, effects of local versus systemic estrogen were studied by using local injections of estrogen in rodents. This study showed that an injection of a minute dose of estrogen to the fat pad adherent very close to the uterus can induce implantation without conferring refractory state to the distant sites of the horn; a systemic estrogen injection later can induce implantation in other uterine regions. This suggests that a small dose of estrogen can locally influence uterine environment (Yoshinaga 1961).

5.3 Uterine Receptivity and Implantation

Corner once said that “the uterine chamber is actually a less favorable place for early embryos to implant than say, the anterior chamber of the eye, except when the hormones of the ovary act upon it and change it into a place of superior efficiency for its new functions” (Corner 1947). In fact, Alexandre Psychoyos first described that differentiation of the uterus to a receptive state is favorable for the development of an embryo and its implantation. He used embryo transfer experiments in

pseudopregnant and delayed-implanting rodent models to conclusively prove this concept. He showed by reciprocal embryo transfer experiments that blastocysts only implant when transferred into hormonally prepared, receptive uteri (Psychoyos 1973). This concept of uterine receptivity is widely accepted and has been confirmed in different species, establishing that all mammals studied so far exhibit a transient, varying duration of uterine receptivity (Dey et al. 2004; Wang and Dey 2006; Cha et al. 2012; Yoshinaga 2013; Cha and Dey 2014).

Acquisition of uterine receptivity approaching blastocyst attachment is reflected in both cellular and molecular changes. The three major uterine compartments (epithelium, stroma, and myometrium) respond uniquely to changing ovarian P_4 and estrogen secretion. In mice and rats, the cooperative interactions between P_4 and estrogen regulate uterine cell proliferation and/or differentiation in a spatiotemporal manner to confer the window of uterine receptivity. There is a gradual loss of apicobasal LE cell polarity and formation of microprotrusions called pinopodes or uterodomes on the apical surface of the LE impending blastocyst attachment (Tachi et al. 1970; Lundkvist and Nilsson 1982; Thie et al. 1996; Nikas and Psychoyos 1997; Murphy 2000). The molecular aspects of uterine receptivity are described below.

5.3.1 Molecular Aspects of Uterine Receptivity

During the peri-implantation period, many uterine factors can significantly affect implantation. For instance, diverse and overlapping gene expression patterns coordinate both uterine receptivity and blastocyst attachment to the LE. Many growth factors and their receptors are expressed within the uterus in a temporal and cell-specific manner, further highlighting the complex molecular landscape necessary for successful initiation of pregnancy.

Two key ovarian hormones estrogen and P_4 primarily execute their functions by nuclear estrogen receptors (ERα and ERβ) and progesterone receptors (PRA and PRB), respectively, in the uterus (O'Malley 1971; Kuiper et al. 1996; Conneely et al. 2002). Studies in mice devoid of each receptor have shown that during the peri-implantation period, a coordinated effort exists between estrogen and P_4 for implantation mediated by their nuclear receptors (Lubahn et al. 1993; Lydon et al. 1995; Curtis et al. 1999; Paria et al. 1999). The expression of ERα and PRA in all major uterine tissue compartments suggests their participation in uterine biology and implantation. Furthermore, studies have shown that reciprocal dialogues between the epithelium and stroma involving these receptors are necessary for appropriate uterine receptivity and implantation.

Estrogen and P_4 execute their uterine functions by inducing and refining multiple paracrine, juxtacrine, and autocrine factors in a spatiotemporal manner. One such factor is leukemia inhibitory factor (LIF) which is critical for implantation and functions as a downstream mediator of estrogen. By binding to its receptor LIFR and partnering with the co-receptor gp130, LIF activates downstream signaling through

signal transducer and activator of transcription 3 (STAT3) (Niwa et al. 1998). The deletion of LIF, *gp130* (*IL6st*), or *stat3* in mice results in implantation failure (Stewart et al. 1992; Song et al. 2000; Pawar et al. 2013; Sun et al. 2013).

Many P_4-responsive genes participate in peri-implantation events. FKBP52, a P_4-inducible immunophilin co-chaperone, is required for optimal PR activity in the uterus, since *Fkbp52−/−* mice are infertile. In addition, the impaired P_4 responsiveness in this knockout strain is reflected in enhanced estrogen-like signaling in the uterus (Tranguch et al. 2005; Yang et al. 2006). Interestingly, injection of excess P_4 can overcome P_4 resistance and lead to successful implantation and pregnancy, depending on the genetic background of mice (Tranguch et al. 2007). In the same vein, steroid receptor co-activator 2 (SRC2, also known as *Ncoa2*) is also recruited by PR and is necessary for proper P_4 action and increasing glycolytic flux during decidualization (Xu et al. 2009; Kommagani et al. 2013), since uterine deletion of *Ncoa2* leads to pregnancy failure (Mukherjee et al. 2006; Han and O'Malley 2014). Indeed, dysregulation of uterine SRC-2 expression has been associated with common gynecological disorders in women of reproductive age (Han et al. 2012).

Implantation failure due to defective uterine receptivity was also found to involve Indian hedgehog (Ihh) signaling (Matsumoto et al. 2002; Lee et al. 2006). Induced by P_4 signaling, *Ihh* is expressed in the epithelium and mediates stromal cell proliferation through interactions with its receptors. Chicken ovalbumin upstream promoter–transcription factor 2 (COUP–TFII, also known as *Nr2f2*), a proposed downstream target of Ihh, is also expressed in the subepithelial stroma (Kurihara et al. 2007). Conditional deletion of *Nr2f2* in PR-expressing tissues leads to infertility due to implantation failure with excessive estrogenic signaling in the epithelium, suggesting that COUP–TFII participates in balancing ER versus PR activities. These results indicate that Ihh executes epithelial–stromal interaction in a paracrine manner for uterine receptivity and implantation. Heart- and neural crest derivative-expressed protein 2 (Hand2) is a P_4-induced stromal transcription factor that was shown to play a role in uterine receptivity and implantation in mice (Li et al. 2011). Mice missing uterine *Hand2* showed implantation failure with increased estrogenic activity and epithelial cell proliferation via FGF–ERK signaling.

Transcription factors that greatly influence uterine receptivity and implantation, but are not directly impacted by estrogen or P_4, have also been reported. One such factor is *Msx1*, an ancient evolutionarily conserved homeobox transcription factor that is transiently expressed on the morning of day 4 of pregnancy (the day of uterine receptivity) in the mouse uterine epithelium (Daikoku et al. 2011). Msx1 is presumed to be important for receptivity due to its transient expression and the complete infertility that results from uterine deletion of *Msx* (*Msx1* and *Msx2*) genes due to failed or defective implantation. This finding was later confirmed (Nallasamy et al. 2012).

Kruppel-like factor 5 (Klf5), a zinc finger–containing transcription factor, is also not directly regulated by ovarian hormones in the uterus but is nevertheless critical for implantation, as mice with uterine deletion of *Klf5* are infertile that results from defective implantation (Sun et al. 2012). In mouse uteri, Klf5 is expressed in the luminal and glandular epithelia until day 5 of pregnancy when decidualization

begins. At this time, *Klf5* expression is seen in proliferating stromal cells surrounding the implantation site, while it is simultaneously downregulated in the epithelium. *Klf5* deletion results in blastocyst entrapment within the uterine lumen long past the implantation phase; however, these mice are surprisingly still capable of initiating some decidualization which is not sustained. This study suggests that epithelial defects due to *Klf5* deficiency fail to transmit signals for appropriate decidualization as evident from reduced expression of *Hoxa10* and *Bmp2* in the stroma. In fact, it has been previously shown that a functional LE is critical for the full-fledged decidual response (Lejeune et al. 1981). Taken together, the results suggest that physical contact between the blastocyst and stroma is not necessarily a requirement to initiate decidualization; unidentified signals from the blastocyst can be transmitted through the epithelium to influence the uterine response.

5.3.2 Molecular Signature for Attachment Reaction and Implantation

The attachment reaction between an implantation-competent blastocyst and receptive uterus is an essential, initial step for implantation. The process of implantation has been thought to involve a proinflammatory response with increased uterine vascular permeability at the site of the blastocyst (Psychoyos 1973; Dey et al. 2004). Psychoyos first showed that vascular permeability at the site of blastocyst can be monitored by intravenously injecting a macromolecular blue dye on day 5 of pregnancy in rodents. This dye binds to serum proteins, and the dye–protein conjugate leaks out at the sites of increased vascular permeability, demarcating the implantation sites (Psychoyos 1961). He identified that blue bands were apparent on day 5 of pregnancy in mice and are one of the earliest visible signs of implantation (Psychoyos 1961). In this respect, histamine, a vascular permeability factor, was considered relevant for implantation in rodents and rabbits (Shelesnyak 1952; Dey et al. 1978, 1979), again suggesting proinflammatory responses during implantation. These studies were then followed by the identification of crucial roles for cyclooxygenase (Cox1 and Cox2)-derived prostaglandins in female reproduction (Pakrasi et al. 1983; Kennedy et al. 2007). Genetic mouse models with *Ptgs2* (encoding Cox2) deletion showed infertility involving defects in ovulation, fertilization, implantation, and decidualization (Lim et al. 1997). Later it was shown that prostaglandins in implantation worked through peroxisome proliferation-activating receptor δ (PPARδ), identifying the first physiological role for this nuclear receptor in a physiologically relevant system (Lim et al. 1999).

Identifying the spatiotemporal sequence of molecular events prior to attachment has also been the subject of investigation. Heparin-binding EGF-like growth factor (HB-EGF), which originates in the implantation-competent blastocyst and LE at site of blastocyst apposition, has emerged as an important molecular link in directing embryo–uterine interactions for the attachment reaction in mice (Das et al. 1994;

Paria et al. 2001). It is expressed exclusively in the LE at the site of blastocyst as both soluble and transmembrane forms several hours before the attachment reaction (Das et al. 1994). Later molecular studies identified paracrine and juxtacrine roles of the soluble and transmembrane forms of HB-EGF in attachment reaction and found positive growth effects on the blastocyst (reviewed in (Lim and Dey 2009)). Notably, global deletion of *Hegf1* produces perinatal lethality (Iwamoto et al. 2003), while its conditional deletion in the uterus results in smaller litter size due to deferred blastocyst implantation (Xie et al. 2007). These results provide evidence that HB-EGF is perhaps not only the first molecular cross talk between the blastocyst and uterus to initiate the implantation process but is also one of the signaling molecules involved in establishing a hierarchy of events between these two different entities. However, certain functions of HB-EGF in implantation can be compensated by another EGF family member amphiregulin (Xie et al. 2007).

In addition to signaling mechanisms that actively promote implantation, there are also processes to prevent physical interaction prior to implantation. Many glycoproteins and carbohydrate ligands and their receptors are expressed in the uterus and blastocyst during the peri-implantation period (Aplin 1997; Kimber and Spanswick 2000). For instance, the long stretch of carbohydrate moieties comprising Muc1 acts as an anti-adhesive masking molecule to provide a physical hindrance between the embryo and LE prior to the receptive phase and attachment reaction (Surveyor et al. 1995). Its proposed role in preventing implantation is consistent with its expression on the apical surface of the mouse LE and its timely downregulation prior to implantation.

Appropriate morphological changes to form implantation chambers (crypts) in the receptive uterus are also a critical aspect of embryo implantation, warranting further investigation. In mice and rats, embryos reside within individual crypts prior to implantation at the antimesometrial (AM) pole. These crypts develop from epithelial evaginations from the primary lumen towards the AM pole, which begin to form on day 3 and are fully formed by day 4 of pregnancy prior to the attachment reaction in mice. Recently, noncanonical Wnt5a-ROR signaling was shown to be critical for this process (Cha et al. 2014a, b). However, the downstream mediators of Wnt5a-ROR signaling which give directional cues to form these crypts remain to be studied.

Overall, many morphogens, transcription factors, signaling pathways, and homeotic proteins have been shown to have crucial roles in uterine receptivity, attachment, and implantation (Cha et al. 2012, 2014a).

5.4 Delayed Implantation

Embryonic diapause is a self-limited phenomenon in which embryos at the blastocyst stage are arrested in growth and metabolic activity in parallel with uterine quiescence. This temporary delay of implantation allows pregnancy to withstand

unfavorable environmental conditions and adapt to a changing photoperiod or maternally derived stimuli such as lactation, thereby allowing pregnancy to resume during conditions favorable to rear young (Mead 1993; Renfree and Shaw 2000; Fenelon et al. 2014). The presumed purpose of delayed implantation is to prolong the gestational period until a time optimal for survival of the offspring.

This reproductive strategy was first identified in lactating rodents by Ferdinand Lataste in the late 1800s (Lataste 1891) and is widespread in the animal kingdom, occurring in >100 mammalian species across seven orders (Renfree and Shaw 2000; Fenelon et al. 2014). The widespread existence of embryonic diapause suggests that there is an evolutionary pressure to safeguard procreation under diverse environmental conditions. Although the endocrine orchestration of diapause varies across species, the underlying molecular mechanism by which the uterus and embryo temporarily achieve dormancy until favorable conditions reactivate the dormant blastocyst and allow implantation to proceed remains largely unknown.

There are two types of delayed implantation: facultative which occurs during lactation and obligatory which happens during every gestation of a species (Yoshinaga and Adams 1966; McLaren 1968; Renfree and Shaw 2000; Fenelon et al. 2014). Facultative delayed implantation can occur during lactation after postpartum mating. The suckling by newly born offspring causes endocrine changes within the mother which temporarily halt embryo development at the blastocyst stage and induce its dormancy within the quiescent uterus, thereby allowing sufficient time to nurse sequential litters (Yoshinaga and Adams 1966). In mice and rats, the suckling stimulus produces increased pituitary prolactin secretion that attenuates ovarian estrogen secretion. When this stimulus is removed, blastocyst reactivation (implantation competency) and implantation can occur (Yoshinaga and Adams 1966; McLaren 1968). In contrast, obligate diapause is prevalent in mustelids, bears, seals, and some wallabies (Fenelon et al. 2014). Species within the mustelid family display periods of embryonic diapause that are variable between individuals but can be in excess of 350 days in the fisher (*Martes pennanti*) or be as brief as 3 weeks in the mink (*Mustela vison*) (Mead 1993; Fenelon et al. 2014).

Irrespective of the type of delay, embryonic diapause is under maternal regulation in all species studied to date. The endocrine milieu resulting from various inducers has been characterized in several diapausing species (Mead 1993; Renfree and Shaw 2000); however, the molecular mechanism which promotes synchronized uterine quiescence and blastocyst dormancy while maintaining implantation competency is poorly understood. The *Msx* family of transcription factors, which have vital roles in fertility, has been shown to play a critical role in embryonic diapause. Uterine *Msx1* expression is sustained during delay and is downregulated upon blastocyst activation. In addition, loss of uterine *Msx1/Msx2* expression results in reduced rates of blastocyst survival and inability to undergo true delay (Cha et al. 2013). Indeed, sustained uterine expression of *Msx* family members is correlated with diapause in three species of evolutionarily divergent mammalian orders: mice

(Eutheria: Rodentia), American mink (Eutheria: Carnivora), and Australian tammar wallabies (Marsupialia: Diprotodontia) with rapid downregulation with impending implantation. These findings suggest that the *Msx* gene family is conserved not only for development and normal implantation but also for maternal regulation of mammalian embryonic diapause.

Global gene expression and proteomics analysis have identified molecular pathways distinguishing blastocyst dormancy and activation in mice, identifying alterations in major functional categories of cell cycle, calcium signaling, adhesion molecules, mitochondrial, and energy metabolic pathways (Hamatani et al. 2004; Fu et al. 2014). An intriguing feature of dormant blastocysts is their activation of autophagy ("self-eating") (Lee et al. 2011), a major cellular catabolic pathway by which macromolecules and organelles are recycled (Mizushima 2007). Dormant mouse blastocysts utilize this pathway to prolong their survival in utero; inhibition of autophagy in an experimental model of delayed implantation is associated with reduced blastocyst survival, although compromised developmental competency is correlated with the length of delayed conditions in mice (Lee et al. 2011).

Delayed implantation has not been shown to occur in certain species, including hamsters, rabbits, guinea pigs, or pigs. However, a recent interspecies embryo transfer study reports that embryos from nondiapausing sheep can undergo dormancy when transferred to a delayed-implanting mouse uterus and could subsequently be reactivated to produce normal offspring upon transfer back into the donor sheep uterus (Ptak et al. 2012). Given their results, the authors suggest that all mammals inherently possess the capacity to undergo embryonic diapause if the uterine environment is conducive to the event with appropriate maternal signals in place. Whether humans and their close primate relatives are capable of undergoing delay is under debate. For further information regarding the embryo and uterus in diapause, please refer to the following references: Mead (1993), Renfree and Shaw (2000), Cha et al. (2014a), Fenelon et al. (2014).

5.5 Decidualization

Following blastocyst attachment with the LE, decidualization is initiated at the antimesometrial site where blastocysts implant. Decidualization is characterized by stromal cell proliferation and differentiation into specialized cell types (termed "decidual cells") with polyploidy and is critical to the establishment of pregnancy in many species. The presumed functions of decidualization include nutritional support for the developing embryo, safeguard the embryo from immunological and other harmful responses from the mother, regulate trophoblast invasion, and direct placentation. A complex interplay of transcription factors, morphogens, cytokines, and signaling pathways is involved in decidualization; regulators for cell cycle and endocycle initiation are of particular importance.

Normally, the implanting blastocyst is the stimulus for decidualization. However, a similar process (deciduoma) can be experimentally induced by intraluminal infusion of various agents including oil, air bubbles, or phosphate buffer solution with BSA in pseudopregnant mice or rats (Dey 1996; Dey et al. 2004). In 1908, Leo Loeb was the first to induce tumorlike deciduoma in the uteri of guinea pigs and described that this event required an endometrium, hormonal conditioning, and a nonspecific stimulus such as glass beads (Loeb 1907, 1908). It was later found that other nonspecific stimuli such as intraluminal infusion of oil, air, or trauma can also initiate decidual reaction (deciduoma) in pseudopregnant or steroid hormonally prepared uteri (Dey 1996). Various aspects of decidualization were further discovered and expanded upon by other investigators including M.C. Shelesnyak, Vincent Defeo, Bruce Moulton, and J.M. Yochim, among others (reviewed in (Cha 2014)). However, there is evidence that the initial uterine reactions induced by nonspecific stimuli are different from those induced by blastocysts (Lundkvist and Nilsson 1982; Paria et al. 2001; Bany and Cross 2006).

A large number of decidual cells undergo endoduplication (polyploidy – repeating rounds of DNA replicating without cytokinesis) (Das 2009). The physiological significance of stromal cell polyploidy during decidualization has yet to be ascertained: the life span of decidual cells during pregnancy is limited and coincides with gestational length; their gradual demise helps to accommodate the rapidly growing embryo. We speculate that polyploidy limits the life span of decidual cells. One of the many functions of the decidua is to support embryonic growth which in turn requires increased protein synthesis. Polyploidy thus may ensure increased synthetic capacity by increasing the number of gene copies for transcription. Regardless, it has been determined that decidual cell polyploidy is critical to pregnancy success at least in mice: inactivation of death effector domain-containing protein (DEDD) results in faulty decidualization with reduced polyploidy and embryonic loss prior to placentation, thereby leading to infertility (Mori et al. 2011) (Fig. 5.1).

5.6 Future Considerations

While several interesting aspects of embryo–uterine interactions in implantation and subsequent pregnancy events have been reported, there is still much to be uncovered. It is now evident that the quality of early pregnancy events (in particular, specific stages of implantation) has profound effects on the later stages of pregnancy and its success. Studies in mutant mice have repeatedly shown that inferior embryo implantation perpetuates adverse ripple effects that lead to defective implantation, abnormal embryo spacing, suboptimal progression through decidualization, and placentation, leading to compromised pregnancy outcome (reviewed in (Cha et al. 2012)).

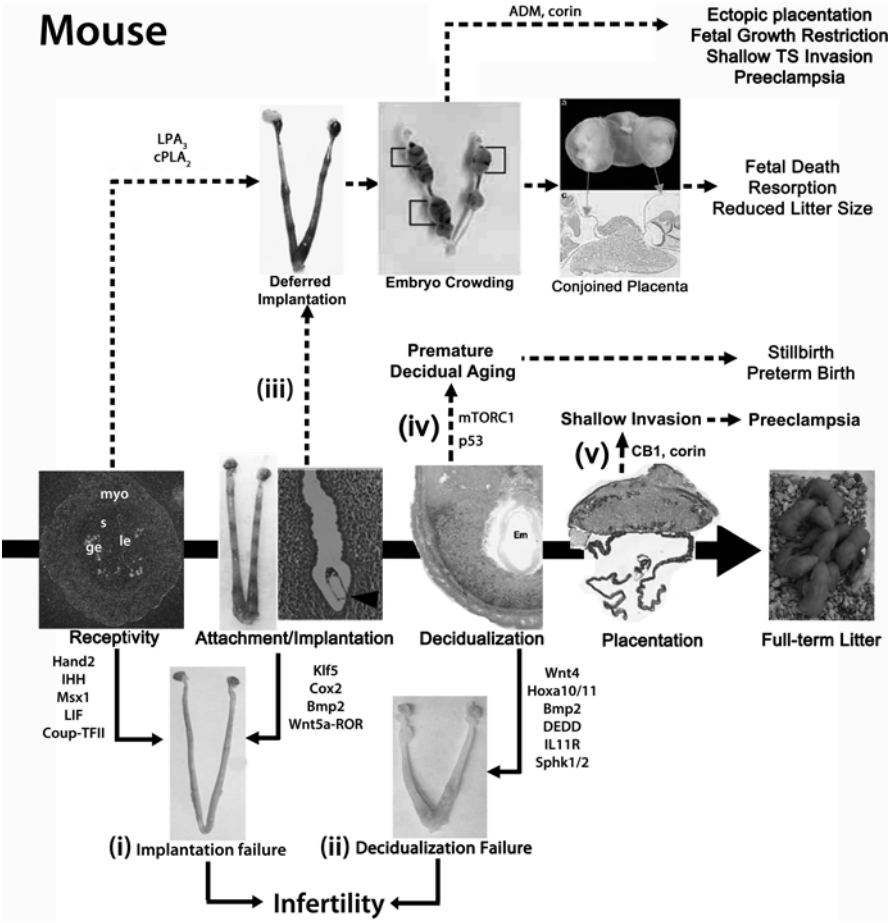


Fig. 5.1 Adverse pregnancy outcomes stemming from aberrant peri-implantation events. Defects in uterine receptivity, attachment reaction, or implantation timing can result in immediate failure of implantation (*i*) or decidualization (*ii*) or can perpetuate adverse ripple effects through the remaining stages of pregnancy. These adverse events can lead to deferred attachment/implantation (*iii*), resulting in embryo crowding, conjoined placenta, placental insufficiency, fetal growth restriction, fetal resorption, and reduced litter size, whereas suboptimal decidualization (*iv*) can lead to premature decidual senescence, resulting in preterm birth with neonatal death. Poor decidualization can lead to abnormal guidance of placentation and shallow invasion (*v*), resulting in preeclampsia. *ADM* adrenomedullin, *Arrowhead*, blastocyst, *Bmp2* bone morphogenetic protein 2, *CB1* cannabinoid receptor 1, *COUP–TFII* chicken ovalbumin upstream promoter–transcription factor-2, *Cox2* cyclooxygenase-2, *cPLA2α* cytosolic phospholipase $A_2\alpha$, *DEDD* death effector domain-containing protein, *Em* embryo, *ge* glandular epithelium, *Hand2* heart- and neural crest derivative-expressed protein 2, *Hoxa10* homeobox A10, *IHH* Indian hedgehog, *IL11R* interleukin 11 receptor α, *Klf5* Kruppel-like factor 5, *le* luminal epithelium, *LIF* leukemia inhibitory factor, *LPA3* lysophosphatidic acid 3, *Msx1* muscle segment homeobox 1, *mTORC1* mammalian target of rapamycin complex 1, *myo* myometrium, *p53* transformation-related protein p53, *ROR receptor tyrosine kinase*-like orphan *receptor, s* stroma, *Sgk1* serum- and glucocorticoid-inducible kinase 1. *Dotted lines*, adverse ripple effects (The images in this figure are adapted from our previous studies (Daikoku et al. 2011; Hirota et al. 2010; Song et al. 2000))

Studies using animal models to determine optimal molecular signatures during uterine receptivity for implantation may be clinically significant in humans, since poor uterine receptivity is a major cause of pregnancy failure in IVF programs. However, in order for this signature to be elucidated in humans, the precise timing of implantation and sequence of implantation events must be identified. Although many molecular players appear similar between mouse and human, the exact timing of their expression during the peri-implantation period in humans is unknown. Studies in subhuman primates may provide more mechanistic information in this regard. Furthermore, molecular programming to initiate the transition from the receptive to refractory phase has not been elucidated. Investigating this transition may allow lengthening of the receptive phase in humans.

In addition to better defining the molecular landscape of uterine receptivity prior to attachment, embryonic signals heralding attachment of the blastocyst to the uterine lining remain to be determined. While increased vascular permeability is considered a marker of implantation in rodents and many other species, this may be the end result of earlier molecular interactions. HB-EGF is considered the earliest known molecular mediator of embryo–uterine interactions; however, additional factors expressed either in sequence or in parallel have yet to be explored. These factors could perhaps be identified by high fidelity or *in situ* mass spectrometry at specific times prior to attachment reaction (Burnum et al. 2009).

Finally, new insights in the epigenetic regulation of chromatin remodeling, gene expression, and long noncoding RNAs (lncRNA) in implantation and decidualization have come to light, such as miRNA regulation of Cox2 (Chakrabarty et al. 2007). Furthermore, miRNA regulation of reproductive organs has been implicated in various stages of pregnancy, such as implantation and parturition timing (Liu et al. 2011; Renthal et al. 2013). DNA methylation in the context of decidual polyploidy has shown to be a requirement in hormone-dependent gene expression, shedding new light on the dynamic gene expression profiles seen in the pregnant uterus under the influence of hormones (Gao et al. 2012). In addition, the transmission of epigenetic programming from mother to future generations has also been studied in the context of dietary deficiencies (Jirtle and Skinner 2007). How the maternal environment influences the reproductive capability of future generations has yet to be investigated.

This chapter is in honor of Roger Short and his seminal contributions to the field of reproduction. After completing a bachelor's degree in veterinary science at Bristol University and earning a PhD in reproductive endocrinology at Cambridge University, Roger Short was fascinated by animal reproduction and became involved with the World Health Organization with special interest in contraceptive research, regulation of reproduction, and human population growth. One of his many interests included the consistent timing of mating behavior in animals in different hemispheres. This observation led to the identification of the influence of light on the pineal gland to trigger the secretion of melatonin. Known to test hypotheses on himself, he tested melatonin as a sleep aid, and it is now regularly used to prevent jet lag. With advances in clock gene biology and recent identification of central and peripheral clocks in reproductive tissues (Miller et al. 2004; Reiter et al. 2014), the

influence of the melatonin, local and central clocks, and their roles in reproduction are worthy endeavors to study. Indeed, with the recent emergence in gene knockout technology, proteomics, mass spectrometry, single-cell analysis, and bioinformatics, much information can be gleaned to study Roger Short's unique observations and adventurous thoughts to further elucidate the orchestration of events required for uterine receptivity and implantation for pregnancy success.

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Chapter 6
The Role of Progesterone in Maternal Recognition of Pregnancy in Domestic Ruminants

Pat Lonergan and Niamh Forde

Abstract Progesterone (P4) secretion by the corpus luteum is critical for the establishment and maintenance of pregnancy and plays a major role in regulating endometrial secretions essential for stimulating and mediating changes in conceptus growth and differentiation throughout early pregnancy. Numerous studies have demonstrated an association between elevated P4 and acceleration in conceptus elongation. Given that larger conceptuses produce more interferon tau, the pregnancy recognition signal in ruminants, it would be reasonable to hypothesize that treatments aimed at increasing peripheral concentrations of P4 should improve pregnancy rate. However, data on the impact of post-insemination supplementation of P4 on pregnancy rates are conflicting and, at best, indicate a modest positive response. Whether a P4-induced increase in conceptus size can improve fertility continues to be an active area of investigation. The aim of this chapter is to review recent data on the role of P4 in conceptus development in ruminants, particularly cattle, and to summarize results from attempts at manipulating endogenous P4 with the aim of improving conceptus survival and pregnancy rate.

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6.1 Introduction

Following ovulation, fertilization of the mammalian oocyte occurs in the oviduct. The resulting embryo moves towards the uterus as it undergoes the first mitotic cleavage divisions. The bovine embryo enters the uterus at about the 16-cell stage on approximately day 4 of pregnancy. It subsequently forms a tight ball of cells, a morula, in which the first cell-to-cell tight junctions occur. By day 7, the embryo has formed a blastocyst consisting of an inner cell mass which, after further differentiation, gives rise to the embryo/fetus, and the trophectoderm, which ultimately forms the placenta. After hatching from the zona pellucida on approximately days 8–9, the spherical blastocyst grows and changes in morphology from a spherical to ovoid shape during a transitory phase preceding the elongation of the trophectoderm to a filamentous form that usually begins between days 12 and 14. The conceptus (embryo and associated extraembryonic tissues) continues to grow and secrete interferon tau (IFNT), the maternal recognition of pregnancy signal in ruminants, which prevents prostaglandin-induced luteolysis and maintains the pregnancy. Unlike primate and rodent embryos which invade the endometrium soon after hatching, ruminant conceptuses remain free-floating for a relatively long period, prior to implantation, which commences at approximately day 19 in cattle.

Up to the blastocyst stage, the embryo is somewhat autonomous (i.e., does not have an absolute requirement for contact with the maternal reproductive tract environment) as evidenced by the fact that blastocysts can be successfully produced in vitro in large numbers using in vitro fertilization (IVF) technology and, following transfer to synchronized recipients, can establish a pregnancy. In a similar vein, in commercial bovine embryo transfer practice, early blastocysts (~7 days old) are typically recovered from superovulated donors and transferred to the uterus of non-pregnant synchronized recipients which, up to that stage, have not seen an embryo. These two facts demonstrate that the development of the early embryo does not absolutely require exposure to the female reproductive tract and also that the reproductive tract does not require exposure to the embryo in order for pregnancy to be established. Indeed, taken to its extreme, it is possible to establish a pregnancy in cattle by transferring embryos as old as 16 days (Betteridge et al. 1980) (i.e., up to the time when the luteolytic mechanisms would normally be initiated), although this would not be practical due to the filamentous nature of the conceptus at this stage. In contrast to the zona-enclosed embryonic stages, the development of the post-hatching and preimplantation conceptus is unequivocally dependent on substances in the uterine lumen that are derived from the endometrium, particularly the uterine glands, for growth and development. Evidence for this comes from the fact that (1) post-hatching elongation does not occur in vitro despite attempts to recapitulate it by growing blastocysts in confined spaces (Brandao et al. 2004; Alexopoulos et al. 2005; Zhao et al. 2015) and (2) the absence of uterine glands in vivo results in a failure of blastocysts to elongate after transfer (Gray et al. 2002).

On the maternal side, the preparation of the uterine luminal epithelium (LE) for the attachment of trophectoderm and implantation in all studied mammals, including

ruminants, involves carefully orchestrated spatiotemporal alterations in gene expression within the endometrium. The steroid hormone, progesterone (P4), plays a critical role in this process. In both cyclic and pregnant cattle, similar changes occur in endometrial gene expression up to the time of maternal recognition of pregnancy, suggesting that the default mechanism in the uterus is to prepare for, and expect, pregnancy (Forde et al. 2011b; Bauersachs et al. 2012). It is only in association with maternal recognition of pregnancy, which occurs by approximately day 16 in cattle, that significant changes in the transcriptomic profile are detectable between cyclic and pregnant endometria (Forde et al. 2011b; Bauersachs et al. 2012). The majority of the changes in the endometrial transcriptome occur in response to increasing amounts of IFNT secreted by the filamentous conceptus. In ruminants, high concentrations of circulating P4 in the immediate postconception period have been associated with an advancement of conceptus elongation, an associated increase in IFNT production, and higher pregnancy rates in cattle (Lamming and Royal 1999; Mann and Lamming 2001; Stronge et al. 2005; McNeill et al. 2006) and sheep (Ashworth et al. 1989; Satterfield et al. 2006).

The aim of this chapter is to review recent results regarding the role of P4 in conceptus development in ruminants, particularly cattle, and to summarize results of attempts at manipulating endogenous P4 with the aim of improving conceptus survival and pregnancy rate. Experiments described in the subsequent sections of this chapter provide unequivocal evidence for the role of P4 in driving conceptus elongation; however, studies investigating the effect of supplementary P4 on pregnancy rates have yielded variable outcomes (Fig. 6.1).

6.2 Importance of Progesterone in the Establishment of Pregnancy

Circulating concentrations of P4 represent a balance between the production of P4 by the corpus luteum (CL) and the metabolism of P4, primarily by the liver. The production of P4 is regulated by the development of the CL after the preovulatory surge of luteinizing hormone, the number of granulosa cells that luteinize into large luteal cells, and the constitutive production of P4 by these cells. The metabolism of P4 is primarily related to the rate of blood flow to the liver (Sangsritavong et al. 2002). Therefore, practical strategies aimed at the manipulation of circulating concentrations of P4 will be most productive by focusing on increasing luteal tissue volume to increase P4 production and/or limiting P4 metabolism (Wiltbank et al. 2014).

While fertilization success following natural or artificial insemination is high (~90 %), a significant proportion of the resulting embryos fail to develop to term. The majority of embryos are lost between fertilization and maternal recognition of pregnancy, which in cattle occurs around day 16 post-estrus (Diskin and Morris 2008), and this loss significantly contributes to reproductive inefficiency in cattle. While poor embryonic survival can be attributed to a variety of factors such as poor

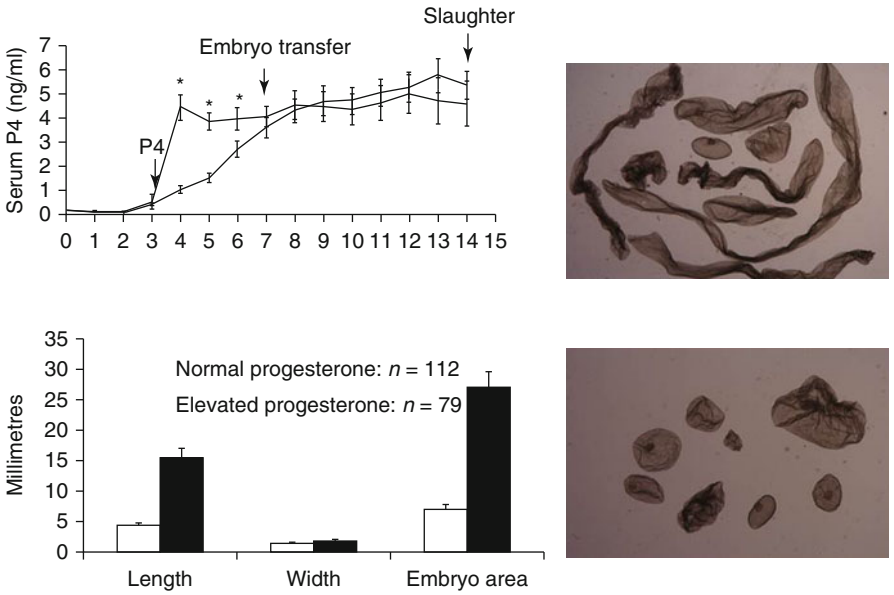


Fig. 6.1 The effect of elevated progesterone on conceptus elongation. Transient elevation of peripheral progesterone concentrations by exogenous administration results in the acceleration of conceptus development. Photographs illustrate elongating bovine conceptuses from the uterus of a recipient heifer with elevated (*top*) or low (*bottom*) serum progesterone, recovered at slaughter on day 14 post-estrus following the transfer of 10 in vitro-produced blastocysts on day 7. Such elongation is entirely maternally driven, as evidenced by the failure of embryos to elongate appropriately in vitro or in vivo in the absence of uterine glands. Note, however, the variation in conceptus length, despite being recovered from the same uterus, suggesting an intrinsic component to the embryo in the elongation process (Modified from Clemente et al. 2009).

follicle and oocyte quality and poor embryo quality and/or poor reproductive tract environment, circulating concentrations of P4 undoubtedly play a role in establishing uterine receptivity and low concentrations, for example, in high-producing dairy cows, may partly contribute to lower fertility in such animals.

Results of several retrospective studies have indicated a positive relationship between circulating concentrations of P4 in the week after breeding and subsequent pregnancy rate (Stronge et al. 2005; Diskin et al. 2006; Parr et al. 2012). Interestingly, there is both a linear and quadratic component to this relationship; that is, too much P4 may lead to a decline in pregnancy rate. Thus, both suboptimal and supraoptimal concentrations of P4 from days 4–7 after AI or a suboptimal rate of increase in the concentration of P4 during this interval is negatively associated with embryonic survival. Using a novel model of high- and low-fertility Holstein-Friesian cows, Cummins et al. (2012) reported that circulating concentrations of P4 were 34 % greater in cows with similar genetic merit for milk production traits, but with extremes of good (Fert+) or poor (Fert-) genetic merit for fertility traits. In a follow-up study, Moore et al. (2014) investigated the factors affecting circulating concentrations of P4 in those cows. Concentrations of P4 were measured from days

1–13. Corpus luteum volume was 41 % greater and mean circulating concentrations of P4 were 79 % greater in Fert+cows compared with Fert- cows. The results indicate that greater circulating concentrations of P4 were primarily due to a greater capacity of CL to secreted P4 rather than differences in the clearance rate of P4 in this lactating cow genetic model of fertility.

6.3 Effect of Progesterone on the Pre-hatching Embryo

As discussed elsewhere in this chapter, the effect of P4 on conceptus development is likely a result of downstream effects of P4-induced changes in gene expression in cells of the uterus (Satterfield et al. 2006; Forde et al. 2009, 2011a) resulting in changes in the composition of uterine lumen fluid or histotroph to which the developing embryo is exposed. Whether any of the effects of P4 are directly on the embryo has been assessed by experiments in which P4 was added to medium during the in vitro culture of embryos. Results of such studies have been varied and contradictory with some authors reporting positive effects of P4 (Merlo et al. 2007; Ferguson et al. 2011), while others have reported no effect (Reggio et al. 1997; Goff and Smith 1998). Overall, however, despite the presence of progesterone receptor (PGR) mRNA on embryos (Clemente et al. 2009), there is little convincing evidence that P4 has a direct effect on the early embryo. In our own laboratory, culture of embryos in vitro in the presence of P4 did not affect the proportion developing to the blastocyst stage in the presence or absence of oviductal epithelial cells (Clemente et al. 2009). This is consistent with the observations of Larson et al. (2011) who failed to observe a direct effect of P4 either from days 1–3 or 4–7 after fertilization. Furthermore, the addition of P4 to culture medium had no effect on conceptus elongation after transfer to synchronized recipients (Clemente et al. 2009). In two other in vivo studies, we failed to demonstrate the effect of elevated P4 on blastocyst development. In the study of Carter et al. (2008), no differences in embryonic development on day 5 or day 7 were observed when beef heifers were supplemented with exogenous P4 from day 3, despite dramatic effects on post-hatching elongation between days 13 and 16 of pregnancy. In a follow-up study, multiple in vitro-produced embryos were transferred to the oviduct of beef heifers that did or did not receive a P4 insert on day 3 after the onset of estrus. There was no effect of P4 on the proportion of embryos that developed to the blastocyst stage by day 7 (Carter et al. 2010).

6.4 Effect of Progesterone on the Post-hatching Blastocyst

In contrast to the lack of a requirement by the embryo for interactions with the female reproductive tract up to the blastocyst stage, the development of the post-hatching and preimplantation conceptus is entirely driven by the uterine

environment. The protracted period of implantation characteristic of ruminants involves rapid proliferation of the trophectoderm cells, which is dependent on substances in the uterine lumen fluid (or histotroph), that are derived from the endometrium, particularly the uterine glands, for growth and development.

Earlier studies in ewes (Wilmut and Sales 1981; Lawson and Cahill 1983) and cows (Garrett et al. 1988b) suggested that maternal P4 regulates early conceptus growth and development. More recent studies confirmed those findings and have begun to unravel the underlying biology (e.g., Satterfield et al. 2006; Forde et al. 2009, 2011a, 2012). In particular, significant progress has been made in clarifying the role of luteal P4 in the successful establishment of pregnancy in sheep and cattle, with particular emphasis on how P4 affects endometrial gene expression and conceptus elongation.

The effects of elevated P4 shortly after conception on the advancement of conceptus elongation have been convincingly demonstrated in cattle and sheep. Garrett et al. (1988b) administered 100 mg P4 on days 1, 2, 3, and 4 of pregnancy which increased concentrations of P4 in peripheral plasma on days 2–5 and significantly larger conceptuses on day 14. Using a P4 implant on day 3 of pregnancy, Carter et al. (2008) significantly elevated concentrations of P4 in plasma until day 8, and this was associated with larger conceptuses recovered at slaughter on day 16. Similarly, when ewes received daily injections of 25 mg P4 from 36 h postmating, blastocyst diameter increased by 220 % on day 9 and at the time of initiation of elongation of blastocysts to a filamentous conceptus on day 12 was advanced (Satterfield et al. 2006); these effects of P4 treatment on blastocyst development were blocked by the administration of RU486, a PGR antagonist.

