

# Studies of uterine secretions and products of primary cultures of endometrial cells in pigs

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The uterus plays a central role in the reproductive biology of mammals. Adaptation of the uterus from an oviparous to a viviparous nature required changes that involved production of a uterine environment that could support the development of the embryo and fetus. Production of a suitable environment includes the synthesis and secretion of products by the uterine endometrium. However, the uterine endometrium is not a single homogeneous unit, but rather consists of several cell populations. Recent accomplishments in cell culture techniques provide a means for examining the contributions and secretory control of different endometrial cell populations. Furthermore, it is possible to recombine specific cell types to study their interaction. It is clear that the luminal epithelium, glandular epithelium and endometrial stroma produce different secretory products. Some secretions (for example uteroferrin) are secreted by only one cell type; others (for example prostaglandins, PGs) are secreted by all types of cell. There is much to be learned about the functions and regulations of endometrial secretions and there are important aspects of the role of the endometrium in pregnancy that present concepts do not address. For example, there is no explanation for the required synchrony between the embryo and uterus before day 10 and the implications of control of the uterine environment by progesterone from day 4 to day 10 are not understood. Almost all of the uterine secretory proteins are produced after day 10. In this review, we consider the protein and prostaglandin products from the different cell populations of the pig endometrium and propose a model to explain the integration of multiple sources of PGs and multiple regulators of PG secretion. Our purpose is to facilitate a more complete understanding of the individual uterine cell populations and a better understanding of how these cell types interact to function as a complete unit.

## Introduction

The endometrium provides an environment that is different from plasma and other extracellular fluids. Important constituents of the uterine luminal environment are the products of *de novo* synthesis and those derived from blood. For blood-derived molecules, the epithelium is the primary rate-limiting barrier for access to the uterine lumen (McCrae, 1988). In addition, some plasma constituents are taken up intracellularly by the basal surface of the epithelium and then released apically (McCrae, 1988). Most of the discussion in this review will deal with endometrial secretory products, but we suggest that models based on growing uterine epithelium on filters *in vitro* that divide the culture medium into basal and apical compartments will be useful for studying the selective transport of molecules across the uterine epithelium.

The uterus is programmed for its role partly by the gonadal hormones of the oestrous cycle (Roberts and Bazer, 1988). The uterus of a virgin gilt is therefore appropriately prepared to receive transplanted embryos and can nurture them after transfers at least as late as day 9 (day 0 = day of onset of oestrus)

(Polge, 1982). A poorly understood aspect is the requirement for embryo-uterine synchrony (Webel *et al.*, 1970; Polge, 1982). Initially, the nongravid (cyclic) uterus will support embryos of a similar day of pregnancy. The changes that fulfil the synchrony requirement of early uterine embryos therefore occur without inputs from the embryos. However, the embryos also modify the endometrium to their own programme, as shown in pigs by the response to conceptus oestrogen on days 11–12 (Geisert *et al.*, 1982a). Pope *et al.* (1990) suggested that production of oestrogen by the most rapidly developing conceptuses advances the uterine environment in a manner that is unacceptable for less developed conceptuses. The nature of the support provided by the endometrium is not completely known, but it continues throughout pregnancy in pigs, in which attachment remains superficial until farrowing (discussed by Roberts and Bazer, 1988).

Questions can be raised about the specific nature of the interaction between uterus and conceptus and the more general concept of providing an environment that is permissive for the conceptus to express its own genetic programme for development. For example, pig embryos can survive and develop to day 11 in the uterus of a gilt ovariectomized on day 6 (Galvin *et al.*, 1990; 1992). The embryos do not survive when ovariectomy is performed on day 4 after mating, probably because ovarian steroids have not yet programmed the environment within the uterus. However, continuous steroid support is clearly not necessary during this early period.

An appropriate question is how similar the embryos developing in a uterus deprived of ovarian steroids are to embryos developing in the uterus of an intact gilt. Embryos in an ovariectomized gilt appear to develop more slowly and are smaller on day 11. This situation is reminiscent of the slow development observed in cultured embryos (Davis, 1985). An emerging view indicates that a certain amount of redundancy occurs in the regulation of conceptus development. Both embryonic and uterine gene products appear to participate, and there is much interest in the potential of uterine growth factors to exert control over some aspects of conceptus growth and development (Simmen and Simmen, 1990). The conceptus may regulate its own development to some extent, but exposure to the uterine environment may be necessary for complete and normal development. Although four-cell embryos will cleave and form blastocysts *in vitro*, they have fewer cells and a reduced developmental potential when transplanted to recipients (Davis, 1985). In addition, there is a limitation on the period for which normal development *in vitro* is possible and blastocyst elongation in culture has not been reported.

