Antiluteolytic mechanisms and the establishment of pregnancy in the pig

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Extended exposure of progesterone and conceptus estrogen influences the vascular compartment of the uterus and expression of many factors, such as prostaglandins (PGs), growth factors, extracellular matrix and adhesion molecules, cytokines and transcription factors. One of the supportive mechanisms by which the conceptus inhibits luteolysis is by changing PG synthesis in favor of luteoprotective PGE2. Alteration in PG synthesis may result from increased PGE synthase (mPGES-1) expression in the trophoblast and endometrium on days 10-13 of pregnancy with simultaneous down-regulation of PGF synthase (PGFS) and prostaglandin 9-ketoreductase (CBR1). Conceptus and endometrial, rather than luteal, synthesis of PGE2, is involved in the process of maternal recognition of pregnancy. However, complex (direct and indirect) actions of estrogen on the CL, including decreased luteal VEGF soluble receptor on day 12 of pregnancy, are important for luteal maintenance. Moreover, conceptus signals affect another lipid signaling component - lysophosphatidic acid receptor (LPA3), as well as HoxA10 and Wnt in the endometrium, to create the appropriate uterine environment for establishment of pregnancy and implantation.

Antiluteolytic mechanisms

In pigs, corpora lutea (CL) regression on days 15-16 of the estrous cycle results from an increase in pulsatile endometrial secretion of prostaglandin F2α (PGF2α) (for review, see Bazer *et al.* 1982). Basal PGF2α release by endometrium increases 3-fold between day 5 and 14 of the estrous cycle (Stepien *et al.* 1999). According to Krzymowski & Stefanczyk-Krzymowska (2008), the PGF2α pulsatile elevation in the blood outflowing from the uterus during luteolysis and shortly afterwards, results from decreased blood flow in the endometrium and excretion of PGF2α and its metabolites to lymph, blood and tissue fluids which accumulate PGs. However, oxytocin, luteinizing hormone (LH) and tumor necrosis factor α should be considered as potential modulators of endometrial PGF2α production during luteolysis (Uzumcu *et al.* 1998, Blitek & Ziecik 2006, Blitek *et al.* 2007).

The porcine CL is unusual among domestic animals because it does not display a luteolytic response to exogenous PGF2α until days 12-13 of the estrous cycle. The insensitivity of early CL to exogenous PGF2α is explained partially by low number of luteal PGF2α receptors (FPr) (Boonyaprakob *et al.* 2003) and a deficiency in post-FPr signaling (Zorrilla *et al.* 2009). Lower FPr concentration on day 14 of pregnancy/pseudopregnancy in comparison to the estrous cycle suggests that conceptus signals may inhibit FPr expression in the CL (Gadsby *et al.* 1993). The

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role of oxytocin in controlling PGF2α secretion is not as well defined as in ruminants (for review see, Ziecik et al. 2006). Systemic infusions of oxytocin-antagonist between days 12 and 20 of the estrous cycle reduce amplitude of PGFM (PGF2α metabolite) pulses, but does not prevent luteolysis (Kotwica et al. 1999). Surprisingly, oxytocin concentration in the uterine lumen significantly increases on days 12-14 of pregnancy when compared to corresponding days of the estrous cycle (Vallet et al. 1998). Moreover, oxytocin is not luteolytic when administered locally to the uterine lumen as it is when administered systemically (Sample et al. 2000) and intra-uterine infusion of oxytocin decreases plasma concentrations of PGFM on day 16 after estrus (Sample et al. 2004).

Maternal recognition of pregnancy (days 11-13) and implantation (days 14-19) are critical for CL maintenance and pregnancy establishment. Maternal recognition of pregnancy occurs simultaneously with rapid transformation of trophoblast from spherical to tubular then filamentous forms between days 10-12 just prior to implantation. During this period, conceptuses secrete elevated levels of estrogens, mainly estradiol-17β. A second, more sustained increase of estrogen secretion is observed between days 15 and 25-30 of pregnancy (Geisert et al. 1990). Inhibition of luteolysis and establishment of pregnancy in pigs require this biphasic pattern of estrogen secretion that results in prolonged luteal life span and progesterone secretion (for review, see Geisert et al. 1990). Systemic estrogen administration on days 11-15 of the estrous cycle prevents CL regression and extends CL life span (Frank et al. 1977). The luteoprotective action of estrogen is complex (Fig. 1). Estrogen stimulates luteal progesterone secretion directly (Conley & Ford 1989) as well as by increasing luteal LH receptor (LHR) concentration (Garverick et al. 1982) and by decreasing PGF2α release from uterus into peripherial circulation (Moeljono et al. 1977, Bazer & Thatcher 1977).