Using a combination of in vitro embryo production and in vivo embryo transfer techniques, we have shown that the effect of P4 on conceptus development is mediated exclusively via the endometrium (Clemente et al. 2009). The addition of P4 to culture medium had no effect on blastocyst formation (Clemente et al. 2009; Larson et al. 2011) or elongation after transfer to synchronized beef recipients (Clemente et al. 2009). Exposure of the uterus to elevated P4 prior to embryo transfer resulted in an advancement in conceptus elongation (Clemente et al. 2009). Most convincingly, the embryo did not need to be present in the uterus during the period of P4 elevation in order to benefit from it (Clemente et al. 2009), strongly suggesting that the effect of P4 is via advancement of the normal temporal changes that occur in the endometrial transcriptome (Forde et al. 2009) resulting in advanced conceptus elongation (Carter et al. 2008; Clemente et al. 2009). In addition, reducing the output of P4 from the CL, for example, by treatment with prostaglandin $F_{2\alpha}$ (PGF) (Beltman et al. 2009; Forde et al. 2012) or by aspirating the contents of the preovulatory follicle just before the expected time of ovulation (O'Hara et al. 2012), results in a delay in the temporal changes in the endometrial transcriptome, causing delayed conceptus elongation in vivo (Forde et al. 2011b, 2012; O'Hara et al. 2012).

6.5 Effects of Progesterone on the Endometrium and Uterine Environment

A prerequisite for establishing uterine receptivity to implantation in all species studied thus far is loss of expression of PGR from uterine LE and then GE (Bazer et al. 2010). Paradoxically, it is sustained exposure of the endometrium to circulating concentrations of P4 that leads to this downregulation of PGR as the luteal phase of the estrous cycle progresses. The concentrations of P4 in circulation modify the loss of expression of PGR in the endometrium such that in animals in which P4 is high, there is early loss of the PGR (Okumu et al. 2010), i.e., uterine receptivity to implantation is established earlier, while conversely, low or suboptimal concentrations of P4 delay loss of the PGR and thus delay establishing uterine receptivity to implantation (Forde et al. 2011a). Thus, in simple terms, it would appear that elevating P4 simply advances the changes in endometrial gene expression which normally occur (Forde et al. 2009) (Fig. 6.2).

The continued exposure of the endometrium to P4 during the luteal phase of the estrous cycle significantly modifies the endometrial transcriptome (Forde et al. 2009, 2011b, 2012) with a large divergence in gene expression in the endometrium (and histotroph composition) between when a zona-enclosed blastocyst (when PGRs are expressed in uterine LE and GE) andan elongated conceptus (when PGR expression is lost from uterine LE and GE) is present. Manipulating circulating concentrations of P4 in vivo significantly alters the expression of genes that encode for secreted proteins in the endometrium, e.g., apolipoprotein A1 (APOA1), connective

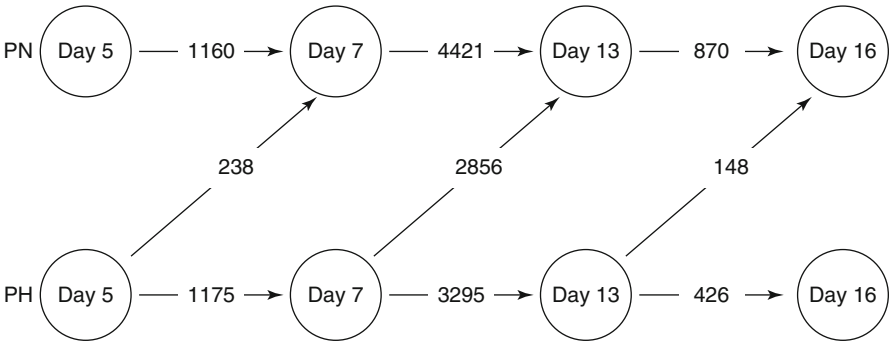


Fig. 6.2 Numbers of differentially expressed genes that are temporally regulated in the endometrium of pregnant heifers with unmanipulated ("normal," PN) or artificially elevated ("high," PH) progesterone concentrations between day 5 and day 16. Note that a day 5 endometrium exposed to elevated progesterone is more similar to a "normal" day 7 endometrium than the corresponding day 5 endometrium. The same applies for day 7 and day 13, indicating that P4 supplementation advances the temporal changes in gene expression which normally occur in the endometrium and which contribute to advanced conceptus development observed on day 13 and day 16 (From Forde et al. 2009).

tissue growth factor (CTGF), lecithin-cholesterol acetyltransferase (LCAT), lipoprotein lipase (LPL), insulin-like growth factor binding proteins 1 (IGFBP1) and 3 (IGFBP3), fatty acid binding protein 3 (FABP3), fibroblast growth factor 10 (FGF10), gastrin-releasing peptide (GRP), mephrin 1B (MEP1B), matrix Gla protein (MGP), neuromedin N (NMN), nephronectin (NPNT), periliphin 2 (PLIN2), and tubulointerstitial nephritis antigen-like 1 (TINAGL1) (Satterfield et al. 2009; Forde et al. 2010, 2013), which are also detectable in the uterine luminal fluid during elongation of the conceptus (Forde et al. 2013) and the pregnancy recognition period (Forde et al. 2014a). Thus, P4, during the luteal phase of the cycle, modifies the uterine environment in a stage-specific manner to provide the optimum environment for the developing conceptus. The differences in the capacity of the uterus to induce elongation of the conceptus are dependent on circulating concentrations of P4 and effects of P4 on the endometrial transcriptome and subsequent modification of the uterine luminal fluid, resulting in either an advance or a delay in conceptus elongation. As a consequence of the different elongation trajectories driven by different concentrations of P4 in circulation, there is a difference in IFNT secretion (Kerbler et al. 1997; Rizos et al. 2012) and thus effects on the pregnancy recognition response in the endometrium. Results from studies of both cattle and sheep demonstrated that stimulation of classical interferon-stimulated genes in uterine GE and stromal cells, but not uterine LE, is greater in those animals in which P4 advanced the pregnancy recognition response in the endometrium.

In addition to proteins, the uterine lumen fluid of ruminants is composed of molecules that are actively transported from the endometrium into the uterine lumen and include glucose, ions, fatty acids, and amino acids (Gao et al. 2009c; Groebner et al. 2011; Meier et al. 2011). In both sheep and cattle, a significant increase in total amino acid content occurs between the post-hatching period of embryo development and the pregnancy recognition period (Gao et al. 2009c; Forde et al. 2014b). The expression of the various cationic, acidic, and neutral amino acid transporters responsible for the transport of these amino acids into the uterine lumen is altered in a temporal and cell-specific manner in both the endometrium and conceptus of sheep during early pregnancy (Gao et al. 2009a, b, c) and is modulated by P4 and/or conceptus IFNT, prostaglandins, and cortisol in vivo (Dorniak et al. 2011, 2012, 2013) with additional data from cattle indicating pregnancy recognition also modifies the expression of these genes in the endometrium (Forde et al. 2014b). The abundance of individual amino acids in the uterine lumen fluid is also modified by circulating concentrations of P4, specifically alanine, arginine, asparagine, and lysine in sheep (Satterfield et al. 2010) and valine (Hugentobler et al. 2010), histidine, and asparagine in cattle (Mullen et al. 2014). These differences are likely due to the modification in endometrial gene expression of their transporters (the solute carrier superfamily of genes, SLCs) by which altering P4 in circulation modifies the expression of acidic (SLC1A1, SLC1A4, SLC1A5), cationic (SLC7A1, SLC7A5, and SLC7A7), as well as neutral (SLC38A2, SLC38A4, SLC38A7, SLC43A2, SLC6A14) amino acid transporters in the endometrium (Forde et al. 2014b).

6.6 Asynchronous Embryo Transfer

The dramatic regulatory effect of the uterus on bovine conceptus development, and the role played by P4, is nicely illustrated in studies comparing the outcome of synchronous and asynchronous embryo transfer. Such synchrony between the needs of the developing embryo and uterine secretions has long been recognized as being critical to the successful establishment of pregnancy (reviewed by Pope 1988). Indeed, embryo transfer studies in sheep and cattle have clearly demonstrated a need for close synchrony between the embryo and the uterine environment of the recipient. Previous studies have established that pregnancy rates are reduced when embryos are greater than 48 h from synchrony with the recipient's uterine environment (Moore and Shelton 1964; Rowson and Moor 1966; Rowson et al. 1972).

Asynchronous transfer of day 7 bovine blastocysts to the uteri of day 5 or day 9 recipients resulted in retarded (5.4 ± 0.4 mm) or advanced (50.4 ± 5.2 mm) conceptuses on day 14, respectively, compared to synchronous controls (day 7 to day 7: 15.7 ± 1.5 mm) or conceptuses derived from AI (12.0 ± 3.3 mm) (Ledgard et al. 2012). Consistent with these observations, Geisert et al. (1991) reported that only 1 of 21 (4.8 %) day 8 bovine blastocysts transferred to a day 5 uterus established pregnancy compared to 50 % in synchronous controls.

The administration of P4 early in the estrous cycle of the recipient can effectively advance uterine receptivity for the transfer of older asynchronous embryos. In sheep, day 6 recipients after early exposure to exogenous P4 supported the development of transferred day 10 blastocysts (Lawson and Cahill 1983). In cattle, embryo transfer to P4-treated recipients (100 mg/day from day 1 to day 4) which showed estrus 72 h after the donor cows (i.e., day 8 blastocysts transferred into a day 5 uterus) resulted in pregnancy rates at day 35 similar to those of synchronous (±12 h) recipients (42.1 vs. 50 %), while, as mentioned above, only approximately 5 % of day 5 asynchronous recipients became pregnant (Geisert et al. 1991).

Similar data have been reported recently by Randi et al. (2015) who transferred multiple day 7 bovine blastocysts to synchronous (day 7) or asynchronous (day 5 or day 9) recipients ($n = 10$ per recipient). The transfer of day 7 blastocysts to a day 5 uterus resulted in fewer conceptuses surviving (20 %) and delayed elongation in those that were recovered. In contrast, transfer to an advanced day 9 uterine environment resulted in the same level of survival as synchronous controls (~50 %), but a dramatic advancement in conceptus elongation, in agreement with the observations of Ledgard et al. (2012). Supplementation of day 5 recipients with P4 from day 3 increased circulating concentrations of P4 and increased conceptus length compared to day 5 controls; however, supplementation with P4 reduced the length of estrous cycles in approximately 50 % of heifers (see below for further discussion).

Together, these studies indicate that P4 stimulates changes within the uterine environment which regulate receptivity and promote embryo survival and conceptus elongation. Manipulating P4 may be one way of strategically regulating the temporal changes that normally occur in the uterine environment in order to allow flexibility in the timing of embryo transfer. Given the above results indicating that transfer

to an advanced uterus (i.e., uterus ahead of the embryo), which has had longer exposure to P4, results in an advancement in conceptus elongation and that such advanced conceptuses produce more IFNT (Kerbler et al. 1997; Rizos et al. 2012), one could reasonably hypothesize that transfer to an advanced uterus would result in improved pregnancy rates. However, interrogation of data from commercial embryo transfer operations does not support that hypothesis (Wright 1981; Donaldson 1985; Hasler et al. 1987; Heyman 1988; Hasler 2001; Rodrigues et al. 2003; Randi et al. 2015). For example, in the study of Randi et al. (2015), 4749 recipients received a single in vitro-produced fresh blastocyst. The overall pregnancy rate was 43.5 %, which is about the norm in such commercial IVF operations. The transfer of a day 7 blastocyst to a synchronous day 7 uterus resulted in a pregnancy rate of 47.3 %. Transfer to a uterus 1 day behind (day 6: 46.6 %) did not affect pregnancy rate. However, transfer to a day 5 (40.8 %) or a day 8 (41.3 %) uterus moderately impacted pregnancy rate, while transfer to a uterus 2 days in advance (day 9: 24.4 %) or 3 days behind (day 4: 27.0 %) dramatically reduced pregnancy rates compared to results from synchronous transfer of blastocysts. Taking the results of all of these studies together, it is clear that the accelerated conceptus elongation associated with the transfer of a blastocyst to an advanced uterus does not translate into an improved pregnancy rate; rather, once synchrony is exceeded by approximately 48 h, pregnancy rates decline appreciably.

6.7 Strategies to Increase Concentrations of Progesterone

Potential beneficial effects of exogenous P4 supplementation on fertility have been acknowledged for a long time (see reviews by Inskeep 2004; Lonergan 2011; Wiltbank et al. 2014). Given the significant volume of data indicating that elevating P4 results in an advancement in conceptus elongation (Garrett et al. 1988b; Carter et al. 2008; O'Hara et al. 2012; O'Hara et al. 2014a, c) and that such advanced conceptuses produce more IFNT (Kerbler et al. 1997; Rizos et al. 2012), one could reasonably hypothesize that such advanced conceptuses would be more likely to establish pregnancy. However, data on the impact of post-insemination supplementation of P4 on pregnancy rate are conflicting and, at best, indicate a modest positive response (Nascimento et al. 2013; Wiltbank et al. 2014).

Several approaches can be taken to increase concentrations of P4 in peripheral blood after AI, including those that (1) increase endogenous function of the existing CL (e.g., strategies which promote growth of the dominant follicle before ovulation, resulting in a larger CL, or luteotrophic treatments which stimulate CL development); (2) induce ovulation of a dominant follicle and formation of accessory CL (e.g., hCG or GnRH administration); or (3) those which supplement P4 directly (e.g., via injection or intravaginal devices). However, results in terms of pregnancy rate are often conflicting or inconclusive and may reflect (1) the timing of treatment, (2) that only a proportion of animals with inherently low P4 benefit from such treatment, (3) that P4 supplementation is less effective in high-producing dairy cows due

to increased liver metabolism, or (4) the lack of sufficient animal numbers and statistical power in many studies to detect effects of treatments.

Dominant follicle size is associated with subsequent CL size (Vasconcelos et al. 2001). Larger CL generally secretes more P4 and this has, in some studies, been associated with improved pregnancy rates. Therefore, strategies which promote growth of the dominant follicle before ovulation and/or stimulate CL development are likely to increase pregnancy rate (Baruselli et al. 2010). Equine chorionic hormone (eCG) incorporated into synchronization protocols has been reported to improve pregnancy rates following fixed-time artificial insemination/embryo transfer, although results for treatment of lactating dairy cows have been less promising than those for heifers or beef cows (Bo et al. 2011).

Human chorionic gonadotrophin (hCG) administration to ovulate a dominant follicle and form an accessory CL has been used widely in an attempt to improve pregnancy rates, albeit with variable results (see Lonergan 2011). In a recent large study, Nascimento et al. (2013) reported the results of two separate analyses that evaluated the effect of hCG treatment post-AI on fertility in lactating dairy cows. The first was a meta-analysis of the combined results of 10 different published studies that used hCG treatment between days 4 and 9 post-AI in lactating dairy cows. Overall, hCG administration increased pregnancies per artificial insemination by 3 percentage points [34 % (752/2,213) vs. 37 % (808/2,184)]. In a subsequent field trial, lactating Holstein cows ($n = 2{,}979$) from six commercial dairy herds received hCG or not on day 5 after a timed AI; pregnancies per AI were greater in cows treated with hCG (40.8 %; 596/1,460) than control (37.3 %; 566/1,519) cows. Surprisingly, the positive effect of hCG was restricted to first-lactation cows.

6.8 Effect of Exogenous Progesterone on Corpus Luteum Life Span

Although the use of exogenous P4 to improve synchrony of embryo transfer and/or advance conceptus elongation as described previously is encouraging, caution is warranted. Paradoxically, depending on the timing of administration, exogenous P4 can have a negative effect on CL life span, resulting in shortened interestrous intervals due to premature CL regression (Ginther 1970; Garrett et al. 1988a; Burke et al. 1994; Pope et al. 1995; O'Hara et al. 2014a; Pugliesi et al. 2014; Randi et al. 2015) while at the same time advancing conceptus development due to the changes induced in the endometrium by P4 (O'Hara et al. 2014a). This situation is clearly not compatible with successful maintenance of pregnancy. In one recent study, Parr et al. (2014) supplemented lactating cows on seven farms with a P4 device from day 4 to day 10; in each farm, pregnancy rate was depressed, presumably due to animals short-cycling.

In cattle, Garrett et al. (1988a) reported that the administration of exogenous P4 from days 1 to 4 shortened the interestrous interval (16.7 d) compared to controls (21.6 d), resulting from an earlier decline in peripheral P4 coincident with an

increase in pulsatile release of PGF/PGFM. In sheep, Pope et al. (1995) showed that the minimal daily dose of P4 required to stimulate advanced development of embryos in ewes (6 mg) is also the dose which produced a reduction in length of estrous cycles, i.e., P4 is both luteolytic and embryotrophic.

The timing of the premature increase in P4 during metestrus is critical to its subsequent effect on luteal life span. Ginther (1970) reported the cycle-shortening effect of exogenous P4 when administered from days 0–3, 1–4, or 2–5. Similarly, Burke et al. (1994) reported that the administration of P4 from days 1–5, but not 4–9, reduced CL life span. Consistent with those results, VanCleeff et al. (1996) reported that P4 supplementation within 2 days of insemination for 7 days suppressed fertility in dairy heifers (18.2 vs. 46.4 %). Interestingly, Ginther (1970) reported that simultaneous administration of P4 and hCG prevented the effect of P4 given on days 1–4 to reduce the length of the estrous cycle.

We have recently shown that a single i.m. injection of hCG as early as day 2 or day 3 after the onset of estrus resulted in a larger CL and increased circulating concentrations of P4 compared to controls (Maillo et al. 2014). It is possible that a combination of exogenous progesterone, to induce the required stimulation of the endometrium and conceptus, and luteotrophic support, such as that provided by hCG, to avoid early CL regression, provides a means of optimizing maternal recognition of pregnancy. Indeed, the administration of hCG at the time of progesterone injections on days 1–4 overcame the negative effect on CL life span (Ginther 1970). In support of this notion, O'Hara et al. (2014b) administered eCG, a glycoprotein secreted by the endometrial cups of pregnant mares with a relatively long half-life of about 2–3 days and with both LH- and FSH-like properties in cattle, to beef heifers on day 3 post-estrus in association with an intravaginal P4 insert which reduced the number of short cycles and increased mean luteal tissue weight and circulating concentrations of P4. However, the number of heifers involved was small and this area of research requires further study. In a subsequent study by Randi et al. (2015), approximately 50 % of day 5 recipients which received a P4 insert from day 3 to day 5 with either eCG or hCG exhibited short cycles, as evidenced by a return to estrus and/or evidence of a fresh ovulation at slaughter.

6.9 Summary and Conclusion

The role of P4 in optimizing uterine receptivity in mammals is unequivocal. Progesterone secreted by the CL is critical for the establishment and maintenance of pregnancy and plays a major role in regulating endometrial secretions that are essential for stimulating and mediating changes in conceptus growth and differentiation throughout early pregnancy. The effects of P4 on conceptus elongation are mediated via changes induced in the endometrium. Results summarized in this chapter support the positive association between circulating concentrations of P4 during metestrus and elongation of the conceptus. Furthermore, they highlight the

paradoxical nature of the effects of P4 in that, when administered early, P4 is associated with short estrous cycles in a proportion of heifers. This highlights the caution that is required when supplementing P4; it may be that strategies aimed at stimulating the development of the endogenous CL, e.g., manipulation of the development of the preovulatory follicle or administration of luteotrophic agents such as hCG rather than supplementation with exogenous P4, will be most effective. Alternatively, a combination of exogenous P4 and luteal support may prove beneficial in achieving balance between the apparent negative effects of P4 supplementation on the development of the CL and the positive effects on conceptus development. Whether a P4-induced increase in conceptus size can improve fertility continues to be an active area of investigation. As pointed out by Geisert et al. (1991), if the progesterone-stimulated increase in growth of the conceptus could be disconnected from the onset of earlier luteolysis in cows, an increase in the success of embryo transfer may be achieved.

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Chapter 7
Implantation and Establishment of Pregnancy in Ruminants

Thomas E. Spencer and Thomas R. Hansen

Abstract The establishment of pregnancy in ruminants occurs during the peri-implantation period and involves the suppression of the endometrial luteolytic mechanism to maintain progesterone production by the corpus luteum (CL). Reciprocal interactions between the elongating conceptus (embryo/fetus and associated extraembryonic membranes) and endometrium culminate in implantation. Antiluteolytic effects of the conceptus are due to the production of interferon tau (IFNT) by the trophoblast that has a paracrine effect to inhibit the upregulation of oxytocin receptors in the endometrial epithelia, thereby disrupting uterine release of luteolytic prostaglandin F2 alpha (PGF) pulses. Additionally, IFNT is released into the uterine vein and has endocrine actions to induce ISGs in peripheral tissues. For example, IFNT may induce luteal resistance to PGF, thereby ensuring survival of the CL and maintenance of pregnancy. Survival of the blastocyst and elongation of the conceptus requires embryotrophic factors from the epithelia of the uterus, and those embryotrophic factors are regulated by ovarian progesterone as well as conceptus-derived factors including IFNT and prostaglandins. This review provides new concepts on mechanisms of the establishment of pregnancy and implantation in ruminants with emphasis on conceptus–maternal signaling associated with elongation of the blastocyst and endometrial responses to the presence of a conceptus.

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7.1 Introduction

The establishment of pregnancy in domestic ruminants (i.e., sheep, cattle, goats) begins at the conceptus stage (embryo/fetus and associated extraembryonic membranes) and includes pregnancy recognition signaling, implantation, and the onset of placentation. Maternal recognition of pregnancy is a phrase coined by Roger Short in 1969 and can be defined as the physiological process whereby the conceptus signals its presence to the maternal system and prolongs lifespan of the corpus luteum (CL) and thus progesterone production. Progesterone acts on the uterus to stimulate and maintain uterine functions that are necessary for early embryonic development, implantation, placentation, and successful fetal and placental development to term. This review summarizes current information on the biology of establishment and maintenance of pregnancy in ruminants with particular emphasis on the peri-implantation stage of conceptus elongation in sheep and cattle. This area of reproductive biology is particularly important in ruminants due to relatively high levels of pregnancy loss during the peri-implantation period. In cattle, estimates indicate that fertilization rate is 90 % with an average calving rate of about 55 %, suggesting an embryonic/fetal mortality of about 35 %; further, 70–80 % of total embryonic loss occurs between days 8 and 16 after insemination (Diskin et al. 2006). Early pregnancy loss is even greater in the high-yielding dairy cattle, which is a major impediment to milk production efficiency (Moore and Thatcher 2006).

7.2 Overview of Peri-implantation Conceptus–Endometrial Interactions in Ruminants

The uterine wall of ruminants can be functionally divided into the endometrium and the myometrium. The adult uterus has an inner endometrium consisting of luminal epithelium (LE), glandular epithelium (GE), stroma (stratum compactum and stratum spongiosum), blood vessels, and immune cells. The endometrium has two distinct areas – aglandular caruncular and glandular intercaruncular. The caruncular areas have LE and compact stroma and are the sites of superficial implantation and placentation (Wimsatt 1950; Amoroso 1951). The establishment of pregnancy in domestic ruminants (sheep, cattle, goats) begins at the blastocyst stage and includes pregnancy recognition signaling, implantation, and placentation (see Guillomot et al. 1993; Guillomot 1995; Spencer et al. 2004b, 2007a, 2008, for review).

As illustrated in Fig. 7.1 the morula-stage ruminant embryo enters the uterus on days 4 to 6 post-mating and then forms a blastocyst that contains an inner cell mass and a blastocoele or central cavity surrounded by a monolayer of trophectoderm. After hatching from the zona pellucida, blastocysts develop into an ovoid or tubular conceptus that begins to elongate on day 12 (sheep) or day 15 (cattle) into a filamentous form that eventually occupies the entire length of the uterine horn. Elongation of the blastocyst is critical for developmentally regulated production of interferon

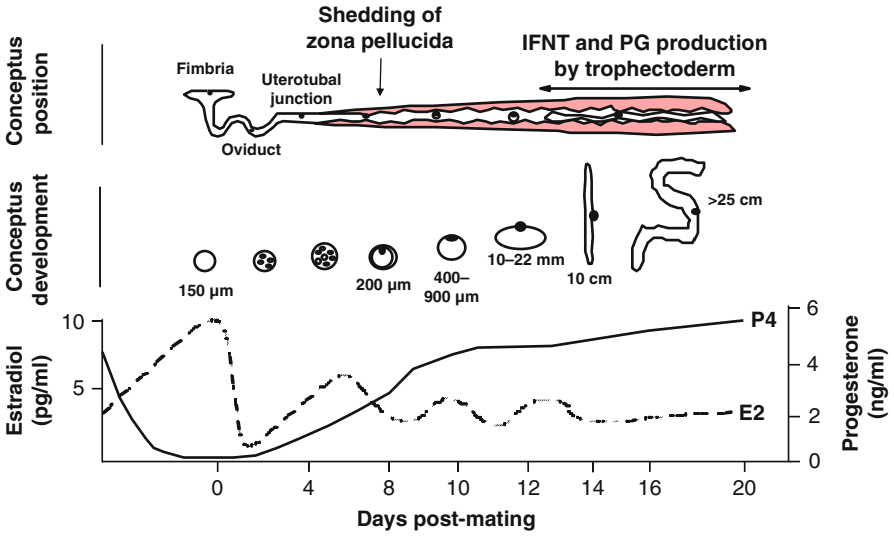


Fig. 7.1 Early pregnancy events in sheep. This schematic summarizes the relative changes in embryo/blastocyst/conceptus development after fertilization in relation to position in the female reproductive tract and circulating levels of ovarian steroid hormones. Fertilization occurs in the oviduct, and the morula-stage embryo enters the uterus on day 4. The blastocyst is formed by day 7, and it hatches from the zona pellucida by day 9. The blastocyst develops from a spherical to a tubular form by days 12 to 13 and then elongates to a filamentous conceptus between days 13 and 19. Elongation of the conceptus marks the beginning of implantation, which involves apposition and transient attachment (days 12 to 16) and firm adhesion by day 16 and is concomitant with the synthesis and secretions of interferon tau (IFNT) and prostaglandins (PG) by the trophectoderm. *E2* estrogen, *P4* progesterone

tau (IFNT), the pregnancy recognition signal, and for implantation (Farin et al. 1989; Guillomot et al. 1990; Gray et al. 2002). Although blastocysts can develop entirely *in vitro*, the overall success of this process and quality of the blastocysts are markedly lower than *in vivo* (Hasler et al. 1995). Moreover, blastocysts must be transferred into a receptive uterus for growth and development into an elongated, filamentous conceptus (Heyman et al. 1984; Flechon et al. 1986; Maddox-Hyttell et al. 2003). Progesterone acts on the uterus to indirectly stimulate preimplantation blastocyst growth and elongation by stimulating the production of embryotrophic factors from the endometrium (Garrett et al. 1988b; Mann and Lamming 2001; Mann et al. 2006; Satterfield et al. 2006). Conceptus elongation involves exponential increases in length and weight of the trophectoderm (Wales and Cuneo 1989) and onset of extraembryonic membrane differentiation, including gastrulation of the embryo and formation of the yolk sac and allantois that are vital for embryonic survival and formation of a functional placenta (Guillomot 1995; Hue et al. 2012). The increase in conceptus length is not due to the geometrical change of trophoblast cell shape, but is likely primarily driven by cell proliferation associated with peculiar plans of cell division or intercalation (Wang et al. 2009).

7.2.1 Sheep

The morula (16–32 cells)-stage embryo enters the uterus from the oviduct on day 4 after mating (day 0 = estrus/mating) (Fig. 7.1). The blastocyst is formed on day 6, and the zona pellucida is shed between days 8 and 9. The zona pellucida is thought to prevent the trophoblast from contacting and attaching to the endometrial LE. The blastocyst is spherical on day 8, measures 200 μm in diameter, and contains approximately 300 cells. By day 10, it measures 400–900 μm in diameter and contains approximately 3000 cells. After day 10, the growth of the blastocyst begins, and it is now termed a conceptus that develops first into an ovoid or tubular and then a filamentous conceptus (Wintenberger-Torres and Flechon 1974).

Between days 9 and 14, no definitive cellular contacts are observed between the trophectoderm and endometrial LE, and the blastocyst can be easily recovered from the uterus by lavage without causing structural damage. Starting on day 12, the spherical or slightly tubular conceptus begins to elongate until it reaches a length of 25 cm or more by day 17 and resembles a long filament composed mainly of extraembryonic trophoblast. By day 13, it reaches a length of 10–22 mm (1–2.2 cm), whereas by day 14, it has elongated markedly and is about 10 cm long. The primitive streak appears at this stage and somites develop soon thereafter. The conceptus, first located in the uterine horn ipsilateral to the CL, elongates into the contralateral horn and may fill more than half of its length on day 17 when only one ovulation has occurred (Rowson and Moor 1966).

Apposition of the conceptus involves the trophectoderm becoming closely associated with the endometrial LE followed by unstable adhesion. After day 14, the filamentous conceptus appears to be immobilized in the uterine lumen, and the trophectoderm maintains close contact with the endometrial LE (King et al. 1982; Guillomot et al. 1993). A close association of the apical membranes of both cell types is observed, although the conceptus can still be recovered intact from the uterus by lavage. Apposition of the blastocyst is ensured by interdigitation of cytoplasmic projections of the trophectoderm cells and uterine epithelial microvilli (Guillomot et al. 1981). In ruminants, the openings of uterine glands are also sites of apposition (Guillomot and Guay 1982; Guillomot et al. 1993). Between the caruncles, the trophoblast develops fingerlike villi or papillae, which penetrate into the mouths of the superficial ducts of the uterine glands at days 15–18 (Guillomot et al. 1981; Wooding et al. 1982). During their short life (they disappear by day 20), these trophoblastic differentiations are hypothesized to anchor the periattachment conceptus and absorb histotrophic secretions of the glands (Guillomot et al. 1981). Similar features were described for the cow conceptus from day 15 of pregnancy, but, curiously, the goat conceptus lacks trophoblast papillae.

On day 16, the trophoblast begins to adhere firmly to the endometrial LE. Uterine lavage to recover the conceptus causes superficial structural damage at this time. The interdigitation of the trophectoderm and endometrial LE occurs in both the caruncular and intercaruncular areas of the endometrium. Adhesion of the trophectoderm to the endometrial LE progresses along the uterine horn and appears to be completed around day 22 (Boshier 1969; Guillomot et al. 1981). Coincident with

apposition and adhesion of mononuclear cells of the trophectoderm to the LE, trophoblast giant binucleate cells (BNC) begin to differentiate by day 16 within the trophectoderm (Wooding 1984) from mononucleate stem cells (Wooding 1992). Migration of BNC to the microvillar junction and then fusion with individual LE cells produce trinucleate fetomaternal hybrid cells (Wooding 1984). Continued BNC migration and fusion with trinucleate cells, together with displacement and/or death of the remaining uterine LE, apparently produce multinucleated syncytial plaques, linked by tight junctions and limited in size to 20–25 nuclei that cover the caruncles (Wooding 1982, 1984, 1992). The syncytial plaques and BNC form specialized structures on the placenta termed cotyledons that interdigitate with the endometrial caruncles of the maternal uterus to form a structure termed a placentome (Igwebuike 2006). Blood flow to the uterus and from the fetus is predominantly routed to the placentomes during later pregnancy, which provides hemotrophic nutrition from the mother to the fetus.

7.2.2 Cattle

Blastocyst growth and conceptus elongation are very similar in cattle and sheep (King et al. 1982), with the major difference that the elongation of the conceptus is initiated later and takes more time. The morula-stage embryo enters the uterus on days 4–6 post-mating and then forms a blastocyst that contains an inner cell mass and a blastocoele or central cavity surrounded by a monolayer of trophectoderm. After hatching from the zona pellucida (days 9–10), the blastocyst slowly grows into a tubular or ovoid form and is then termed a conceptus (Guillomot 1995; Hue et al. 2012). In cattle, the hatched blastocyst forms an ovoid conceptus between days 12–14 and is only about 2 mm in length on day 13. By day 14, the conceptus is about 6 mm and reaches a length of about 60 mm (6 cm) by day 16. It is 20 cm or more in length by day 19. Thus, the bovine blastocyst/conceptus doubles in length every day between days 9 and 16 with a significant increase (~10-fold) in growth between days 12 and 15 (Betteridge et al. 1980; Berg et al. 2010). After day 19 in cattle, the elongating conceptus is adhered to the LE and starts the process of placentation (Guillomot et al. 1981). Many aspects of placentation are similar in cattle and sheep, although some differences have been noted in placentome morphology and cellular architecture (Wooding and Wathes 1980; King and Atkinson 1987; Wooding 1992).

7.3 Maternal Recognition of Pregnancy

Maternal recognition of pregnancy in ruminants (sheep, cattle, goats) requires that the conceptus elongate and produce IFNT, which is the pregnancy recognition signal (see Spencer et al. 1996b; Roberts et al. 1999, 2008; Spencer and Bazer 2002, for review). The antiluteolytic effects of IFNT result in the maintenance of

the CL and, hence, secretion of progesterone that is essential to maintain a uterine environment that supports events critical to the successful development of the conceptus to term.

7.3.1 Luteolytic Mechanism

Domestic ruminants are spontaneous ovulators that undergo uterine-dependent estrous cycles until the establishment of pregnancy (Wathes and Lamming 1995; McCracken et al. 1999; Spencer and Bazer 2002). The estrous cycle is dependent on the uterus, because it is the source of the luteolysin, prostaglandin F2 alpha (PGF2α). During the estrous cycle, the endometrium releases oxytocin-induced luteolytic pulses of PGF2α that result in functional and structural regression of the ovarian CL, termed luteolysis. In sheep, the source of luteolytic PGF2α pulses is the endometrial LE and superficial ductal glandular epithelium (sGE) (Gray et al. 2000a), because they express the oxytocin receptors (OXTR) (Wathes and Lamming 1995) and prostaglandin-endoperoxide synthase 2 (PTGS2), a rate-limiting enzyme in the synthesis of prostaglandins (Charpigny et al. 1997b; Simmons et al. 2010).

As illustrated in Fig. 7.2, the luteolytic mechanism that develops in the endometrial LE and superficial GE (sGE) involves sequential effects of progesterone, estrogen, and oxytocin, acting through their respective receptors (McCracken et al. 1984; Spencer et al. 1996b; Spencer and Bazer 2002). At estrus (day 0), estrogens from the antral follicle(s) increase uterine *estrogen receptor alpha* (*ESR1*), *progesterone receptor* (*PGR*), and *OXTR* expression (Wathes and Hamon 1993; Spencer and Bazer 1995); however, PGF2α is not secreted, because OXT is not present due to the absence of a CL. During early diestrus, progesterone from the newly formed CL stimulates the accumulation of phospholipids in LE/sGE that can liberate arachidonic acid for the synthesis and secretion of PGF2α. Progesterone levels increase and act via PGR to "block" expression of *ESR1* and *OXTR* in the endometrial LE and sGE (McCracken et al. 1984). During most of diestrus, the expression of *ESR1* and *OXTR* is not detected between days 5 and 11 of the cycle. The promoter of the ovine *OXTR* gene contains several SP1 elements that appear to mediate responsiveness to ligand-activated ESR1 (Fleming et al. 2006). Continuous exposure of the uterus to progesterone for 8–10 days downregulates the expression of *PGR* in endometrial LE/sGE after days 11–12 (Spencer et al. 1995b), allowing for rapid increases in the expression of *ESR1* on days 12 and 13 followed by *OXTR* on day 14 (Hixon and Flint 1987; Spencer et al. 1995a). PTGS2 is also upregulated between days 10 and 12 post-estrus/mating (Charpigny et al. 1997b; Simmons et al. 2010). Oxytocin, secreted from day 9 of the estrous cycle and pregnancy from the posterior pituitary and/or CL, then induces the release of luteolytic PGF2α pulses between days 14 and 16 (Wathes and Lamming 1995). The CL undergoes regression, allowing the ewe to return to estrus and complete the 17-day estrous cycle. Thus, progesterone is paradoxically involved first in suppressing and then inducing the development of the endometrial luteolytic mechanism in cyclic ewes. The timing of the PGR

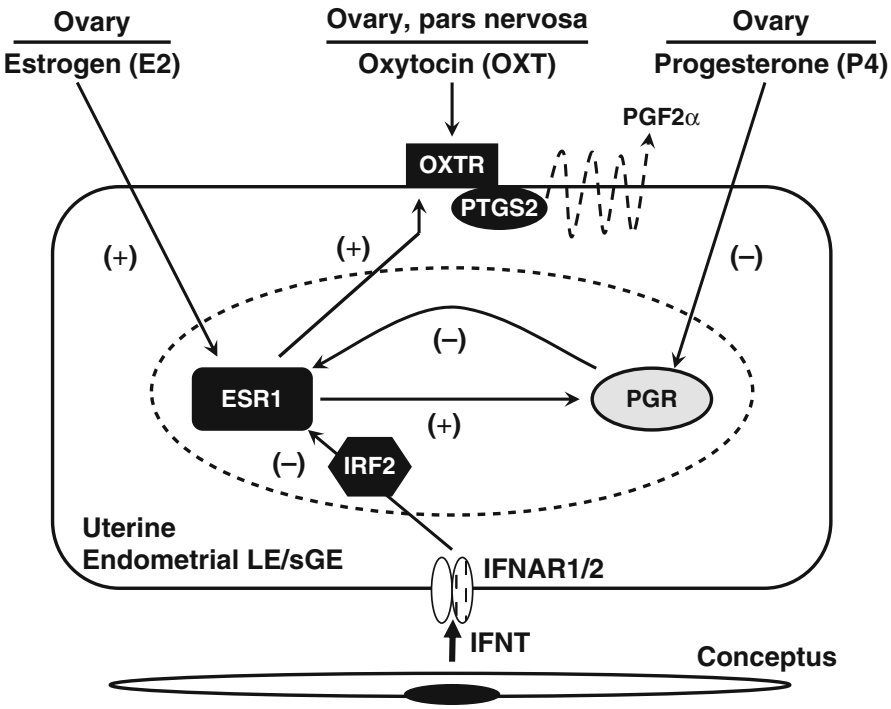


Fig. 7.2 Schematic illustrating hormonal regulation of the endometrial luteolytic mechanism and antiluteolytic effects of the conceptus on the ovine uterine endometrium. During estrus and metestrus, the expression of oxytocin receptors (*OXTR*) by uterine luminal and superficial ductal glandular epithelia (*LE/sGE*) increases in response to estrogens from the ovarian follicles that first stimulate the expression of estrogen receptor alpha (*ESR1*) and estrogens act via ESR1 to increase OXTR. Progesterone receptors (*PGR*) are expressed by LE/sGE during metestrus and diestrus, but low systemic levels of progesterone are insufficient to act via PGR to suppress ESR1 and OXTR gene expression. During early diestrus, endometrial ESR1 and estrogen are low, but progesterone levels begin to increase with the formation of the corpus luteum (*CL*). Progesterone acts through the PGR to suppress ESR1 and OXTR synthesis for 8 to 10 days. Continuous exposure of the endometrium to progesterone eventually downregulates PGR gene expression in the endometrial LE/sGE by days 11 to 12 of the estrous cycle. The loss of PGR terminates the progesterone block to ESR1 and OXTR formation. Thus, ESR1 appears between days 11 and 12 post-estrus, which is closely followed by increases in OXTR on days 13 and 14. The increase in OXTR expression is facilitated by increasing secretion of estrogens by ovarian follicles. In both cyclic and pregnant ewes, oxytocin is released from the posterior pituitary and ovarian corpus luteum beginning on day 9. In cyclic ewes, OXT binds to OXTR on LE/sGE and increases the release of luteolytic pulses of prostaglandin F2α (*PGF2α*) to regress the CL through a PTGS2-dependent pathway. In pregnant ewes, interferon tau (*IFNT*) is synthesized and secreted by the elongating conceptus beginning on day 10 of pregnancy. IFNT binds to type I IFN receptors (*IFNAR*) on the endometrial LE/sGE and inhibits transcription of the ESR1 gene through a signaling pathway involving interferon regulatory factor 2 (*IRF2*). These antiluteolytic actions of IFNT on the ESR1 gene prevent OXTR formation, thereby maintaining the CL and progesterone production required for the establishment and maintenance of pregnancy. *E2* estradiol, *ESR1* estrogen receptor alpha, *IFNAR* type I IFN receptor, *IFNT* interferon tau, *IRF2* interferon regulatory factor 2, *OXT* oxytocin, *OXTR* oxytocin receptor, *P4* progesterone, *PGF* prostaglandin F2α, *PGR* progesterone receptor, *PTGS2* prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)

downregulation by progesterone appears to determine when the luteolytic mechanism develops in the endometrium. This hypothesis is supported by the finding that exogenous progesterone administration during metestrus decreased the interestrus interval in sheep and cattle (Woody et al. 1967; Garrett et al. 1988a) and that treatment of cyclic sheep with RU486, a PGR antagonist, during the early luteal phase extended the interestrus interval (Morgan et al. 1993).

7.3.2 Pregnancy Recognition

Embryo transfer experiments in sheep initially defined the period of maternal recognition of pregnancy by finding that the conceptus must be present in the uterus prior to the onset of luteolysis to extend CL lifespan (Moor et al. 1969). Moor and Rowson (Moor and Rowson 1966a; Moor and Rowson 1966b) found that a conceptus must be present in the uterus by days 12 or 13 of the cycle in order for a successful pregnancy to be obtained following embryo transfer. Removal of conceptuses from the uteri of ewes before day 13 of pregnancy had no effect on estrous cycle length, whereas removal after that time resulted in extension of CL lifespan past day 17 (Moor and Rowson 1964, 1966a; Moor et al. 1969). Thus, maternal recognition of pregnancy in the ewe occurs around days 12 and 13.