As pointed out by Roberts and Bazer (1988), there has been much speculation about the roles of the constituents of the uterine fluids "... but few definitive results have emerged". All these considerations draw attention to the need for experimental models that permit studies of the interactions between the endometrium and the conceptus and between the cell types within the endometrium. We believe that such models may be developed using the cells of the porcine endometrium harvested by enzymatic dispersion and with the aid of sieve separation. In this review, we consider the products of the pig endometrium and the expression of specific cell types when grown in primary cultures. We have not attempted to passage pig endometrial cells and do not know what effect that might have on their secretion of various products.

### Endometrial Cells in Primary Culture

The endometrium is composed of two distinct epithelial populations (luminal and glandular) and a stroma that contains a variety of cell types. These cell types, the conceptus, and the potential interactions are illustrated (Fig. 1).

Procedures for harvesting and growing pig endometrial cells *in vitro* have been described (Zhang *et al.*, 1991). These initial methods proved less than satisfactory for luminal epithelial cells. More recently, Y. Zhang (unpublished) in our laboratory has modified these procedures. To improve the yield and growth of luminal epithelial cells, we currently digest the cells at room temperature with dispase (0.48%) for 20 min followed by digestion with dispase (0.48%)/pancreatin (1.25%) for 2 h. The flask is shaken vigorously every 20–30 min. This solution is then removed and the tissue rinsed two to three times. The initial solution and rinses contain the luminal epithelial cells. The cells are pelleted by centrifugation (200 g for 10 min). The remaining endometrial tissue is cut into small strips (approximately 1 mm in diameter) and processed for glandular and stromal cell isolation (Zhang *et al.*, 1991).

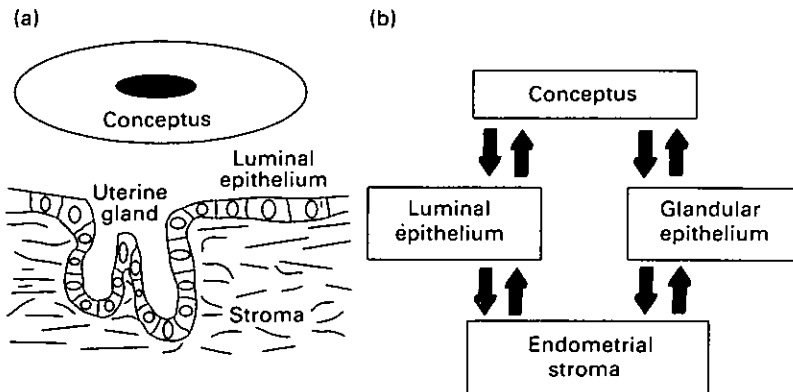


Fig. 1. (a) The tissues contributing to the uterine luminal environment and (b) the potential interactions between the tissues.

It is necessary to determine the secretory products of endometrial cells, the regulation of those secretions, and how these phenomena compare with the situation *in vivo* to characterize these cultures fully. Only limited information is available to describe the secretions of cultured endometrial cells. Here, we present a brief review of two classes of endometrial secretion, proteins and prostaglandins, and for each class, we present the available evidence for cell-type specific secretion.

### Proteins Secreted by the Pig Endometrium

Information concerning protein secretions of the pig endometrium is summarized (Tables 1–3). A variety of proteins has been identified and information on the proposed functions of some of the proteins is available. Most of these proteins first appear during the peri-attachment period (days 11–14). Most of the proteins are probably regulated by progesterone, and secretion of some proteins is further modified by oestrogen (Geisert *et al.*, 1982a, b). The uterine secretory proteins are useful markers for describing the phenotype of endometrial cells in culture. Conversely, culture of endometrial cells may provide information about their secretion and functions.