Estrogen receptor (ESR) expression in luminal (LE) and glandular epithelium (GE) of the endometrium (Geisert et al. 1993) and in the conceptus (Kowalski et al. 2002) coincides with estrogen secretion from the conceptus, which suggests both autocrine and paracrine responses. Both sufficient conceptus estrogen secretion (less than 2 conceptuses in each horn results in pregnancy failure) and timing of endometrial exposure to estrogen is critical for establishment of pregnancy. Early administration of estrogen on days 9-10 of pregnancy results in embryonic loss and altered endometrial expression of many genes, during later pregnancy, probably causing desynchronization of the uterine environment and conceptus implantation (Geisert et al. 2006). Included among the altered genes is an enzyme involved in PG synthesis (prostaglandin-endoperoxide synthase 2; PTGS2).

Endometrial function and conceptus development during the peri-implantation period of pregnancy are uniquely regulated through interacting effects of progesterone from the CL and estrogen from the conceptus. Estrogen is suggested to be involved in changes of uterine secretory activity (Geisert et al. 1982), increased blood flow (for review see, Bazer et al. 1982), endometrial edema (Laforest & King 1992), and regulation of endometrial expression of many factors (Fig. 1). However, other reports indicate that concentrations of many proteins within the uterine lumen during early pregnancy are independent of the presence of the conceptus (Vallet et al. 1998, Kayser et al. 2006).

Factors involved in PG synthesis and signaling during inhibition of luteolysis and pregnancy establishment

Concentrations and peak amplitude of PGF2α in utero-ovarian vein plasma are higher in cyclic than in pregnant gilts on days 12-17 (Bazer & Thatcher 1977, Moeljono et al. 1977). Moreover, uterine flushings of pregnant pigs contain higher amounts of PGF2α than those from
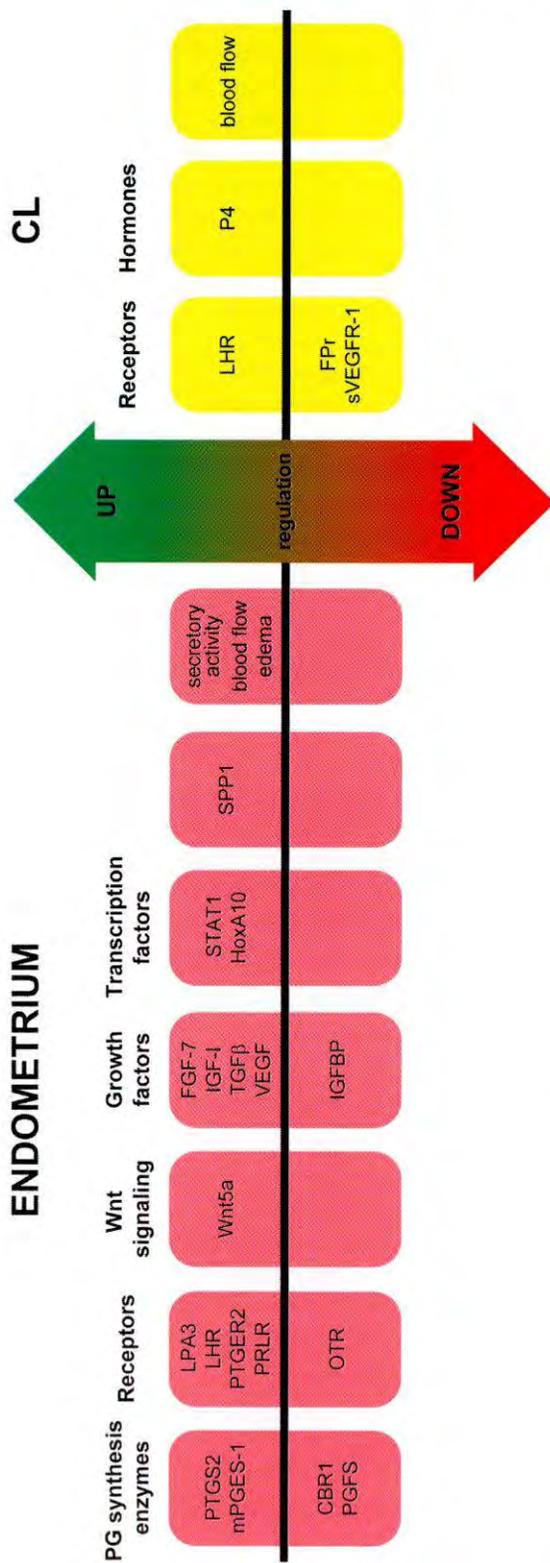
Fig. 1 Changes induced in the endometrium and the CL by conceptus estrogen and/or occurring simultaneously with increased conceptus estrogen secretion. PTGS2 - prostaglandin-endoperoxide synthase 2, mPGES-1 – PGE synthase, CBR1 - prostaglandin 9-ketoreductase/carbonyl reductase, PGFS - PGF synthase, LPA3 - lysophosphatidic acid receptor, LHR - luteinizing hormone receptor, PTGER2 – PGE2 receptor, PRLR – prolactin receptor (Young *et al.* 1990), OTR - oxytocin receptor, Wnt5a - wingless-type MMTV integration site family member, FGF-7 - fibroblast growth factor-7, IGF-I - insulin-like growth factor-I, TGFβ - transforming growth factor β, VEGF - vascular endothelial growth factor, IGFBP – IGF binding protein, STAT1 - signal transducer and activator of transcription 1 (Joyce *et al.* 2007), HoxA10 – homeobox A10, SPP1 – secreted phosphoprotein 1, FPr – PGF2α receptor, sVEGFR-1 – VEGF soluble receptor, P4 -progesterone.