7.3.3 Discovery of Interferon Tau (IFNT)

Homogenates of day 14–15, but not day 21–25, conceptuses extended CL lifespan and the interestrous interval when infused into the uterus of cyclic ewes (Rowson and Moor 1967; Ellinwood et al. 1979; Martal et al. 1979), suggesting that the conceptus secreted an antiluteolytic protein that was produced for a limited amount of time before day 20. The antiluteolytic substance was heat and protease labile (Rowson and Moor 1967; Martal et al. 1979). Godkin et al. (1984b) subsequently demonstrated that intrauterine injections of conceptus secretory proteins from day 15–16 conceptuses would extend the interestrous interval when administered to cyclic ewes between days 12 and 14.

In order to identify the antiluteolytic protein(s), ovine conceptuses at different stages of development where cultured in the presence of radioactive amino acids and *de novo* synthesized proteins identified by two-dimensional polyacrylamide gel electrophoresis and fluorography (Godkin et al. 1982). In sheep, the major product synthesized and released was a protein of low molecular weight (17–20 kDa). Because it was the first major protein secreted by the trophoblast of the developing ovine conceptus, the protein was later designated as "ovine trophoblast protein one or oTP-1" (Godkin et al. 1984a). Synthesis of oTP-1 was not detectable by day 23 conceptuses, which correlated with the inability of conceptus homogenates from this day to extend the interestrous interval of cyclic ewes. Intrauterine injections of purified oTP-1 into the uterus of cyclic ewes, between days 12 and 14, extended the

interestrous interval and maintained progesterone production by the CL (Godkin et al. 1984b). These studies suggested that oTP-1 was the sole antiluteolytic factor present in the total array of conceptus secretory proteins. In an elegant experiment, Vallet et al. (1988) demonstrated that oTP-1 was the sole antiluteolytic protein of those secreted by the trophoblast of the ovine conceptus.

Imakawa et al. (1987) and Stewart et al. (1987) identified oTP-1 as a member of the type I interferon alpha (IFNA) family of proteins based on protein and DNA sequencing technologies. Homology between the 172-amino-acid ovine oTP-1 and the 165-amino-acid bovine *IFNA1* mRNA and protein is 63 % and 50 %, respectively (Roberts 1991; Roberts et al. 1991). However, homology with the 172-amino-acid bovine *IFN omega 1* (*IFNW1*) mRNA and protein was 85 % and 72 %, respectively, suggesting that oTP-1 was a distinct subgroup of the type I IFN family (Imakawa et al. 1987). Because of the unique developmental expression of oTP-1 by the trophoblast and its relatedness to other type I IFNs (alpha, beta, omega), oTP-1 was classified as IFNT by the International Cytokine and Interferon Society (Roberts 1991). Cattle and sheep possess three copies of *IFNT* in their genomes (Hansen et al. 1991). Recent RNA-sequencing data found that two conceptus *IFNT* genes are expressed in the trophoblast of cattle (Sakurai et al. 2013b). It is now clear that the *IFNT* are unique to the ruminant ungulates, having diverged from ones encoding its closest relative, *IFNW*, about 36 million years ago at a time when the ruminant species themselves began to emerge as a separate lineage within the artiodactyl order (Roberts et al. 1997). It is tempting to assume that IFN production and its ability to trigger particular downstream signaling pathways in the endometrium enabled the superficial implantation and placentation of the pecoran ruminants to evolve successfully (Roberts et al. 2008).

7.3.4 Expression of IFNT

Immunocytochemical studies found that IFNT is confined to mononuclear cells of the trophectoderm (Godkin et al. 1984a; Guillomot et al. 1990). *In situ* hybridization analysis of conceptuses also localized *IFNT* mRNA exclusively to trophectoderm cells, and expression was not detected in the extraembryonic endoderm, yolk sac, allantois, or embryo proper (Farin et al. 1989; Guillomot et al. 1990). During maternal recognition of pregnancy, the mononuclear cells of the conceptus trophectoderm synthesize and secrete IFNT between days 10 and 21–25 with maximal production on days 14–16 (Bazer et al. 1992; Roberts et al. 1999). On day 15, ovine conceptuses release greater than 100 μg of the protein in culture in a 24-h period (Ashworth and Bazer 1989b). Concentrations of *IFNT* mRNA in the conceptus appear to peak around day 14 in sheep and day 20 in cattle (Hansen et al. 1988; Stewart et al. 1989). Ashworth and Bazer (Ashworth and Bazer 1989a) detected low amounts of IFNT as early as days 8 and 10 of pregnancy. *In situ* hybridization analyses of *IFNT* mRNA in ovine conceptuses confirmed the protein production results with mRNA detected as early as days 10 and 11 with maximum expression after day 13 and a decline after day 17 (Farin et al. 1989, 1990, 1991; Guillomot et al. 1990).

The reduction in *IFNT* gene expression occurs after the conceptus has adhered to the epithelium during definitive placentation. Thus, IFNT is transiently produced by the conceptus, and the expression is highest prior to the formation of OXTR in the endometrial epithelium on days 13 to 14 in cyclic or nonpregnant ewes.

IFNT expression is unique in at least four respects when compared to other type I IFNs: It is confined to the ruminant ungulates, there is lack of viral inducibility, expression is restricted to the embryonic trophectoderm, and high-level synthesis is sustained over several days and then terminates (Roberts et al. 2008). The cellular and molecular mechanisms that regulate *IFNT* gene expression in the mononuclear trophectoderm are only partially understood (see Roberts et al. 2008). Elements that control tissue and temporal expression are in the 5'-flanking region of the intronless *IFNT* genes and are highly conserved across the ruminant species (Leaman et al. 1994). Interestingly, the arrest of *IFNT* gene expression occurs in regions of the mononuclear trophectoderm that have established cellular contacts with the LE during the implantation process (Guillomot et al. 1990). The molecular mechanism of *IFNT* gene silencing may involve transcriptional repressors, such as eomesodermin, that are upregulated in trophectoderm cells adhered to the LE that culminates in implantation (Sakurai et al. 2013a).

7.3.5 Antiluteolytic Effects of IFNT

The most unique biological effect of IFNT is its antiluteolytic activity in ruminants. Intrauterine injections of ovine IFNT into sheep (Godkin et al. 1984b; Vallet et al. 1988; Ott et al. 1993), as well as cattle (Knickerbocker et al. 1986a; Knickerbocker et al. 1986b; Thatcher et al. 1986; Meyer et al. 1995; Thatcher et al. 2001) and goats (Newton et al. 1996), abrogate the development of the endometrial luteolytic mechanism and extend CL lifespan and the interestrous interval. It must be noted that the same mechanism(s) involved in IFNT action in sheep may be slightly different from those in the cow (Thatcher et al. 1992; Hansen et al. 1999). Therefore, the discussion of detailed mechanisms of the antiluteolytic actions of IFNT on the endometrium is limited to sheep given that less is known about those aspects of IFNT action in cattle.

IFNT appears to be the sole factor produced by the conceptus that prevents the development of the endometrial luteolytic mechanism (Vallet et al. 1988). In sheep, IFNT does not act to stabilize *PGR* expression in the endometrial epithelium during pregnancy (Spencer and Bazer 1995, 1996; Spencer et al. 1995b). As illustrated in Fig. 7.2, IFNT acts in a paracrine fashion on endometrial LE/sGE to suppress the transcription of *ESR1* and *OXTR* genes (Spencer and Bazer 1996; Spencer et al. 1996a; Fleming et al. 2001), thereby abrogating the development of the endometrial luteolytic mechanism. The increases in *ESR1* and *OXTR* gene expression detected in the LE/sGE on days 11–17 post-estrus in cyclic sheep do not occur in pregnant sheep (Spencer and Bazer 1995) or in cyclic sheep infused with recombinant ovine IFNT (Spencer et al. 1995c). By inhibiting increases in *OXTR* expression, IFNT prevents endometrial production of luteolytic pulses of PGF2α. However, IFNT does not inhibit basal production of PGF2α, which is higher in pregnant than cyclic

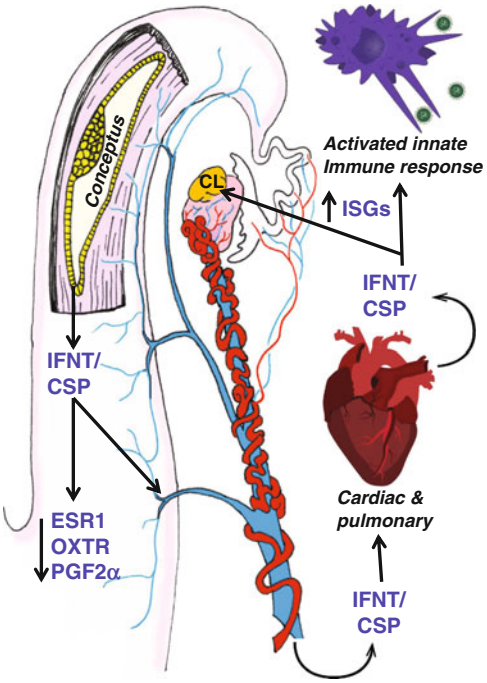


Fig. 7.3 Endocrine action of pregnancy in ruminants. IFNT is a major conceptus secretory protein (*CSP*) that is released by the expanding and elongating blastocyst. IFNT has been show to suppress the upregulation of ESR1, which leads to the suppression of OXTR, disruption of pulsatile PGF2α release, and antiluteolytic action on the corpus luteum. Because the bovine OXTR gene does not contain estrogen response elements, the mechanism disrupting PGF may be slightly different. Regardless, paracrine action of IFNT alters PGF2α pulses in both sheep and cattle and, thereby, protects the CL so that it can continue to produce progesterone, which supports the production of histotroph and further development and attachment of the conceptus. In addition to activating ISGs in the endometrium, IFNT, in addition to other CSP, may be released into the uterine vein to act in peripheral/endocrine action on immune cells and the corpus luteum. The consequences of activated innate immune responses during the establishment of pregnancy in ruminants are unknown and need to be clarified as functionally important or simply consequential to massive release of IFNT by the developing conceptus. Concerns with the utility of detection of ISGs in blood cells as indicators of pregnancy center on massive induction of these same ISGs in response to viral infections and other inflammatory responses (i.e., bacterial infections such as mastitis in dairy cows). However, the endocrine action of pregnancy and IFNT when inducing ISGs in the CL may be relevant to the establishment of luteal resistance to PGF2α. This is certainly implicated through studies demonstrating resistance of the CL induced by endocrine delivery of IFNT in response to both endogenous PGF2α and exogenous PGF2α

ewes, and the conceptus and IFNT do not affect PTGS2 expression in the endometrial LE/sGE (Charpigny et al. 1997b; Kim et al. 2003b; Simmons et al. 2010). Thus, the antiluteolytic actions of IFNT are to prevent increases in epithelial *ESR1* and *OXTR* gene expression, which are estrogen responsive, by directly inhibiting transcription of the *ESR1* gene. The precise cellular and molecular mechanisms involved in IFNT inhibitory actions on the ovine *ESR1* gene are not fully known but involve IFN regulatory factor 2 (IRF2) (Fleming et al. 2001) (Fig. 7.3).

Unlike the promoter for the ovine *OXTR* gene, the bovine *OXTR* gene lacks a classical palindromic estrogen response element (Bathgate et al. 1998), and no change in *ESR1* expression was observed in the uterine epithelia of pregnant as compared with nonpregnant cattle (Robinson et al. 1999). Thus, pregnancy and IFNT can apparently alter *OXTR* mRNA expression independent of ESR1 in the endometrium of cattle. Of note, IRF2 can regulate the expression of the bovine *OXTR* gene (Telgmann et al. 2003), suggesting a common role as an effector of IFNT antiluteolytic actions manifest on the endometrium to establish pregnancy.

7.3.6 Endocrine Actions of IFNT

For many years, IFNT was not thought to be released from the uterus and was believed to have only paracrine effects on the endometrium, because it was not detected in peripheral blood. However, *ISG* mRNAs were found to be upregulated in peripheral blood monuclear cells (PBMC) in response to pregnancy in both sheep (Yankey et al. 2001) and cattle (Han et al. 2006; Gifford et al. 2007). The impact of pregnancy on induction of ISGs in blood cells was intriguing, especially in light of opinion that IFNT was not released from the uterus into peripheral circulation. Exactly how PBMC became activated to express ISGs was unknown; however, 674 genes were upregulated and 721 genes were downregulated in PBMC from pregnant compared with nonpregnant cattle on day 18 (Hansen et al. 2010a). Importantly, many of the upregulated genes were ISGs, suggesting that IFNT exited from the uterus and had an endocrine effect on maternal tissues. Schalue-Francis et al. (Schalue-Francis et al. 1991) reported very low levels of antiviral activity in uterine vein blood of pregnant sheep. Next, significant antiviral activity was found in uterine vein blood from day 15 pregnant sheep (Oliveira et al. 2008). This antiviral activity was shown to be specifically induced by IFNT because preadsorption of IFNT using anti-IFNT antibody eliminated antiviral activity in uterine vein blood from day 15 pregnant sheep (Bott et al. 2010). Based on antiviral activity, the amount of IFN released from the uterus was estimated to be approximately 200 μg per 24 h. Further, uterine venous blood had 500- to 1000-fold higher concentrations of bioactive IFN than uterine arterial blood on day 15 of pregnancy. Thus, Bott and coworkers (Bott et al. 2010) concluded that IFNT exited the uterus in sheep and could be detected in uterine vein blood on day 15 of pregnancy. Indeed, IFNT has been identified in the uterine vein blood of early pregnant sheep by mass spectrometry as well as radioimmunoassay (T. R. Hansen, unpublished results).

7.3.6.1 Regulation of CL Function by IFNT

Moor and Rowson (Moor and Rowson 1966a) and Mapletoft and coworkers (Mapletoft et al. 1976b) described a local effect of the conceptus in maintaining the ipsilateral but not contralateral CL in ruminants. Those studies were interpreted to

indicate that the conceptus does not have a systemic effect on the CL. However, the CL of pregnancy is much more resistant to lytic effects of PGF2α (Inskeep et al. 1975; Mapletoft et al. 1976a; Pratt et al. 1977; Silvia and Niswender 1984). Thus, the elongating conceptus could have endocrine effects on the CL during early pregnancy in sheep.

Pregnant and cyclic ewes have very different patterns of PGF2α release in the blood between days 12–16 post-ovulation (Thorburn et al. 1972; Zarco et al. 1988a). Cycling ewes released PGF2α in a pulsatile manner, while pregnant ewes lack the pulsatile pattern but have higher basal circulating concentrations (Peterson et al. 1976; Zarco et al. 1988b). Higher levels of PGF2α are found in the uterine vein of day 13 pregnant as compared to cyclic ewes (Wilson et al. 1972). Although IFNT clearly inhibits the uterine production and release of luteolytic pulses of PGF2α, PGF2α synthesis by the endometrium is not inhibited, and there is a possibility that the CL produces PGF2α (Silva et al. 2000). Thus, mechanisms inducing resistance of the CL to PGF2α may need to be activated during early pregnancy to prevent luteolysis.

As found in the PBMC, ISGs are upregulated in the CL in pregnant sheep and cattle (Oliveira et al. 2008; Bott et al. 2010; Yang et al. 2010). For example, *ISG15* mRNA levels were much higher in CL from day 15 pregnant compared with non-pregnant ewes. Likewise, ISG15 protein and its ISGylated protein targets also were upregulated in CL in response to pregnancy, predominantly in large luteal cells on day 15 of pregnancy, with diminished but significant localization to small luteal cells. IFNT, but not PGE2, treatment of small, large, and mixed luteal cells from day 10 cyclic ewes induced *ISG15* expression (Antoniazzi et al. 2013; Romero et al. 2013). Further, intrauterine injections of recombinant ovine IFNT (roIFNT) induced ISG expression in the CL of cyclic ewes (Spencer et al. 1999b). These studies strongly supported the idea that IFNT exited the uterus of early pregnant sheep and had an endocrine effect on the CL and many other maternal tissues.

In order to examine the potential endocrine actions of IFNT, osmotic pumps were implanted into day 10 cyclic ewes and 200 μg of roIFNT was infused into the uterine vein each day. *ISG15* mRNA was upregulated in the ipsilateral and contralateral CL as well as in the endometrium and liver (Oliveira et al. 2008; Bott et al. 2010). When the uterine vein of cyclic ewes was infused with roIFNT from day 10 to day 17 post-estrus, the interestrous interval was extended to greater than 32 days, whereas cyclic ewes infused with bovine serum albumin returned to estrus by day 19. Thus, endocrine delivery of IFNT into the uterine vein for 7 days was able to block luteolysis from endogenously produced PGF2α. Further, Bott and coworkers (Bott et al. 2010) demonstrated that delivery of 200 μg of roIFNT into the uterine vein would protect the CL from the luteolytic actions of PGF2α. More recently, 24 h infusion of only 20 μg of roIFNT per day into the uterine vein or subcutaneously into the neck on days 10–11 of the estrous cycle was able to significantly protect the CL from the lytic action of PGF2α exogenously administered on day 11 (Antoniazzi et al. 2013). Collectively, these results strongly support the idea that resistance of the CL in pregnant sheep to luteolytic PGF2α is due to the endocrine actions of IFNT, perhaps by protecting the integrity and steroidogenic machinery and/or

attenuating apoptosis in the CL of pregnancy (Hansen et al. 2010a; Antoniazzi et al. 2013; Romero et al. 2013).

The endocrine effects of IFNT on CL function are less established in cattle, but *ISG* mRNAs are upregulated in PBMC in response to pregnancy in cattle (Han et al. 2006; Gifford et al. 2007; Hansen et al. 2010a). Indeed, ISGs may be useful as an early pregnancy test in cattle (Han et al. 2006; Gifford et al. 2007; Green et al. 2010; Pugliesi et al. 2014). However, concerns with utility of detection of ISGs in blood cells as indicators of pregnancy center on massive induction of the same ISGs in response to viral infections and other inflammatory responses, i.e., bacterial infections such as mastitis in dairy cows (Hansen et al. 2010b; Smirnova et al. 2012).

7.3.7 IFNT Regulation of Endometrial Function and Conceptus Elongation

In addition to antiluteolytic effects on the endometrium, IFNT induces or enhances the expression of ISGs in the endometrium of both early pregnant sheep and cattle that are hypothesized to regulate uterine receptivity for conceptus elongation and implantation (Hansen et al. 1999, 2010a; Spencer et al. 2008; Bazer et al. 2009a). The actions of IFNT are mediated by the interferon (alpha and beta) receptor (IFNAR), which is composed of two subunits, IFNAR1 and IFNAR2 (Hansen et al. 1989). To test the hypothesis that IFNT and its receptor have biological roles in conceptus elongation, an *in vivo* loss of function study was recently conducted by inhibiting IFNT or IFNAR1/2 mRNA translation in the trophectoderm of the ovine conceptus using morpholino antisense oligonucleotides (MAO) delivered via osmotic pumps from days 8–14 post-mating (Brooks and Spencer 2014). Elongating, filamentous-type conceptuses were recovered from day 14 ewes receiving a control morpholino or IFNAR MAOs. In contrast, severely growth-retarded and malformed conceptuses were recovered from IFNT MAO-infused ewes. Those conceptuses contained abnormal trophectoderm cells that were apoptotic. Available studies support the idea that IFNT is a critical regulator of conceptus elongation and its effects are most likely indirectly mediated by IFNT-stimulated embryotrophic factors from the endometrium.

7.3.7.1 Classical Type I IFN-Stimulated Genes in the Endometrium

A number of transcriptional profiling and proteomic experiments conducted with human cells, ovine endometrium, bovine endometrium, and bovine peripheral blood lymphocytes have elucidated classical ISGs induced by IFNT during pregnancy (Hansen et al. 1999; Spencer et al. 2007a, 2008; Ott and Gifford 2010; Forde et al. 2011;

Bauersachs et al. 2012). The development of a bovine endometrial cell line called BEND cells allowed study of signal transduction following treatment with IFNT *in vitro*. Using these cells, it was demonstrated that IRF1 and STAT1, STAT2, and STAT3 proteins were phosphorylated in response to IFNT (Perry et al. 1999; Thatcher et al. 2001). Also, specific binding of IRF1 to the bovine ISG15 gene promoter (ISRE) was described using shift and supershift transcription factor/promoter assays (Perry et al. 1999). The effects of IFNT in the bovine endometrium are not as well understood compared to ovine endometrium in terms of nonclassical ISGs and associated signal transduction, but recent studies have started to unravel those effects in cattle (Forde et al. 2011; 2012; Bauersachs et al. 2012).

In vivo studies revealed that the majority of classical ISGs are induced in the endometrial stroma and glands as well as the myometrium of the ovine uterus during early pregnancy (Johnson et al. 1999b, 2001; Choi et al. 2001, 2003; Song et al. 2007). The lack of classical ISG expression in the endometrial LE or sGE during pregnancy may be a critical mechanism preventing immune rejection of the semi-allogeneic conceptus (Choi et al. 2003). One challenge has been to determine which of the large number of classical ISGs induced in the endometrium by IFNT has a biological role in conceptus elongation and implantation, as traditionally the main function of type I IFN is to inhibit viral infection and has primarily been associated with cellular antiviral responses (Pestka 2007). It is likely that the classical ISGs induced by IFNT in the endometrium have biological roles in conceptus implantation and establishment of pregnancy by actions on the trophectoderm (Imakawa et al. 2006) or modulation of immune cells at the conceptus–maternal interface (Hansen 1995, 2007, 2013; Hansen et al. 1999).

7.3.7.2 Nonclassical IFNT-Stimulated Genes in the Endometrium

Transcriptional profiling of human U3A (STAT1-null) cells and ovine endometrium and candidate gene analyses were used to discover novel "nonclassical" ISG in the endometrial LE during pregnancy (Kim et al. 2003a; Song et al. 2005; Gray et al. 2006; Satterfield et al. 2006; Song et al. 2006). Subsequently, a series of transcriptomic and candidate gene studies found that IFNT stimulates the expression of a number of elongation- and implantation-related genes that are initially induced by progesterone specifically in the endometrial LE, sGE, and(or) GE (Spencer et al. 2007a, 2008; Bazer et al. 2009a, b). None of these genes are classical type I ISGs and thus can be referred to as "nonclassical or novel" ISG. Indeed, IFNT stimulation of these nonclassical ISG requires initial induction in the endometrial epithelia by progesterone. Importantly, all of the nonclassical ISGs encode factors whose actions on the trophectoderm (proliferation, migration, attachment and (or) adhesion, nutrient transport) would be or are important for conceptus elongation (see Spencer et al. 2004a, 2008; Bazer et al. 2011, 2012a; Dorniak et al. 2013, for review).

7.4 Functional Role of Endometrial Secretions in Implantation and Establishment of Pregnancy in Ruminants

All mammalian uteri contain endometrial epithelia that synthesize and secrete or transport a complex array of proteins and related substances termed "histotroph" (Wimsatt 1950; Amoroso 1952; Bazer 1975), that is, a complex mixture of enzymes, growth factors, cytokines, lymphokines, hormones, transport proteins, and other substances. Evidence from human, primate, and subprimate species during the last century supports an unequivocal role for secretions of endometrium as primary regulators of conceptus survival, development, production of pregnancy recognition signals, implantation, and placentation (reviewed in Bazer et al. 1979; Roberts and Bazer 1988; Gray et al. 2001a; Burton et al. 2002; Filant and Spencer 2014). The microvillous epithelial cells of the uterine lumen present a high secretory activity during the luteal phase of the cycle and at the beginning of implantation (Guillomot et al. 1981). The sheep trophoblast appears to be the site of intense pinocytotic activity that increases as the blastocyst develops and elongates (Wintenberger-Torres and Flechon 1974). Indeed, blastocyst growth into an elongated conceptus does not occur *in vitro*, as it requires secretions supplied by the endometrium of the uterus (Betteridge and Flechon 1988; Gray et al. 2001c; Lonergan 2011).

7.4.1 Uterine Gland Knockout (UGKO) Ewe Model

The UGKO ewe model is produced by continuous administration of a synthetic, nonmetabolizable progestin to neonatal ewes from birth to 8 weeks of age (Bartol et al. 1999; Gray et al. 2000b). This inappropriate exposure to a progestin permanently ablates the differentiation and development of the glandular epithelia (GE) from LE in the endometrium and produces an UGKO phenotype without altering the development of myometrium or other Müllerian duct-derived female reproductive tract structures or the hypothalamic–pituitary–ovarian axis (Gray et al. 2000b, 2001b). The endometrium is devoid of middle to deep endometrial glands, and the LE surface area is markedly reduced. UGKO ewes exhibit recurrent early pregnancy loss in which the blastocyst fails to elongate. Transfer of blastocysts from normal fertile ewes into the uteri of timed recipient UGKO ewes does not ameliorate this defect (Gray et al. 2001c). Morphologically normal blastocysts are present in uterine flushes of bred UGKO ewes on days 6 and 9 after mating, but not on day 14 (Gray et al. 2001c, 2002). On day 14, uterine flushes of mated UGKO ewes contain either no conceptus or a severely growth-retarded tubular conceptus. Therefore, histotrophic secretions from the endometrial epithelia, particularly the GE, are required for peri-implantation blastocyst survival and conceptus elongation in sheep.

Available results indicate that the defects in blastocyst survival and elongation in UGKO ewes are not due to alterations in the expression of steroid receptors, mucin

glycoprotein 1 (MUC1), or adhesive integrins on the endometrial LE or to the responsiveness of the endometrium to the conceptus pregnancy recognition signal IFNT (Gray et al. 2001b, 2002). However, when uterine flushes of day 14 bred UGKO ewes were analyzed for the presence of osteopontin (OPN or secreted phosphoprotein 1) and glycosylated cell adhesion molecule 1 (GLYCAM1) proteins, which are adhesion proteins secreted primarily by GE (Johnson et al. 1999a; Spencer et al. 1999a), very low levels of OPN and GLYCAM1 were found in UGKO as compared to normal day 14 pregnant ewes (Gray et al. 2002). Therefore, the reduction or absence in adhesion proteins of endometrial epithelial origin was proposed to be a cause of recurrent pregnancy loss in the UGKO ewe. Given the complexity of uterine luminal fluid, undoubtedly a number of other factors are deficient in the UGKO uteri that act on the conceptus to stimulate trophoblast survival and proliferation.

7.4.2 Embryotrophic Factors in the Uterine Lumen Regulating Conceptus Elongation

The uterine luminal fluid contains histotroph that governs elongation of the conceptus via effects on trophectoderm proliferation and migration as well as attachment and adhesion to the endometrial LE (Spencer et al. 2007b, 2008; Bazer et al. 2010). Histotroph is derived primarily from transport and (or) synthesis and secretion of substances by the endometrial LE and GE, and it is a complex and rather undefined mixture of proteins, lipids, amino acids, sugars (glucose, fructose), ions, and exosomes/microvesicles (Bazer 1975; Gray et al. 2001a; Koch et al. 2010; Bazer et al. 2012b; Burns et al. 2014). The recurrent early pregnancy loss observed in uterine gland knockout (UGKO) ewes established the importance of uterine epithelial-derived histotroph for support of conceptus elongation and implantation (Gray et al. 2001c). Available evidence supports the idea that ovarian P4 induces the expression of a number of genes, specifically in the endometrial epithelia, that are then further stimulated by factors from the conceptus (e.g., IFNT, PGs, cortisol) as well as the endometrium (e.g., PGs and cortisol) (Dorniak et al. 2013; Brooks et al. 2014). The genes and encoded hormones, cytokines, and other functional mediators in the endometrial epithelia elicit specific changes in the intrauterine histotrophic milieu necessary for conceptus elongation (Spencer et al. 2007b, 2008; Bazer et al. 2010; Forde and Lonergan 2012; Dorniak et al. 2013). The outcome of the progesterone-induced changes in the uterus during the estrous cycle or pregnancy is to modify the intrauterine milieu, such as an increase in select amino acids, glucose, cytokines and growth factors, and adhesion proteins in histotroph, for support of blastocyst growth into an ovoid conceptus and its elongation to form a filamentous conceptus (see Spencer et al. 2008; Bazer et al. 2010; Forde and Lonergan 2012; Dorniak et al. 2013; Brooks et al. 2014). Factors from the endometrium may also stimulate the expression of IFNT in the conceptus trophectoderm (Roberts et al. 2003; Michael et al. 2006; Ealy and Yang 2009; Kim et al. 2011). Several recent reviews catalogue

the endometrial contributions to uterine luminal fluid that functions in conceptus elongation in ruminants (Roberts et al. 2008; Spencer et al. 2008; Bazer et al. 2010; Forde and Lonergan 2012; Bauersachs and Wolf 2013; Dorniak et al. 2013; Ulbrich et al. 2013; Brooks et al. 2014; Lonergan and Forde 2014).

7.5 Prostaglandins and Conceptus Elongation

The conceptus and endometrium synthesize a variety of PGs during early pregnancy in both sheep and cattle (Lewis et al. 1982; Lewis and Waterman 1983, 1985; Lewis 1989; Charpigny et al. 1997a, b). The endometrium and uterine lumen contain substantially more PGs during early pregnancy than the estrous cycle (Ellinwood et al. 1979; Marcus 1981; Ulbrich et al. 2009). The dominant cyclooxygenase expressed in both the endometrium and trophectoderm of the elongating conceptus is PTGS2 (Charpigny et al. 1997a, b). Although the antiluteolytic effects of IFNT are to inhibit the expression of the *OXTR* in the endometrial LE/sGE of early pregnant ewes, it does not impede the upregulation of PTGS2, a rate-limiting enzyme in PG synthesis, in the endometrium (Charpigny et al. 1997b; Kim et al. 2003c; Simmons et al. 2010). In the bovine uterus, PTGS2 is also not downregulated in the endometria of early pregnant cattle, but rather is upregulated by IFNT (Arosh et al. 2004; Emond et al. 2004). Further, IFNT acts as a molecular switch that stimulates PGE2 production in the bovine endometrium (Krishnaswamy et al. 2009). In sheep, PTGS2 activity in the endometrium is stimulated by IFNT, and PTGS2-derived PGs were found to mediate, in part, the effects of progesterone and IFNT on the endometrium of the ovine uterus (Dorniak et al. 2011b, 2012). Indeed, type I IFNs were found to stimulate phospholipase A2 activity and synthesis of PGE2 and PGF2α in several different cell types over 25 years ago (Fitzpatrick and Stringfellow 1980; Fuse et al. 1982).

Prostaglandins are essential for conceptus elongation, as intrauterine infusions of meloxicam, a selective PTGS2 inhibitor, prevented conceptus elongation in early pregnant sheep (Simmons et al. 2010; Dorniak et al. 2011a). Elongating conceptuses of both sheep and cattle synthesize and secrete more PGs than the underlying endometrium (Lewis et al. 1982; Lewis and Waterman 1983; Lewis 1989). Thus, PG levels are much greater in the uterine lumen of pregnant when compared with cyclic or nonpregnant cattle (Ulbrich et al. 2009). In sheep, Charpigny and coworkers (Charpigny et al. 1997a) found that PTGS2 was abundant in day 8 to 17 blastocysts/conceptuses, whereas PTGS1 was undetectable. There was a 30-fold increase in PTGS2 content per protein extract between days 10 and 14, corresponding to a 50,000-fold increase in the whole conceptus, and PTGS2 protein in the conceptus then declined substantially after day 16 to undetectable levels by day 25 of pregnancy. Given that membrane and nuclear receptors for PGs are present in all cell types of the ovine endometrium and conceptus during early pregnancy (Cammas et al. 2006; Dorniak et al. 2011a), PTGS2-derived PGs from the conceptus likely have paracrine, autocrine, and perhaps intracrine effects on endometrial function and conceptus development during early pregnancy.

Both PGI2 and PGJ2 can activate nuclear peroxisome proliferator-activating receptors (PPARs) (Desvergne and Wahli 1999). PGI2 is a ligand for PPARD, and PGD2 spontaneously forms 15-deoxy-Δ12,14-PGJ2 within cells that is a ligand for PPARG (Forman et al. 1995; Kliewer et al. 1995; Lim et al. 1999; Lim and Dey 2000). The expression of prostacyclin (PGI2) synthase (PTGIS), PGI2 receptors (PTGIR), PPARs, and RXRs in the uteri and conceptuses of sheep during early pregnancy has been well documented (Cammas et al. 2006). Recently, *in utero* loss-of-function studies of PPARD and PPARG in the ovine conceptus trophectoderm were conducted using morpholino antisense oligonucleotides (MAO) that inhibit mRNA translation (Brooks and Spencer 2014). Elongating, filamentous-type conceptuses were recovered from ewes infused with a control morpholino or PPARD MAO. In contrast, PPARG MAO resulted in severely growth-retarded conceptuses or conceptus fragments with apoptotic trophectoderm. In order to identify PPARG-regulated genes, PPARG ChIP-Seq and RNA-Seq were conducted using day 14 ovine conceptuses. These analyses revealed candidate PPARG-regulated genes involved in biological pathways including lipid and glucose uptake, transport, and metabolism. Collectively, results support the hypothesis that PTGS2-derived PGs and PPARG are essential regulators of conceptus elongation in sheep with specific roles in trophectoderm survival and proliferation. Of note, the expression of *PTGS2* in biopsies of day 7 bovine blastocysts is a predictor of the successful development of that blastocyst to term and delivery of a live calf (El-Sayed et al. 2006). Further, pregnancy rates were substantially reduced in heifers that received meloxicam, a partially selective inhibitor of PTGS2, on day 15 after insemination (Erdem and Guzeloglu 2010). A recent study supports the hypothesis that the day 13 conceptus secretes PGs that act locally in a paracrine manner to alter gene expression in the endometrium prior to pregnancy recognition in cattle (Spencer et al. 2013).

7.6 Conclusion

The antiluteolytic effects of IFNT in sheep involve paracrine effects on the endometrium and endocrine effects on the CL that culminate in maternal recognition of pregnancy and maintenance of progesterone, the unequivocal hormone of pregnancy. The production of sufficient IFNT to establish pregnancy is dependent on conceptus elongation. The individual, additive, and synergistic actions of progesterone, IFNT, and PGs regulate the expression of elongation- and implantation-related genes in the endometrial epithelia. Progesterone, IFNT, and PGs are essential regulators of conceptus elongation in sheep and likely cattle. The outcome of carefully orchestrated changes in endometrial gene expression is secretion or transport of substances (e.g., glucose, amino acids, proteins) from the endometrium into the uterine lumen that govern conceptus survival and elongation via effects on trophectoderm proliferation, migration, attachment, and adhesion. Recent studies indicate that some, but not all, of the same mechanisms, pathways, and factors that regulate conceptus elongation in cattle are conserved with sheep (Bauersachs et al. 2008;

Spencer et al. 2008; Forde et al. 2011; Forde and Lonergan 2012). One important area of future research is determining which endometrial genes and products are critical determinants of uterine receptivity and early pregnancy success. This knowledge should be useful to develop genetic tools essential to select animals for enhanced fertility. Improvement of functional traits using conventional approaches of quantitative genetics is difficult, because most reproductive traits are complex (polygenic) with low heritability (Weigel 2006; Veerkamp and Beerda 2007). McMillan and Donnison (1999) summarized a novel approach for experimentally identifying high and low fertility heifers based on early pregnancy success using serial transfer of *in vitro*-produced embryos. Of note, those investigators suggested that a failure in the mechanism involved in conceptus elongation and maternal recognition of pregnancy was a major cause of early pregnancy loss in low fertility heifers (McMillan and Donnison 1999; Peterson and Lee 2003). Accordingly, the selected high fertility heifers would have a uterus that was superior in the ability to support the growth and development of the conceptus. Thus, natural variation in early pregnancy rates in cattle can be used to define genes and pathways important for the implantation and establishment of pregnancy (Minten et al. 2013). Other ruminant models to understand endometrial receptivity and pregnancy loss include (a) the UGKO ewe (Gray et al. 2002), (b) heifers versus cows (Berg et al. 2010), (c) nonlactating versus lactating cows (Cerri et al. 2012), (d) advanced versus delayed post-ovulatory rise in progesterone (Lonergan 2011; Forde and Lonergan 2012), and (e) recessive lethal mutations that manifest in defective conceptus elongation and/or epiblast formation (Charlier et al. 2012). A systems biology approach is necessary to understand the multifactorial phenomenon of early pregnancy loss and provide a basis for new strategies to improve pregnancy outcomes, fertility, and reproductive efficiency in ruminants.

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Chapter 8
Implantation and Establishment of Pregnancy in the Pig

Rodney D. Geisert, Gregory A. Johnson, and Robert C. Burghardt

Abstract Establishment of pregnancy in the pig is initiated through the release of estrogens from the rapidly elongating conceptuses. Release of estrogens from the developing conceptuses alters the movement of endometrial prostaglandin F2α from being released into the vasculature (endocrine secretion) to sequestering in the uterine lumen (exocrine secretion). Rapid trophoblast elongation, which is unique to the pig, may be triggered through production of interleukin 1β2 (IL1B2) by conceptuses. Trophoblast elongation through the uterine horns provides the mechanism to allow conceptus–endometrial interactions essential for the implantation, placentation, and maintenance of pregnancy in the pig. This chapter provides current information on conceptus signaling pathways and endometrial responses to those conceptus factors leading to establishment of pregnancy.

8.1 Introduction

Although early stages of zygote cleavage to formation of the blastocyst are similar to that of many other mammalian species, the biological processes initiated for the establishment and maintenance of the porcine pregnancy are unique following blastocyst hatching on day 8 of gestation. To establish and maintain pregnancy, pig blastocysts must first migrate within and between the long horns of the bicornuate uterus. The classical studies of Dziuk and others (Dziuk et al. 1964; Dhindsa et al. 1967; Polge and Dziuk 1970) established that intra- and inter-uterine migration of

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blastocysts is initiated shortly after hatching from the zona pellucida and continues until the time of rapid trophoblast elongation on day 11–12 of gestation. Uterine migration is stimulated through conceptus estrogen and prostaglandin (PG) synthesis quite possibly through the induction of histamine release from the underlying endometrium (Pope et al. 1982, 1986b) and/or actions of lysophosphatidic acid (Seo et al. 2012b). Uterine migration and spacing of blastocysts serve to not only provide sufficient surface area for attachment of the diffuse, epitheliochorial placenta of individual conceptuses from day 13 to 18 of gestation but provide sufficient interface with the endometrium to prevent luteolysis. The lungs of the pig do not metabolize prostaglandin F2α (PGF2α) as efficiently as ruminants (Davis et al. 1979) providing both a systemic and local uterine vascular pathway for CL regression (Del Campo and Ginther 1973; Ginther 1981). Therefore, it is essential that conceptuses cover the uterine surface area as rapidly and completely as possible at this early stage of pregnancy since leaving greater than one quarter of a uterine horn unoccupied results in greater loss of pregnancy in the pig due to release of adequate PGF2α to regress CLs on both ovaries (Dhindsa and Dziuk 1968b). Moreover, maintenance of a unilateral pregnancy during the first 3 weeks of gestation is a very rare occurrence in the pig (du Mesnil du Buisson 1961).

Migration and equidistant uterine spacing of the developing blastocysts between days 8 and 12 of gestation play an essential role in setting up the critical biological events involved with maternal recognition of pregnancy in the pig. These key biological events include (1) downregulation of progesterone receptor (PR) from the luminal (LE) and glandular (GE) epithelia, (2) rapid elongation of conceptus trophoblast (day 12) to deliver the conceptus signals across the uterine endometrial surface and provide surface area for placental attachment and growth, (3) conceptus synthesis and release of estrogens to alter uterine movement of PGF2α away from the uterine vasculature (endocrine secretion) and into the uterine lumen (exocrine secretion), (4) trophoblast adhesion and attachment to the uterine surface, (5) increased uterine blood flow and vascularity to the site of conceptus attachment, and (6) spatiotemporal conceptus/endometrial signaling for endometrial secretion of enzymes and their inhibitors, proteins, growth factors, and cytokines needed for continued growth and differentiation of the conceptus and regulation of the maternal immune system. The following review is intended not only to provide information from the current literature for understanding the biological mechanisms for establishment of pregnancy in the pig but also to offer a brief historical perspective of the seminal papers that provided the groundwork upon which much research has been focused over the past 40 years.

8.2 Timing of Luteolysis and Conceptus Attachment

8.2.1 Luteolysis

Endometrial release of PGF2α into the uterine vasculature between days 14 and 17 of the estrous cycle of pigs (Moeljono et al. 1977; Shille et al. 1979) induces CL regression shortly after day 15. One interesting aspect concerning the induction of

luteolysis in the pig compared to other domestic farm species is the refractoriness of the CL to PGF2α until after day 11 of the estrous cycle. Administration of PGF2α before day 12 of the estrous cycle or early pregnancy does not result in luteolysis (Gadsby et al. 2006) which is associated with the lower abundance of receptors for PGF2α (FP) on luteal cells. Thus, shortening the length of the estrous cycle or estrous synchronization with PGF2α or its analogues is not effective in the pig. However, although not an easy or practical method for estrous synchronization, repeated administration of PGF2α analogues from day 5 to 10 of the estrous cycle will induce premature luteolysis and shortens the estrous cycle by about 7 days (Estill et al. 1993). The refractoriness of the pig CL to PGF2α before day 12 is certainly not a problem with the timing of endometrial release during the normal estrous cycle.