Uteroferrin is an iron-containing protein secreted by the progesterone-stimulated uterine glands of the pig endometrium and is secreted during the peri-implantation period. Secretion of uteroferrin increases markedly after day 30, is maximal at day 60, and then declines (Basha *et al.*, 1979; Simmen *et al.*, 1988b). Uteroferrin mRNA expression follows a similar pattern, except that the message remains abundant in late gestation but translation decreases (Simmen *et al.*, 1988b). Uteroferrin functions in iron transport to the fetus and has colony-forming unit activity (Bazer *et al.*, 1991) and acid phosphatase activity. The latter property is not believed to function in the uterus but provides a convenient method for assaying the protein. Uteroferrin is secreted by glandular epithelial cells *in vitro* as indicated by western analysis of the culture medium and by the presence of acid phosphatase activity in the medium (Zhang *et al.*, 1991). Culture media of the other two cell populations have not provided evidence for uteroferrin secretion. Uteroferrin secretion has been localized to the uterine glands *in vivo* by immunocytochemical methods (Fazleabas *et al.*, 1985).

Another uterine protein that appears to function in nutrient transport is retinol-binding protein (RBP). RBP is thought to control the supply of vitamin A to the conceptus (Adams *et al.*, 1981) and its expression appears to be induced by progesterone and modulated by oestrogen from the conceptus (Trout *et al.*, 1992). Recently Groothuis and Davis (unpublished), using endometrial cell cultures and western analysis, found evidence for RBP in the culture media of glandular and luminal epithelial cells harvested from sows on day 13 of pregnancy.

The mRNAs for both insulin-like growth factor II (IGF-II) and its binding protein (IGFBP-2) have been demonstrated in both types of epithelial cell and in the endometrial stroma (Simmen *et al.*, 1990) using enzymatic cell separation techniques. Expression of IGFBP-2, but not IGF-II, is induced by progesterone.

Table 1. Secretory proteins of the pig uterus: lysosomal enzymes

Protein	Cellular origin	Hormonal regulation	Properties	Postulated functions	Reference(s)
Uteroferrin	Glandular epithelium	Progesterone-induced and secretion is oestrogen modulated	MW = 35 000 Basic pl Iron binding, acid phosphatase activity and colony forming unit activity	Transfer of iron from uterus to conceptus; haematopoiesis	Chen <i>et al.</i> (1975) Basha <i>et al.</i> (1979) Renegar <i>et al.</i> (1982) Baumbach <i>et al.</i> (1984) Raub <i>et al.</i> (1985) Simmen <i>et al.</i> (1988a) Bazer <i>et al.</i> (1991) Simmen <i>et al.</i> (1991) Roberts <i>et al.</i> (1976)
Lysozyme	—	Progesterone-induced	MW = 14 000 Basic pl Cleaves $\beta$ 1, 4-glycosidic linkages of bacterial peptidoglycans	Antibacterial	Hansen <i>et al.</i> (1985)
$\beta$ -Hexosaminidase	—	Progesterone-induced	MW = 82 000–89 000 2 isoforms pl = 5.5 and 8.0	—	—
Cathepsin B <sub>1</sub>	—	Progesterone-induced	Wide range of proteolytic activity	—	Roberts <i>et al.</i> (1976)
Cathepsin D	—	Progesterone-induced	Wide range of proteolytic activity	—	Roberts <i>et al.</i> (1976)
Cathepsin E	—	Progesterone-induced	Wide range of proteolytic activity	—	Roberts <i>et al.</i> (1976)
Cathepsin L	—	May be progesterone-induced	MW = 40 000–45 000 pl = 6.0–6.5 Hydrolyses collagen and elastin	Facilitates endometrial growth	Blair <i>et al.</i> (1991)

Table 2. Secretory proteins of the pig uterus: growth factors and related peptides

Protein	Cellular origin	Hormonal regulation	Properties	Postulated functions	Reference(s)
IGF-I	—	Progesterone-induced and oestrogen-modulated	MW = 7000–8000 Related to insulin Promotes cell division and differentiation and tissue morphogenesis	Regulates endometrial remodelling	Simmen <i>et al.</i> (1990) Simmen <i>et al.</i> (1992a)
IGF-II	Luminal and glandular epithelium and stroma	—	MW = 7000–8000 Related to insulin Promotes cell division and differentiation and tissue morphogenesis	Mediates endometrial growth and differentiation	Simmen <i>et al.</i> (1990) Simmen <i>et al.</i> (1992a)
IGFBP-2	Luminal and glandular epithelium and stroma	Progesterone-induced	—	Modulates IGF activity	Simmen <i>et al.</i> (1990)
ULFM	—	—	MW = 4800	—	Simmen <i>et al.</i> (1992a)
EGF-like peptide	—	—	Mitogenic in uterine stroma cells MW < 10 000	—	Simmen <i>et al.</i> (1988a) Simmen <i>et al.</i> (1989) Simmen <i>et al.</i> (1988a)

IGF: insulin-like growth factor; IGFBP: insulin-like growth factor binding protein; ULFM: uterine luminal fluid nitrogen; EGF: epidermal growth factor.

Table 3. Other secretory proteins of the pig uterus

Protein	Cellular origin	Hormonal regulation	Properties	Postulated functions	Reference(s)
Uteroferrin-associated polypeptides	—	Progesterone-induced	3 Isoforms MW = 40 000, 46 000, 50 000 Basic pI	Maintains high MW uteroferrin in stable, active conformation	Baumbach <i>et al.</i> (1986)
Retinal-binding protein	Luminal and glandular epithelium	Progesterone-induced and oestrogen-modulated	MW = 20 000–22 000 pI = 6.0–6.5 Binds vitamin A	Supplies vitamin A to the conceptus	Adams <i>et al.</i> (1981) Clawitter <i>et al.</i> (1990) Trout <i>et al.</i> (1992)
Plasmin/trypsin inhibitor	Luminal and upper glandular epithelium	Progesterone-induced and oestrogen-modulated	MW = 14 500 Basic pI Inhibits trypsin plasmin and chymotrypsin	Controls proteolytic activity in the uterus	Mullins <i>et al.</i> (1980) Fazleabas <i>et al.</i> (1982) Fazleabas <i>et al.</i> (1983) Fazleabas <i>et al.</i> (1985)
Antileukoproteinase	Luminal and glandular epithelium	Progesterone-induced and oestrogen-modulated	MW = 14 000 Basic pI Hydrolyses elastase and cathepsin G	Maintains placental cell membrane integrity	Farmer <i>et al.</i> (1990) Simmen <i>et al.</i> (1991) Simmen <i>et al.</i> (1992b)
$\beta$ -Endorphin	Luminal and glandular epithelium	Progesterone-induced	Inhibits NK cell activity	Immune function	Li <i>et al.</i> (1987)
Met-enkephalin	—	Progesterone-induced	Enhances oestrogen binding by uterine epithelial cells	Enhances action of conceptus-derived oestrogen	Li <i>et al.</i> (1992) Li <i>et al.</i> (1991)

NK cell: natural killer cell

Further evidence for the involvement of growth factors in endometrial function comes from the report of Zhang *et al.* (1992), who found that both the stromal cells and glandular epithelial cells have epidermal growth factor receptors (EGF-R). These receptors are functional because EGF stimulates prostaglandin (PG) secretion by both types of cell. The ligand for this receptor may be transforming growth factor  $\alpha$  (TGF- $\alpha$ ) synthesized by the conceptus (Vaughan *et al.*, 1992), an EGF-like peptide in the uterine secretions (Simmen *et al.*, 1988a), or another ligand from sources that are not yet identified.

A plasmin/trypsin inhibitor has been localized in the luminal and shallow glandular epithelium of the pig endometrium (Fazleabas *et al.*, 1985). The inhibitor is induced by progesterone, and its secretion is further modulated by oestrogen (Fazleabas *et al.*, 1982).