8.2.2 Endometrial Epithelial Progesterone Receptor

The increase in endometrial production and release of PGF2α is consistent with the fold increases in expression of endometrial prostaglandin-endoperoxide synthase 1 (PTGS1) and PTGS2 mRNAs and proteins during the period of luteolysis (day 13–15) in the pig (Ashworth et al. 2006). Timing for the increase in PTGS in the endometrium and pulsatile release of PGF2α during the estrous cycle is regulated by the duration (8–10 days) of stimulation by progesterone from the CL and cellular localization of the progesterone receptor (Geisert et al. 1992). The role of progesterone in the timing of luteolysis or opening the "window of receptivity" for elongation and attachment of the trophoblast to the uterine LE during pregnancy occurs through the cell-specific expression of steroid receptors within the uterine LE and GE and stroma. Uterine stromal and myometrial cells express progesterone receptor (PGR) throughout the estrous cycle and pregnancy, but a clear spatiotemporal association exists between the downregulations of PGR (specifically PGRA) in the endometrial LE and GE after day 10 of the estrous cycle or pregnancy (Geisert et al. 1994; Spencer and Bazer 2004). Although the downregulation of PGR in uterine LE and GE has been clearly established in a number of mammalian species (see Geisert et al. 2012), the mechanism involved with cell-specific loss of PGR from the uterine epithelia has not been established.

8.3 Maternal Recognition of Pregnancy

8.3.1 Conceptus Estrogens

The timing for maternal recognition of pregnancy (i.e., extension of CL lifespan) in the pig was first determined through the classical approach of flushing conceptuses from the uterus. Removal of the pig conceptuses before day 11 of gestation resulted in a normal 21-day interestrous interval, while flushing conceptuses on or after day

12 extended the interestrous interval to 25–28 days (Dhindsa and Dziuk 1968a). The demonstration by Kidder et al. (1955) that administration of diethylstilbestrol on day 11 of the "estrual cycle" extended the interestrous interval to 25 days was the first indication that estrogen might be involved with maintenance of CL function in the pig. However, with their focus on the anterior pituitary and only the ability to observe the ovaries at that time, they incorrectly concluded that extension of length of the estrous cycle was due to luteinization of ovarian follicles. Years later, studies by Gardner et al. (1963) and others (see review Geisert et al. 1990) confirmed that administration of estradiol or estrone to gilts on day 11 of the estrous cycle extends CL lifespan.

The capacity of early (day 11–18) porcine conceptuses to synthesize and release estrogens was first reported by Perry et al. (1973) and confirmed in subsequent studies (Gadsby et al. 1980; Fischer et al. 1985). Uterine luminal content of estrogens increases rapidly during the period of conceptus elongation on day 12 of gestation and declines between days 13 and 14 followed by a second prolonged increase in estrogen production by conceptuses from day 15 to 18 of gestation (Zavy et al. 1980; Geisert et al. 1982a). The biphasic changes in synthesis of estrogen by pig conceptuses are reflected in the uterine luminal fluid, utero-ovarian vein blood (Zavy et al 1980; Ford et al. 1982), and the peripheral circulation of pregnant gilts (Robertson and King 1974; Stone and Seamark 1985). The biological relevance of the biphasic release of conceptus estrogens on extension of CL lifespan was demonstrated through administration of estrogen to cyclic gilts. Studies indicated that administration of estrogen on day 11 of the estrous cycle extended the interestrous interval to approximately 25 days. However, prolong exposure to exogenous estrogens (day 11–15) extends CL function beyond 60 days (Frank et al. 1977). The need for exogenous estrogen at day 11 (period of conceptus elongation) and day 14–18 (trophoblast attachment and second surge of estrogen release) to maintain CL function beyond 25 days indicated the necessity for the two phases of conceptus estrogen release (Geisert et al. 1987). The need for the short-term increase in conceptus estrogen during elongation and the sustained increase in estrogen production after attachment of the trophectoderm to uterine LE is consistent with the prolonged maintenance of CLs in gilts with conceptuses flushed from the uterine horns on or after day 18 of gestation (Dhindsa and Dziuk 1968a; Ford et al. 1982).

Synthesis and secretion of estrogens by the pig conceptuses provide the maternal signal for not only CL maintenance during pregnancy but open the window of endometrial receptivity for implantation. Following downregulation of PGR from the endometrial LE and GE, estrogen receptor (ESR1) in uterine LE and GE is upregulated (Geisert et al. 1993) which provides the pathway for the cell-specific responses to conceptus estrogens released on day 12. However, the maternal uterine environment is sensitive to the precise timing of conceptus estrogen stimulation (Geisert et al. 2004). Exposure of pregnant pigs to environmental estrogens (e.g., mycotoxins in moldy corn) or administration of estrogen prior to day 10 of gestation results in total embryonic loss before day 30 (Long et al. 1983; Pope et al. 1986a). Administration of estrogen prior to the window of receptivity for conceptus elongation and attachment results in fragmentation and degeneration of the conceptuses

between days 14 and 18 of pregnancy (Gries et al. 1989). Conceptus degeneration results from a breakdown in the surface glycocalyx of uterine LE (Blair et al. 1991), alterations in uterine protein secretion, and aberrant endometrial transcriptional activity during the period of conceptus attachment (Ashworth et al. 2012b). Thus, the utilization of estrogen as one of the major signals to maintain CL throughout pregnancy in the pig makes the pig susceptible to the disruptive effects of environmental estrogens during early pregnancy. Not only can exposure of mated females to environmental estrogens disrupt early embryonic development and survival, but it induces a prolonged (60–110 days) period of pseudopregnancy.

8.3.2 Luteostatic Mechanism of CL Maintenance

The luteostatic "endocrine–exocrine theory" of pregnancy in pigs (Fig. 8.1) was first proposed by Bazer and Thatcher (1977). The endocrine–exocrine model is based on: (1) reduction of PGF2α in the utero-ovarian veins (Moeljono et al. 1977) and peripheral plasma (Shille et al. 1979) (endocrine secretion) of pregnant compared to cyclic gilts between days 12 and 18, (2) increased uterine luminal content of PGF2α in uterine flushing (exocrine secretion) of pregnant gilts versus cyclic gilts on day 11–15 (Zavy et al 1980), and (3) administration of estrogen to cyclic gilts reducing PGF2α in the utero-ovarian vein (Frank et al. 1977) and increasing endometrial luminal content of PGF2α over tenfold (Frank et al. 1978; Gross et al. 1988). Thus, conceptus estrogens do not directly inhibit endometrial synthesis of prostaglandins (PGs) but cause sequestration of PGs in the uterine lumen where PGF2α can be metabolized to the inactive 15 keto-13, 14 dihydroprostaglandin F2α metabolite before being released into the utero-ovarian vein (Ziecik et al. 2011). Further evidence in support of the endocrine–exocrine model came from *in vitro* endometrial perfusion studies which indicated a reorientation of PGF secretion into the uterine lumen of pregnant pigs through effects of estrogen- and prolactin-mediated calcium cycling (Gross et al. 1988, 1990).

In addition to the alteration of PG movement from the uterine endometrium, release of estrogen from the expanding conceptuses can have a direct luteotrophic effect on the CL (Conley and Ford 1989) as well as stimulating a localized increase in endometrial vascular permeability (Keys and King 1988, 1995) and an overall increase in uterine blood flow (Ford et al. 1982; Ford and Stice 1985). During conceptus elongation and the early peri-implantation period, the endometrium increases the release of a number of growth factors and cytokines such as epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-1), fibroblast growth factor 7 (FGF7), vascular endothelial growth factor (VEGF), interleukin 6 (IL-6), transforming growth factor beta (TGFβ), and leukemia inhibitory factor (LIF) (see review Bazer et al. 2010; Geisert et al. 2014). Endometrial release of EGF, FGF7, LIF, and IGF-1 is specifically enhanced in the epithelium during the period of conceptus elongation and estrogen secretion. Conceptus estrogens also increase endometrial expression of genes for aldo–keto reductase (Seo et al. 2014a), interleukin 1

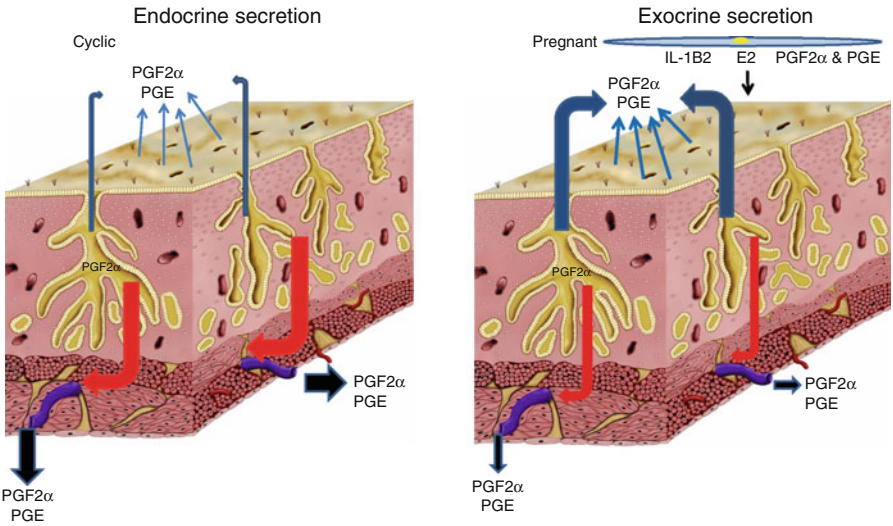


Fig. 8.1 The luteostatic "endocrine–exocrine" model for maternal recognition of pregnancy in the pig. In cyclic gilts, the loss of PGR in the uterine epithelia by day 10 of the estrous cycle is temporally associated with increased expression of PTGS1 and PTGS2 by uterine LE and GE which results in the release of PGF2α and some PGE. In the absence of conceptuses or estrogen stimulation, the majority of the PGF2α produced is released into the uterine capillary bed (endocrine direction) and then into the uterine vein and general circulation to exert luteolytic effect on both ovaries. The systemic effect of PGF2α results as only 30 % of PGF2α is metabolized in one passage through the lungs. In pregnant gilts, conceptuses release estrogens stimulating secretion of the majority of the endometrial PGF2α into the uterine lumen where it is metabolized by the conceptus trophoblast to inactive form or PGE2. In addition, IL1B2 produced by the conceptuses may increase expression of endometrial PGE synthase-1 to increase the ratio of PGE2 to PGF2α and sequester prostaglandins in the uterine lumen or the endocrine release of PGE2 may support CL function during early pregnancy

receptor accessory protein (IL1RAP) (Seo et al. 2012a), lysophosphatidic acid receptor 3 (LPAR3) (Seo et al. 2008), secreted phosphoprotein 1 (SPP1) (White et al. 2005), and stanniocalcin1 (STC1) (Song et al. 2009).

8.3.3 Rapid Elongation of the Conceptus

The capacity of pig conceptuses to secrete estrogen clearly provides the major mechanism by which the movement of PGF2α is altered from endocrine to exocrine by the uterine endometrium. However, in order for conceptus estrogens to reduce PGF2α release into the utero-ovarian vein, the conceptuses must first cover the majority of the uterine surface area after day 11 of pregnancy (Dhindsa and Dziuk 1968b). Heuser and Streeter (1929) were the first to document the spherical, tubular, and filamentous morphological forms of the pig conceptus. In addition to pig conceptuses expanding across the uterine surface to inhibit luteolysis, trophoblast

expansion provides sufficient surface area for nutrient transfer through the trophectoderm initially and then the diffuse, epitheliochorial placenta.

The peri-implantation pig conceptus undergoes a remarkable transformation from a 1–2 mm sphere to a 9–10 mm long ovoid shape between days 10 and 12 of pregnancy and then rapidly remodels to tubular and filamentous forms by elongating at 30–40 mm/h to >100 mm in length in 1–2 h (Anderson 1978; Geisert et al. 1982b). Perry (1981) stated that "the rapidity of elongation and reduction in diameter that accompanies it suggest that it is by deformation rather by cell division." The rapid alteration in conceptus morphology within the uterine horns does occur through cellular migration and remodeling of the trophectoderm and endoderm following differentiation of the epiblast mesoderm (Geisert et al. 1982b; Mattson et al. 1990). Many of the morphological forms of the conceptus can be found within the same uterus (Anderson 1978) indicating that development of pig conceptuses within a litter is not necessarily uniform and that may contribute to some of the early embryonic losses that occur in pigs (Pope 1994). Upon completion of rapid elongation, the filamentous conceptuses continue to elongate to 60 cm by day 13 and reach a length of 1 m by day 18 of gestation. Because the placental membranes of pigs do not overlap, females with a high ovulation rate have conceptuses that must compete for adequate uterine space for development and survival to term. The variation in conceptus development on day 12 may provide some of the selection pressure for survival of conceptuses that are first to elongate and establish their surface area for implantation and placentation in the uterus (Pope 1994).

Although growth of the pig conceptuses is regulated through release of uterine growth factors during early development (Geisert et al. 2014), rapid trophoblast elongation and estrogen secretion are triggered by conceptus development and cellular differentiation (Fig. 8.2). Mesoderm differentiation and outgrowth from the epiblast are markers for the increase in steroidogenesis of the developing spherical conceptuses (Yelich et al. 1997; Conley et al. 1992, 1994). The growth and expansion of mesoderm between the trophectoderm and underlying extraembryonic endoderm may provide the cellular interactions needed to initiate the elongation process (Fig. 8.2). Cellular alterations in junctional complexes of the trophectoderm and migration of endodermal cells involved in conceptus elongation occur at the epiblast and extend down the "elongation zone" to the tips of the tubular conceptus (Geisert et al. 1982b). Epiblast production of FGF4 and the activation of mitogen-activated protein kinases (MAPK) through trophectoderm expression of fibroblast growth factor 2 (FGFR2) could induce expression of bone morphogenetic protein 4 (BMP4) by the mesoderm (Valdez Magaña et al. 2014). It is quite possible that paracrine secretion of BMP4 from the developing mesoderm initiates pathways to induce the cellular changes required for localized migration of the underlying endoderm and modification of microfilaments and junctional complexes (Mattson et al. 1990) making the overlying trophectoderm layer more fluid for rapid remodeling during elongation. Certainly, Perry's (1981) suggestion that "conceptus elongation occurs in much the same way as does a ball of plasticene rolled under the hand" fits with the centralized (epiblast) localization of cell migration and shifting of cells needed for rapid transformation of ovoid to filamentous forms of the conceptuses (Fig. 8.2).

8.3.4 Expression of Interleukin 1 Beta (IL1B) by Conceptuses

Analyses of the transcriptome of developing spherical, ovoid, tubular, and filamentous pig conceptuses have provided information concerning genes involved during this critical period in development (Ross et al. 2003a, b, 2009; Blomberg et al. 2005, 2006). Although transcriptome profiling identified a number of genes that are up- or downregulated during this critical period of conceptus transformation, *IL1B* is the most abundantly expressed transcript during the time of tubular to filamentous transition for pig conceptuses (Ross et al. 2009). Expression of *IL1B* during the period when pig conceptuses transition to filamentous morphology was first described by Tuo et al. (1996). Because the rapid increase in *IL1B* mRNA and IL1B protein expression during conceptus elongation is immediately followed by a loss of transcript expression, IL1B was proposed as a possible candidate for initiating the cellular signaling pathway for conceptus remodeling (Fig. 8.2). Interestingly, the pig conceptus expresses a novel isoform, *IL1B2*, which resulted from gene duplication (Mathew et al. 2015). Expression of conceptus IL1B2 is specific to the pig conceptus as the transcript is not detected in peripheral tissues or in other mammals. Protein sequences of IL1B and IL1B2 are 85 % identical. Substitutions in amino acids may affect caspase-1 cleavage sites needed for IL1B2 secretion and biological activity. In addition, differences in the promoter region may contribute to specific expression of *IL1B2* in the pig conceptus. IL1B promotes motility during human cytotrophoblast formation and induces secretion of urokinase plasminogen activator involved with endometrial invasion (Prutsch et al. 2012). Conceptus production of plasminogen activator is associated with the period of conceptus elongation, estrogen synthesis, and IL1B2 release in pigs (Fazleabas et al. 1983). IL1B can also stimulate phospholipase A2 (PLA2) (Kol et al. 2002) which increases membrane fluidity through the release of arachidonic acid from the phospholipid bilayer of the cell membrane. The increase of plasminogen activator and PLA2 activity during elongation of the conceptus (Davis et al. 1983) would be consistent with a role for IL1B2 in the induction of rapid cellular remodeling. The release of arachidonic acid from the conceptus trophoblast contributes to increases in secretion of PGs from the conceptus during and following elongation as conceptus expression of PTGS2 also increases (Wilson et al. 2002). However, PLA2 release of arachidonic acid appears to be the critical event in promoting membrane fluidity, as inhibition of PTGS1 and PTGS2 (downstream of PLA2) does not block conceptus elongation (Geisert et al. 1986).

8.3.5 Endometrial Stimulation by IL1B2

Although the dramatic increase in *IL1B2* mRNA expression by pig conceptuses only occurs during the period of rapid elongation (day 11–12), IL1B2 protein is detectable in the uterine lumen from day 12 to 18 of gestation (Ross et al. 2009).

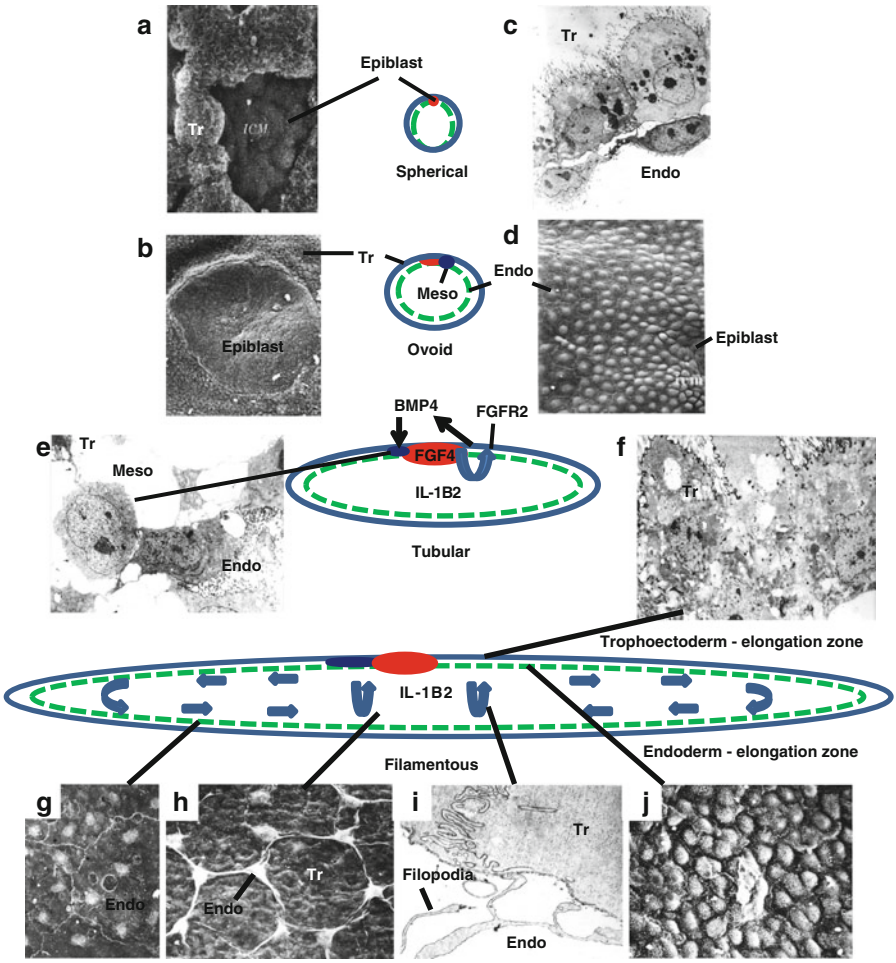


Fig. 8.2 Elongation of the trophoblast of pig conceptuses involves cellular differentiation and rapid remodeling of the trophectoderm (*Tr*) and endoderm (*Endo*) between days 11 and 12 of pregnancy. The trophectoderm covering (Rauber's layer) the early spherical (2–4 mm) conceptus is removed (**a**) exposing the epiblast (*ICM*) on the surface by the 5 mm spherical stage (**b**). The early spherical conceptus is composed of outer rounded cuboidal trophectoderm with a closely attached layer of endoderm on its basement membrane (**c**). The endoderm facing the inner blastocele of the conceptus forms a continuous layer from the epiblast (**d**). Differentiation of conceptus mesoderm (*Meso*) is evident after the 5 mm spherical conceptus stage when estrogen and IL1B2 production are first detected (**f**). Epiblast production of FGF4 is proposed to stimulate fibroblast growth factor receptor 2 (*FGFR2*) receptors present in the trophectoderm to stimulate BMP4 production required for differentiation of mesoderm cells. Increased expression of IL1B2 mRNA and protein by tubular conceptuses is proposed to trigger cellular movement and remodeling during elongation of the pig conceptus. Near the epiblast, cellular junctional complexes of the trophectoderm undergo a shift to allow cell movement and transition of those cells to a columnar shape (**e**) and the underlying endoderm cells form filopodia (**h**, **i**) that pull the overlying trophectoderm toward the elongation zone. Movement of the spindle-shaped endodermal cells toward the elongation zone tightly compacts the endoderm cells together (**j**). With the alteration in shape of trophectoderm and increased density of the endoderm in the elongation, the cells are moved toward the tips of the elongating conceptus returning the cells to normal pattern (**g**) as the process repeated until elongation is complete

Salivary lipocalin (SAL1), which functions as a transporter of hydrophobic compounds in biological fluids, is induced by IL1B and secreted by the uterine glands during conceptus elongation in the pig (Seo et al. 2011). Conceptus-induced secretion of SAL may play an important role in binding to lipids, PGs, and lysophosphatidic acids for implantation, placentation, and establishment of pregnancy in the pig (Seo et al. 2008).

Pro-inflammatory effects of the IL1family of cytokines on tissue are well established (Dinarello 2009). Tissue responses to IL1B are regulated through two receptors (IL1R1 functional, IL1R2 pseudo-receptor), receptor accessory protein and receptor antagonists (Dinarello 2009). Binding of IL1B2 to IL1R1 on the epithelial surface initiates a cascade of signaling pathways through activation of the inducible transcription factor, nuclear factor kappa-B (NFKB) (Hayden and Ghosh 2012). NFKB plays an essential role in sensing and adapting to alterations in the microenvironment of the immune system at the level of tissues and epithelia (Wullaert et al. 2011). Inactive dimers of NFKB are sequestered in the cytoplasm until receptor binding triggers phosphorylation and release of IKB (a regulatory protein inhibitor of κB) allowing NFKB translocation to the nucleus to effect gene transcription (Dinarello 2009).

Downregulation of PGRA in the endometrial LE and GE during the peri-implantation period not only plays a role in the loss of mucin-1 expression to open the window for conceptus attachment to uterine LE but also removes PGR-dependent inhibition of NFKB activation (Ross et al. 2010; Mathew et al. 2011). There is a clear spatiotemporal relationship between PGR downregulation and NFKB activation by IL1B2 from pig conceptuses (Mathew et al. 2011). Conceptus elongation and IL1B2 release stimulate nuclear translocation of NFKB in LE adjacent to conceptuses during the establishment of pregnancy (see review Geisert et al. 2014) Nuclear of translocation of the NFKB dimers stimulates transcription pathways involved in inflammation, cell adhesion, cytokine release, anti-apoptotic factors, and immunoreceptors (Hayden and Ghosh 2012). Many cytokines (TNFα, IL1, IL2, IL6, IL12, LIF, and GMCSF), chemokines (IL8 and RANTES), and PTGS2 are transcriptionally regulated by NFKB (Ali and Mann 2004). Activation of inflammatory pathways in the endometrium must be tightly regulated in order to prevent severe inflammation leading immunological rejection of the conceptus. Estrogen has the capacity to act as either an antagonist (Quaedackers et al. 2007) or agonist of NFKB activity (King et al. 2010). The presence of ESR1 in the endometrial LE and GE and tight coupling of conceptus estrogen synthesis and release with expression of *IL1B2* would modulate the pro-inflammatory reaction of the uterus during elongation and placental attachment (see review Geisert et al. 2014).

Production of estrogen and IL1B2 by pig conceptuses enhances endometrial production of LIF and PTGS2. Endometrial LIF is associated with implantation in a number of species and increases on day 12 of pregnancy in the pig (see review Geisert et al. 2014). Inhibition of PG synthesis during the pre-implantation period causes early embryonic loss (Kraeling et al. 1985). IL1B stimulates endometrial PG synthesis and release, which, in addition to conceptus estrogens, may induce the pregnancy-specific increase in endometrial and uterine luminal content of PGE2

and PGF2α (Franczak et al. 2010; Seo et al. 2012a, 2014a). Increases in PGE2 production may occur through stimulation of endometrial PGE synthase-1(PGES-1) by conceptus IL1B2 (Franczak et al. 2010). An increase in the ratio of conceptus and endometrial PGE2 to PGF2α has been proposed to function as part of the luteostatic mechanism to sequester and metabolize PGF2α in the uterine lumen (Waclawik et al. 2009). In addition, an increase in PGE release into the utero-ovarian vein (Fig. 8.1) may have a direct luteotrophic action on the CL (Christenson et al. 1994). The PG transporters, ATP-binding cassette, subfamily C, member 4 (ABCCA4), and solute carrier organic anion transporter family, member 2A1 (SLCO2A1) are induced by IL1B and expressed in a temporal- and cell-specific manner within the endometrium of pregnant pigs (Seo et al. 2014b). Expression and cellular localization of the PG transporters within the endometrial LE and GE add support to the endocrine–exocrine model for the establishment and maintenance of pregnancy.

8.3.6 Secretion of Interferons by Conceptuses

Immediately following conceptus elongation and the rapid decline in IL1B2 gene expression, pig conceptuses express and secrete interferon gamma (INFG) and delta (INFD) between days 12 and 20 of gestation when there is a 567-fold increase in IFNG mRNA during the transition from spherical to day 14 filamentous conceptuses (La Bonnardière et al. 1991; Cencic and La Bonnardière 2002; Ross et al. 2009). Indeed, pig conceptuses are unique in secreting both type I and type II IFNs during the peri-implantation period of pregnancy. In contrast to sheep conceptuses which secrete type I IFN tau (IFNT), the pregnancy recognition signal in ruminants (Spencer et al. 2007), IFNs produced by pig conceptuses do not appear to be antiluteolytic (Harney and Bazer 1989; Lefèvre et al. 1998). Although pig conceptus IFNs are not known to influence pregnancy recognition, paracrine and autocrine effects of IFND and IFNG are suggested by localization of both the type I IFN receptor1 (IFNAR1, which binds pig IFND) and the type II IFN gamma receptor 1 (IFNGR1) on endometrial epithelial and conceptus trophectoderm (Niu et al. 1995; Lefèvre et al. 1998; D'andrea and La Bonnardiere 1998). When conceptus secretory proteins containing IFND and IFNG were infused into uteri of pseudopregnant pigs, uterine secretion of prostaglandin E2 was increased (Harney and Bazer 1989), as was expression of several IFN-responsive genes in the endometrium, including but not limited to signal transducer and activator of transcription 1 (STAT1) and STAT2, interferon regulatory factor 1 (IRF1), swine leukocyte antigens 1, 2, 3, 6, 7, and 8 (SLAs 1, 2, 3, 6, 7, 8), and beta 2 microglobulin (B2M) (Joyce et al. 2007a; Joyce et al. 2007b; Joyce et al. 2008; Johnson et al. 2009). Ka et al. (2009) reported that SLA-DQA, a major histocompatibility complex (MHC) class II gene, is expressed in the uterine endometrium at the time of conceptus implantation in pigs, and using endometrial explant cultures from day 12 of the estrous cycle, determined that expression of SLA-DQA and SLA-DQB mRNAs increased in response to IFNG (Kim et al. 2012).

Secretion of estrogens, IFND, and INFG by pig conceptuses could coordinate STAT1 activation in uterine LE and stroma to induce cellular pathways for trophoblast attachment and regulation of the maternal immune response to the semi-allogenic conceptuses (Joyce et al. 2007a, b). Interestingly, with the decline in IL1B2 secretion, there is an increase in endometrial IL18 expression during the period of trophoblast attachment from day 13 to 18 of gestation (Ashworth et al. 2010b). Previously referred to as interferon inducing factor, IL18 is a member of the pro-inflammatory IL1B family. A pregnancy-specific increase in endometrial caspase-1 expression increases release of IL18 into the uterine lumen which could stimulate INFG and IFND production by conceptuses. The switch from conceptus IL1B2 to endometrial IL18 production parallels the second sustained increase in conceptus estrogen production which suggests that estrogen modulates the endometrial response to the pro-inflammatory cytokines.

Fragmentation and loss of conceptuses following premature exposure of pregnant gilts to estrogen (day 9) are associated with lack of expression of IFNG by conceptuses required to activate STAT1 in stromal cells (Joyce et al. 2007b). The failure of IL18 to accumulate in the uterine lumen of estrogen-treated gilts is consistent with its role in stimulating IFNG production by pig conceptuses (Ashworth et al. 2010b). These studies illustrate the spatiotemporal sensitivity of the endometrial/conceptus interface to the interplay between estrogen and the expression of endometrial IL18 and IFNG by conceptuses during the period of implantation/placentation and maintenance of pregnancy in the pig.

8.4 Adhesion Cascade for Implantation

8.4.1 Implantation

Implantation of the conceptus and development of a placental connection to the maternal circulation are strong evolutionary advantages of eutherian mammals. The placenta (chorion, allantois, and amnion) forms the interface between the microcirculatory systems of the mother and conceptus and functions for efficient, sustained, and high-throughput exchange of nutrients, respiratory gases, and metabolic wastes, and it protects the growing embryo/fetus and is a source of hormones. Due to its recent appearance in the evolutionary record, a considerable variability exists among species relative to histogenesis and organization of the placenta (Carter and Enders 2013). The placental membranes are formed from components of the blastocyst and embryo; however, both the embryo and maternal endometrium begin to form components of the placenta as soon as the conceptus trophectoderm attaches to the endometrium. Indeed, the placental trophoblast interaction with maternal tissues remains extensive in all species. Blastocysts of some species, including primates and rodents, are invasive and penetrate the epithelial layer of the endometrium. Pigs, however, employ a vastly different strategy, and demonstrate a true epitheliochorial placentation in which there is no displacement or

invasion of the maternal epithelium and the conceptus remains within the uterine lumen throughout gestation (Burton 1992). The changes that occur at the interface between trophectoderm and uterine LE during the initial stages of epitheliochorial placentation in pigs, day 13–26, have been elegantly described by Dantzer (1985). Throughout implantation, the glycocaylx that extends from the apical surface of the uterine LE is thicker than the glycocaylx at the surface of conceptus trophectoderm. On days 13 and 14, the uterine LE develops protrusions that become enclosed by caps of trophectoderm cells that serve to physically immobilize the conceptus; and by day 14, there is close apposition between the apical plasma membranes of trophectoderm and uterine LE cells. Interdigitating microvilli form between these plasma membranes through days 15 and 16, and then, the interface becomes increasingly complex as it functionally transitions from histotrophic to histotrophic and hemotrophic nutrient transport over day 15–20. This transition is characterized by the development of apical domes on the uterine LE that are closely related to the trophectoderm and provide long cytoplasmic extensions into a luminal space between the apical domes. Finally, adhesion transitions into placentation through ever-increasing development of interdigitating microvilli between trophectoderm and uterine LE that extends into the peripheral zone by day 26 of gestation.

8.4.2 Trophoblast Attachment and Adhesion

The term "implantation" is somewhat of a misnomer for the pig but is, nevertheless, used to describe the initial stages of placentation in this species. Despite differences in duration of the pre-implantation period, protracted in the pig, and type of implantation, the initial stages of implantation/placentation are common across species and are characterized as the "adhesion cascade for implantation" (Dantzer 1985; Guillomot 1995; Burghardt et al. 2002). The phases of this adhesion cascade in pigs include (1) shedding of the zona pellucida and elongation of the conceptus trophectoderm, (2) precontact and conceptus trophectoderm orientation to the uterine LE, (3) apposition of trophectoderm to uterine LE, (4) adhesion of the apical surface of trophectoderm to the apical surface of uterine LE, and (5) development of interdigitating microvilli between trophectoderm and uterine LE (Fig. 8.3). As this cascade concludes, adhesion seamlessly transitions to the progressive formation of epitheliochorial placentation that supports fetal–placental development throughout pregnancy (Johnson et al. 2014).

During the peri-implantation period of pregnancy, uterine LE and conceptus trophectoderm develop adhesion competency in synchrony to initiate the adhesion cascade within a restricted period of the uterine cycle termed the "window of receptivity" (Fazleabas et al. 2004; Spencer et al. 2007; Bazer et al. 2011). Similar to other species, this window is orchestrated through the actions of progesterone and estrogen to regulate locally produced cytokines, growth factors, cell surface glycoproteins, cell surface adhesion molecules, and extracellular matrix (ECM) proteins

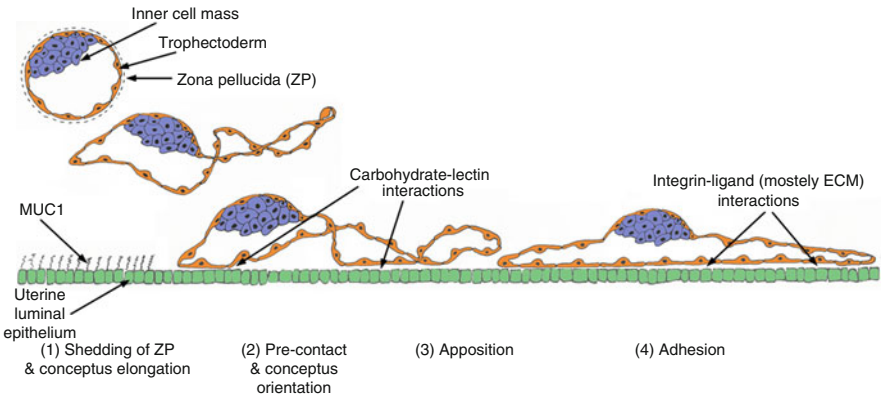


Fig. 8.3 The initial stages of implantation are common across species and are characterized as the "adhesion cascade for implantation." The phases of this adhesion cascade in pigs include (*1*) *elongation* of conceptus trophectoderm after *shedding of the zona pellucida*; (*2*) downregulation of MUC1 at the apical surface of uterine LE to expose potential, but not yet identified, low-affinity carbohydrate–lectin binding molecules that mediate *precontact* and conceptus trophectoderm *orientation* to the uterine LE; (*3*) replacement of low-affinity contacts by a more stable and extensive repertoire of adhesive interactions between integrins and maternal ECM to mediate *apposition* and attachment of trophectoderm to uterine LE; (*4*) integrin receptors expressed at the apical surface of uterine LE cells bind to Arg-Gly-Asp (RGD) and non RGD amino acid sequence-containing ECM molecules and bridge to another complement of potential integrin receptors expressed at the apical surface of conceptus trophectoderm cells to mediate conceptus trophectoderm *adhesion*; and (5) development of *interdigitating microvilli* between uterine LE and trophectoderm to stabilize the trophectoderm–uterine LE interface for exchange of nutrients and gases (not illustrated in the figure)

in the pig (Johnson et al. 2009). Conceptus synthesis and release of estrogens modulate uterine gene expression (Johnson et al. 2009). The importance of estrogen to implantation of pig conceptuses is underscored by the fact that premature exposure of the pregnant uterus to estrogen on days 9 and 10 results in degeneration of all pig conceptuses by day 15 (Ashworth et al. 2006). Progesterone has a clear role in initiating the adhesion cascade for implantation in pigs; however, the mechanism by which progesterone regulates uterine epithelial cell functions remains a paradox. As long ago as 1973, studies of endocrine regulation of expression of uteroferrin secreted by the uterine epithelia of pigs revealed a requirement for long-term treatment with progesterone indicating that effects of progesterone are not mediated by a "classical" steroid receptor-type mechanism of action (Knight et al. 1973). Based on current evidence, long-term treatment with progesterone is required in order to downregulate PGR in uterine epithelia as a prerequisite to epithelial cell proliferation, gene expression, and differentiated functions. Similar to all species studied, progesterone downregulates expression of PGR in the uterine epithelia of pigs after day 10 of pregnancy, immediately prior to the time when the endometrium becomes receptive to implantation (Geisert et al. 1994; Bazer et al. 2008; Bailey et al. 2010). As previously indicated, uterine stromal cells express PGR throughout gestation, suggesting that effects of progesterone on PGR-negative uterine epithelia may be

mediated through an indirect pathway whereby PGR-positive stromal cells stimulate expression of a progestamedin(s) that, in turn, modulates function of epithelial cells (Bazer et al. 2012). Although proposed progestamedins such as FGF10 and hepatocyte growth factor are expressed by the uterine stromal cells of sheep, no presumptive progestamedin has been detected in the stroma of pigs, and FGF7 is actually expressed by the uterine LE during the peri-implantation period in response to estrogen after progesterone downregulates PGR (Chen et al. 2000a, b; Ka et al. 2000). An alternative possibility is that downregulation of PGR in uterine epithelia removes an unidentified intrinsic "block" to differentiated functions in these epithelia (Spencer et al. 2004a). Regardless of the specific mechanism involved, downregulation of PGR in uterine LE is temporally associated with alterations in the expression of anti-adhesive components, mainly MUC1, an intrinsic transmembrane mucin within the glycocalyx of LE that sterically inhibits attachment of the conceptus (Brayman et al. 2004). In vivo administration of progesterone to cyclic gilts results in the loss of MUC1 from the apical surface of uterine LE (Bowen et al. 1996). It is accepted that in all mammals, initial conceptus attachment requires this loss of MUC1.

Downregulation of MUC1 exposes potential low-affinity carbohydrate ligand binding molecules including selectins and galectins and perhaps heparan sulfate proteoglycan, heparin binding EGF-like growth factors, cadherins, integrins, and CD44, which are proposed to contribute to initial attachment of conceptus trophectoderm to uterine LE (Kimber et al. 1995; Kimber and Spanswick 2000; Spencer et al. 2004b; Johnson et al. 2014). The involvement of carbohydrate–lectin interactions during the adhesion cascade of pigs has not been investigated. However, it is likely that filamentous porcine conceptuses undergo a series of attach-and-release events between carbohydrates and lectin receptors at the apical surfaces of trophectoderm and uterine LE that result in maximal apposition of these tissues, similar to the "rolling and tethering" that occur during leukocyte adhesion to the endothelium for extravasation of leukocytes (Kling et al. 1992), and proposed for the initial attachment of human blastocysts to the uterine wall (Red-Horse et al. 2004). In support of this idea, other domestic farm species, goats and sheep, exhibit prominent expression of H-type 1 antigens and glycosylation-dependent glycam 1, respectively, at the uterine–conceptus interface during implantation (Powell et al. 2000; Gray et al. 2004). These low-affinity contacts are then replaced by a more stable and extensive repertoire of adhesive interactions between integrins and maternal ECM which appear to be the major contributors to stable adhesion at implantation (Hynes 1987; Ruoslahti and Pierschbacher 1987; Aplin et al. 1994; Burghardt et al. 1997, 2002; Johnson et al. 2001; Lessey 2002). Integrins are dominant glycoproteins in many cell adhesion cascades (Kling et al. 1992). They are transmembrane glycoprotein receptors composed of non-covalently bound α and β subunits that promote cell–cell and cell–ECM adhesion, cause cytoskeletal reorganization to stabilize adhesion, and transduction signals through numerous signaling intermediates (Giancotti and Ruoslahti 1999; Albelda and Buck 1990). There are 18 α- and 8 β-subunits capable of dimerizing to form 24 different heterodimer combinations that can bind to numerous extracellular

ligands including a variety of ECM proteins (Albelda and Buck 1990; Humphries et al. 2006; Gallant et al. 2005). Integrin receptors expressed at the apical surface of uterine LE cells are capable of binding to Arg-Gly-Asp (RGD) and non RGD amino acid sequence-containing ECM molecules and bridge to another complement of potential integrin receptors expressed at the apical surface of conceptus trophectoderm cells.

At present, it is known that eight integrin subunits, α1, α3, α4, α5, αv, β1, β3, and β5, are expressed at the apical surface of both uterine LE and conceptus trophectoderm. Luminal epithelial expression of α4, α5, and β1 increases during the period of maternal recognition of pregnancy, and similar increases in expression of these integrins can be stimulated in the uteri of cyclic pigs through treatment with progesterone. Further, α4, α5, αv, β1, β3, and β5 have been localized to porcine implantation sites on day 12–15 of gestation in pigs (Bowen et al. 1996). These subunits potentially give rise to the integrin receptors αvβ1, αvβ3, αvβ5, α4β1, and α5β1 that likely function as part of an adhesion cascade that serves to generate both stable adhesion between trophectoderm and LE and activation of outside-in signal transduction (Burghardt et al. 1997). Integrin-mediated adhesion results in dynamic macromolecular complexes, termed focal adhesions (FAs) which are composed of heterodimeric transmembrane integrin receptors that connect ECM proteins to the actin cytoskeleton along with a diverse array of cell signaling intermediates (Sastry and Burridge 2000; Wozniak et al. 2004; Larsen et al.. 2006). It is noteworthy that immunofluorescence staining has revealed that αv and β3 integrin subunits co-localize with altered distribution of talin (an intracellular signaling intermediate within FAs) within large aggregates at the junction between trophectoderm and uterine LE on day 20 of gestation which is approximately midway through the process of attachment for implantation and placentation in pigs (Erikson et al. 2009). The size and nature of these aggregates are reminiscent of the well-characterized FAs that form at the base of cultured cells as they attach to ECM on a rigid substrate at their basal surfaces (Sastry and Burridge 2000; Burghardt et al. 2009). Five ligands capable of engaging integrin receptors to induce assembly of focal adhesions have been characterized at sites of implantation in pigs. The inter-α-trypsin inhibitor heavy chain-like (IαIH4) protein contains a von Willebrand type A domain, a recognition site for the αvβ3 integrin receptor (Geisert et al. 1998). The latency-associated peptide (LAP) of transforming growth factor beta (TGFB) binds to αvβ1, αvβ3, and αvβ5 (Massuto et al. 2009a). Fibronectin is capable of binding α4β1, α5β1, and αvβ3 (Bowen et al. 1996). Vitronectin is a major ligand for αvβ3 (Bowen et al. 1996). Finally, osteopontin [OPN, also known as secreted phosphoprotein 1 (SPP1)] is the most promiscuous of the ligands and it interacts with αvβ1, αvβ3, αvβ5, and α4β1 (Johnson et al. 2003).