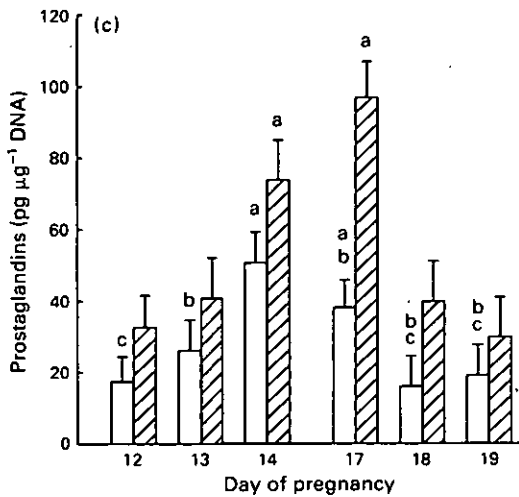
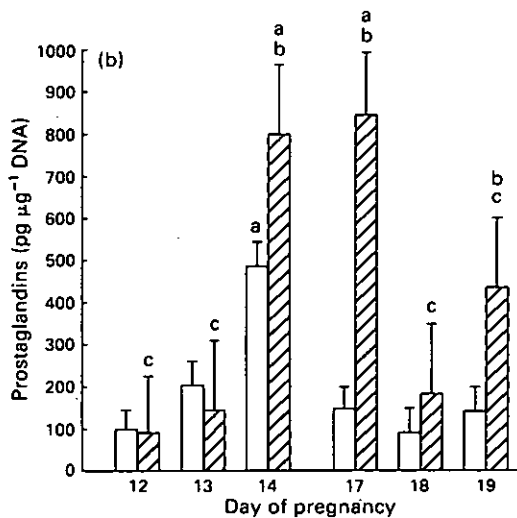
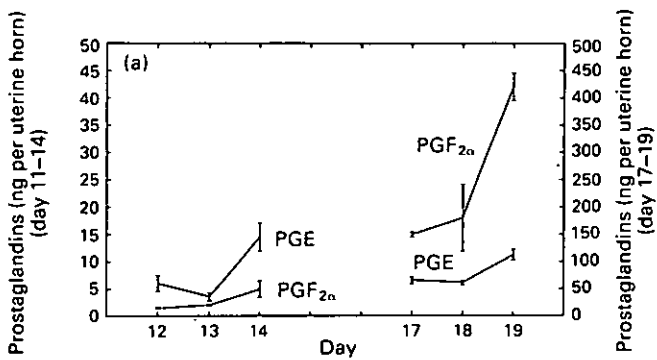
The opioid peptides represent another class of protein secreted by the pig endometrium. There is evidence for both  $\beta$ -endorphin (Li *et al.*, 1987, 1992) and *met*-enkephalin secretion (Li *et al.*, 1991).  $\beta$ -Endorphin is predominantly localized in the apical aspect of the luminal and glandular epithelium (Li *et al.*, 1992). These opioids are induced by progesterone and are found during the peri-implantation period (Li *et al.*, 1992).

The cellular origin of most of the uterine secretory proteins has not been determined. Separated cell types can be useful for determining cell-type specific secretion and has already been used to identify the localization of mRNAs for IGFs and IGFBP-2 (Simmen *et al.*, 1991). Culture of these cell types will also be useful for determining the regulation of cell-type specific secretion. For example, progesterone induces uteroferrin secretion *in vivo* (Roberts and Bazer, 1988), and Zhang *et al.* (1990) observed that glandular epithelial cells secrete more uteroferrin, measured as acid phosphatase activity, when progesterone is present in their culture medium.

### Prostaglandin Secretion by the Pig Endometrium

The endometrium is well known as a source of prostaglandins (PGs). The literature on endometrial production of PGs in pigs began over 17 years ago with the report of Patek and Watson (1976). Much attention has focused on the role of PGs in the uterine regulation of regression of the corpus luteum. Relatively little is known about the potential for PGs to regulate events associated with development of the pig conceptus and its attachment to the luminal epithelium. The roles for PGs in implantation in rodents have been the focus of attention (Kennedy, 1983; Malathy *et al.*, 1986; Gupta *et al.*, 1989). It is clear that in rats and mice the production of various prostanoids is tightly regulated at about the time of implantation and that both the blastocyst and the endometrium contribute to the prostanoid environment at the implantation site. Although the pig conceptus is less invasive in its attachment to the endometrium, many of the same signals may be operating at the attachment sites. Perhaps these events have received less attention in pigs because implantation is not easily monitored and spans several days. Other problems are the need to remove the uterus to monitor the progress of attachment and the need for careful work with the electron microscope to determine the earliest events. This is a physiological process that is ripe for the development of models *in vitro*.

All the cell types in the uterus and the conceptus secrete PGs. The complexity of this situation can be addressed by the separation and culture of individual endometrial cell types. In this section, we will outline our observations on the milieu of PGE and PGF *in vivo* and the production of these prostaglandins during the peri-implantation period by endometrial explants and the different types of endometrial cells in culture. The first attachment of the conceptus to the endometrium occurs on day 13 (Keys *et al.*, 1986), beginning in the region of the embryonic disc and extending towards the extremities of the chorion (Keys and King, 1990). All stages of attachment, including interlocking microvilli, are present at the earliest attachment (Keys and King, 1990), and the process of attachment continues at least to day 26 (Dantzer, 1985). We have collected data over two periods: at initial attachment on day 13 (peri-attachment) and during progressive attachment from day 17 to day 19. Paria and Rosenkrans (1988) determined the PG environment in the uterine lumen during these two periods, the accumulation of PGs in the culture medium of endometrial explants and the content of PGs in the endometrial tissue (Fig. 2). In other studies, we determined the PG secretory characteristics of endometrial cells harvested on different days of pregnancy (Zhang and Davis, 1991 and unpublished) (Fig. 3).





Prostaglandins in the uterine flushings present a dynamic picture, with an approximately tenfold increase in the amounts of PGs recovered from the uterine lumen between the peri-attachment period and the later period of progressive attachment. An additional difference is seen in the relative amounts of the two prostaglandins: PGE is relatively more abundant during the first period at initial attachment but, later, PGF predominates quantitatively. These data reflect contributions from both endometrium and conceptus. The endometrium itself shows an increased content and release of both PGs during an 8 h culture of explanted tissue. This result could reflect both endometrial production and a contribution of conceptus PGs that are taken up by the endometrium and released during culture. We therefore examined the PG secretion of individual types of cell after growth *in vitro* to determine the specific contribution of the endometrium (Fig. 3). These data provided some interesting insights. First, the glandular epithelium produces more PGF than PGE, whereas the stroma produces more PGE than PGF. This relative difference in secretion is also present when cells are incubated with [<sup>3</sup>H]arachidonic acid and the radioactive products determined (Blair, unpublished). Second, these relationships are altered by the conceptus. When the endometrial cells are harvested from pregnant pigs, the PGE:PGF secreted by stromal cells is increased over that observed when the cells are harvested from cyclic females (Fig. 4) (Zhang *et al.*, 1991). We recently repeated this comparison and obtained similar results (P. Groothuis, unpublished). Conceptus signals therefore result in an alteration in stromal cell PG synthesis that the cells 'remember' for a few days in culture. Longer studies have not been conducted.

The data from two studies addressing the question of effects of day of pregnancy are presented (Fig. 3). The initial studies of Zhang and Davis (1991) indicated that the day of pregnancy on which the cells are harvested affects their PG secretion. The PGE secretion by stromal cells harvested on day 13 increased markedly over the secretion by cells harvested on the 2 previous days. In another study with cells harvested on days 13 and 14 (Zhang and Davis, unpublished), it appeared that the heightened production of both PGs may extend to day 14. These observations probably reflect regulations in the pregnant uterus. Basha *et al.* (1979) observed that the secretory activity of pig endometrial explants depended on the physiological status of the donor. Furthermore, the similarity of the profiles of the endometrial explants and the glandular cells is consistent with the glandular cells being a major contributor to the luminal prostaglandin environment.

At present we can only speculate about the functions of these PG profiles, but their proximity to initial attachment of the conceptus to the endometrium is intriguing. PGE receptors have been detected in the pig endometrium (Kennedy *et al.*, 1986). The vascular effects associated with initial attachment include increased blood flow (Ford *et al.*, 1982a, b) and increased vascular permeability (Keys *et al.*, 1986; Keys and King, 1988). PG involvement in these responses to the attaching conceptus is tenable, and PGs have been implicated in a similar phenomenon in the peri-implantation uterus of other species (Kennedy, 1983). Furthermore, treatment of gilts with indomethacin, a PG synthase inhibitor, interferes with pregnancy (Kraeling *et al.*, 1985).

PGE and PGF are secreted by multiple cell types, and there are multiple regulators of their secretion. Cell types also vary in their response to some of the regulators (Zhang and Davis, 1992; Zhang *et al.*, 1992) and endometrial cell types differ in their relative production of PGE and PGF (Fig. 2). Because PGE and PGF differ in many of their actions, PGE:PGF may integrate information from different sources to contribute to an appropriate environment in each micro-region of the uterine lumen. Such a potential system is illustrated (Fig. 5) and might be particularly effective for subtly shifting micro-environments to fine-tune responses within a complex milieu. Presently, this concept is hypothetical but could explain how multiple endometrial cell types and the conceptus can produce and respond to the same signals without confusion.