The IαIH4 is part of the kallikrein–kininogen–kinin protease system. Both IαIH4 protein expression and kallikrein enzymatic activity increase within the uterine environment during the peri-implantation period of pigs (Geisert et al. 1998; Vonnahme et al. 1999). The IαIH4 can directly engage the αvβ3 integrin receptor, as well as interact with hyaluronic acid within the ECM to aid in the initial stages of implantation. However, it is hypothesized that the primary role of IαIH4 during

implantation is to act in concert with bikunin to stabilize the surface glycocalyx of the uterine LE during conceptus attachment (Hettinger et al. 2001). The LAP associates with TGFB to form an inactive homodimer called the small latent complex which remains in the cell until it is bound by another protein called latent TGFB-binding protein to form the large latent complex (LLC) that is then secreted into the ECM (Lawrence 1996). After being secreted, LAP is cleaved from the LLC by proteases to release active TGFB (Jenkins et al. 2006). In pigs, TGFB1, TGFB2, and TGFB3, as well as their receptors, TGFBRI and TGFBRII, are expressed by conceptus trophectoderm and by uterine LE between days 10 and 14 of gestation; and acting through LAP, TGFB increases fibronectin synthesis, cell adhesion to fibronectin, and the formation of FAs in a porcine trophectoderm cell line (Jaeger et al. 2005). In addition, when LAP is infused into the uteri of pregnant pigs, conceptuses failed to implant, suggesting that infused LAP competed with the endogenous LLC for binding to integrins expressed on trophectoderm (Massuto et al. 2009b). In support of this idea, aggregates of LAP, β1, β3, and β5, have been detected at the porcine conceptus trophectoderm–uterine LE interface, suggesting functional adhesion complexes that support conceptus attachment during porcine implantation (Massuto et al. 2009a). Both fibronectin and vitronectin have been detected at sites of conceptus attachment in the pig (Bowen et al. 1996), and the glycosylation variant of fibronectin, oncofetal fibronectin (oFN), is constitutively expressed by porcine conceptus trophectoderm as well as uterine LE throughout gestation (Tuo and Bazer 1996). Fibronectin and vitronectin are prototype cell adhesion proteins, and fibronectin recognizes as many as 10 different integrin receptors to generate different signal transduction functions depending upon the specific integrin receptor involved (Johansson et al. 1997; Humphries et al. 2006). It is noteworthy that the human conceptus trophoblast produces oFN so precisely at sites of trophoblast contact with endometrium that it has been referred to as "trophoblast glue" (Feinberg et al. 1994), and fibronectin is the leading candidate for conceptus adhesion molecule in rodents (Armant 2005).

The most extensively studied ECM adhesion protein for implantation in pigs is osteopontin (Johnson et al. 2003, 2014). Osteopontin is a multifunctional secreted acidic member of the small integrin-binding ligand *N*-linked glycoprotein (SIBLING) family of ECM proteins (Denhardt and Guo 1993; Butler et al. 1996; Sodek et al. 2000). Regarding implantation, studies have focused on the ability of OPN to support integrin-mediated cell adhesion and cell migration (Senger et al. 1994). It is likely that the expression of no other ECM protein is conserved across different species to the degree that has been observed for OPN, which is expressed abundantly within the conceptus–uterine environment of humans, mice, rabbits, sheep, goats, and pigs (Johnson et al. 1999a, b; Garlow et al. 2002; Apparao et al. 2003; Mirkin et al. 2005; Joyce et al. 2005; White et al. 2006). In pigs, expression of OPN is initially induced by conceptus estrogens in discrete regions of the uterine LE juxtaposed to the conceptus trophectoderm just prior to implantation on day 13, expands to the entire uterine LE by day 20 when firm adhesion of conceptus trophectoderm to uterine LE occurs, and remains high at this interface throughout pregnancy (Garlow et al. 2002; White et al. 2005). In vitro affinity chromatography and

immunoprecipitation experiments illustrated that OPN directly binds the αvβ6 integrin heterodimer on porcine trophectoderm cells and the αvβ3 integrin heterodimer on uterine LE cells (Erikson et al. 2009). In addition, OPN binding promotes dose- and integrin-dependent attachment of trophectoderm and uterine LE cells and stimulates haptotactic trophectoderm cell migration, meaning that cells migrated directionally along a physical gradient of nonsoluble OPN (Erikson et al. 2009). Interestingly, immunofluorescence staining of tissue sections of porcine implantation sites revealed FAs containing these same integrins distributed in a similar cell-type-specific pattern to that suggested by in vitro binding to OPN. The αv integrin subunit staining revealed large aggregates at the junction between trophectoderm and uterine LE, suggesting the formation of FAs at the apical surfaces of both conceptus trophectoderm and uterine LE that facilitate conceptus attachment to the uterus for implantation. The β3 subunit, however, appeared in aggregates on the apical surface of uterine LE, but not trophectoderm, which supports results from affinity chromatography studies indicating direct in vitro binding of αvβ3 on uterine LE to OPN (Erikson et al. 2009). Finally, OPN-coated microspheres co-localize with the αv integrin subunit and talin at FAs present on the apical domain of porcine trophectoderm cells in vitro (Erikson et al. 2009). Collectively, results indicate that OPN binds integrins to stimulate integrin-mediated FA assembly, attachment, and cytoskeletal force-driven migration of conceptus trophectoderm cells to promote implantation of conceptuses in pigs.

8.5 Conclusion

A great deal of knowledge has been obtained on the establishment of pregnancy in the pig since Roger Short first coined the phrase “maternal recognition of pregnancy” (Short 1969). Rapid elongation of conceptus trophoblast following down-regulation of PGR in the uterine LE and GE provides the mechanism for trophectoderm to achieve contact with the endometrial surface of the long uterine horns. Estrogen synthesis and release by pig conceptuses induce exocrine secretion of PGs to prevent CL regression and induce transcriptional factors that stimulate endometrial changes essential to attachment to the uterine LE and secretion of nutrients, growth factors, cytokines, and immune factors by LE and GE for maintenance of pregnancy to term. The unique production of IL1B2 by pig conceptuses provides not only the trigger for trophoblast elongation but also activation of additional transcription factors for regulation of angiogenesis and immune regulation which are balance by its spatiotemporal release of estrogen. Molecular analyses of the interactions between the developing pig conceptuses and the endometrium are not nearly as advanced as for the mouse (see Chap. 5). Changes in endometrial transcriptome during early stages of conceptus attachment to uterine LE are just beginning to widen our understanding of the critical factors involved with establishment and maintenance of pregnancy in the pig (Samborski et al. 2013; Chen et al. 2015; Kiewisz et al. 2014). The emergence of microRNAs as

posttranscriptional players in regulating gene function has added a new area of investigation to enhance understanding of conceptus–endometrial interactions (Krawczynski et al. 2015). Utilization of CRISPR technology will now allow knockout of select genes in research to determine the network of endometrial and conceptus factors, such as estrogen and IL1B2, that are critical for establishment of pregnancy.

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Chapter 9
Pregnancy Recognition and Implantation of the Conceptus in the Mare

Claudia Klein

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 PGF2α 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 PGF2α 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 PGF2α. 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

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 trophectoderm 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 F2α 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 F2alpha (PGF2α) 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 PGF2α 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 F2α production in the equine endometrium. Mares given a synthetic analog of prostaglandin F2α during the mid-luteal phase responded with a two-phase increase in circulating concentrations of PGFM (13,14-dihydro-15-keto-PGF2α, the main metabolite of PGF2α); the first peak within 45 min, attributed to administration of exogenous PGF2α, 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 PGF2α production by the endometrium triggered by exogenous PGF2α. In vitro studies using endometrial explant cultures and cultured epithelial and stromal cells confirmed that exposure to PGF2α increased PTGS2 expression and stimulated release of PGF2α (Okuda et al. 2014).

A hallmark of maternal recognition of pregnancy in the mare is reduced endometrial secretion of PGF2α in the presence of a conceptus during the expected time of luteolysis. In this regard, concentrations of PGF2α 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, PGF2α 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 PGF2α 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 PGF2α 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 PGF2α 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 PGF2α 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 PGF2α. Although uterine flushings of pregnant mares contain no detectable amounts of PGF2α on Days 12, 14, and 16 after ovulation, the abundance of PGF2α 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 PGF2α 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 PGF2α would be to reduce binding sites for PGF2α. During early pregnancy, binding capacity of the corpus luteum for PGF2α is high until Day 18. However, as of Day 20 of pregnancy-binding capacity of the CL for PGF2α begins to decline (Vernon et al. 1979). The hypothesis that reduced affinity of the corpus luteum to bind PGF2α 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 PGF2α 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 PGF2α 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 PGF2α 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 H2 (PGH2) 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 PGF2α 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 PGF2α 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 PGF2α, 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 PGF2α 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 PGF2α 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 PGF2α 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 uteroovarian vein and the ovarian artery in the mare are clearly separate, resulting in a systemic pathway of luteolysis, i.e., PGF2α released by the endometrium reaches the ovaries via the systemic circulation (Ginther 1974). Consequently, PGF2α 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 PGF2α and 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 PGF2α could be caused by conceptus-derived estrogens (Stout and Allen 2001), since estrogens can stimulate secretion of PGF2α 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 PGF2α and 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, PGF2α or PGE2, is the stimulator of uterine contractions. The addition of flunixin meglumine to Day-14 conceptuses in culture significantly reduced secretion of PGE2, but not PGF2α, indicating that Day-14 conceptuses do not produce PGF2α 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 PGE2 is the conceptus-derived prostaglandin stimulating uterine contractility required for conceptus mobility. The actions of 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 PGF2α 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 trophectoderm 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 antiadhesive 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 trophectoderm/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 bioavailability 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 trophectoderm/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 trophectoderm and extraembryonic endoderm just beneath the embryonic disk (Walters et al. 2000). Stage-specific expressions of 17β-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-norandrorstenedione 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 antiadhesive 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 (Grunig et al. 1995). Invasive chorionic girdle cells express high levels of paternal major histocompatibility complex class I (MHCI) 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).

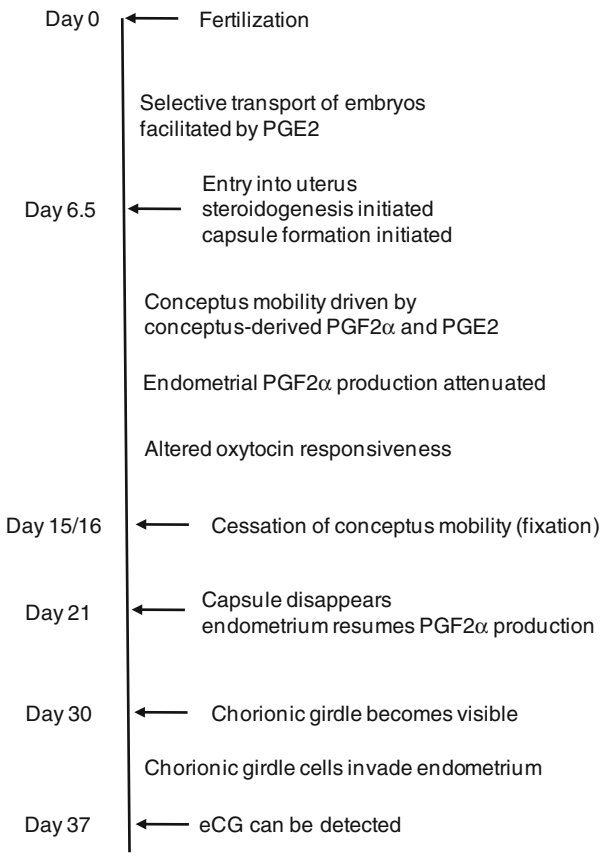


Fig. 9.1 Timeline of events during early pregnancy in the mare

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 PGF2α 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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Chapter 10
Implantation and Establishment of Pregnancy in Human and Nonhuman Primates

Ren-Wei Su and Asgerally T. Fazleabas

Abstract Implantation and the establishment of pregnancy are critical for the propagation of the species, but yet remain the limiting steps in human and primate reproduction. Successful implantation requires a competent blastocyst and a receptive endometrium during a specific window of time during the menstrual cycle to initiate the bilateral communication required for the establishment of a successful pregnancy. This chapter provides an overview of these processes and discusses the molecular mechanisms associated with implantation of the blastocyst and decidualization of the uterus in primates.

10.1 Introduction

Reproduction is absolutely essential for the propagation of every species from generation to generation. In most mammalian species, a zygote is formed in vivo following fertilization of an egg by a sperm. After the egg is successfully fertilized in the fallopian tube, it travels through the fallopian tube toward the uterus, during which time the fertilized egg divides and develops into a multicellular structure termed a blastocyst. It then adheres itself to the endometrium, which is the initial step in the process of implantation (Wilcox et al. 1999).

Implantation and the establishment of pregnancy are critical for human reproduction. Implantation failure is responsible for significant pregnancy loss in the human (Koot and Macklon 2013; Koot et al. 2012; Norwitz et al. 2001). Understanding the mechanisms associated with the implantation process and other events during early pregnancy will significantly improve the success of assisted reproductive technologies (ART).

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10.2 Implantation

In humans, as well as other nonhuman primates, implantation is one of the most interesting biological events and marks the first biological interaction of the blastocyst with the uterus during the establishment of pregnancy. In humans, one in every six couples is subfertile, and 25 % of those are classified as unexplained infertility. ART offers many subfertile couples effective treatment, but implantation failure remains the rate-limiting step: only around 25 % of transferred blastocysts will successfully implant (Edwards 2006). Both a competent blastocyst and a receptive endometrium are key components required for successful implantation of the blastocyst (Cha et al. 2012).

10.2.1 Window of Implantation

Driven by estrogen and progesterone, the two primary steroid hormones produced by the ovaries, the human endometrium undergoes cyclic changes in morphology and function during the menstrual cycle (Cha et al. 2012). However, during the menstrual cycle, the uterus is only receptive to the blastocyst for implantation for a short window of time. In humans, implantation occurs during the mid-secretory phase, between cycle days (CD) 20 and 24, or 6–10 days after ovulation (Blesa et al. 2014; Donaghay and Lessey 2007; Psychoyos 1973), which is a temporally framed period called the window of implantation (WOI). During the WOI, the endometrium which has been primed by estrogen and progesterone is characterized by changes that are collectively termed endometrial receptivity (Lessey 2011). During the past decades, intensive morphological, histological, and molecular studies have defined the "signature" of the WOI in many species (Cheong et al. 2013; Diaz-Gimeno et al. 2014; Lessey 2011).

10.2.1.1 Morphological and Histological Characteristics

Histological dating of the endometrium has been performed for over 60 years following the Noyes criteria (Noyes et al. 1950). Using these established criteria, histological features of the receptive endometrium include the glands becoming more irregular with a papillary or sawtooth appearance, movement of vacuoles to a supranuclear position of epithelial cells with the possibility of the contents being visible within the uterine lumen, the uterine stroma becoming looser and more edematous, and decidualization of the uterine stroma (Diaz-Gimeno et al. 2013).

Pinopods or uterodomes are the hairlike microvilli of epithelial cells which transiently fuse to form a single flowerlike membrane projection which appears only on the luminal surface of endometrial epithelial cells during the WOI (Nikas 1999; Nikas and Aghajanova 2002). Pinopods found in human endometria are

proposed as a marker of uterine receptivity to implantation (Gordon 1975; Psychoyos 1986). Pinopods can be separated into developing pinopods, fully developed pinopods, and regressing pinopods according to their morphology (Nikas and Aghajanova 2002). Pinopods have also been observed in uteri of baboons (Nikas 1999). The association of pinopods with endometrial receptivity has been correlated with implantation outcomes in a clinical study: all patients with abundant pinopods (>50% of endometrial surface) became pregnant, while pregnancy rates were lower than 50 % in the patients with moderate numbers of pinopods group (20–50 %), and none of the patients with few pinopods (<20 %) were pregnant (Nikas and Aghajanova 2002). Other evidence that supports a correlation between numbers of pinopods and uterine receptivity to implantation is the expression of potential molecular markers of receptivity on the pinopods (Aplin et al. 1996; Nardo et al. 2003).

Epithelial plaques are an early endometrial response in primates to implantation of the blastocyst and the initiation of pregnancy. The epithelial plaque reaction is a morphological transformation of epithelia lining the uterine lumen and necks of the uterine glands characterized by the hypertrophy, hyperplasia, and rounded acinar multicellular pad (Enders 1991). The presence of epithelial plaques is common among primates, and it has been described in several Old and New World Monkeys such as rhesus monkeys (Enders 1991; Ramsey et al. 1976), baboons (Enders et al. 1997; Fazleabas et al. 1999b), green monkeys (Owiti et al. 1986), cynomolgus monkeys (Enders et al. 1996), and dusky leaf monkeys (Burton 1980), whereas this type of structural change has not been reported for humans and great apes. The epithelial plaque reaction of the endometrium has been observed as early as 1 day after implantation in primary implantation sites of rhesus monkeys and baboons (Enders et al. 1983; Tarara et al. 1987). The epithelial plaque reaction induced by chorionic gonadotropin affects the entire luminal surface of the endometrium in baboons compared to a reaction restricted to the site of implantation in pregnant baboons (Fazleabas et al. 1999a). Additionally, there are few reports of spontaneous epithelial plaque reactions in the secretory phase of the menstrual cycle in nonpregnant primates (Kaspareit et al. 2004). The absence of epithelial plaques is associated with infertility in the bonnet monkey (Rosario et al. 2005)

10.2.1.2 Molecular Characteristics

Steroid hormone nuclear receptors in humans and other primates, both estrogen (ESR1) and progesterone receptors (PGR), decline in uterine epithelial cells at the time of implantation while persisting in the stromal compartment of the uterus (Brenner et al. 1990). The mechanism responsible for this downregulation of PGR is progesterone dependent in the macaque (Dorofeyeva 1975).

Mucin 1 (MUC1) is a highly glycosylated polymorphic mucin-like protein. In the human endometrium, MUC1 is produced and secreted by the epithelium as a large and extended transmembrane glycoprotein, which continues to be expressed

in the luteal phase and peri-implantation period of pregnancy in a progesterone-dependent manner (Hey et al. 1994). In baboons, however, MUC1 displayed strong surface expression on days 5–8 postovulation or following treatment with estrogen and progesterone, and its expression decreases in the late secretory phase (Hild-Petito et al. 1996).

Integrins are transmembrane glycoproteins with alpha and beta subunits which mediate cell-to-cell and cell-to-extracellular matrix (ECM) adhesion as heterodimers. Three integrins were discovered that have unique expression patterns which correlate with the WOI in women: α1β1, α4β1, and αvβ3 (Lessey 2002). The localization of αvβ3 on the pinopods on the apical surface of the luminal epithelium at the time of uterine receptivity suggests a role for this integrin in initializing implantation (Aplin et al. 1996; Lessey 2002).

Osteopontin (OPN, also known as secreted phosphoprotein 1, SPP1) is a glycoprotein produced by endometrial epithelia and secreted into the uterine lumen at the time of implantation where it binds to the αvβ3 integrin present on the surface of uterine luminal epithelia (Apparao et al. 2001). OPN is also the only common differentially expressed gene from five microarray studies that identified the genomic signature of endometrial receptivity to implantation of the blastocyst (Mirkin et al. 2005).

Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is expressed in a cycle-dependent manner by luminal epithelial cells of the human endometrium in response to both estrogen and progesterone (Lessey et al. 2002). HB-EGF is expressed by the uterine luminal epithelium when fully developed pinopods are localized to the surface of pinopods during the menstrual cycle which suggests its role in the ability of the endometrium to interact with the blastocyst during implantation (Stavreus-Evers et al. 2002).

Transcriptomics signature Since the beginning of the twenty-first century, a number of studies have been performed to identify the global transcriptomic signature of the endometrium during the WOI of natural menstrual cycles (Borthwick et al. 2003; Carson et al. 2002; Kao et al. 2002; Mirkin et al. 2005; Riesewijk et al. 2003) and controlled ovarian stimulated menstrual cycles (Horcajadas et al. 2005; Mirkin et al. 2004). The results offer an opportunity to develop a database of endometrial genes expressed uniquely during the WOI, even though there are significant variations among the studies due to differences in experimental design, sample collection, data analyses, and statistical methods (Horcajadas et al. 2007). Based on the transcriptomic signature elucidated from these studies, a customized endometrial receptivity array (ERA) that contains 238 endometrial receptivity-related genes was created as a genome-based diagnostic tool for assessing human endometrial receptivity to implantation (Diaz-Gimeno et al. 2011). This ERA is superior in accuracy and reproducibility to the traditional histological dating method as a diagnostic tool for endometrial receptivity (Diaz-Gimeno et al. 2013) and offers a chance for clinicians to personalize the time of transfer of a blastocyst(s) during ART procedures (Diaz-Gimeno et al. 2014).

10.2.2 Process of Implantation

Histological examination of human uteri during early pregnancy revealed distinct patterns of blastocyst adherence to the endometrial surface and underlying stroma: the process of implantation could be classified into three stages: apposition, adhesion, and invasion (Hertig et al. 1956; Lindenberg 1991).

10.2.2.1 Apposition and Attachment

Apposition is the very first connection between the blastocyst and the endometrium, during which the human blastocyst finds a location at which to implant, guided by the maternal endometrium. At this stage the blastocyst is able to be dislodged from the uterine surface by flushing the uterine lumen without damage to the blastocyst (Bischof and Campana 1996; Sharma and Kumar 2012). The attachment phase is much stronger and initiates a physical connection between the blastocyst and the endometrium. During attachment, direct contact occurs between the endometrial epithelium and the trophoblast, and the blastocyst cannot be dislodged (Bischof and Campana 1996; Sharma and Kumar 2012; Valles and Dominguez 2006).

The apposition and attachment of the trophoblast to the endometrial epithelium is unique in that it occurs via respective apical cell membranes of the endometrium (Lindenberg 1991). On the blastocyst, the inner cell mass (ICM) usually faces the endometrium. Nevertheless, the area of the blastocyst at which apposition occurs is not dependent on the orientation of the ICM. Instead, the ICM migrates along the inside face of the trophoblast to align itself to the side of apposition. In short, the entire surface of the blastocyst has the potential to form the apposition and attachment site to the endometrium (Sharma and Kumar 2012; Vinatier and Monnier 1990).

There is significant communication between the blastocyst and the endometrium during apposition and attachment phases of implantation which is mediated by receptor-ligand interactions. For example, L-selectin is a protein expressed on the surface of trophoblast cells, and an in vitro model of implantation provided evidence to support the hypothesis that L-selectin mediates apposition of the blastocyst to the uterine epithelium by interacting with its carbohydrate ligands (Genbacev et al. 2003). MUC1 was later identified as a major scaffold for L-selectin to bind to the surface of the endometrium (Carson et al. 2006). Another example of receptor-ligand pairing that mediates blastocyst-endometrial communication is integrin αvβ3 and its ligand OPN which are both spatially and temporally expressed on the surface of endometrial epithelium when attachment occurs (Apparao et al. 2001; Lessey 2002). The integrin heterodimer αvβ3 is also present on the surface of human trophoblast cells; therefore, it is likely that integrin αvβ3 and its ligand OPN participate in trophoblast endometrial recognition during attachment (Reddy and Mangale 2003).

10.2.2.2 Invasion

Invasion of the uterine endometrium by the blastocyst is a process during which trophoblast cells penetrate the endometrial epithelium and invade into the endometrial stroma to reach maternal blood vessels. In humans and nonhuman primates, as attachment is established, trophoblast cells penetrate the endometrial epithelium and reach the matrix layer under the epithelial cells, known as the basement membrane (BM), via intercellular gaps between neighboring endometrial epithelial cells without destroying them (Carson et al. 2000). This is defined as interstitial implantation (Bischof and Campana 1996).

The formation of invadopodia, the thin folds of trophoblast cells between the adjacent endometrial epithelial cells, leads to degradation of the basement membrane and extracellular matrix (ECM), allowing trophoblast cells to reach the endometrial stromal compartment (Giudice 1999). The activated gelatinases (metalloproteinases, MMPs) play a primary role in degrading matrices during this process, and different types of integrins guide the invading trophoblast through different layers of cells and matrices within the endometrium (Bischof and Campana 1996).

The process of invasion in humans and nonhuman primates is followed by syncytialization, during which the protrusions of trophoblast cells that migrate into the endometrium continue to proliferate, differentiate, and fuse to become a new type of cell: syncytiotrophoblasts (STB). The rest of the trophoblast cells, surrounding the inner cell mass, are referred to as cytotrophoblasts (CTB). STB cells invade the endometrium to guide the entire blastocyst as it invades into the endometrium. In humans, the blastocyst is completely embedded within the endometrial stroma at 8 days after ovulation and the entry site is covered by fibrin, over which the endometrial epithelial cells grow and embed the blastocyst in the endometrium (Bischof and Campana 1996). In nonhuman primates such as gibbons, gorillas, and chimpanzees, implantation is also invasive, like humans, whereas in baboons and macaques, implantation is superficial (Carter and Pijnenborg 2011; Houston 1969).

In STB, fluid-filled spaces, known as lacunae, separated by trabeculae appear, transforming the STB into a spongelike material (Bischof and Campana 1996). Eventually, the STBs come into contact with maternal blood vessels, and maternal blood is trapped within the lacunae, forming a basic oxygen and nutrition transfer unit for the developing conceptus (embryo/fetus and placenta). The CTBs grow into the trabeculae of STB to form the primary chorionic villi which are considered to represent the initiation of placentation (Dockery et al. 2000).

10.3 Decidualization

10.3.1 Decidua

The word decidua is from the Latin “de” which means down and “cadere” which means to fall and is so named since the uterine decidua is shed after parturition. The decidua is classified into three different types in the human uterus during pregnancy relative to the developing conceptus: the region of the decidua directly beneath the site of implantation

forms the decidua basalis; the region that overlies the developing conceptus and separates it from the uterine cavity is the decidua capsularis; and the rest of decidua is the decidua vera or decidua parietalis (Pritchard et al. 1985). Both the decidua basalis and the decidua capsularis can be invaded by trophoblast cells and chorionic villi of the conceptus, but only the decidua basalis supports the formation of the discoid placenta in middle and late pregnancy while the rest of the tissue undergoes degeneration later in pregnancy (Pritchard et al. 1985). The decidua in great apes resembles that of humans, whereas Old World monkeys such as baboons and macaques do not have the decidua capsularis due to their superficial implantation (Carter and Pijnenborg 2011).

10.3.2 Functions of Decidua

The main cell type of the decidua is decidualized endometrial stromal cells, which can provide nutritional support for the implanting blastocyst. Apart from the decidual stromal cell, other cell types within the decidual tissue include hematopoietic cells, macrophages, an abundance of uterine natural killer (uNK) cells, and monocytes (Dunn et al. 2003). Associated with these cells are uterine glands and small blood vessels, including spiral arteries, which support the maternal blood supply to the growing conceptus. Functionally the decidua plays a central role in the establishment and maintenance of pregnancy.

10.3.2.1 Controlled Trophoblast Invasion

Implantation and placentation in the human involves deep invasion of trophoblast cells into the maternal uterine architecture. The decidua is the compartment of the uterus with which trophoblast cells interact with during invasion. The decidua forms a dense cellular matrix that, on the one hand, generates a local microenvironment to promote trophoblast attachment and invasion and, on the other hand, limits the extent of aggressive invasion of trophoblast cells (Gellersen et al. 2007; Gellersen and Brosens 2014).

The extracellular matrix formed by decidual cells is the target of trophoblast invasion. Recent studies showed that decidual cells actively engage in the process of invasion by encapsulating the blastocyst (Gellersen and Brosens 2014). Trophoblast invasion requires proteolytic degradation and remodeling of the decidual extracellular matrix. Matrix metalloproteinases (MMPs) secreted by trophoblast cells are able to degrade the extracellular matrix of the decidua (Librach et al. 1991; Shimonovitz et al. 1994). Based on recent studies, decidualized stromal cells also produce MMPs, and the capacity of MMP secretion of these cells is comparable to that of trophoblast cells (Anacker et al. 2011; Weimar et al. 2013). In in vitro culture models, decidualized human endometrial stromal cells (HESCs) surrounding the blastocyst migrate away to accommodate the outgrowth of the trophoblast (Grewal et al. 2010; Grewal et al. 2008). Furthermore, elastin microfibril interfacer 1, a connective tissue glycoprotein produced by the decidua, can attract migrating extravillous trophoblast cells (Spessotto et al. 2006).

Alternatively, decidual cells and their microenvironment play a role to limit over-invasion of the trophoblast to protect the endometrium from invasive damage. Conditioned medium from human decidual cells inhibits growth of cultured BeWo choriocarcinoma cells as well as invasion of trophoblast cells (Graham and Lala 1991; Lewis et al. 1993). The actions of MMPs are inhibited by tissue inhibitors of metalloproteinase (TIMPs). In humans, TIMPs abolish trophoblast invasion (Librach et al. 1991). *TIMP-3* mRNA expression is upregulated in decidualized endometrial stromal cells by progesterone treatment both in vivo and in vitro (Higuchi et al. 1995), whereas endometrial *TIMP-1* and *TIMP-2* mRNA expression is not dependent on the stage of the menstrual cycle. Additionally, decidual cells secrete transforming growth factor-β (TGF-β) to inhibit the production of MMPs by trophoblast cells (Graham et al. 1992).

10.3.2.2 Protection of the Conceptus from Maternal Immune Rejection

The decidua also plays an integral role in ensuring immune tolerance toward the semi-allogeneic fetal-placental unit and protects the conceptus from the mother's immune system. The physiological mechanisms are mediated by immune cells, particularly uterine natural killer (uNK) cells and regulatory T cells (Tregs), which increase in their numbers during early pregnancy (Fu et al. 2013; Tilburgs et al. 2008; Xiong et al. 2010).

The uterine NK cells are often considered to be cytotoxic, killing virally infected cells and cancer cells (Biron 2010). However, this killing function is lost in the uterus during pregnancy: the uNK cells play a supportive role. Differentiating resident stromal cells, but not trophoblast cells, have been demonstrated to play a critical role in the recruitment of $CD56^{bright}/CD16^{-}$ uNK cells at the maternal-conceptus interface (Rieger et al. 2004; Vassiliadou and Bulmer 1998). uNK cells promote immune tolerance and successful pregnancy by dampening inflammatory Th17 cells via IFN-γ (Fu et al. 2013). The uNK cells can also inhibit the function of T cells by expressing immunomodulatory molecules such as galectin-1 and glycodelin A (Koopman et al. 2003). Galectin-1 is known as an inhibitor of T-cell proliferation and survival, and it promotes apoptosis of activated T cells in the decidua (Kopcow et al. 2008). Glycodelin A inhibits T-cell activation through its ability to interact with the tyrosine phosphatase receptor CD45 on the T-cell surface (Rachmilewitz et al. 2003; SundarRaj et al. 2008). The uNK cell-mediated T-cell regulatory response is lost in patients who experience recurrent spontaneous abortions (RSA) due to extensive local inflammation (Fu et al. 2013).

Tregs are $CD4^{+}CD25^{+}$ T cells, which suppress the activity of other immune cell types and are involved in downregulating immune responses (Campbell and Koch 2011). Tregs produce immunosuppressive cytokines such as IL-10 for exerting immune tolerance (Hara et al. 2001). Tregs are essential for the maintenance of pregnancy. The decrease in these cells and its product IL-10 in the decidua is associated with RSA which involves an inflammatory response and is accompanied by an increase in Th17 cells (Plevyak et al. 2002; Sasaki et al. 2004). Since IL-10 signaling

in Tregs is required for suppression of Th17 cell-mediated inflammation (Chaudhry et al. 2011), it is plausible that Tregs regulate immune tolerance in decidua during early pregnancy by suppressing Th17 cell-mediated inflammation via its product IL-10.

Besides immune cells, decidualized stromal cells are able to induce apoptosis of activated T cells by inducing expression of Fas ligand (FasL) (Kayisli et al. 2003). Thus, the decidua acts as a gatekeeper that controls immune tolerance during pregnancy by blocking T cells that would otherwise attack the developing conceptus.

10.3.3 Decidualization

Decidualization is the differentiation of elongated fibroblast-like mesenchymal cells in the uterine stroma to rounded epithelioid-like cells during the menstrual cycle and pregnancy. This process is one of the most critical and remarkable events that occurs within the endometrium of human and nonhuman primates during pregnancy (Kim et al. 1999a). This morphological change in humans is initiated in the luteal phase and begins with stromal cells surrounding the spiral arteries in the upper two-thirds of the endometrium regardless of the presence or absence of a conceptus (Ramathal et al. 2010). This initial change is referred to as predecidualization.

Once implantation of the blastocyst occurs, the reaction persists and spreads beyond the perivascular regions and becomes the decidua of pregnancy. The decidual reaction continues throughout pregnancy. Not only do the decidual cells increase in size throughout pregnancy, but the percentage of stromal cells in the decidua increases progressively from 9.8 % in early pregnancy to 57.8 % at term (Wewer et al. 1985). In contrast, baboons and macaques do not undergo a predecidual reaction during the menstrual cycle (Enders 1991; Kim et al. 1999a; Ramsey et al. 1976). However, the stromal cells undergo extensive modification following the establishment of pregnancy to form the decidua in baboons and macaques (Enders 1991). This decidualization process is slower in the baboon compared to the human, but earlier than that seen in the macaque (Carter et al. 2015). Decidualization of HESCs as well as baboon uterine stromal cells can be induced in vitro in the presence of hormones and cyclic adenosine monophosphate (cAMP) (Kim et al. 1998). Additionally, human uterine fibroblast (HuF) cells obtained from full-term decidua (maternal side of placenta) are able to differentiate morphologically and biochemically in vitro (Richards et al. 1995). HuF cells are used extensively to study decidual function due to the fact that these cells are readily available in larger numbers than those from endometrial biopsies (Afshar et al. 2012; Richards et al. 1995; Strakova et al. 2000).

Ultrastructural studies of human decidual cells indicate characteristics of epithelioid cells: progressive enlargement, rounding of the nucleus, increased number and complexity of nucleoli, expansion of the rough endoplasmic reticulum and Golgi complex, cytoplasmic accumulation of glycogen and lipid droplets, and dense membrane-bound secretory granules (Wynn 1974). In between these cells, adherens junctions, but

not true desmosomes, are found and the arrangement of gap junctions in these cells may be helpful for trophoblast invasion (Lawn et al. 1971). Extracellular matrix (ECM) proteins produced by decidualized stromal cells include decorin, laminin, type IV collagen, fibronectin, and heparin sulfate proteoglycans (Gellersen et al. 2007).

The characteristics of decidual cells described previously are also characteristics of secretory cells (Kim et al. 1999a). The major secretory products of decidual cells are prolactin (PRL) and insulin-like growth factor binding protein-1 (IGFBP1), which have been used widely as marker genes for decidualization (Gellersen et al. 2007). Other secretory molecules from decidual cells include interleukin-11 (IL-11), epidermal growth factor (EGF), heparin-binding epidermal growth factor (HB-EGF), LEFTY2, activin A, and neuropeptides (Dimitriadis et al. 2005). In the past decades, microarray studies have been used to identify transcriptional changes during differentiation of stromal cells to decidual cells. Based on results of those studies, decidualization has been described as a process of sequential reprogramming of functionally related changes including ECM organization, cell adhesion, cytoskeletal organization, signal transduction, metabolism, stress responses, cell cycle progression, differentiation, and apoptosis (Gellersen et al. 2007).

10.3.4 Molecular Mechanisms

The decidualization process encompasses changes that commence in response to the actions of progesterone. Circulating concentrations of progesterone increase during the secretory phase of the menstrual cycle and remain elevated during pregnancy. Progesterone acts by binding and activating its nuclear receptor, PGR. In the stromal compartment of the human endometrium, PGR-A is the dominant isoform (Chen et al. 2009). It is highly expressed in stromal cells throughout the menstrual cycle and in pregnancy, whereas its expression in epithelial cells decreases after ovulation (Chen et al. 2009). Progesterone regulates the expression of IGFBP1 and decidual PRL via PGR-A (Christian et al. 2002a; Gao et al. 2000). Silencing of downstream progesterone-regulated genes and PGR co-regulators such as *HOXO10*, *KLF9*, and *FKBP52* leads to decrease in expression of markers of decidualization, *PRL* and *IGFBP1*, in HESC cells (Lu et al. 2008; Pabona et al. 2010; Yang et al. 2012). However, decidualization of the superficial layer of the endometrium is only apparent in vivo approximately 10 days after the increase in circulating concentrations of progesterone (de Ziegler et al. 1998). The induction of decidualization of HESC in vitro with progesterone or progesterone and estradiol is relatively slow (Brar et al. 1997). The addition of cAMP rapidly accelerates decidualization of HSEC cells (Brosens et al. 1999).

The concentration of cAMP is higher in endometrial biopsies taken during the secretory phase compared with proliferative phase of the menstrual cycle (Bergamini et al. 1985) and reaches a peak in the late-secretory phase in response of prostaglandin E2 (PGE2) (Tanaka et al. 1993). In pregnancy, human chorionic gonadotropin (hCG) further enhances concentrations of cAMP and decidualization of endometrial stromal cells (Tang and Gurpide 1993). In cultured HESCs, cAMP alone increases the expression of *PRL*, but for only a few days (Brosens et al. 1999).

Further induction and stabilization of PRL, a decidualization marker requires the presence of both cAMP and progesterone (Brosens et al. 1999). The effect of cAMP during in vitro decidualization can be blocked with a PKA inhibitor (Brar et al. 1997). Thus, cooperation of progesterone and cAMP is necessary and sufficient for decidualization. Progesterone, via PGR-A, together with cAMP and genes under regulation of progesterone/cAMP, such as *HOXO10*, *FOXO1*, *BMP2,* and *WNT4,* comprise a critical network for decidualization of endometrial stromal cells (Li et al. 2007, 2013; Lu et al. 2008; Takano et al. 2007; Vasquez et al. 2015).

Based on in vivo studies in the baboon, the process of decidualization can be separated into two distinct phases. The first phase is manifested by the expression of alpha smooth muscle actin (α-SMA) in the differentiation of endometrial stromal cells, which is regulated by chorionic gonadotropin (CG) and progesterone (Christensen et al. 1995). This change in cytoskeletal reorganization is associated with binding of decidual integrins to the ECM, which also undergoes extensive remodeling in response to CG (Fazleabas et al. 1997a). The second phase of decidualization requires the presence of a conceptus and is manifested by the downregulation of expression of α-SMA and the induction of IGFBP1 (Kim et al. 1999a).

Results from high-throughput analysis revealed that genes and proteins involved with the cell cycle, apoptosis, transcription and translation, metabolism, inflammatory response, cell structure remodeling, and paracrine signaling are differently regulated during decidualization of human endometrial cells (Brar et al. 2001; Popovici et al. 2000). MicroRNAs also play a role during decidualization (Estella et al. 2012; Qian et al. 2009). As a result of differentiation, decidual cells acquire unique biochemical and cellular properties that enable them to support implantation of the blastocyst. Therefore, the decidualization of stromal fibroblasts within the human endometrium is a requirement for successful pregnancy.

10.4 Endometrial Response to Blastocyst/Conceptus Signals

In humans, the presence of a blastocyst/conceptus alters the endometrial phenotype compared to the normal menstrual cycle in the mid-secretory phase. This altered phenotype of endometrium during the cycle of conception includes the persistent increase of stromal edema, predecidual formation, and the absence of leukocytic infiltration compared to the normal menstrual cycle (Hertig 1964; Karow et al. 1971; Wentz et al. 1986). These observations suggest the importance of embryonic signals for the development of uterine receptivity to facilitate implantation.

10.4.1 Endometrial Response to Chorionic Gonadotropin (CG)

Chorionic gonadotropin (CG), the major embryonic signal in the primate, is a glycoprotein hormone synthesized and secreted by the trophoblast cells. The CGβ mRNA is detected as early as the 6–8 cell stage of the human embryo, but measureable levels of CG protein are present in culture medium of the late blastocyst

(Srisuparp et al. 2001). In vivo, CG is first detectable in maternal serum during the window of implantation and increases rapidly along with the establishment and progress of early pregnancy (Alfthan and Stenman 1996). In primates, CG acts as a luteinizing hormone (LH) superagonist, extending the lifespan of the corpus luteum to sustain progesterone production to support the pregnancy. In baboons, pregnancy-associated serum CG activity is detectable by day 15 of gestation, peaks at day 27, and returned to baseline values by day 51. Lower levels of CG are associated with spontaneous abortion (Fortman et al. 1993). Besides the ovarian response to CG, the endometrium also responds to embryonic CG, since LH/CG receptor is expressed in the primate endometrium (Reshef et al. 1990; Cameo et al. 2006). In vivo studies in the baboon from our laboratory showed that CG alters the morphology and biochemical activity of the endometrium (Banerjee and Fazleabas 2011; Fazleabas et al. 1999a; Hausermann et al. 1998). Transcriptomics analysis identified genes influenced by CG in the baboon and identified pathways that are involved in embryo attachment, extracellular matrix remodeling, and modulation of the immune response around the implanting blastocyst (Banerjee and Fazleabas 2010; Sherwin et al. 2007).

10.4.1.1 Epithelial Response

Our laboratory has utilized the baboon as a nonhuman primate model to investigate endometrial responses to embryonic CG. CG was infused into the uterine cavity of cycling baboons from day 6 to day 10 postovulation to mimic normal blastocyst transit (Fazleabas et al. 1999a; Jones and Fazleabas 2001). As a result of CG treatment, an epithelial plaque reaction was observed in the luminal epithelium (Fig. 10.1) (Fazleabas et al. 1999a; Jones and Fazleabas 2001). Endometrial glandular structures in response to CG infusion resemble those observed in the pregnant baboon at the same age of gestation. Glandular secretions including glycodelin, a major secretory protein of uterine glands during the secretory phase and pregnancy, also increase in response to CG (Fig. 10.1) (Fazleabas et al. 1997a, b; Hausermann et al. 1998).

Several in vitro studies also support the role of CG in inducing changes in endometrial epithelial cells in the baboon and human. Prostaglandin E2 (PGE2) induces cAMP in endometrial stromal cells to promote their predecidualization response during the secretory phase (Tanaka et al. 1993). According to results from our and other laboratories, treatment of both human and baboon endometrial epithelial cells with CG induces expression of cyclooxigenase-2 (COX2, coded by *PTGS2*) and prostaglandin E synthase (PGES), two enzymes that control the synthesis of PGE2 (Banerjee et al. 2009; Zhou et al. 1999), as well as the production of PGE2 (Srisuparp et al. 2003). The response of endometrial epithelial cells to CG and the downstream PGE2 production occurs through the CG receptor LHCGR, a seven transmembrane G protein-coupled receptor, and the inositol phosphate-dependent mitogen-activated protein kinases (MAPK) pathway (Banerjee et al. 2009). Interestingly, treatment with CG failed to induce production of cAMP in endometrial epithelial cells, but

leads to a release of PGE2 which induces cAMP production in stromal cells (Srisuparp et al. 2003; Tanaka et al. 1993), suggesting the role of epithelial cells mediating the stromal response to embryonic CG. The mechanism by which this response is initiated is summarized in Fig. 10.1a. Furthermore, CG downregulates expression of its receptor in baboon endometrial epithelium, but upregulates LHCGR in stromal cells surrounding spiral arteries (Cameo et al. 2006), indicating a shift in the endometrial response to CG from epithelium to stroma which is driven by CG itself.

10.4.1.2 Stromal Response

In the baboon model, the first detectable molecular response to CG in uterine stromal cells is an increase in the expression of α-SMA in the subepithelial region in early gestation after implantation and after CG infusion, indicating that remodeling of the stromal cell cytoskeleton is necessary for decidualization of endometrial stromal cells (Fazleabas et al. 1999a; Jones and Fazleabas 2001; Strakova et al. 2005). This stimulation has been attributed to binding of ECM proteins to integrin heterodimers on stromal cells (Fazleabas et al. 1997a). The remodeling of the cytoskeleton of stromal cells is essential for their differentiation. In vivo, in the absence of α-SMA, endometrial stromal cells in baboons are not able to predecidualize until induced by CG signaling from the implanting blastocyst or infusion of CG

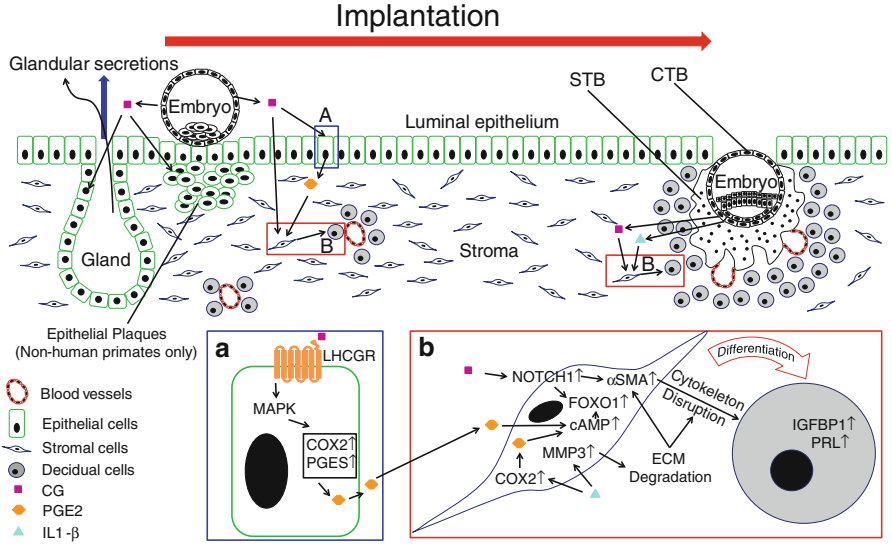


Fig. 10.1 Endometrial responses to the embryonic signal in primates. Epithelial and stromal cells respond to chorionic gonadotropin (*CG*) and interleukin 1 β (*IL-1β*) during the establishment of pregnancy in the primates. Inserts *A* and *B* reflect the changes in epithelial and stromal cell responses which are described in detail in expanded form in Panels (**a**) and (**b**)

into the uterine lumen (Enders 1991; Kim et al. 1999a; Ramsey et al. 1976). However, these stromal cells can be induced to decidualize by cAMP and steroid hormones since they induce expression of α-SMA during in vitro culture (Kim et al. 1998). Furthermore, disruption of actin filaments by cytochalasin D sensitizes the cultured baboon endometrial stromal cells response to inducers of decidualization characterized by expression of IGFBP1 within 24 h after treatment (Kim et al. 1999b) compared to 6 days under standard conditions (Kim et al. 1998). Stromal cells isolated from the endometrium primed by embryonic or infused CG in vivo exhibit the decidualization response in vitro as rapidly as cytochalasin D-sensitized stromal cells (Kim et al. 1999b), indicating the importance of CG to initiate decidualization of endometrial stromal cells. Expression of the decidualization marker IGFBP1 is also regulated by the conceptus and CG (Fazleabas et al. 1997b). In early gestation (day 28), LHCGR is expressed by stromal cells around spiral arteries where decidualization is initiated, indicating that those cells are a direct target of CG to induce initiation of decidualization (Cameo et al. 2006). Responses of endometrial stromal cells to CG during decidualization are summarized in Fig. 10.1b.

Full differentiation of endometrial stromal cells requires a decrease in abundance of α-SMA to allow for an increase in the expression of IGFBP1. In vivo, α-SMA disappears between days 32 and 40 of pregnancy, which is the time when expression of IGFBP1 is detectable (Christensen et al. 1995; Strakova et al. 2005; Tarantino et al. 1992). In vitro, an increase in IGFBP1 is associated with the decrease in expression of α-SMA (Kim et al. 1998, 1999b). This decrease in α-SMA is also associated with a decrease in expression of LHCGR in later stages of gestation (days 40–50) and at the completion of in vitro decidualization (Cameo et al. 2006). Collectively, the embryonic signal CG first induces expression of α-SMA to promote remodeling of the cytoskeleton of stromal cells and differentiation of endometrial stromal cells around spiral arteries via its membrane receptor. Subsequently the decrease in CG signaling appears to be necessary for the completion of decidualization. Our recent studies demonstrated that NOTCH1, a membrane receptor of Notch signaling, may mediate CG-regulated decidualization (Afshar et al. 2012; Su et al. 2015).

10.4.2 NOTCH1 Acts Downstream of Chorionic Gonadotropin (CG)

CG is believed to rescue the endometrium from its apoptosis cascade, which usually occurs at the end of each menstrual cycle, and direct it toward a decidualization response. CG inhibits this apoptotic fate of endometrial cells (Lovely et al. 2005) and, with ovarian hormones, differentiates them into the decidualized phenotype (Jasinska et al. 2006). CG prevents apoptosis by inducing anti-apoptosis genes like BCL-2 (Jasinska et al. 2006).

Notch signaling is a highly conserved pathway across most multicellular organisms. It plays an important role in cell-cell communication and mediates cell fates such as proliferation, differentiation, and apoptosis (Rizzo et al. 2008). Notch signaling is associated with four transmembrane receptors (Notch 1–4) and five transmembrane ligands of the jagged/delta-like families (Afshar et al. 2012). Activation of Notch signaling is generally initiated by interactions between adjacent cells expressing receptor and ligand. This results in a series of receptor-mediated cleavage events and the release of the Notch intracellular domain (NICD) which translocates to the nucleus where it binds and activates the Notch family transcription factor, recombination signal binding protein Jκ (RBP-Jκ). RBP-Jκ then initiates the expression of Notch target genes, such as the "hairy enhancer of split" (Hes) and Hes-related (Hey) transcription factor families (Su et al. 2015).

Notch signaling mediates cellular processes that are essential for successful decidualization. Expression of NOTCH1 and its target α-SMA are both induced by CG in baboon endometrial stromal cells in vivo. Silencing of NOTCH1 in human uterine fibroblast (HuF) cells cultured in vitro leads to the impairment of decidualization, suggesting that NOTCH1-α-SMA mediates CG function in rescuing endometrial stromal cells from apoptosis and differentiating them to decidual cells (Afshar et al. 2012). In vivo CG infusion upregulates expression of both NOTCH1 and α-SMA in human endometrium, which further supports our findings from studies of the baboon model (M.R. Strug and A.T. Fazleabas, unpublished data). Furthermore, activation of Notch signaling is regulated by progesterone which indicates a significant interaction between the CG-Notch pathway and progesterone signaling during decidualization (Afshar et al. 2012). Additional studies have shown that silencing NOTCH1 in HuF cells inhibits decidualization only during the initiation of the differentiation process. To inhibit decidualization of HuF cells in vitro, NOTCH1 must be silenced before the induction of decidualization, whereas silencing NOTCH1 three days after induction of decidualization does not inhibit the expression of decidualization markers (Su et al. 2015). Furthermore, comparative microarray analysis indicated that Forkhead box protein O1 (FOXO1) is a downstream target of NOTCH1 during in vitro decidualization, since FOXO1 and its specific target genes are downregulated when NOTCH1 is silenced during in vitro decidualization (Su et al. 2015). FOXO1 is one of the earliest genes induced during decidualization (Brar et al. 2001; Christian et al. 2002b). A number of in vitro experiments conclusively demonstrated the importance of FOXO1 for the induction of decidualization (Buzzio et al. 2006; Grinius et al. 2006; Labied et al. 2006). Overexpression of FOXO1 in human endometrial stromal cells can induce expression of IGFBP1 and PRL independent of cAMP and hormones induced in vitro decidualization (Buzzio et al. 2006; Christian et al. 2002b; Kim et al. 2005; Takano et al. 2007). A recent study demonstrated that FOXO1 is functionally required for the binding of PGR to genomic targets during decidualization (Vasquez et al. 2015). These results demonstrate that NOTCH1 acts downstream of CG and plays a critical role during the decidualization response of endometrial stromal cells by regulating the expression of its targets, α-SMA and FOXO1 (Fig. 10.1b).

On the other hand, NOTCH1 is downregulated at the completion of decidualization (Afshar et al. 2012) similar to α-SMA and LHCGR expression, which is necessary for the induction of IGFBP1 (Cameo et al. 2006; Kim et al. 1998). Results of our most recent studies indicated that constitutively active Notch signaling by overexpressing the NOTCH1 intracellular domain (N1ICD) prevents HuF cells from undergoing decidualization (R. Su and A.T. Fazleabas, unpublished data). The necessity for the decrease in NOTCH1 expression for the completion of decidualization may be because decidualization depends on cAMP stimulation, sustained PKA activity, and CREB activation (Gellersen and Brosens 2014; Kusama et al. 2014), but N1ICD sequesters nuclear CREB and inhibits cAMP/PKA mediated signaling (Hallaq et al. 2015).

In summary, NOTCH1 initially mediates a survival signal in the uterine endometrium in response to CG from the implanting blastocyst together with progesterone, so that menstrual sloughing is averted. Subsequently, NOTCH1 downregulation may be critical for the complete transition of stromal fibroblasts to decidual cells, which is essential for the establishment of a successful pregnancy.

10.4.3 Endometrial Response to Interleukin-1β

Implantation has been characterized as an inflammatory response, and IL-1β is a key regulator of this response (Fazleabas et al. 2004). Cytotrophoblast cells isolated from first trimester placentae release more IL-1β then those from second and third trimester in culture (Librach et al. 1994), and co-culture of cytotrophoblast cells with HuF cells induces them to decidualize (Jasinska et al. 2004). Numerous studies have provided evidence of the importance of IL-1β as an embryonic signal that affects endometrial responses in primates.

Our laboratory previously reported that IL-1β induces expression of COX2 and PGE2 synthesis in human and baboon endometrial stromal cells (Strakova et al. 2000), which is believed to subsequently increase cAMP in stromal cells and induce decidualization (Fazleabas et al. 2004). Indeed, induced expression of the decidualization marker IGFBP1 by IL-1β in the presence of steroid hormones is blocked by COX-2 inhibitor in human and baboon endometrial stromal cells (Strakova et al. 2000). Induction of IGFBP1 expression by cAMP with steroid hormones is not affected by inhibition of COX-2 which supports the inference that cAMP acts downstream of IL-1β-COX2-PGE2 signaling during decidualization. Interestingly, cAMP prevents decidualization induced by IL-1β which suggests a negative crosstalk between IL-β- and cAMP-induced decidualization responses (Strakova et al. 2000, 2002).

The remodeling of the cytoskeleton of stromal cells is essential to the differentiation of stromal cells (Kim et al. 1998). Cytoskeleton changes can be induced by the disruption of the ECM (Fazleabas et al. 1997a). In baboon endometrial stromal cells, IL-1β induces expression and synthesis of MMP3, which can degrade the ECM (Strakova et al. 2003). This induction of MMP3 is regulated via the MAPK

pathway and is critical for decidualization. Inhibition of MMP3, using doxycycline or specific MMP-3 inhibitor N-isobutyl-N-(4-methoxyphenylsulfonyl) glycyl hydroxamic acid (NNGE), suppresses the induction of decidualization by IL-1β and hormones (relaxin, estradiol-17β, and medroxyprogesterone acetate) (Strakova et al. 2003). The expression of MMP3 and degradation of ECM may contribute to the decrease in expression of the cytoskeleton protein α-SMA that is induced by IL-1β and hormones during decidualization (Strakova et al. 2000). Additionally, expression of IGFBP1 can be induced in stromal cells close to the apical surface by in vivo infusion of IL-1β in the presence of CG which further supports a role for IL-1β from the blastocyst in regulating decidualization of endometrial stromal cells during implantation (Strakova et al. 2005). The postulated roles of IL-1β during decidualization are summarized in Fig. 10.1.

10.5 Summary

Successful implantation and decidualization are necessary for providing required maternal support and protection of the developing conceptus. Human reproduction is highly inefficient compared to other primates. In women the average chance of pregnancy is only 15 % per cycle during their reproductive lifespan (Hjollund et al. 2000). In ART, only around 25 % of transferred embryos will successfully implant (Edwards 2006). Understanding the processes and mechanisms required for implantation and the establishment of pregnancy can help improve outcomes of ART. In support of results of studies on the role of CG in modulating the receptive endometrium as discussed in Sect. 10.4, intrauterine injection of CG before embryo transfer significantly improves implantation and pregnancy rates following ART (Mansour et al. 2011). Thus, an understanding of endometrial response to embryonic cells in primates may provide insight into improving pregnancy rates in women who are infertile.

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Chapter 11
The Dog: Nonconformist, Not Only in Maternal Recognition Signaling

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Abstract Although similar at the molecular and cellular levels, endocrine mechanisms governing reproductive function in the domestic dog (*Canis familiaris*) differ markedly at the regulatory level from those known in other domestic animal species. Some of the events, e.g., the lack of luteolysis in the absence of pregnancy, resulting in similar luteal function and, therefore, hormonal profiles in early pregnant and nonpregnant animals, are species-specific. Consequently, no early gestation marker has so far been identified for the dog. Following implantation, relaxin of fetal placental origin can be detected and used for pregnancy diagnosis. Characterized by the lack of an active luteolytic principle from intra- or extra-luteal sources, the canine reproductive cycle appears to represent a "basic" form of mammalian reproductive function with apparently reduced opportunities for facilitating fecundity and hastening reproduction. Nevertheless, in the dog some kind of mechanism for synchronization between blastocyst development and uterine preparation for pregnancy must have evolved in order to support gestation. Driven by this assumption, studies including our recent investigations have been initiated aimed at characterizing some of the embryo-mediated effects of the preimplantation embryo on the canine uterus. Moreover, the lack of a uterine luteolysin and consequently the absence of a need to develop an antiluteolytic strategy make the dog an interesting model for investigating early evolutionary mechanisms involved in the preparation for implantation and ensuring embryo survival. These mechanisms result in an inverse relationship between the duration of pregnancy and of the nonpregnant cycle in the dog, compared with all other domestic animal species.

Dedicated to the memory of Professor Patrick W. Concannon, PhD, a pioneer in the sciences of small animal reproduction

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11.1 Introduction

Despite increasing scientific interest and considerable progress in understanding canine reproduction, knowledge concerning the establishment of canine pregnancy is still extremely limited compared with other domestic animal species. This seems surprising considering that the dog is one of the most important pets and the closest human companion sharing a common environment with us and, moreover, having also become one of the best models for studying multifactorial human diseases (Starkey et al. 2005). On the other hand, however, because canine reproduction displays a sexual cycle with characteristics that are highly divergent from those of other domestic animals, its study requires specific research approaches. Thus, in contrast to polyestrous (seasonal or aseasonal) domestic animal species, such as cattle, pigs, sheep, or even other domestic carnivores, e.g., cats, the domestic dog (*Canis familiaris*) is classified as being "aseasonal monoestrous" because it ovulates only once per breeding season, an activity which is separated from the next estrus by a period of sexual inactivity, referred to as anestrus. The duration of this obligatory period of sexual quiescence varies considerably among bitches of different breeds and individuals within a breed and can be as long as 36 weeks or as short as 11 weeks (Okkens and Kooistra 2006; Concannon 2011). Consequently, the small number of cycles and pregnancies within a canine lifetime provides scientists with fewer investigative possibilities and yields less information on mechanisms regulating the species-specific reproductive patterns.

11.1.1 "The Nonconformist"

The definition of maternal recognition of pregnancy coined originally by Roger V. Short (1969), describing the strategies observed in different species for preventing luteolysis and sustaining the luteal life span beyond the cyclical activity, does not apply to the dog, in which a similar luteal phase duration is observed during pregnancy and in nonpregnant cycles. A more general definition appears more suitable for this species, designating the maternal recognition as a morphological and functional relationship between the uterus, the embryo, and the corpus luteum (CL) which is the sole source of progesterone in the dog (Concannon et al. 1989). The more intimate embryo- and feto-maternal contact starting following implantation and placentation is important for maintenance of pregnancy and also plays a role at term (Kowalewski et al. 2010, 2011b; Gram et al. 2013, 2014a, b).

Thus, based on reports and reviews from our laboratory and that of other researchers, including the pioneering work from Bischoff (1845), who for the first time initiated studies concerning early canine development, and some of our unpublished data, here we present an overview of current knowledge on the canine-specific endocrine mechanisms characterizing luteal function and early uterine embryo-maternal contact, as well as the role of the placental feto-maternal interactions during establishment and maintenance of canine gestation.

11.2 Luteal Phase

Progesterone (P4) is indispensable for successful pregnancy outcomes. Its luteal production is continuously required for the establishment and maintenance of canine gestation, as there is no steroidogenic activity within the canine placenta (Hoffmann et al. 1994; Nishiyama et al. 1999). This emphasizes the central role played by the CL in regulating canine reproduction.

11.2.1 Endocrine Patterns during Pregnancy and Pseudopregnancy

The endocrine and molecular mechanisms regulating luteal function in the dog have been extensively discussed recently (Hoffmann et al. 2004b; Concannon 2011, 2012; Papa and Hoffmann 2011; Kowalewski 2012, 2014; Kowalewski et al. 2013). A schematic representation of the most important regulatory events is shown in Fig. 11.1.

Follicular luteinization can be observed as early as 6 days before first significant LH increase (LH surge) and is reflected in slowly increasing P4 levels from basal values of 0.2–0.4 ng/ml to the levels of 0.6–1.0 ng/ml observed at the preovulatory LH surge (Concannon 2009). Coincident with the onset of the LH surge (0.5–3 days

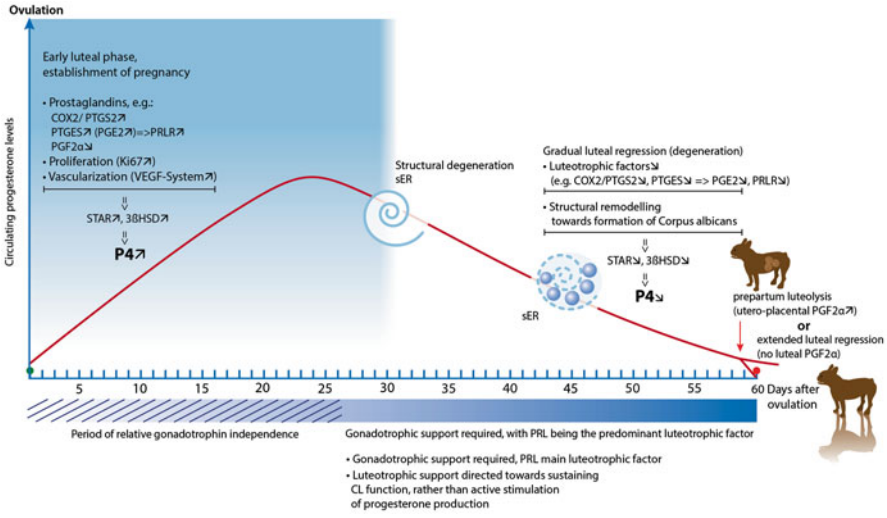


Fig. 11.1 Schematic representation of the most important hormonal mechanisms regulating canine luteal function (Modified from Kowalewski et al. (2014b)). A detailed explanation is provided in the text. *COX2/PTGS2* cyclooxygenase 2 (PTGS2), *PTGES* PGE2 synthase, *PRLR* PRL receptor, *STAR* steroidogenic acute regulatory protein, *3βHSD (HSD3B2)* 3β-hydroxysteroid-dehydrogenase, *sER* smooth endoplasmic reticulum, *VEGF* vascular endothelial growth factor

after the estrogen peak), the preovulatory luteinization becomes very intense and P4 increases rapidly, concomitantly with still decreasing estrogen levels (Fig. 11.2). Ovulation takes place at 48–60 h (2–3 days) after the initial LH surge (Concannon 2009) and is accompanied by relatively high circulating P4 levels of about 5 ng/ml (Concannon et al. 1989) (Figs. 11.1 and 11.2). The phenomenon of preovulatory luteinization, described for the first time in the dog by Bischoff in his work (Bischoff 1845), is not unique to the dog and, even if not that intense, can also be observed, for example, in pigs, rodents, and primates. As recently shown, analogous to other species, ovulation is associated with high PGE2 and PGF2α concentrations in the forming CL (Tsafriri et al. 1972; Iesaka et al. 1975; Kowalewski et al. 2014a), implicating their involvement in this process. Following ovulation, and before the end of estrus (male acceptance), the structural formation of the CL begins. The remaining long-lasting phase of luteal activity is commonly referred to in the literature as diestrus. Progesterone concentrations rise rapidly and vary widely between individual animals, reaching maximal circulating levels of 30–35 ng/ml (sometimes even up to 80 ng/ml or higher) within 15–30 days (Concannon et al. 1989; Concannon 2011). The turning point of luteal steroidogenic activity is indicated by the onset of

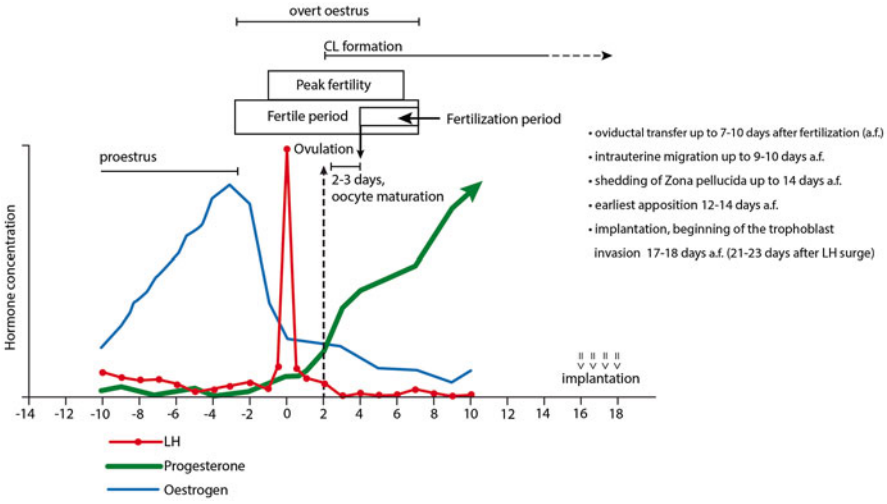


Fig. 11.2 The timeline of endocrine pre- and postovulatory events and timing of early embryonic development in the dog (Modified after England and Pacey (1998)). A detailed explanation is provided in the text. Briefly, the strong preovulatory luteinization results in relatively high circulating progesterone levels of about 5 ng/ml at the time of ovulation, which takes place 48–60 h (2–3 days) after the first significant LH increase above the basal level (referred to as LH surge). The latter occurs 0.5–3 days after the estrogen peak. Following ovulation and before the end of male acceptance (overt estrus), the structural formation of the CL begins. The long-lasting phase of luteal activity is commonly referred to as diestrus. The oocyte maturation and completion of first meiotic division is delayed in the dog and takes place 2–3 days after ovulation (i.e., 4–5 days after the initial preovulatory LH surge). The fertilization period is long and variable due to the extended life span of intrauterine spermatozoa (up to 7 days) and oviductal oocytes (7–8 days including up to 5 days, which follow the oocyte maturation). (*a.f.* after fertilization)

a slow luteal regression as becomes obvious by the gradually decreasing P4 levels. Especially following implantation and placenta formation, the mean circulating P4 levels tend to be numerically but not statistically higher in pregnant dogs (Steinetz et al. 1989), precluding the use of P4 levels as an endocrine marker for pregnancy detection. These diverging P4 levels observed during the second half of gestation, compared with nonpregnant cycles, are likely to result from the increased prolactin (PRL) levels also measured during the same period approximately 4–6 weeks following the LH surge (reviewed in (Concannon 2009; Kowalewski et al. 2014a)). This enhanced PRL secretion could be initiated by the simultaneously or slightly earlier (days 25–30 from the preovulatory LH surge) increasing secretion of relaxin from placental syncytiotrophoblast (Klonisch et al. 1999). Such a PRL-releasing role for relaxin was reported for pigs and monkeys (Bethea et al. 1989; Li et al. 1993). Similarly as for P4, the high peripheral PRL concentrations observed in overtly pseudopregnant bitches also preclude using this hormone as a reliable marker for detection of pregnancy. Instead, placental relaxin is the only suitable endocrine marker of pregnancy.

At approximately day 60 of the luteal life span, the P4 profiles, which up until then are similar in pregnant and nonpregnant dogs, start to diverge. At this time, the steep P4 decline, which is observed in pregnant dogs, signals the initiation of prepartum luteolysis (Nohr et al. 1993). This is accompanied by a concomitant increase in PGF2α concentrations in the maternal circulation, indicating the role of PGF2α during both luteolysis and parturition. The *placenta fetalis* appears to be the main source of this PGF2α increase (Kowalewski et al. 2010; Gram et al. 2013, 2014b).

Interestingly, the nonpregnant uterus does not exert any effect on luteal function, since normal ovarian cyclicity is maintained following hysterectomy (Hoffmann et al. 1992). In other words, there is no acute uterine luteolytic mechanism in the absence of pregnancy. Moreover, any luteolytic function of prostaglandins produced by the CL could be ruled out (Hoffmann et al. 1992; Kowalewski et al. 2006b, 2009; Gram et al. 2013). As a result thereof, a physiological pseudopregnancy can be observed, characterized by the aforementioned similar luteal life span in pregnant and nonpregnant animals, until shortly before parturition.

The lack of an active luteolytic principle indicates that luteal regression in dogs is a passive degenerative process and a part of the inherently controlled luteal life span. This apparently biologically preprogrammed aging process allows extension of the luteal phase in nonpregnant dogs beyond the time equivalent to parturition. Consequently, luteal function in pseudopregnant bitches fades out slowly, generating gradually decreasing P4 concentrations. This, following the highest P4 concentrations measured at the mid-luteal phase, can even last as long as 1–3 months. Once peripheral P4 reaches levels below 1 ng/ml, the canine reproductive cycle, per definition, enters anestrus (see review Kowalewski 2012, 2014). In contrast to the actively regulated prepartum luteolysis, which is associated with strong apoptotic signals occurring concomitantly with the prepartum PGF2α increase, during the extended luteal regression only sporadic apoptotic signals can be observed (Hoffmann et al. 2004b; Luz et al. 2006; Kowalewski 2014).

Furthermore, estrogens seem to be entirely of luteal origin, as suggested from the absence of detectable placental aromatase activity (Hoffmann et al. 1994; Nishiyama et al. 1999) and the time-dependent luteal expression of aromatase (Papa and Hoffmann 2011). Displaying variable serum concentrations, the profile of estradiol production during diestrus/pregnancy parallels to some extent that of P4. It increases slightly after the end of estrus, is higher in the mid-luteal phase, and decreases thereafter. Importantly, the mid-diestrus increase is not pregnancy-specific and the rapid prepartum drop, which additionally suggests its ovarian origin (Hoffmann et al. 1994; Onclin et al. 2002), is striking. Similarly striking is the lack of prepartum increase of cortisol. Its erratic presence in the maternal circulation seems not to be mandatory for normal parturition and was linked to maternal stress (Hoffmann et al. 1994). However, as suggested by Concannon and collaborators (Concannon et al. 1978), the cortisol levels observed in the peripheral circulation may not reflect possible increases at the uterine and/or placental levels. Both the estradiol and cortisol secretion patterns further emphasize the differences in mechanisms of endocrine control of canine pregnancy compared with most other domestic animal species.

11.2.2 Morphological Aspects and Regulatory Mechanisms

The exceptionally intense preovulatory luteinization of ovarian follicles in dogs is mirrored in a strong folding of proliferating theca interna layers. Unlike in most other species investigated so far, morphologically and ultrastructurally the canine CL consists of only one steroidogenic cell type. The cellular origin of the canine CL was, until lately, a subject of scientific debate (Concannon 2011; Kowalewski 2014). However, our recent histological documentation of postovulatory follicles revealed the presence of strongly proliferating theca cells associated with vascular structures and separated by remnants of basement membrane from the not yet vascularized luteinizing granulosa cells (Kowalewski et al. 2014a). Nevertheless, further investigations are needed that would elucidate the process of development of the uniform CL cell population.

The early luteal phase, which follows ovulation, is characterized by strong proliferative and vasculogenic activity driven, at least in part, by hypoxia (Papa et al. 2014) and associated with increased infiltration of immune cells (Hoffmann et al. 2004a), cumulatively leading to continuously and rapidly increasing steroidogenic activity. The latter is directly reflected in elevated expression of steroidogenic acute regulatory (STAR) protein and 3-β-hydroxysteroid-dehydrogenase (3βHSD, HSD3B2) (Kowalewski et al. 2006a; Kowalewski and Hoffmann 2008), which is translated into dynamically rising luteal P4 output as discussed above. During this time, the CL is responsible for providing P4 required for facilitating uterine responsiveness for embryo implantation. Around implantation, which in dogs takes place at approximately day 17–18 after mating (Amoroso 1952; Kehrer 1973), the canine CL is already well developed, reaching the mature stage characterized by maximal steroidogenic activity, shortly thereafter, at about day 20–25 after ovulation. Acting at the level of their receptors, P4 and estrogens are among the luteotrophic factors,

and treatment with an antiprogestagen at any time during gestation unequivocally results in luteolysis (Kowalewski et al. 2009). Although both PRL and LH are luteotrophic, with PRL being the predominant luteotrophin as early as day 25 after the preovulatory LH peak, early luteal development appears to be characterized by a period of transitional gonadotropic independence (see review Kowalewski 2014). During this time, locally produced (intra-CL) prostaglandins, and especially PGE2, appear to be among the most important luteotrophic factors (Kowalewski et al. 2006b, 2008a, 2009). The development of the CL is associated with increased PGE2 and low PGF2α luteal content (Kowalewski et al. 2014a).

Recently, in addition to our *in vitro* studies (Kowalewski et al. 2013) with dispersed canine lutein cells, compelling evidence for the luteotrophic function of prostaglandins in canine CL has been provided *in vivo* by applying a selective cyclooxygenase 2 (COX2/PTGS2) inhibitor during early diestrus (Janowski et al. 2014; Kowalewski et al. 2014a). This treatment resulted in significantly decreased levels not only of the respective PGE2-synthase (PTGES)-encoding gene but also in suppression of luteal PGE2 production. The impaired CL function was associated with suppressed STAR expression, clearly indicating the causality between PTGS2 function and PGE2 synthesis in the canine CL (Kowalewski et al. 2014a). Additionally, as indicated by both our *in vivo* and *in vitro* experiments, PGE2 seems to be involved indirectly in the luteotrophic function of PRL by stimulating expression of its receptor (PRLR). This seems to be a new regulatory pathway not previously described for species more dependent than dogs on LH for luteal maintenance, e.g., cattle and pigs (Kowalewski et al. 2014a).

The slowly ongoing luteal regression appears to be a process of both cellular degeneration and structural remodeling. The role of luteotrophic support during this period of the luteal life span seems to be in sustaining CL function, rather than in active stimulation of P4 production, thereby supporting maintenance of canine gestation. The first signs of structural degeneration can be observed ultrastructurally as early as day 30 after ovulation. At this time, the smooth endoplasmic reticulum (sER) exhibits whirl-like structures, moves toward the periphery of the lutein cell, and at approximately day 45 encircles large lipid droplets (Fig. 11.1). This, together with large lipid vacuoles observed all over the cytoplasm, can be seen as a further indication of fatty degeneration (Hoffmann et al. 2004b). Further structural remodeling and proliferation of connective tissue components complete the process of progressive reduction of luteal function and corpus albicans formation.

11.3 Early Embryonic Development

Exact knowledge of oocyte maturation and timing of embryonic development is mandatory for fully understanding reproductive physiology; yet in the bitch the data concerning these events are largely imprecise. This is mostly due to the different and imprecise “starting points” concerning several endocrinological, morphological, and behavioral events related to the time of ovulation, such as the LH surge, peripheral progesterone levels, acceptance of the male, or cytological appearance

(see Holst and Phemister 1971; Archbald et al. 1980; Renton et al. 1991; Bysted et al. 2001; Concannon et al. 2001; Hatoya et al. 2006).

Oocyte maturation, and completion of the first meiotic division, which is uniquely delayed in the dog, takes place 2–3 days after ovulation, i.e., 4–5 days after the preovulatory LH surge (Concannon et al. 1989) (Fig. 11.2). Oocyte maturation in *Canidae* is completed within the oviduct, unlike all other mammals in which this process occurs within the ovarian follicle; this complicates efforts to study fertilization and early embryo development in vitro in the dog.

Moreover, the uterus, and not the oviductal ampulla or isthmus, seems to be the major sperm reservoir in the bitch (Bischoff 1845; Pacey et al. 2000; Rijsselaere et al. 2014). The "fertilization window," i.e., the time period when a bitch can be successfully mated, is relatively long and variable. Thus, mating can occur as early as 5 days before ovulation or as late as 6 days afterward (Fig. 11.2). This is because of the extended life span of intrauterine spermatozoa (up to 7 days, considerably longer than in most other domestic species) and of oviductal oocytes (7–8 days, including up to 5 days, which follow the delayed oocyte maturation) (Bischoff 1845; Concannon 2009). Also, embryonic development in dogs is slow. It takes longer for embryos to reach the uterotubal junction and enter the tip of uterine horns, 7–10 days following fertilization, compared to 3–4 days in other species (Holst and Phemister 1971; Concannon 2009), at this time being as early as the 16 cell stage, but usually morulae or early blastocysts (Holst and Phemister 1971). This agrees with the earliest descriptions by Bischoff (1845), who never observed the uterine presence of embryos before day 8 after mating.

The subsequent intrauterine transcornual migration and distribution of embryos take up to 9–10 days (Shimizu et al. 1990; Bysted et al. 2001) and are not influenced by the number of oocytes ovulated from either of the ovaries (Tsutsui et al. 2002). Blastocysts can still be enclosed by the zona pellucida as late as day 19 after the LH surge (around 14 days after oocyte maturation/fertilization) (Concannon et al. 2001). The apposition of blastocysts to the uterine epithelium can, however, already be observed as early as between days 12 and 14 after oocyte maturation (fertilization), and the associated uterine swellings, implantation, and, shortly afterward, invasion, normally occur by days 17–18 after fertilization (Amoroso 1952; Kehrer 1973). The latter, taking place at the end of the first third of gestation, is rather late considering the entire length of canine pregnancy (Fig. 11.2).

11.4 Role of Progesterone in Implantation and Placentation in Dogs

By regulating the secretory activity of the endometrium and stimulating expression of genes involved in uterine receptivity both in cyclic and pregnant animals, P4, at least in part, indirectly regulates embryonic development by maintaining the uterus in a state of physiological receptivity for the conceptus (see review Dorniak and Spencer 2013). By expressing the so-called "decidualization markers", P4 is

responsible for the morphological remodeling processes of the endometrium during decidua formation. Adequate levels of circulating P4 are essential for successful establishment of early pregnancy and, if too low, can lead to embryo loss in some species, e.g., in cattle (Mann and Lamming 2001; Lonergan 2011). Other than that, the canine uterus is normally exposed to P4 levels, which by far exceed those required for establishment and maintenance of pregnancy. As reported by Concannon (Concannon et al. 2001; Concannon 2009), exogenous supplementation with P4 in bitches that were ovariectomized as early as at day 14 after the LH surge maintained pregnancy even though serum concentrations of this hormone were chronically below 5 ng/ml; the PRL concentrations following implantation did not differ from the levels expected during normal pregnancy (Concannon et al. 2001). Therefore, based on this study, it has been concluded that P4 is the predominant, if not the only, luteal steroid needed for implantation and placentation in previously estrogenized bitches. Unlike in humans and several domestic animal species, and as already indicated above, there is no large increase in estrogens during canine pregnancy, and levels observed in the second half of gestation never exceed those observed shortly before ovulation (Concannon et al. 2001). The use of natural P4 in these experiments precludes any possible estrogenic effect exerted by synthetic hormones. However, it should be noted that the synthetic capabilities of canine embryos toward conversion of progesterone or androgens to estrogens are not known.

11.5 Preimplantation Embryo-Maternal Communication

Being the only domestic animal species devoid of an active luteolytic principle in the absence of pregnancy, and therefore exhibiting similar hormonal profiles during early pregnancy and pseudopregnancy, the dog appears to lack an important regulatory mechanism that would facilitate reproductive events and allow for faster procreation, i.e., by shortening the nonpregnant cycle. Needless to say, this situation strongly contrasts with what is observed in livestock, in which cyclicity is maintained due to periodic secretion of a uterine luteolysin, i.e., PGF2α. Consequently (reviewed elsewhere), to allow successful establishment of pregnancy, different strategies for the maternal recognition of pregnancy have evolved to prevent luteolysis and preserve endogenous progesterone concentrations by extending the luteal life span. Briefly, in ruminants this task is fulfilled by IFN-τ produced by the early embryo (Bazer et al. 1991; Spencer and Bazer 2004). Being produced between days 10 and 21–25, its function, among others, is to suppress PGF2α production by inhibiting ERα and oxytocin receptor (OXTR) expression. In pigs, the trophoblast produces estrogens, which are responsible for redirection of uterine PGF2α secretion from endocrine to exocrine secretion and thus prevent luteolysis, which normally occurs on days 15–16 of the estrous cycle (Bazer and Thatcher 1977; Spencer and Bazer 2004). This switch from endocrine to exocrine secretion starts with embryonic estrogen production between days 10 and 12 (Bazer 1989). Nevertheless, in the dog, as in these other species, there must be some kind of synchronization between

blastocyst development and uterine preparation for pregnancy in order to support gestation, even though its mechanism is not through suppressing luteolysis.

Some earlier studies addressed the biochemical and endocrine milieu characteristic of early canine gestation in attempts to find new early pregnancy detection markers. A major change in the circulating levels of acute phase proteins was found on days 28–37 after fertilization in the blood of pregnant animals, but not in corresponding healthy nonpregnant bitches (Evans and Anderton 1992). Similarly, concomitantly with increased levels of relaxin, strongly elevated concentrations of fibrinogen and serum C-reactive protein-like (CRP-LI) were found in pregnant dogs between days 21 and 30 after the LH surge and maintained until day 50 (Eckersall et al. 1993; Concannon et al. 1996). However, acting as mediators of inflammation, these acute phase proteins may be triggered by infections or trauma. Thus, the rise observed at pregnancy cannot be seen as pregnancy-specific, since it is apparent that dogs suffering from some infections could be falsely classified as being pregnant (Evans and Anderton 1992).

At the uterine level, efforts were undertaken to characterize secretion of proteins synthesized during pregnancy. Uteri collected from dogs during early nonpregnant diestrus were compared with their counterparts obtained from dogs prior to embryos traversing the uterotubal junction and during their free-floating phase prior to implantation. Two major protein complexes, designated as cP5 and cP6, were detected as differentially expressed; one of them (cP6) proved highly similar to retinol binding proteins (Buhi et al. 1992, 1993, 1995). While their secretion differed by day, it did not differ by status, i.e., in pregnant vs. nonpregnant dogs.