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Fig. 2. (a) Prostaglandin content of the luminal flushings from one uterine horn on various days of pregnancy. Flushings were centrifuged (2000 g) for 15 min at 5°C and supernatants assayed. (b) (□) PGE and (▨) PGF in the culture medium of endometrial explants harvested on the indicated days of pregnancy and incubated for 8 h as described by Rosenkrans *et al.* (1990). Means within PG type without common superscripts are significantly different ( $P < 0.01$ ). Data are expressed as  $\text{pg } \mu\text{g}^{-1} \text{ DNA}$  in the explant. (c) Endometrial content of PGs. Tissue was frozen in the presence of indomethacin ( $10 \mu\text{g ml}^{-1}$ ) and later thawed, homogenized, extracted with ethyl acetate and (□) PGE and (▨) PGF in the extract determined by radioimmunoassay. Means within PG type without common superscripts are significantly different ( $P < 0.01$ ).

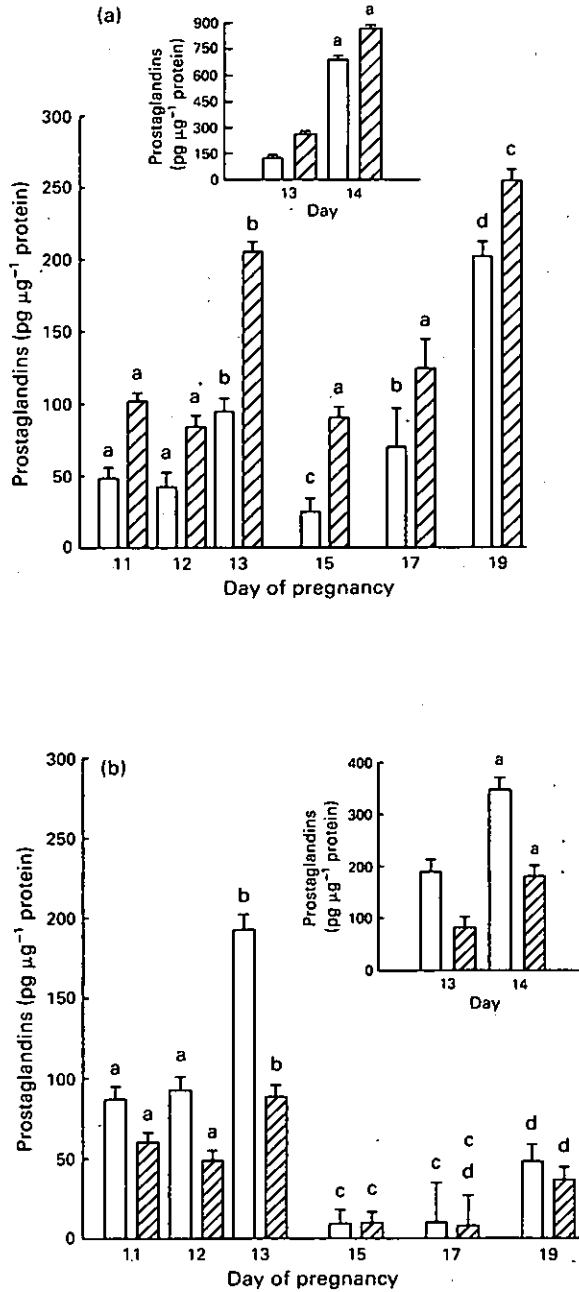


Fig. 3. Secretion of (□) PGE and (▨) PGF into the culture medium by cells harvested on various days of pregnancy. Data from Zhang and Davis (1991) are presented in the larger graphs, and the insets depict results of an unpublished study by Zhang and Davis. Data are expressed per μg cell protein and represent PGs secreted during 24 h. (a) Cultures of glandular cells. (b) Cultures of stromal cells. Means within PG type without common superscripts are significantly different ( $P < 0.05$ ).