More recently, some insight into immunological processes possibly involved in regulating embryo-maternal contact was provided by investigating the expression of several cytokines and growth factors in the early pregnant, preimplantation uterus (day 10 of gestation), and comparing their expression in corresponding nonpregnant uteri. Among the most interesting findings was the expression of CD8, IL4, and IFNγ mRNA, which seemed to be targeted to the preimplantation uterus, whereas the expression of CD4, TNFα, and IL6 was found abundantly in the nonpregnant uterus (Schafer-Somi et al. 2008). Additionally, in the same study, transcripts encoding for TGFβ, IL2, IL10, and LIF were only detected in the early pregnant uterus. In another study from the same group, the expression of Fas ligand (FasL) and its receptor (Fas) mRNA did not differ between early pregnant and nonpregnant uteri (Schafer-Somi et al. 2012). On the other side, the 10-day-old embryos tested positively for several factors, such as GM-CSF, IL1β, IL6, IL8, LIF, and CD4 (Schafer-Somi et al. 2008) (Fig. 11.3). Although most of these data were generated utilizing a qualitative transcriptional approach and thus require further confirmation, they clearly indicate differential, possibly embryo-mediated, regulation of uterine function and modulation of the uterine immune response in order to avoid embryo rejection and facilitate implantation, which is known to occur in other species.

In addition to these earlier studies, lately we have investigated the uterine expression of several target genes potentially involved in the process of early decidualization (the so-called “decidualization markers”) (Kautz et al. 2014). The uterine response to the presence of free-floating embryos during the preimplantation stage

of pregnancy (days 10–12 of gestation, determined by uterine flushings) was investigated. The majority of embryos, 63 %, were hatched blastocysts while the remaining 37 % were unhatched blastocysts. Dogs inseminated but determined as nonpregnant in uterine flushing were used as controls in these studies. The effects of seminal plasma alone were not separately investigated.

Being implicated in various cellular activities like proliferation and differentiation, insulin-like growth factors 1 and 2 (IGF1 and IGF2) and PRL are among the most prominent decidualization markers (Irwin et al. 1994; Ramathal et al. 2010). Functionally, the mitogenic activity of IGF1 and IGF2 is mediated mainly through their type 1 receptor (IGF1R) (see review Yu et al. 2011). As for the canine uterus, increased preimplantation expression of IGF2, but not of IGF1, was found. While it was not affected at the level of transcript expression, IGF1R was clearly detectable and abundantly expressed at the protein level during early pregnancy. Similarly, at

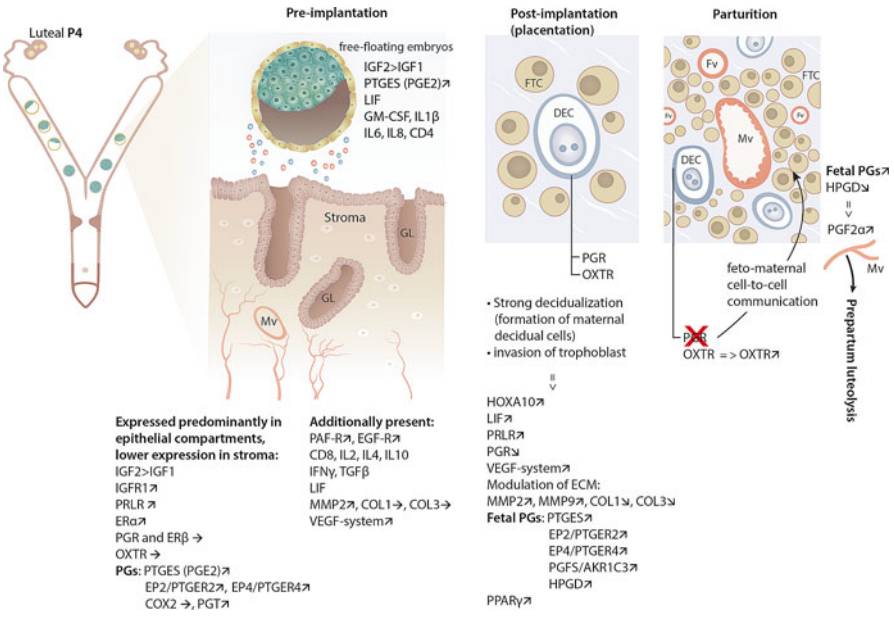


Fig. 11.3 Schematic representation of signals participating in embryo- and feto-maternal communication in the dog and underlying effects exerted on the pregnant canine uterus. Preimplantation, effects exerted by free-floating embryos; *arrows* indicate uterine expression of different factors compared with their expression levels in the nonpregnant uterus. Post-implantation and at parturition, schematic pictures represent fragments of the canine endotheliochorial placenta; maternal decidual cells (*DEC*) are present in a direct contact with fetal trophoblast cells (*FTC*). DEC are the only cells expressing progesterone receptor (*PGR*) within the canine placenta. Following implantation, utero-placental expression of several regulatory factors is indicated. The role of decidual cells in the maintenance of pregnancy and their involvement in the endocrine cascade within the canine placenta resulting in prepartum output of luteolytic PGF2α are presented. Thus, blocking PGR function in the placenta materna (decidual cells) leads to activation of fetal PGs synthesis. A detailed explanation is provided in the text. *Mv* maternal blood vessel, *GL* endometrial glands, *Fv* fetal vessels, *PGs* prostaglandins

the same time, IGF2 expression was higher than that of IGF1 in the hatched embryos, suggesting a possible predominant role of IGF2 during the establishment of early canine pregnancy. Both IGF1 and IGF2 were below the detection limits in the unhatched embryos (Kautz et al. 2014). The presence of early preimplantation embryos was also associated with increased expression of receptors for two other important growth factors, i.e., platelet-activating factor (PAF) and epidermal growth factor (EGF) (Schafer-Somi et al. 2013) (Fig. 11.3).

Interestingly, in contrast to rodents and primates (Prigent-Tessier et al. 1999; Tseng and Mazella 1999) in which PRL is one of the most prominent decidualization markers involved, e.g., in regulating endometrial glandular secretory activity (Jabbour et al. 1998), the canine uterus does not exhibit a strong capacity to express PRL. Its expression at the transcript level was low and frequently below the detection limit in our studies. Thus, the uterus does not seem to contribute strongly to the overall circulating PRL in the dog. However, the highly upregulated PRLR expression (Kowalewski et al. 2011b; Kautz et al. 2014) could serve to compensate for low PRL expression and thereby to locally increase its relative availability. The expression of LH receptor, which was recently suggested to be involved in implantation in mice (Gridelet et al. 2013), was significantly reduced in the early pregnant canine uterus (Kautz et al. 2014).

As for the steroidogenic hormone receptors, ERα was significantly upregulated in early pregnant canine uteri compared with nonpregnant diestrus controls, while ERβ and progesterone receptor (PGR) remained unaffected (Kautz et al. 2014). Similarly to the situation with PRL, the increased expression of ERα could possibly counteract the low availability of estradiol of luteal origin during canine pregnancy (Fig. 11.3). The oxytocin receptor (OXTR) could be involved in mechanisms regulating the distribution and positioning of free-floating embryos before attachment and is a known mediator of local prostaglandin effects. As for the canine uterus, its uterine expression varied greatly between early pregnant and nonpregnant bitches, so no further conclusions about its role could be drawn (Gram et al. 2014a; Kautz et al. 2014). It is noteworthy that, throughout the above-cited studies (Kautz et al. 2014), stronger uterine signals for the protein expression of the respective genes (IGF1, IGF2, IGFR1, ERα, ERβ, PGR, OXTR) were observed in the endometrial epithelial compartments, i.e., in luminal and glandular epithelium, compared with the weaker signals found in the uterine stromal cells. This was interpreted as an indication of the early stage of uterine differentiation observed at the beginning of pregnancy (preimplantation stage), which is further supported by the unaltered expression of E-cadherin (CDH1) (Kautz et al. 2014), a cell adhesion protein known for controlling migratory activity in different cell types.

11.5.1 Prostaglandins

Concerning the prostaglandin family members, our attention was especially drawn to the increased expression of prostaglandin transporter (PGT) and of PTGES and its two G protein-coupled receptors, PTGER2 and PTGER4, observed in the early

pregnant canine uterus (Kautz et al. 2014) (Fig. 11.3). This interest has been further strengthened in our recent studies on the biological function and expression of PTGES in uterine and placental tissues throughout canine gestation (Gram et al. 2014b). Based on its subcellular localization bound to the endoplasmic reticulum, the only currently available canine PTGES, which was cloned in our laboratory (GenBank: NM_001122854; Kowalewski et al. 2008a), appears to be a microsomal protein corresponding to the inducible microsomal isoform known from other species (Gram et al. 2014b). In addition, as presented above, being one of the most important luteotrophic factors in the dog, acting mostly through the cAMP/PKA signaling pathway, PGE2 was shown to accelerate decidualization mediated by P4 and cAMP (Brar et al. 1997). Similar effects were observed in rats (Kennedy and Doktorcik 1988). Furthermore, by inhibiting IL2 and expression of its receptor in human decidua, PGE2 was shown to block activation of maternal leukocytes with potential anti-trophoblast function, thereby revealing an immunosuppressive but embryo-protective effect (Parhar et al. 1989). The embryo- and luteo-protective role of endometrial PGE2 has been discussed for pigs, in which additionally, species-specifically, estrogen also increases PGE2 secretion (see review Ziecik et al. 2011). Similar effects could also apply to the canine species as suggested by the concomitantly and abundantly expressed PTGES levels in hatched compared to unhatched preimplantation embryos (Kautz et al. 2014). Together with the strongly increased PTGES expression in the course of cAMP-induced decidualization of canine uterine stromal cells isolated from early dioestrus(own data, unpublished), there is emerging evidence identifying PGE2 as an important factor involved in canine decidualization. This effect could be amplified by the local PGE2 feedback loop since PTGER2 and PTGER4 are known mediators of cAMP/PKA pathway-coupled effects. The local effects of prostaglandins could be additionally coordinated by the clearly detectable expression of endometrial HPGD (15-hydroxyprostaglandin dehydrogenase) that is responsible for biochemical deactivation of prostaglandins (Gram et al. 2013; Kautz et al. 2014). Finally, as also observed in our previous experiments (Kautz et al. 2014), together with IGF2, the expression of PTGES was abundant in hatched embryos flushed from preimplantation canine uteri, indicating a possible functional interplay between these two entities as embryo-derived factors regulating canine decidualization (Fig. 11.3).

11.5.2 Structural Remodeling

Remodeling of uterine matrix is essential for preparation of invasive growth of the cytotrophoblast, especially when the highly invasive canine placentation type is considered. As an integral part of it, extracellular matrix proteins (ECM), which are secreted by the decidualizing uterus in response to the changing uterine milieu and which interact with other components of the uterine environment, are critical players during implantation and placentation. Several processes important for cell adhesion, migration, and differentiation are mediated by these components, such as integrins, e.g., aberrant expression of some of the integrin family members is

associated with infertility and recurrent pregnancy loss (Lessey et al. 1995; Liu et al. 2012).

Information concerning the nature and composition of ECM proteins in the canine uterus is, however, still lacking. The only available data indicate an upregulated messenger expression encoding for integrins-α2b, −β2, and −β3, as well as higher uterine activity of matrix metalloproteinases, especially MMP2, in response to free-floating embryos (Beceriklisoy et al. 2007; Bukowska et al. 2011). The significantly elevated activity of uterine MMPs positively correlates with the activity of both MMP2 and MMP9 in blood serum, which is higher than in nonpregnant animals (Schafer-Somi et al. 2005). These metalloproteinases were predominantly localized in vascular endothelial and smooth muscle cells, myometrium, and glandular epithelium. In our recent study, which is ongoing, we have characterized the uterine expression and distribution patterns of collagens (COL) 1, −3, and −4 as major components of the uterine stromal ECM. While modulated throughout gestation, their expression did not change significantly in response to early, free-floating preimplantation embryos. Interestingly, this was in contrast to the significantly increased expression of extracellular matrix protein 1 (ECM1) and decreased expression of fibronectin 1 (FN1) in embryo-exposed uteri(own data, unpublished). Moreover, as a part of induced structural remodeling, increased uterine vascularization was indicated by elevated expression of vascular endothelial growth factors (VEGF) −165, −182, and −188 and one of its receptors, particularly VEGFR-2 (Bukowska et al. 2011; Schafer-Somi et al. 2013).

11.5.3 Perspectives

It needs to be emphasized that all of the aforementioned studies (Schafer-Somi et al. 2008, 2012, 2013; Bukowska et al. 2011; Gram et al. 2013; Gram et al. 2014a, b; Kautz et al. 2014) describe changes in the uterine response to free-floating canine embryos between days 10 and 12 of gestation. This corresponds to pregnancy stages in other species, i.e., ruminants and pigs, when the embryonic antiluteolytic signals are initiated. Thus, it appears plausible that expanding our knowledge about canine pregnancy can bring about a better general understanding of embryonic signals, which perhaps diverged early on in dogs during evolution, that modify the uterine milieu and serve to support embryo survival and successful establishment of pregnancy.

In line with this, in our ongoing study, microarray DNA analysis of genes differentially expressed in the canine preimplantation uterus was performed. Global transcriptome analysis using a custom-designed Agilent microarray revealed 433 differentially expressed genes (DEG) (false recovery rate 10 %) between the two groups (early pregnant vs. nonpregnant), 332 of which were upregulated and 101 downregulated (Kowalewski MP, unpublished data). Several functional terms specifically overrepresented for either group of genes (up- or downregulated) were identified by bioinformatics analysis. The strongest overrepresentation was found for functional terms related to inflammatory response, ECM, cell signaling, positive regulation of cell motion, and cell migration. Among the downregulated genes,

higher functional variation was noted, resulting in fewer functional terms obtained, e.g., focal adhesion or ECM-receptor interaction. Among the genes significantly upregulated were indoleamine-2,3-dioxygenase (IDO), allograft inflammatory factor 1 (AIF1), chemokine ligand 16 (CXCL16), chemokine receptors 6 and 7 (CXCR6 and CXCR7), liver X receptor (LXR), and prostaglandin D receptor (PTGDR), whereas expression of the gene encoding for pappalysin-2 (PAPPA2) was downregulated during early pregnancy. The latter is a metalloproteinase known for local regulation of IGF bioavailability. These comprehensive transcriptome changes characteristic of the early pregnant canine uterus identified in our study provided us with a valuable resource for targeted studies related to, e.g., the morphological, biochemical, and immunological remodeling processes of uterine tissues.

11.6 Embryo-Maternal Communication during Implantation and Placentation

Implantation and uterine receptivity to blastocysts in the dog are associated with increased mRNA expression of HOXA10 and LIF (Guo et al. 2009; Schafer-Somi et al. 2009b), well-recognized factors pertinent to implantation and both involved in uterine preparation and the attachment reaction. Whereas the epithelial expression of HOXA10 mediates its roles during uterine receptivity, being expressed in stromal cells, it is functionally involved in the decidualization process (see review Zhang et al. 2013). Their increased expression during decidua formation and placentation in bitches was associated with high MMP activities (Beceriklisoy et al. 2007), participating in structural remodeling processes and facilitating trophoblast invasion. This seems to involve modulation of the composition of stromal collagens, as observed in our ongoing study(Kowalewski MP, unpublished data). Thus, the expression of COL1 and COL3 was lower at the placentation sites when compared with their expression at interplacental sites, i.e., parts of the uterine wall not attached to the placenta. Whereas COL1 represents the tougher type of collagen, COL3 is characteristic of reticular types of fibers, indicating the proliferative activity of fibroblastic tissues. The association of COL3 with proliferative events could explain its generally higher expression at interplacental sites during the second half of gestation (Kowalewski MP, unpublished data).

As expected, decidua formation and placentation were associated with increased vascularization as indicated by elevated expression of the VEGF system (Schafer-Somi et al. 2013) and some of the components of the endothelin system, such as endothelin receptor B (ETB), known to be a strong vasodilator (Kowalewski MP, unpublished data). The local immunomodulation was characterized by elevated MHCII levels (Schafer-Somi et al. 2009a).

Following placentation, a further increase in PRLR was noticed and appeared to be specific to the invasion sites, as concluded from its lower expression at interplacental sites (Kowalewski et al. 2011b). In addition to the epithelial components of

uterine structures, it was localized in trophoblast cells. Besides involvement in uterine secretory activity, only recently PRL was shown to stimulate migration and invasion of human trophoblasts in vitro (Stefanoska et al. 2013). This could also apply to the canine species.

Concerning the prostaglandin system, PTGES maintained its clearly detectable expression as observed prior to implantation (Kowalewski et al. 2010; Gram et al. 2014b). Interestingly, concomitantly, the uterine PGF2α synthase (PGFS/AKR1C3) was strongly induced. Belonging to the aldo-keto reductases family of enzymes, PGFS/AKR1C is the only canine-specific PGF2α synthase known so far (GenBank: NM_001012344; Kowalewski et al. 2008b) and is responsible for the direct conversion of PGH2 to PGF2α (Gram et al. 2013). At placental sites, both synthases, together with their respective receptors (EP2/PTGER2, EP4/PTGER4 and FP), were colocalized in the uterine glands and in invading trophoblast cells (Kowalewski et al. 2010; Gram et al. 2013, 2014b). Interestingly, their expression in placental compartments was associated with the increased presence of peroxisome proliferator-activated receptor gamma (PPARγ) in fetal trophoblasts (Kowalewski et al. 2011a). Together with PGR and estrogen receptors, PPARγ is a nuclear hormone receptor. It can act as an endogenous receptor for various factors such as cyclooxygenase-, lipooxygenase-, or epoxygenase-derived metabolites of arachidonic acid (see review Komar 2005). Among these, PPARγ can act as an alternative receptor for prostaglandins. PPARγ was shown to modulate the biochemical and morphological differentiation of trophoblast in, e.g., human, rat, and mouse placentas (Barak et al. 1999; Schaiff et al. 2000; Wang et al. 2002). Thus, cumulatively, these data clearly implicate the potential role of fetal prostaglandins during placental development and trophoblast invasion in the dog. Locally, i.e., in the placenta, the activity of prostaglandins could be regulated at the level of increased HPGD and decreased PTGS2 availability, explaining their low observed peripheral levels, especially at the time of highly increased PGFS/AKR1C3 expression (Gram et al. 2013). Importantly, placental HPGD was shown to be a P4-responsive gene in humans (Patel et al. 1999).

11.6.1 Decidual Cells and the Role of Placental Feto-Maternal Communication in Pregnancy Maintenance

The intense invasion of trophoblast cells observed on the way to formation of the canine endotheliochorial placenta is associated with a strong decidualization process. This leads to the formation of highly specialized maternally derived decidual cells, which are the only cells of the canine placenta expressing progesterone receptor (Vermeirsch et al. 2000; Kowalewski et al. 2010). Together with the maternal vascular endothelial cells, decidual cells are able to resist the strong invasiveness and MMP-mediated proteolytic activity of the fetal trophoblast.

As in other species, following implantation, the continuous exposure of the endometrium to P4 seems to result in suppression of PGR expression, as suggested

from its decreased expression both at the placentation sites and in the interplacental compartments following implantation (Schafer-Somi et al. 2009b; Kowalewski et al. 2010, 2014b). Although decreased, its expression and function in decidual cells have a pivotal role in the regulation of utero-placental function in dogs. Thus, altering PGR function, e.g., by using a selective blocker, results in activation of utero-placental prostaglandin synthesis. This, within the placenta through upregulated fetal PTGS2 expression, leads to the prepartum PGF2α release (Kowalewski et al. 2010). The mediating role of oxytocin in this process is suggested by the decidual cell-targeted expression of its receptor, OXTR, positively responding to the suppression of PGR function rather than expression, which was not altered in normal and antiprogestagen-induced parturition (Kowalewski et al. 2010; Gram et al. 2014a) (Fig. 11.3). Therefore, in dogs the underlying feto-maternal communication at the level of maternal decidual cells and fetal trophoblast seems to play an important signaling function, both during the maintenance of pregnancy and in the process of prepartum release of placental PGF2α (Fig. 11.3).

11.7 Conclusions

Many of the herein presented evolutionarily determined features of the species-specific physiological mechanisms governing reproductive function in the domestic dog appear unusual. Among these, certainly, the lack of a classical maternal pregnancy recognition signal is one of the most interesting. On the other hand, many of the regulatory processes observed at the cellular level are not unlike those in other species, e.g., the progesterone-dependent structural and functional transformation of the uterus. Apparently, early preimplantation canine embryos are already capable of transmitting signals to the uterus, further modulating its biochemical and endocrinological milieu. These signals, not being directed toward suppression of endocrine PGF2α function to avoid luteolysis, primarily induce proliferative, secretory, and immunomodulatory effects. The embryo-induced structural changes of uterine tissues do not seem to be strongly pronounced at this early stage of pregnancy. Cumulatively, however, the resulting functional remodeling of the uterus serves to facilitate apposition, implantation, and the subsequent more intimate physical embryo-maternal contact during placentation and trophoblast invasion.

Nevertheless, canine uterine physiology is far from being well understood. Better understanding of underlying mechanisms and the functional interplay between different regulatory pathways could provide a basis for better understanding of mechanisms involved in the etiopathogenesis of some frequently occurring diestrual uterine disorders resulting from dysregulated endocrine responses to hormonal stimuli, such as the cystic endometrial hyperplasia-pyometra complex. The knowledge acquired regarding the molecular mechanisms of canine decidualization, e.g., those that are PGE2-dependent, could be useful in developing new contraceptive strategies involving disruption of the decidualization process as an alternative to surgical spaying of bitches, which can result in long-term negative effects. It could

also serve to improve the poor outcome of IVF procedures in canids, which may result from an inappropriate environment for oocyte maturation using protocols derived from other research animal models. Finally, unveiling the mechanisms, regulating placental feto-maternal communication at the end of canine gestation, and being responsible for induction of parturition would offer new possibilities for developing therapeutic strategies to improve breeding management and, thereby, the well-being of our small animal patients.

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Chapter 12
Embryonic Diapause and Maternal Recognition of Pregnancy in Diapausing Mammals

Marilyn B. Renfree

Abstract The dynamic nature of early embryonic growth is at odds with the phenomenon of mammalian embryonic diapause, because embryos in diapause are in a state of suspended animation of varying duration. The signals that control embryonic diapause differ between species, but in all cases, it acts to synchronise reproduction with external factors to maximise the survival of the offspring.

This chapter provides an overview of current understanding of the control of embryonic diapause, with an emphasis on the three species about which most is known, namely, the mouse, the mink and the tammar wallaby.

12.1 Introduction

In his perceptive introduction to the symposium on Maternal Recognition of Pregnancy, Heap (1979) commented that it would be a mistake to assume that a form of maternal recognition of pregnancy is absent in non-mammalian vertebrates (and by extension of his argument, in marsupials) and further asked whether the mother recognises the presence of an embryo during delay (or embryonic diapause) or whether the embryo withholds evidence of its existence during diapause. The answers to some of these questions are now at hand, although there is still much to learn about the fine details of the control of embryonic diapause.

Embryonic diapause is a period of suspended animation of the mammalian embryo at the blastocyst stage. Importantly, the uterus is also quiescent, and it is clear that there is a great deal of crosstalk between blastocyst at the entry into diapause, the maintenance of diapause and the reactivation from diapause. Successful implantation can only occur during the so called "window of implantation" (Ma

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et al. 2003). Approximately 130 mammalian species have diapause, of which 38 are marsupial, but the morphological and molecular changes that take place during the onset and reactivation from diapause have been examined in very few (Fenelon et al. 2014a; Renfree and Calaby 1981). Rodents, especially the mouse, have been the primary model, but two other species, namely, the mink *Mustela vison* and the tammar wallaby *Macropus eugenii* have been extensively studied (Cha and Dey 2014; Dey and Lim 2006; Dey et al. 2004; Fenelon et al. 2014a; Lopes et al. 2006; Mead 1993; Murphy 2012a, b; Renfree and Shaw 2000, 2014; Tyndale-Biscoe and Renfree 1987). Interestingly, the first species in which diapause was recognised was the roe deer, *Capreolus capreolus*, but it is the only confirmed ungulate with diapause although there have been suggestions that Pere David's deer (*Elaphurus davidianus*) also may have a period of diapause (Brinklow and Loudon 1993). Diapause is widespread in carnivores and pinnipeds, more limited in the bats, but they have additional reproductive strategies including delayed fertilisation and delayed development (Badwaik and Rasweiler 2001; Rasweiler and Badwaik 1997; Wimsatt 1975). Only one or two species of edentates have diapause, but few have been fully studied (Renfree and Calaby 1981). Only one subfamily of shrews and one species of mole use diapause so it is also uncommon in insectivores.

Diapause is controlled by seasonal or lactational signals, or both within a species. In diapausing mammals, the entry into diapause is controlled by either a change in day length, the presence of sucking young or the availability of nutrition. Traditionally, diapause has been divided into obligate or facultative states, but as we learn more about the similarities of the molecular controls at the uterine-blastocyst interface, these terms create an artificial separation of the environmental, physiological and metabolic signals that occur across mammals. The endocrinology underlying lactational and seasonal diapause is well understood in several species (Cha et al. 2012, 2013; Fenelon et al. 2014b; Murphy 2012a, b; Renfree and Shaw 2000, 2014), but the precise details of the molecular changes that occur in the uterus and blastocyst are still being discovered. Regardless, it is the corpus luteum that is central to the control of diapause, and it is the mediator of the external signals that hold the embryo in quiescence. This review will not repeat the wealth of information in these many excellent reviews but will endeavour to highlight some of the advances in our understanding.

12.2 Entry into Diapause and Endocrine Control

After fertilization, conceptuses of diapausing mammals reach the blastocyst stage before entering diapause. In some, like the roe deer (Aitken 1981), there is a continued slow growth of both corpus luteum and blastocyst throughout the period of diapause, but in most, there is a complete cessation of growth and cell division. In the western spotted skunk, *Spilogale putorius latifrons*, the blastocyst slowly increases in size a few days before activation (Enders et al. 1986; Mead 1981). In the tammar, there are two controlling switches for diapause entry. In the first half

of the year, a month after the December summer solstice, the process begins with birth, a post-partum estrus and cleavage of the conceptus until it reaches the blastocyst stage whereupon it enters diapause so long as there is a sucking young present in the pouch. If the pouch young is lost before the winter solstice in June, the blastocyst reactivates. However, later in the year, only photoperiod is sufficient to cause reactivation, and in the wild animal, this does not happen until December and after the longest day. On or about December 22, the eyes and the pineal gland sense the change to shortening days, and the changed melatonin secretion is sufficient to precisely reactivate all the animals carrying blastocysts (Tyndale-Biscoe and Renfree 1987), with births about 1 month later beginning the cycle again. Photoperiod also controls diapause in the mink, but lactation does not (Lopes et al. 2004). In the tammar, both sucking and decreasing photoperiod result in elevated prolactin which inhibits the corpus luteum (so it is luteostatic), whilst in the mink, the elevated prolactin, as a result of lengthening days, has a luteotrophic effect (Fig. 12.1).

Entry into diapause was long debated as being caused by the release of an inhibitor (e.g. Spindler et al. 1999; Weitlauf 1994) because blastocysts transferred to quiescent uteri themselves entered quiescence, but there remains no direct evidence of this. In contrast, it appears that it is more likely to be due to the absence of a stimulator (Spindler et al. 1999; Thornber et al. 1981). The cannabinoid anandamide has been suggested as a negative regulator as it is downregulated in the mouse uterus during reactivation (Wang et al. 2003).

Both the tammar and mink blastocyst are surrounded by multiple acellular layers. The tammar does not implant at all, but the placenta attaches to the uterine epithelium at around 18 days after reactivation, and the mink embryo does not implant until some days after reactivation, so the uterine secretions are the only means of transferring signals controlling diapause (Fenelon et al. 2014b; Murphy 2012a, b; Renfree 1973; Renfree and Shaw 2000, 2014; Shaw and Renfree 1986). Due to the importance of this, recent studies have focussed on the analysis of the uterine secretions before, during and after diapause.

12.3 Maintenance of Diapause

Diapause is maintained by a variety of mechanisms. With its 11-month suspension of development, the tammar wallaby has the longest diapause of any mammal so far described but remarkably that can be extended even further. Exogenous progesterone reactivates diapausing blastocysts (Renfree and Tyndale-Biscoe 1973), and when given to ovariectomised animals 2 years after the removal of the ovaries, the blastocysts reactivate (Tyndale-Biscoe and Hearn 1981). This must be amongst the longest time a mammalian blastocyst has remained viable. The fisher, *Martes pennanti*, also has a post-partum estrus and a long period of delay of 9 months (Frost et al. 2005; Mead 1993), and the badger blastocyst is in diapause for 10 months (Canivenc and Bonnin 1980).

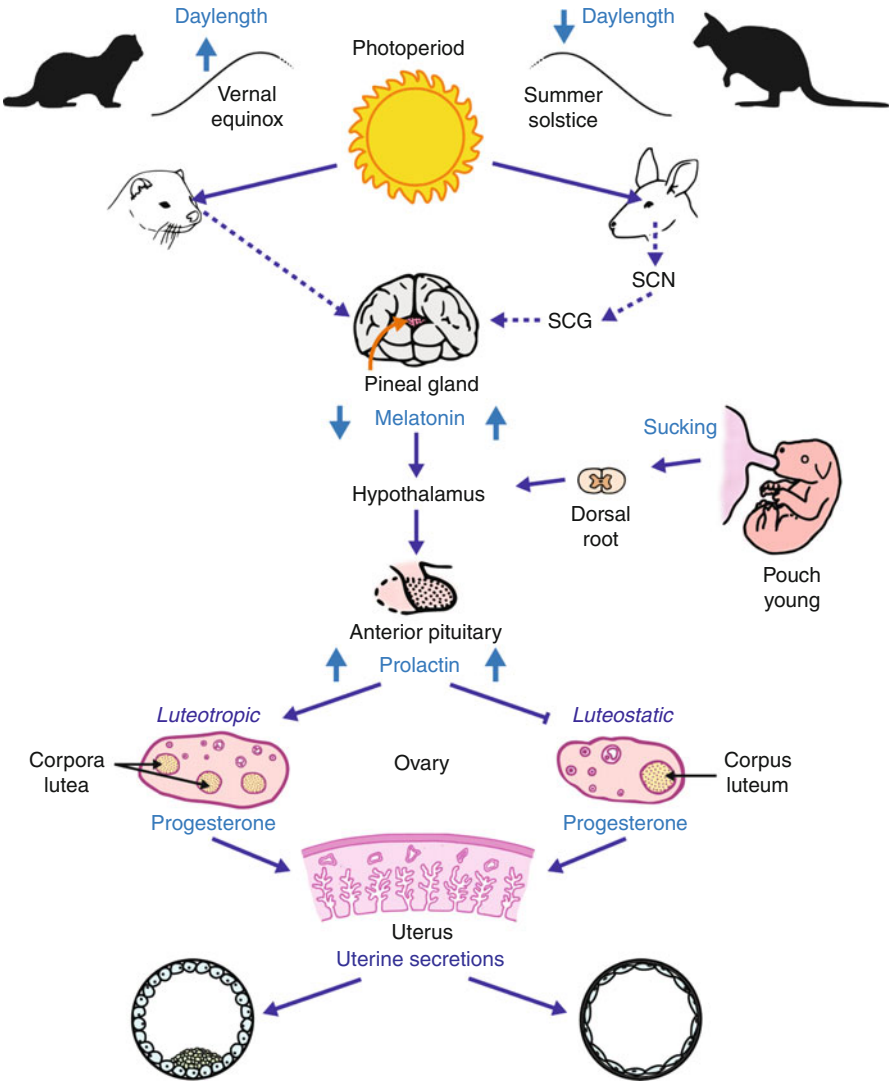


Fig. 12.1 Photoperiodic and lactational control of diapause in the mink (LHS) and tammar (RHS). In the tammar, the sucking stimulus upregulates prolactin, inhibiting the corpus luteum, initiating and maintaining diapause in the first half of the year. In the second half of the year, photoperiod mediated via melatonin maintains the diapause in response to the duration of melatonin secretion. After the summer solstice (December 22 in the southern hemisphere), the increasing night length reactivates the blastocyst remarkably synchronously and births occur at the end of January. In the mink, photoperiod associated with the spring equinox decreases melatonin secretion and releases prolactin from inhibition. Increased prolactin activates the corpora lutea, which releases progesterone (and other factors) that terminate diapause. In both cases, the growth factors and cytokines in the uterine secretions are highly conserved and mediate the crosstalk between blastocyst and uterine epithelium. *SCN* suprachiasmatic nucleus, *SCG* superior cervical ganglion

In mice and rats, the period of delay is quite short and variable depending on the number of sucking pups. Prolactin is also an important regulator: when rats are treated with the dopamine agonist, bromocriptine, diapause is inhibited and premature implantation occurs, suggesting that lactation-induced hyperprolactinaemia is responsible for the maintenance of delay (Flint and Renfree 1982). A single intramuscular injection of bromocriptine results in reactivation of the diapausing blastocyst in the tammar (Tyndale-Biscoe 1979). However, although both depend on prolactin, it has opposite effects: in rats, prolactin stimulates progesterone secretion by the corpus luteum during diapause, whereas in the tammar, removal of prolactin allows the corpus luteum escape from its luteostatic inhibition.

During diapause, there are no obvious metabolic changes, no cell division and in most no increase in size. It appears that blastocysts are arrested in G1 phase of the cell cycle, but it is not possible to distinguish between the G0 and G1 phase on DNA content (Surani 1975). Quiescent cells have therefore not been characterised in diapausing embryos for stage of the cell cycle but a new technique describes the potential to measure this using a fusion protein (consisting of mVenus and a mutant cyclin-dependent kinase inhibitor). It will be of interest to apply this to the cells of mammalian diapausing blastocyst cells as it is more likely that they are held in G0.

12.4 Reactivation and Molecular Control of Embryonic Diapause

In most diapausing species including the mouse, estrogen is the nidatory stimulus that causes an increase in uterine secretory activity. However, both progesterone and estrogen are required to stimulate endometrial proliferation and the production of various cytokines that can have both autocrine and paracrine actions to regulate the preimplantation embryo and prepare the endometrium for implantation (Sharkey 1998). In marsupials, or at least in macropodids (the kangaroos and wallabies), progesterone alone is needed to reactivate the dormant blastocyst (Renfree and Shaw 2000). In the tammar and the mink, once progesterone begins to be secreted again, the uterus becomes secretory and the blastocyst resumes development (Fig. 12.1). This is in contrast to the mouse, which requires an estrogen surge for reactivation to occur.

Once the reactivation signal has been received, mitoses increase in mice within 12 h at a time that pyruvate is the main energy source, but after 16 h, glucose is the main energy source (Spindler et al. 1996). In the tammar after reactivation, the process is slower, and there is no significant increase in carbohydrate uptake or production for the first 5 days before a switch to lactate production occurs (Spindler et al. 1995, 1998). In the tammar, RNA synthesis also increases by day 5 (Spindler et al. 1998, 1999).

There is a growing list of known growth factors and cytokines present in the uterus, and all have been shown to influence the development and growth of the

preimplantation embryo in eutherian mammals. Some of these presumably control the arrested growth that occurs in diapause. These include epidermal growth factor (EGF and HB-EGF), leukaemia inhibitory factor (LIF), insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), platelet-activating factor (PAF), transforming growth factor β (TGF-β), interleukin-1ß (IL1B), bone morphogenic protein-2 (BMP2), prostaglandin synthetase PTGS2 (COX2), fibroblast growth factor (FGF), signalling molecules of the wingless (WNT) family and the transcriptional regulators Msx1 and Msx2 (Cha and Dey 2014). Many of these are also present in the blastocyst, and the expression of a number of them is now known to coincide with reactivation.

HB-EGF is produced in the luminal epithelium of the uterus at the site where the blastocyst will attach. It is upregulated at reactivation (Hamatani et al. 2004) and in the endometrium 6–7 h before implantation (Das et al. 1994). Blastocyst signalling to the uterus prepares it for implantation, since HB-EGF is upregulated in the endometrium by the blastocyst which then binds to its receptors ERBB1 and ERBB4 on the blastocyst. However, although blastocysts need to have hatched from the zona before attachment to the luminal epithelium, this is not needed to induce HB-EGF because the induction begins before zona dissolution. Bovine blastocysts also have this EGF receptor-ligand system during the time of trophoblast elongation (Kliem et al. 1998), so clearly EGFs have a wider role than just during diapause. Vascular endothelial growth factor (VEGF) induces endothelial proliferation and vascularization in the uterus and is upregulated in the mink at reactivation (Lopes et al. 2003, 2006) and in the tammar (MB Renfree, H Clark, G Shaw, LJ Parry, SR Frankenberg, AJ Pask, unpublished results).

Leukaemia inhibitory factor (LIF) has multiple roles in regulating blastocyst implantation, diapause and blastocyst viability in mice (Batlle-Morera et al. 2008; Hondo and Stewart 2005; Nichols et al. 2001). Estrogen stimulates the release of LIF from the endometrial glands. LIF activates the STAT3 pathway as well as the ERK pathway. LIF is secreted into the uterine lumen where it binds to two heterodimeric LIF transmembrane receptor complexes (LIFRs) and gp130 (Rosario et al. 2014). LIF is required for implantation in mice, binding to a heterodimer of LIF receptor and IL6ST that are expressed in the blastocyst (Nichols et al. 1996, 2001). In the absence of LIF, mouse blastocysts enter diapause but blastocysts lacking gp130 do not survive (Hondo and Stewart 2005). *LIF* mRNA is expressed at very low levels during diapause, and its expression increases in the endometrium at the termination of diapause in the mouse, mink, Western spotted skunk and the wallaby (Bhatt et al. 1991; Hearn 2005; Hirzel et al. 1999; Passavant et al. 2000; Song et al. 1998; Stewart et al. 1992) (MB Renfree, H Clark, G. Shaw, SR Frankenberg, CM Hearn AJ Pask, unpublished results). In the skunk, LIF receptor β (LIFRB) increases in the uterus when blastocysts resume development, apparently under prolactin control (Passavant et al. 2000). In the mouse, *LIF* expression appears to be under estrogenic control and can replace the estrogen injection to induce reactivation (Chen et al. 2000). LIF induces changes in gene expression, downregulating 54 genes in the first hour after treatment, and upregulating 256 genes including *Sox*, *Kfl*, *Hes*, *Hey* and *Hox* families of transcription factors (Rosario et al. 2014). Thus, LIF has

multiple roles and activates pathways in the luminal epithelium of the uterus to induce a dynamic and complex network of changes essential for reproduction (Rosario et al. 2014) and has an important role in embryonic diapause.

The muscle segment homeobox-1 (*MSX-1* and *MSX-2*) genes are highly conserved transcriptional regulators of some of the uterine implantation factors, and uterine receptivity requires downregulation of *Msx1* expression (Cha et al. 2013; Daikoku et al. 2011). Lack of Msx1 affects uterine receptivity by disrupting Wnt signalling through Wnt5a (Cha et al. 2013; Nallasamy et al. 2012). Interestingly, it appears that the function of *Msx* is to limit uterine stress-mediated inflammatory responses and so to maintain diapause (Cha et al. 2015). Msx1 and Msx2 are found in the uteri of mice, mink and tammar wallabies during embryonic diapause (Cha et al. 2013; Fenelon et al. 2014a; Renfree and Shaw 2014). In mice without diapause, *Msx* expression is transient early on day 4pc, but during diapause, it is highly expressed, and in its absence, there is reduced blastocyst recovery and survival (Cha and Dey 2014; Cha et al. 2013). Msx and LIF interact, but in *LIF* knockout mice, *Msx1* continues to be expressed (Cha et al. 2013; Daikoku et al. 2011). LIF injection induces implantation and downregulates *Msx1/2* expression (Cha et al. 2013). In mice and mink, the predominant gene is *Msx1*, but in the tammar, it is *MSX2* that remains high during diapause but decreases steadily up to day 5 after reactivation. The *MSX* genes are highly conserved in mammals, but these two genes may have developed subtly different actions as diapause evolved. Since these divergent mammals have been separated for at least 160 million years (Luo et al. 2011), this appears to be an ancestral mechanism that may be widespread in diapausing species.

Platelet-activation factor, or PAF as it is now known, is a phospholipid that is present in the endometrium. PAF receptor (PTAFR) interacts with PAF in the embryos of rodents, rabbits and humans (Ammit and O'Neill 1991; Jin and O'Neill 2011; O'Neill 1985, 1991, 2005). PAF may be important for maternal recognition of pregnancy since it stimulates embryonic metabolism, cell proliferation and viability (O'Neill 1991). PAF production and release is dependent on progesterone and estradiol (Chami et al. 1999; Li et al. 1999). In the diapausing tammar embryo, PAF is low, but endometrial PAF increases around the time of reactivation (Fenelon et al. 2014b; Kojima et al. 1993). It has a reciprocal expression pattern with *MSX* (Renfree and Shaw 2014). It is present in culture media from endometrial cultures and although variable appears to increase at the time when the first mitoses are observed after blastocyst reactivation (Kojima et al. 1993; Spindler et al. 1996). The release of endometrial PAF appears to upregulate *PTAFR* expression which is internalised with local cytoplasmic expression in the perinuclear region of the blastocyst cells at reactivation (Fenelon et al. 2014b). Whilst this does suggest that endometrial PAF is involved in reactivation, there is as yet no information as to whether it is essential.