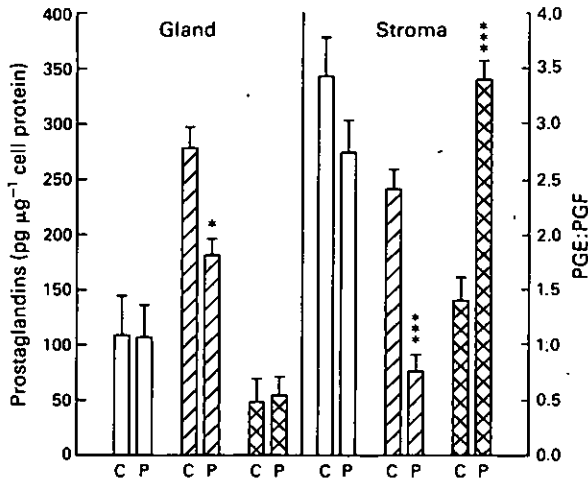


Fig. 4. Secretion of prostaglandins ( $\square$ ) PGE, ( $\boxtimes$ ) PGF and ( $\boxtimes$ ) PGE:PGF by endometrial cells harvested from sows on day 13 of pregnancy (P) or the oestrous cycle (C). Data represent production of the PGs during 24 h. Pregnancy effects indicated by \* ( $P < 0.05$ ) and \*\*\* ( $P < 0.001$ ). (Redrawn from Zhang *et al.*, 1991a.)

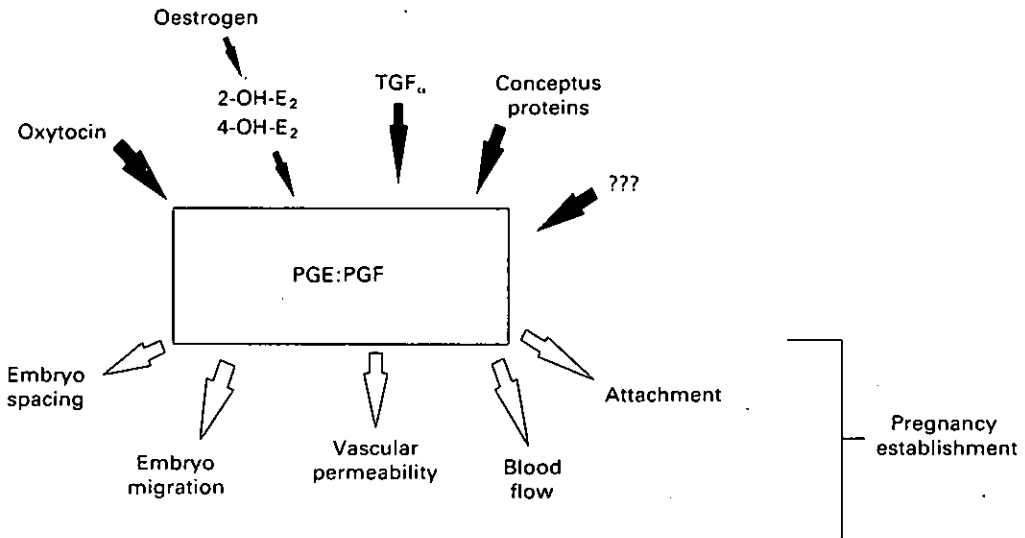


Fig. 5. A proposed model to explain the regulation and action of multiple sources of prostaglandins (PGE:PGF). The figure illustrates multiple regulators of PG secretion (solid arrows) and multiple sites of action of secreted PGs (open arrows). 2-OH-E<sub>2</sub>: 2-hydroxy oestradiol; 4-OH-E<sub>2</sub>: 4-hydroxy oestradiol; TGF $\alpha$ : transforming growth factor  $\alpha$ .

### Conclusions

Study of the pig endometrium has provided many insights into the physiology of early pregnancy. However, much remains to be learned about the functions and regulations of endometrial secretions. In addition, important aspects of the role of the endometrium in pregnancy are not addressed by present concepts. For example there is no explanation for the required synchrony between the embryo and uterus before day 10, and the implications of progesterone control of the uterine environment from day 4 to day

10 are not understood. Almost all of the uterine secretory proteins appear after day 10. During later stages of pregnancy, how does the endometrium respond to the conceptus when space per conceptus is 'roomy' versus 'crowded'? Are there endometrial adaptations to the crowded situation that can be exploited to increase litter size? These examples illustrate that the endometrium and its secretions will remain fertile areas for research for some time.

Contribution No. 93-408-J from the Kansas Agricultural Experiment Station. Part of the research in this paper was supported by NIH grant HD 26762-02.

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