The polyamines putrescine, spermidine and spermine are factors that regulate cell cycling and protein synthesis, and uterine polyamine-related genes appear to be important for embryo implantation (Igarashi and Kashiwagi 2010; Mandal et al. 2013; Zhao et al. 2008). These are candidates for controlling the resumption of development after diapause. Polyamine synthesis is rate limited by the ornithine

decarboxylase-1 (*ODC1*) gene (Lefèvre et al. 2011a, b). They have many functions in reproduction including in spermatogenesis, sperm motility, fertilization, onset of puberty and folliculogenesis (Lefèvre et al. 2011b). ODC1 and putrescine are induced by estradiol-17β and are upregulated at reactivation in the mouse (Van Winkle and Campione 1983) and in the mink (Fenelon et al. 2014a; Lefèvre et al. 2011a, c; Murphy 2012b).

Polyamines may act by inhibiting cell proliferation and arrest of the cell cycle in diapause (Fenelon et al. 2014a; Lefèvre et al. 2011a; Lopes et al. 2004). Since polyamines are essential regulators of cell proliferation and growth, evidence for their role in diapause comes from experimental manipulation of mink diapause. Ornithine decarboxylase inhibitor treatment reduces polyamine levels in the uterus, and in the mink, it rearrests cell proliferation even after reactivation only as long as the inhibitor is given: if withdrawn, the embryos resume development (Lefèvre et al. 2011a). Similarly, mink trophoblast cells *in vitro* do not proliferate. Administration of this inhibitor also arrests embryo development in the mouse, rat and hamster (Fozard et al. 1980; Galliani et al. 1983; Lopez-Garcia et al. 2008; Reddy and Rukmini 1981) showing that polyamines are critical factors in the reactivation of diapausing blastocysts. Anandamide and other cannabinoids also appear to have a role in cessation of embryonic development in diapause. They are downregulated in the mouse uterus during reactivation and conversely inhibit reactivation *in vitro* (Wang et al. 2003).

12.5 Genes and miRNAs in Diapause and Reactivation

There are many more factors yet to be discovered. A suite of genes have been identified in the diapausing mouse blastocyst and after reactivation using microarray analysis (Hamatani et al. 2004; Hondo and Stewart 2005). Only 229 (1 %) of the >20,000 genes examined were differentially expressed between blastocysts in diapause (80 genes) and reactivated blastocysts (149 genes) (Hamatani et al. 2004). These 229 genes consisted of major functional categories, including the cell cycle, cell signalling, adhesion molecules and metabolic pathways. One of the earliest genes upregulated at implantation is interleukin-1 (IL-1) of embryonic origin which modulates endometrial cell responsiveness (Bourdiec et al. 2013). In mink, there are 123 genes differentially expressed between diapause and reactivation. Almost half of the genes appear to be secreted products including those involved in cell proliferation, homeostasis, protein folding, electron transport and the innate immune response (Lefèvre et al. 2011c). Specific proteins involved in chromatin and tissue remodelling are changed at reactivation. SPARC (secreted protein acidic and cysteine rich), a secreted glycoprotein, increases, perhaps as a result of progesterone secretion from the reactivation of the corpus luteum (Lefèvre et al. 2011c), and HMGNI (high mobility group nucleosome binding domain 1), a chromatin remodelling factor, is also upregulated in the uterine epithelium at reactivation.

MicroRNAs may also be involved, since miRNAs suppress translation. There are 45 differentially expressed miRNAs between diapause and reactivation, 38 of which are downregulated at reactivation (Liu et al. 2012). Of nine members of the tumour suppressor miRNA family *lethal* (*let*) *7*, five are downregulated at reactivation of mouse-diapausing embryos (Liu et al. 2012). Let 7 inhibits attachment, and its targets include genes that regulate cell proliferation (Gurtan et al. 2013). The gene data set has now been extended by a proteomic analysis of mouse blastocysts during diapause and after activation (Fu et al. 2014). This study of 6000 mouse blastocysts identified 2255 proteins that have differential regulation of the protein translation, aerobic glycolysis, pentose phosphate pathway, purine nucleotide biosynthesis, glutathione metabolism and chromatin remodelling. Interestingly, reactivation is accompanied by activation of mitochondria and of the endosome-lysosome system (Fu et al. 2014).

12.6 Evolutionary Origins of Diapause

Although each species has its own specific diapause controls, there are enough factors conserved to suggest that diapause may have been an ancestral condition in mammals (Ptak et al. 2012). Transfer of sheep blastocysts to the uteri of mice in diapause induces a period of quiescence of the sheep blastocyst until reimplantation into sheep uteri (Ptak et al. 2012), and these authors suggest that the potential for diapause is not restricted to the species where it is known to occur. Ptak et al. (2013) further agree with the suggestion by Tarin and Cano (1999) that human conceptuses may be able to enter diapause. As yet there is not widespread acceptance of these suggestions.

12.7 Maternal Recognition of Pregnancy in Diapausing Species

Most embryos in diapause, by definition, are totally quiescent so there is no obvious uterine response to their presence. However, once they reactivate, the blastocyst engages in considerable "crosstalk" with the uterus that in most species results in changes to the uterine milieu and implantation, effectively a delayed maternal recognition. The nature of the signal in mammals is varied, from the HCG of humans to the interferon tau (IFN-τ) of ungulates which prevents luteolysis (Flint 1995). This delay of maternal recognition of pregnancy seems likely in the roe deer at least, because it enters diapause after hatching, but at a stage when other ruminants do not produce IFN-τ and when there is no IFN-τ detectable in the uterine secretions (Flint et al. 1994). It is therefore likely that there is no maternal recognition of pregnancy

in the formal sense in any delayed species, but that instead it occurs later when reactivation occurs.

This is certainly the case in the tammar wallaby but the timing is even later. Maternal recognition of pregnancy was long thought of as a eutherian-specific character because the signals occur early in pregnancy and around the time of implantation. In most marsupials, since there is no "implantation" or erosion of the uterine endometrium, but rather an attachment of the placental membranes to the uterine epithelium, it was tacitly assumed that these mammals had no maternal recognition of pregnancy. Further, the fact that the estrous cycle progresses uninterrupted in marsupials further hindered interpretation of specific embryo-maternal interactions. Closer examination of the changes in uterine endometrial proliferation and secretion in the tammar shows there is indeed a maternal recognition of pregnancy that is a direct response to the presence of an expanded embryonic vesicle in the gravid uterus, although the specific signalling molecule(s) is not yet identified (Renfree 1972, 2000). Thus, the answer to Brian Heap's question is that the embryo appears to withhold evidence of its existence during diapause, but that a maternal recognition of pregnancy occurs later after reactivation at a time that is species specific.

12.8 Summary and Outlook

Understanding the way in which the mammalian embryo can be held in a state of suspended animation has progressed in just a few selected species, including non-traditional laboratory species. Whilst the external factors are now well understood, the increasing number of cytokines and growth factors emanating from both the uterine epithelium and the trophoblast and their cellular effects are still being discovered. In addition, the roles of the specific uterine proteins are only now being subjected to proteomic and transcriptomic analysis. However, there are potential therapeutic applications for holding cells in quiescence, such as in certain tumours, but these opportunities have yet to be recognized. Continued studies of this amazing phenomenon are awaited with interest.

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Chapter 13
Predicting Embryo Presence and Viability

K.G. Pohler, J.A. Green, T.W. Geary, R.F.G. Peres, M.H.C. Pereira, J.L.M. Vasconcelos, and M.F. Smith

Abstract Pregnancy establishment, followed by birth of live offspring, is essential to all mammals. The biological processes leading up to pregnancy establishment, maintenance, and birth are complex and dependent on the coordinated timing of a series of events at the molecular, cellular, and physiological level. The ability to ovulate a competent oocyte, which is capable of undergoing fertilization, is only the initial step in achieving a successful pregnancy. Once fertilization has occurred and early embryonic development is initiated, early pregnancy detection is critical to provide proper prenatal care (humans) or appropriate management (domestic livestock). However, the simple presence of an embryo, early in gestation, does not guarantee the birth of a live offspring. Pregnancy loss (embryonic mortality, spontaneous abortions, etc.) has been well documented in all mammals, especially in humans and domestic livestock species, and is a major cause of reproductive loss. It has been estimated that only about 25–30 % of all fertilized oocytes in humans result in birth of a live offspring; however, identifying the embryos that will not survive to parturition has not been an easy task. Therefore, investigators have focused the identification of products in maternal circulation that permit the detection of an embryo and assessment of its well-being. This review will focus on the advances in predicting embryonic presence and viability, *in vivo*.

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13.1 Introduction

The establishment and maintenance of pregnancy is dependent upon bidirectional communication between the embryo and maternal system. Consequently there are products of the embryo, endometrium, and corpus luteum during pregnancy that end up in the maternal circulation and may be used to detect the presence and viability of an embryo or fetus. Early detection of a viable embryo/fetus is important in human obstetrics as well as for reproductive management of both livestock (e.g., cattle) and captive species. In humans, early detection of an embryo allows for appropriate attention to the welfare of the embryo and mother. During recent years there has been a focus on how the maternal environment can influence embryo/fetal development in humans as well as other species. Notably, some of these changes can alter the health and well-being of an individual later in life (known as "prenatal programming"; (Heijmans et al. 2008; Du et al. 2010)). In livestock species, the early detection of an embryo or fetus facilitates management strategies, such that nonpregnant females can be rebred as soon as possible. The early detection of pregnancy in captive animals also facilitates appropriate management practices such as removal of a male and (or) dietary changes.

In addition to detecting the presence of an embryo, monitoring embryonic viability is important due to the relatively high incidence of embryonic mortality in mammals (i.e., 25–30 %). Embryonic mortality is a major limitation to fertility in mammals and has been divided into early embryonic and late embryonic mortality. In cattle, early embryonic mortality generally occurs before day 28 (Table 13.1), whereas late embryonic/fetal mortality occurs after day 28 (Table 13.2). In cattle, the majority of early embryonic mortality is reported to occur before maternal recognition of pregnancy (i.e., days 15–16 post-insemination). Although early embryonic mortality in livestock has been an area of intense investigation for a number of years, our understanding of the molecular, cellular, and physiological mechanisms associated with pregnancy establishment and maintenance has been limited by an inability to accurately monitor the presence of an embryo from fertilization until

Table 13.1 Incidence of early embryonic mortality (EEM; ≤day 28 of gestation) in cattle

Cattle type	No. studies	Specific days of EEM	Incidence of EEM Mean (range)	Reference
Beef heifers	2	2–16	21.8 % (4.5–43.7 %)	[a]
Dairy heifers	1	2–6	28.9 %	[b]
Beef cows	3	2–16	35.6 % (10.5–70 %)	[c]
Dairy cows (lactating)	1	2–7	46.3 %	[d]
Dairy cows (nonlactating)	3	2–7	33.3 % (13.8–46.9 %)	[e]

[a]Maurer and Chenault (1983), Dunne et al. (2000)
[b]Sartori et al. (2002)
[c]Maurer and Chenault (1983), Breuel et al. (1993), Ahmad et al. (1995)
[d]Sartori et al. (2002)
[e]DeJarnette et al. (1992), Dalton et al. (2001), Sartori et al. (2002)

Table 13.2 Incidence of late embryonic mortality (LEM; > day 28 of gestation) in cattle

Cattle type	No. studies	No. animals	Incidence of LEM Mean (range)	Day of gestation	Reference
Beef heifers	4	1250	4.2 % (4–5 %)	30–90	[a]
Dairy heifers	2	203	5.4 % (4–6 %)	30–80	[b]
Beef cows	3	2570	10 % (6.5–14 %)	25–65	[c]
Dairy cows (lactating)	11	4680	14 % (3.2–42.7 %)	27–60	[d]

[a]Lamb (2002), Kill et al. (2013)
[b]Dunne et al. (2000), Silke et al. (2002)
[c]Stevenson et al. (2003), Perry et al. (2005), Pohler et al. (2013)
[d]Vasconcelos et al. (1997), Cartmill et al. (2001a, b), Thompson et al. (2010) Ricci et al. (2015)

implantation begins around day 7 in humans (Hay et al. 1986; Lenton and Woodward 1988) or when active placentation begins about days 20–23 in cattle (Assis Neto et al. 2010; Aires et al. 2014). Therefore, the ability to detect the presence and viability of a preimplantation embryo is important from both scientific and management viewpoints. The purpose of this chapter is to review the methods for detecting the early presence and viability of a preimplantation and postimplantation embryo (*in vivo*), with emphasis on humans and cattle.

13.2 Detecting the Presence of an Embryo

13.2.1 Ultrasonography

The gold standard for determining pregnancy and confirming the presence of a viable embryo is with ultrasound. In women, transvaginal ultrasound can be used to detect a gestational sac and embryonic heartbeat approximately 33 days after the last menstrual period which is detectable with a transabdominal ultrasound about a week later (Coyne and Raine-Fenning 2010). In cattle, transrectal ultrasonography can be used between days 26 and 29 to diagnose pregnancy and visualize a discernable heartbeat (Pierson and Ginther 1984; Kastelic et al. 1988; Beal et al. 1992). Today, ultrasound is considered the only visual indicator of pregnancy in cattle and is used for comparison with all recent attempts at diagnosing earlier pregnancy in this review.

13.2.2 Early Pregnancy Factor

Early pregnancy factor (EPF) or early conception factor was first reported by Morton et al. (1974) in pregnant mice, and EPF has subsequently been reported in the serum of pregnant sheep (Morton et al. 1979), pigs (Koch et al. 1983), cattle (Yoshioka et al. 1995), horses (Ohnuma et al. 2000), red deer (Lash et al. 1997), and humans (Morton et al. 1977). EPF was identified as a homologue to chaperonin 10 (Cavanagh and Morton 1994; Morton 1998) and is believed to suppress the maternal immune

system, based on its activity in a rosette inhibition test (Morton et al. 1987; Morton 1998). In addition, EPF has growth factor activity and is believed to be an embryonic survival factor (see review Morton et al. 1992). In this regard, when pregnant mice were passively immunized against EPF, the pregnancy was terminated (Athanasas-Platsis et al. 1989, 1991). Prior to implantation, EPF is reportedly produced by the ovum in response to fertilization (Cavanagh et al. 1982), and postimplantation EPF is believed to be produced by the placenta (Morton 1984). Other sources of EPF include platelets in response to stimulation by platelet-activating factor, tumor cells, and regenerating liver cells (Morton 1998). Although some have suggested that EPF may be used to diagnose pregnancy in cattle, its usefulness in this regard is highly questionable (Cordoba et al. 2001). The practicality of diagnosing pregnancy prior to maternal recognition may have limited utility in a number of species due to the high incidence of early embryonic mortality in mammals. However, in cattle that are intensively managed (e.g., dairy cows), the ability to determine that a cow is not pregnant before initiation of spontaneous luteolysis would allow for resynchronization of ovulation and rebreeding at an earlier time than is currently possible.

13.2.3 INFT Stimulated Gene Expression in Ruminants

In ruminants, interferon tau (IFNT) is the primary signal for maternal recognition of pregnancy (Bazer et al. 2009; Dorniak et al. 2013). IFNT is secreted by the bovine trophoblast beginning around day 14 after insemination. It binds to a type I interferon receptor and inhibits expression of the estrogen receptor within the uterine epithelium. Inhibition of estrogen receptor transcription prevents an increase in uterine epithelial expression of oxytocin receptor, which prevents circulating oxytocin from initiating secretion of large episodic pulses of the uterine luteolysin, prostaglandin $F_{2\alpha}$ (Bazer et al. 2009; Dorniak et al. 2013). IFNT was thought to act exclusively on the endometrium; however, recent evidence indicates that IFNT is released into the vasculature and has an endocrine action on ovine peripheral mononuclear blood cells (PMBC) as well as the corpus luteum (Oliveira et al. 2008; Bott et al. 2010; Hansen et al. 2010). Although IFNT has both paracrine and endocrine roles in maternal recognition of pregnancy, circulating concentrations of IFNT are too low to be detected in circulation with current assay technology. Therefore, expression of IFNT-stimulated genes in PMBC has been investigated. The upregulation of interferon-stimulated gene expression in bovine and ovine PMBCs has been investigated as the basis for the potential development of an early pregnancy test in these species (Han et al. 2006; Gifford et al. 2007; Stevenson et al. 2007; Green et al. 2010). For example, on days 16, 18, and 20 after insemination in dairy cows, expression of interferon-stimulated genes (ISG, e.g., ISG-15, Mx1, and Mx2) were increased in PBMC of pregnant compared to nonpregnant cows (ISG-15 on days 18 and 20, Mx1 on day 20, and Mx2 on days 16, 18, and 20), whereas expression of other ISGs (IFN regulatory factor 1, IFN regulatory factor 2, and β2 microglobulin in PBMC) was not different (Gifford et al. 2007). Green et al. (2010)

reported differential expression of ISGs between pregnant and nonpregnant dairy cows and heifers; however, the use of ISGs to accurately diagnose pregnancy on day 18 after insemination was greater for heifers compared to cows. Since IFN-stimulated genes are not unique to pregnancy, the measurement of IFN-stimulated genes in PMBCs has proven to be more useful for identifying nonpregnant animals than it has for detecting pregnant animals.

13.2.4 Use of Steroids to Detect Pregnancy

Progesterone, a steroid hormone secreted by the corpus luteum, is essential for maintenance of pregnancy in mammals and can be detected in plasma/serum, milk, saliva, feces, and urine. Differences in serum concentrations of progesterone between pregnant and nonpregnant animals occur between the predicted time of spontaneous luteolysis and subsequent formation of a new corpus luteum in nonpregnant animals; this period represents a time in which one can measure differences in circulating concentrations of progesterone between nonpregnant and pregnant animals. The preceding divergence in concentrations of progesterone in circulation or milk formed the basis for an indirect measure of pregnancy in cattle, sheep, buffalo, horses, goats, and other species. A close association between the concentrations of progesterone in milk and plasma during the estrous cycle has been reported in dairy cows (Laing and Heap 1971; Heap et al. 1973). In cattle, there is a significant divergence in serum or milk concentrations of progesterone between nonpregnant and pregnant cows around days 20–24 post-insemination (Sasser and Ruder 1987). The accuracy associated with diagnosing pregnancy correctly on the basis of concentrations of progesterone in milk varied from 60 to 100 % (Sasser and Ruder 1987; Nebel 1988), whereas the accuracy of detecting that a cow is not pregnant based on the concentration of progesterone in milk varied from 81 to 100 % (Sasser and Ruder 1987; Nebel 1988). The reason for this discrepancy in the accuracy of the milk progesterone test is when progesterone is low between days 20 and 24 post-insemination; there is a high probability that luteolysis has occurred and the animal is not pregnant. Alternatively, the concentration of progesterone in milk may remain elevated in a nonpregnant cow between days 20 and 24 due to a longer luteal phase in cows having three versus two follicular waves, a persistent corpus luteum following uterine infection, or a luteal or luteinized cyst. Because these measures are compared to pregnancy determined at a later time, it is also likely that some early embryonic mortality existed among the cows included in the data, which may have also reduced the accuracy of diagnosing pregnancy based on progesterone in milk or circulation. The establishment of pregnancy in undomesticated species has been determined by measuring progesterone or a progestin metabolite in urine or feces of a variety of species, including big cats (Umapathy et al. 2013). However, serum concentrations of progesterone are not particularly effective at monitoring embryonic viability or the precise timing of embryonic death when it occurred after ultrasound confirmation (Pohler et al. 2013).

High correlations between blood flow in the corpus luteum (CL) and serum concentration of progesterone secretion in cattle between days 18 and 21 of gestation using Doppler ultrasonography may provide some usefulness as a chute side measure of CL viability and thus receptivity to maternal recognition of pregnancy (Ginther 2007). Detection of luteolysis using Doppler ultrasonography can be used to identify nonpregnant cattle as early as day 15, but better accuracy for the determination of pregnant cattle is achieved between days 18 and 21 (Siqueira et al. 2013; Pugliesi et al. 2014; Scully et al. 2015). While CL blood flow is an indirect measure of CL viability and serum concentrations of progesterone, it is an indirect measure that a viable embryo is present and maternal recognition of pregnancy has occurred.

Although progesterone is the primary steroid that has been used to predict pregnancy in a number of species, circulating concentrations of estrone sulfate have also been employed for this purpose (Sasser and Ruder 1987). Estrone sulfate is the form of estrogen produced by the conceptus and sulfated by the endometrium that can be detected in serum or milk (cattle) after the following days of gestation in pigs (days 20–29; (Robertson and King 1974)), sheep (day 100; (Thimonier et al. 1977)), and cattle (day 100; (Holdsworth et al. 1982)).

13.2.5 Extracellular microRNAs and Pregnancy Detection

MicroRNAs (miRNA), a class of small noncoding RNAs (approximately 22 nucleotides in length), have been shown to regulate gene expression and play critical roles in many biological systems. Generally speaking, RNAs, including miRNAs, are localized in the cytoplasm, but they can also be found in extracellular microvesicles, which may provide a mechanism for cell-to-cell communication (Valadi et al. 2007). Overall, extracellular miRNAs are encapsulated in lipid vesicles, which are released as microparticles (e.g., exosomes; (Raposo and Stoorvogel 2013)), or bound to protein, in which case the miRNA is complexed with specific proteins (e.g., high-density lipoprotein; (Vickers et al. 2011)). Exosomes are small microvesicles, ranging in size from 50 to 100 nm in diameter (Heijnen et al. 1999), and have been identified in a variety of biological fluids: urine (Pisitkun et al. 2004), amniotic fluid (Keller et al. 2007), serum (Gilad et al. 2008), human saliva, plasma, and breast milk (Lasser et al. 2011). Circulating miRNAs may serve as potential biomarkers for determinants of health status and disease (see review Reid et al. 2011). For the purpose of this review, we will focus on exosomal-derived miRNAs in the maternal circulation that are pregnancy specific.

In relation to reproduction, exosomal microRNAs have been associated with reproductive cancers, male and female reproductive tract function, and stages/health of pregnancy. Numerous microRNAs have been shown to be pregnancy specific in normal human tissue (Bentwich et al. 2005; Liang et al. 2007), and more recently, Gilad et al. (2008) demonstrated that placental-derived microRNAs can be detected in sera of pregnant, but not nonpregnant patients. All placental microRNAs

mentioned above were found at increased concentrations in sera from pregnant patients and tended to increase with gestational age. Furthermore, it was demonstrated that three placental specific microRNAs (miR-526a, 527, and 520d-5p) detected in maternal circulation could accurately distinguish pregnancy status (Gilad et al. 2008) in women, thus providing a potential biomarker of pregnancy or pregnancy complications. Luo et al. (2009) demonstrated that the preceding circulating microRNAs are most likely products of human villous trophoblasts that express and secrete miRNAs into maternal circulation via exosomes. Subsequently, there have been several reports in humans that support pregnancy-specific exosomal-derived miRNAs in the maternal circulation of women (Miura et al. 2010; Kotlabova et al. 2011). An examination of the two studies identified at least five miRNAs in common that showed a significant increase in maternal circulation throughout pregnancy, followed by a rapid decrease after parturition.

Most of the investigation on the relationship between circulating microRNAs and pregnancy has been carried out in humans, which have a highly invasive placenta type, allowing for a plausible mechanism for placental specific material to enter the maternal circulation. Although it is known that fetal DNA can be found in the maternal circulation of domestic animals, it is not clear whether pregnancy-specific miRNAs are present. A recent study provided evidence that pregnancy-specific miRNAs maybe present in circulation of mares. Cameron et al. (2012) reported differences in specific exosomal-derived miRNAs between pregnant and nonpregnant mares. To date, there have been no other published reports of pregnancy-specific circulating miRNAs in domestic livestock species. However, there is evidence of miRNAs in extracellular microvesicles (potential exosomes) of uterine flushings in a ruminant species (i.e., sheep). In pregnant and cycling ewes, extracellular microvesicles, collected via uterine flushing on day 14, contained differential amounts of miRNAs and proteins (Burns et al. 2014). In addition preliminary data from our labs suggest that at days 17 and 24 of gestation, circulating microRNAs may provide a useful marker of embryonic presence (Pohler et al., unpublished data). Further research is needed in domestic animals to determine if pregnancy-specific extracellular miRNAs exist and if they can provide an accurate marker of embryonic presence and viability.

13.2.6 Detection of Placental Products to Monitor the Presence of an Embryo

The placenta is a multifaceted organ that has a vital role in the establishment and maintenance of pregnancy. Along with transferring nutrients and protecting the fetus, the placenta serves as an endocrine organ throughout pregnancy. The early embryo has been shown to produce a wide range of factors *in vitro* (Gardner et al. 2001); however, many of these molecules never reach a sufficient concentration *in vivo* for the detection in maternal circulation. In humans, detection of human chorionic gonadotropin (hCG) in circulation is the most commonly utilized marker of

early pregnancy. *In vitro*, trophoblast cells from the developing embryo have been reported to produce hCG as early as 7 days postfertilization (Shutt and Lopata 1981; Fishel et al. 1984; Lachlan and Lopata 1988). *In vivo*, detection of hCG in circulation, produced by the developing embryo, has been reported to occur as early as 6.5–9.5 days after the preovulatory gonadotropin surge, which coincides with the initiation of implantation (Hay et al. 1986; Lenton and Woodward 1988). The ability to predict pregnancy loss based on circulating concentrations of hCG will be discussed in more detail in the section below.

In the ruminant placenta there is a unique cell type (giant binucleated trophoblast cells) that constitutes 15–20 % of the fetal placental epithelium (Anthony et al. 2010). Binucleated cells become visible around days 19–20 of gestation in cattle and secrete a number of hormones and proteins including placental lactogen (PL) and pregnancy-associated glycoproteins (PAGs; see Fig. 13.1; (Wooding et al. 2005)). PAGs are members of a relatively large gene family and are abundantly expressed in the placenta of species within the *Cetartiodactyla* order (even-toed ungulates) and can be detected in circulation and milk (Wallace et al. 2015). There is significant variation in the spatial and temporal expression of PAGs in species having an epitheliochorial and synepitheliochorial placenta. In ruminants, PAGs have gained considerable attention for the detection of pregnancy. Sasser et al. (1986) reported detectable levels of pregnancy-specific protein B (PSPB; PAG1) in the maternal circulation and developed a specific radioimmunoassay, which successfully detected pregnancy in cattle (Sasser et al. 1986), sheep (Ruder et al. 1988) and goats (Humblot et al. 1990). There has been interest in detecting other PAGs for pregnancy detection throughout gestation. Green et al. (2005) reported the establishment of an ELISA-based test for PAGs produced during early pregnancy that have a relatively short half-life (4.3 days). In the preceding study, PAGs were detected in all cattle by day 28 of gestation, PAG concentrations peaked around the time of parturition, and following parturition PAGs were undetectable by 8 weeks' postpartum in 38 out of 40 cows. Using a similar assay platform, Pohler et al. (2013) reported an even shorter half-life for PAGs (~36 h) and determined that the first significant increase in circulating PAGs occurred on day 24 of gestation. The difference in reported half-life could be due to differences in clearance of PAGs in maternal blood during pregnancy versus the postpartum period. There are many members of the PAG family, and the presence of distinct circulating forms at different stages of gestation may account for the difference in observed half-lives between these studies. In cattle, circulating concentrations of PAGs have been shown to be influenced by a number of factors including breed, weight, parity, fetal sex, fetal number, fetal birth weight, and sire of the fetus, along with pregnancy stage and status (Patel et al. 1997; Lobago et al. 2009). Overall, PAGs have been shown to be an accurate tool for pregnancy diagnosis in cattle, sheep, goats, buffalo, and elk (Sasser et al. 1986; Sousa et al. 2006; Szafranska et al. 2006; Silva et al. 2007; Pohler et al. 2013), and several assay platforms are commercially available for both blood and milk (Leblanc 2013).

Placental lactogen (PL) has been identified and characterized in a number of species including primates, rodents, and domestic ruminants. In humans, PL was

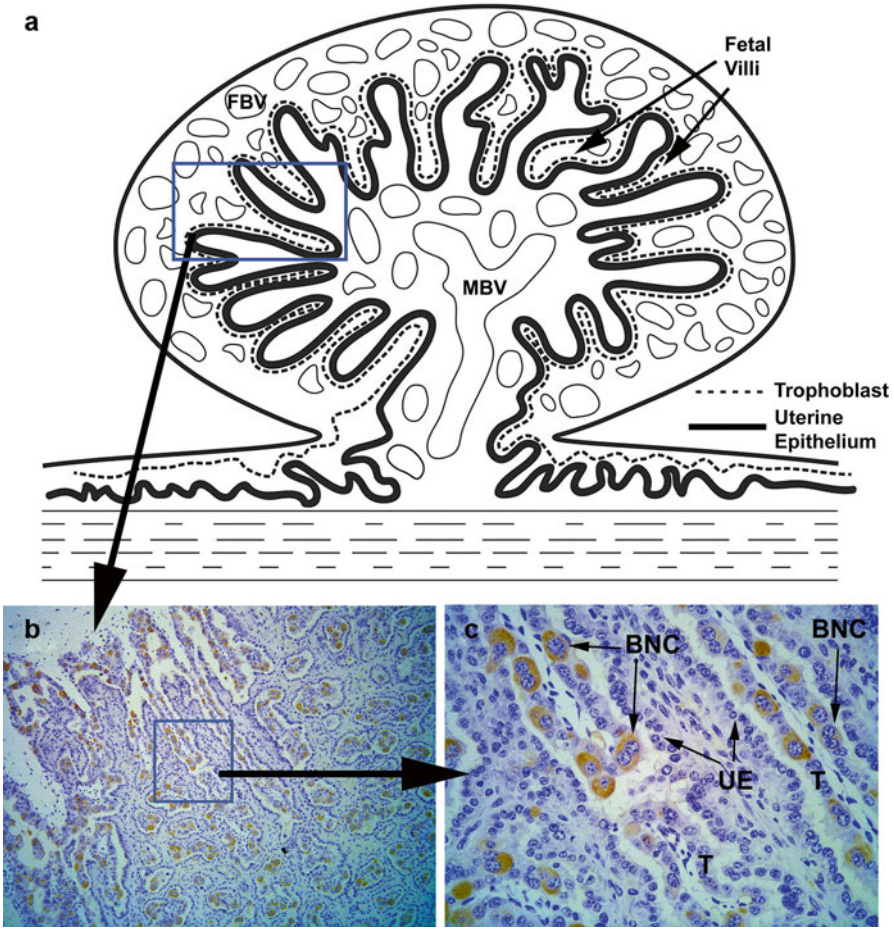


Fig. 13.1 The image illustrates the presence of trophoblast giant "binucleated" cells at the placenta-uterine interface in ruminant ungulates. (**a**) A stylistic drawing of a placentome, which consists of fetal villi (derived from trophoblasts and associated connective tissues) interdigitated with maternal uterine tissue. Both the fetal and maternal sides of the placentome become highly vascularized; the maternal blood vessels (*MBV*) and fetal blood vessels (*FBV*) are illustrated in the drawing. The convoluted contact area between the fetal placental trophoblasts and the uterine epithelia is represented by dashed and solid lines, respectively. (**b**) A section taken from a day 60 bovine placentome illustrates the interface between the trophoblasts of the placental villi and the maternal uterine epithelia. The section was incubated with an antibody raised against bovine PAG10; the resulting staining pattern reveals the presence of giant binucleated trophoblasts that comprise 15–20 % of the total trophoblast population. Magnification: 100×. (**c**) A higher magnification image of the boxed region shown in panel (b). The giant binucleated cells (*BNC*) are revealed by the immunostaining with the PAG10 antibody. The trophoblast (*T*) and uterine epithelium (*UE*) layers are indicated. Magnification: 400×

reported in circulation as early as 6 weeks of gestation and rose during the first and second trimesters, reaching a peak concentration during the third trimester (Samaan et al. 1966). Similar to PAGs, ruminant PL is produced by binucleated cells and secreted into the maternal and fetal circulation. Circulating PL is detectable in the maternal circulation of both sheep and cattle starting about day 50 of gestation (Kappes et al. 1992). Due to the low abundance of circulating PL during early to mid-gestation, little effort has been placed on using PL as a method of pregnancy diagnosis; however, later in pregnancy concentrations of PL are positively correlated with placental mass and fetal number (Gootwine 2004).

13.3 Monitoring Embryo Viability

So far, this review has focused on measurable factors, primarily in the maternal circulation, that can be used to detect the presence of an embryo. However, during the early stages of gestation, the presence of an embryo does not necessarily result in a successful pregnancy. As previously mentioned, the incidence of early embryonic mortality is relatively high during early gestation (e.g., humans = 20 %; (Macklon et al. 2002); cattle = 25 %; (Sartori et al. 2002)). Being able to predict or assess embryonic viability may provide an opportunity to intervene and to perhaps prevent embryonic mortality. Alternatively, the ability to identify an animal likely to lose a pregnancy would permit rebreeding of the animal earlier than would normally be possible. This section will focus on some methods for monitoring embryonic viability during the preimplantation and postimplantation periods.

13.3.1 Preimplantation Embryo Viability

During the preimplantation stage, the embryo is undergoing cleavage stage divisions, migrating from the oviduct into the uterus, and frequently interacting with the maternal environment to signal its presence. There are very few *in vivo* markers of embryonic mortality in the maternal circulation during the preimplantation period. However, there have been multiple reports of *in vitro* markers of embryonic viability that predict either developmental competence or pregnancy outcome. Specifically, in human IVF systems, a number of predictive methods have been reported during the first few days of embryo culture in relation to developmental competence. A simple marker, such as time of first cleavage in humans, has been reported to predict pregnancy success. Embryos undergoing cleavage in the first 25–27 h following fertilization resulted in higher pregnancy rates following transfer versus embryos that had not experienced early cleavage within this time frame, *in vitro* (Salumets et al. 2003).

Along with morphological characterization of developing embryos, alterations in early embryo-conditioned culture media are often correlated with the developmental potential of embryos. Houghton et al. (2002) reported distinct amino acid

turnover patterns in media between embryos of high developmental versus low developmental competence. In this particular study, Ala, Arg, Gln, Met, and Asn flux predicted blastocyst potential with >95 % accuracy. Other measures of nutrient metabolism have also provided potential predictors of embryonic viability. Lane and Gardner (1996) have shown in mice that glycolytic activity, as measured by the percentage of glucose converted into lactate, was a successful measure of embryonic developmental competence and viability. *In vitro* embryos with low glycolytic activity resulted in increased pregnancy rate compared to embryos with higher glycolytic activity. Similarly, bovine blastocysts that incorporated low levels of glucose in culture for 20 h resulted in increased *in vivo* development compared to embryos that did not utilize glucose, *in vitro* (Renard et al. 1980). However, when tested in humans, glucose metabolism did not differ between viable and nonviable embryos following IVF and embryo transfer, suggesting that in humans, glucose metabolism is not a predictive measure of embryonic viability (Jones et al. 2001).

Another example is platelet-activating factor (PAF), a signaling phospholipid that has been shown to be an indicator of embryo viability and a predictor of pregnancy outcome in humans. Human embryo-conditioned culture media following normal IVF demonstrated a strong correlation between PAF concentrations and pregnancy outcome. Patients with either medium or high levels of PAF resulted in significantly higher pregnancy rates compared to patients with low PAF (Roudebush et al. 2002). Similar data have also been shown in mice (Ryan et al. 1989, 1990) along with supplementation of PAF to embryo culture which increased the number of cells in expanded blastocysts and increased their ability to implant *in vivo* (Ryan et al. 1990).

13.3.2 Postimplantation Embryo Viability

Placental products that are measureable in maternal circulation provide obvious targets as markers of embryonic viability. As previously mentioned, hCG and PAGs serve as an accurate measure of pregnancy diagnosis in humans and ruminants, respectively, and are easily measured by RIA or ELISA. In humans, early pregnancy detection by hCG has become a common practice. There seem to be clear differences in the circulating concentrations of hCG that are correlated with the viability of the embryo and pregnancy success. In patients who experienced embryonic mortality, lower concentrations of hCG in maternal serum have been detected (Lenton et al. 1982; Yovich et al. 1986; Liu and Rosenwaks 1991; Bjercke et al. 1999). Furthermore, Lenton et al. (1988) reported two types of embryonic mortality based on the pattern of hCG secretion: (1) termination of implantation or (2) implantation that is delayed. In both cases, the authors reported either normal or delayed rises in the concentration of hCG in maternal circulation, followed by a failure in the exponential rise of hCG over the next few weeks of gestation.

Pregnancy-associated glycoproteins have also been reported to serve as a marker of embryo/fetal viability and potentially even a marker of placental function in cat-

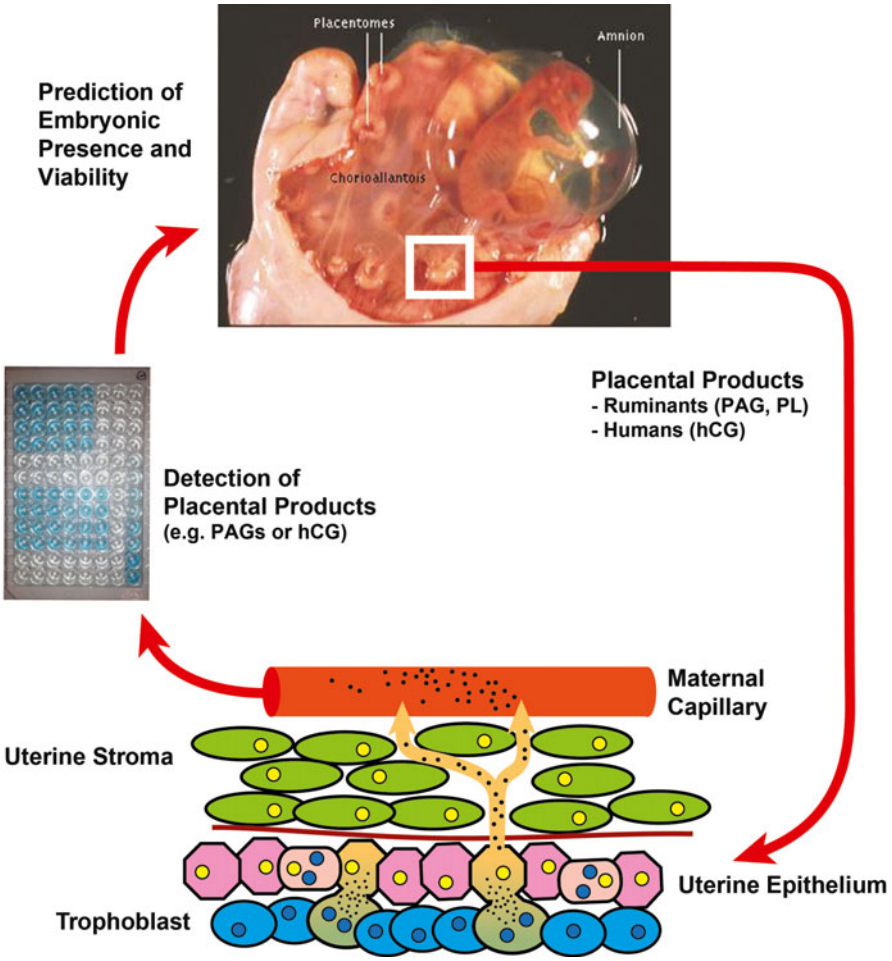


Fig. 13.2 Placentation in ruminants leads to the formation of placentomes which are responsible for nutrient transport and direct communication between the developing fetus and maternal environment. The figure shows production of pregnancy-associated glycoproteins (*PAGs*) and placental lactogen (*PL*) by binucleated trophoblast cells (BNC) within a placentome of the ruminant placenta. BNCs fuse with uterine epithelial cells to form trinucleated cells, and PAGs and PL subsequently enter the maternal circulation. The human placenta (not pictured here), which is more invasive and histologically distinct from the ruminant placenta, produces human chronic gonadotropin (*hCG*) which also enters the maternal circulation. These placental products can be detected with different assay platforms (e.g., ELISA, RIA, etc.) and used to identify the presence of a conceptus and assessment of placental function. The overall working hypothesis is that placental products can be used to monitor conceptus presence and well-being (The placenta image was obtained from the *Placentation in Ruminants* website (Colorado State University; http://www.vivo.colostate.edu/hbooks/pathphys/reprod/placenta/ruminants.html) and was used with the permission of R. Bowen)

tle (Perry et al. 2005; Pohler et al. 2013). Beef cattle, experiencing embryonic mortality between day 28 and day 72 of gestation, had significantly lower circulating concentrations of PAGs at day 28 compared to cows that maintained a successful pregnancy to day 72 (Pohler et al. 2013). In the preceding study, embryos at day 28, in both the embryonic mortality and embryonic maintenance groups, had viable heartbeats, suggesting that on day 28 all embryos were viable in both groups based on ultrasound. There have been similar reports on circulating PAG concentrations and embryonic mortality in lactating dairy cattle (Humblot et al. 1988; Thompson et al. 2010; Breukelman et al. 2012) and sheep (Wallace et al. 1997). Collectively, these data suggest that measurement of circulating PAGs in ruminant ungulates may serve as a useful animal management tool for identifying pregnant animals that are likely to undergo loss of the fetus. See Fig. 13.2 for a working hypothesis of how placental products could potentially be used for monitoring embryonic and placental viability.

13.4 Summary

In summary, there are a number of ways to determine the presence of an embryo in many mammals; however, the ability to predict the success of a pregnancy during early gestation remains difficult. As research advances and our understanding of uterine and placental interactions become more defined, the ability to develop models to successfully predict embryonic survivability will hopefully follow. Ideally, research in this area will lead to novel biomarkers early in pregnancy that can determine overall pregnancy success.

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