cyclic animals (Zavy *et al.* 1980). This may be explained by the proposed theory that during maternal recognition of pregnancy, conceptus estrogen redirects PGF2α secretion from the uterine venous drainage (endocrine) to the uterine lumen (exocrine) (Bazer & Thatcher 1977). A part of the putative antiluteolytic mechanism could also be the retrograde transfer of PGF2α from venous blood and uterine lymph into the uterine lumen and ability of uterine veins and arterial walls to accumulate PGF2α (Krzymowski & Stefanczyk-Krzymowska 2004). However, Hunter & Poyser (1982) suggested that exocrine redirection of uterine PGF2α secretion may not provide a full explanation for maintenance of the CL in pregnancy in pigs, nor may this route of sequestering the luteolytic hormone always be effective.

Conceptus and/or endometrium PGE2 was suggested to have a role in CL protection against luteolytic PGF2α. In contrast to estrogen, intraluteal administration of PGE2 protects individual CL against luteolytic effect of simultaneously administered PGF2α (Ford & Christenson 1991). PGE2 is capable of extending CL life span (Akinlosotu *et al.* 1986). Interestingly, progesterone content is elevated in the blood plasma or luteal tissue after uterine infusions of PGE2 (Akinlosotu *et al.* 1986) or when increased secretion of PGE2 was observed in the gravid uterus (Christenson *et al.* 1994). The infusion of PGE2 into the ovarian artery increases the concentration of progesterone in ovarian venous blood on day 13 and 14 of pregnancy (Stefanczyk-Krzymowska *et al.* 2006). Moreover, higher PGE2/PGF2α ratio protects against the luteolytic effect of PGF2α on luteal cells collected on days 10-12 of the estrous cycle and stimulates progesterone and estradiol-17β secretion by these luteal cells (Gregoraszczuk & Michas 1999).

On days 11-13 of pregnancy, at the time of maternal recognition of pregnancy, the PGE2/PGF2α ratio in the uterus and uterine vein increases, suggesting that PGE2 helps to overcome the luteolytic effect of PGF2α, thus preventing CL regression (Davis & Blair 1993, Christenson *et al.* 1994). Therefore, another potential mechanism by which the conceptus helps to prevent luteolysis is by changing PG synthesis in favor of the luteoprotective PGE2 (Fig. 2), since the endometrium and trophoblast synthesize elevated amounts of PGE2 before implantation (Waclawik *et al.* 2006, Waclawik & Ziecik 2007).

PGs are synthesized by PTGS and specific terminal prostaglandin synthases: PGE synthase (mPGES-1) and PGF synthase (PGFS). Moreover, PGE2 can be converted into PGF2α by prostaglandin 9-ketoreductase/carbonyl reductase (CBR1). Our recent results *in vitro* indicate that estrogen stimulates PGE2 synthesis through increase of PTGS2 and mPGES-1 gene expression and decreases the content of enzymes involved in PGF2α production (PGFS and CBR1) in the endometrium on days 11-12 after estrus (Waclawik *et al.* 2009). It is consistent with findings that mPGES-1 expression is relatively high whereas PGFS and CBR1 expression is low in the endometrium between days 10-13 of pregnancy compared to the implantation period (Waclawik *et al.* 2006, Waclawik & Ziecik 2007). Similar, but more pronounced alterations of PG enzyme expression were found in conceptuses (Fig. 3, Waclawik & Ziecik 2007). Selective changes in PG synthesis enzymes during early pregnancy correspond with estrogen secretion by the conceptus. Endometrial mPGES-1 expression exhibits a biphasic profile, similar to conceptus estrogen. By contrast, rapid increase of PGFS and CBR1 after initiation of implantation corresponds with the decline in conceptus estrogen synthesis. Accordingly, estrogen elevates PGE2 content in the uterus (Geisert *et al.* 1982) and therefore may be an important factor regulating the PGE2/PGF2α ratio during early pregnancy.

Our recent studies indicate the existence of a putative PGE2 autoamplification loop in the endometrium that can additionally contribute to the increase of PGE2/PGF2α ratio during the peri-implantation window. PGE2 acting through endometrial PGE2 receptor (PTGER2) elevates secretion of this prostanoid and expression of the enzymes involved in PGE2 synthesis in the endometrium. Interestingly, endometrial PTGER2 is higher during implantation and can be regulated by estrogen (Waclawik *et al.* 2009).
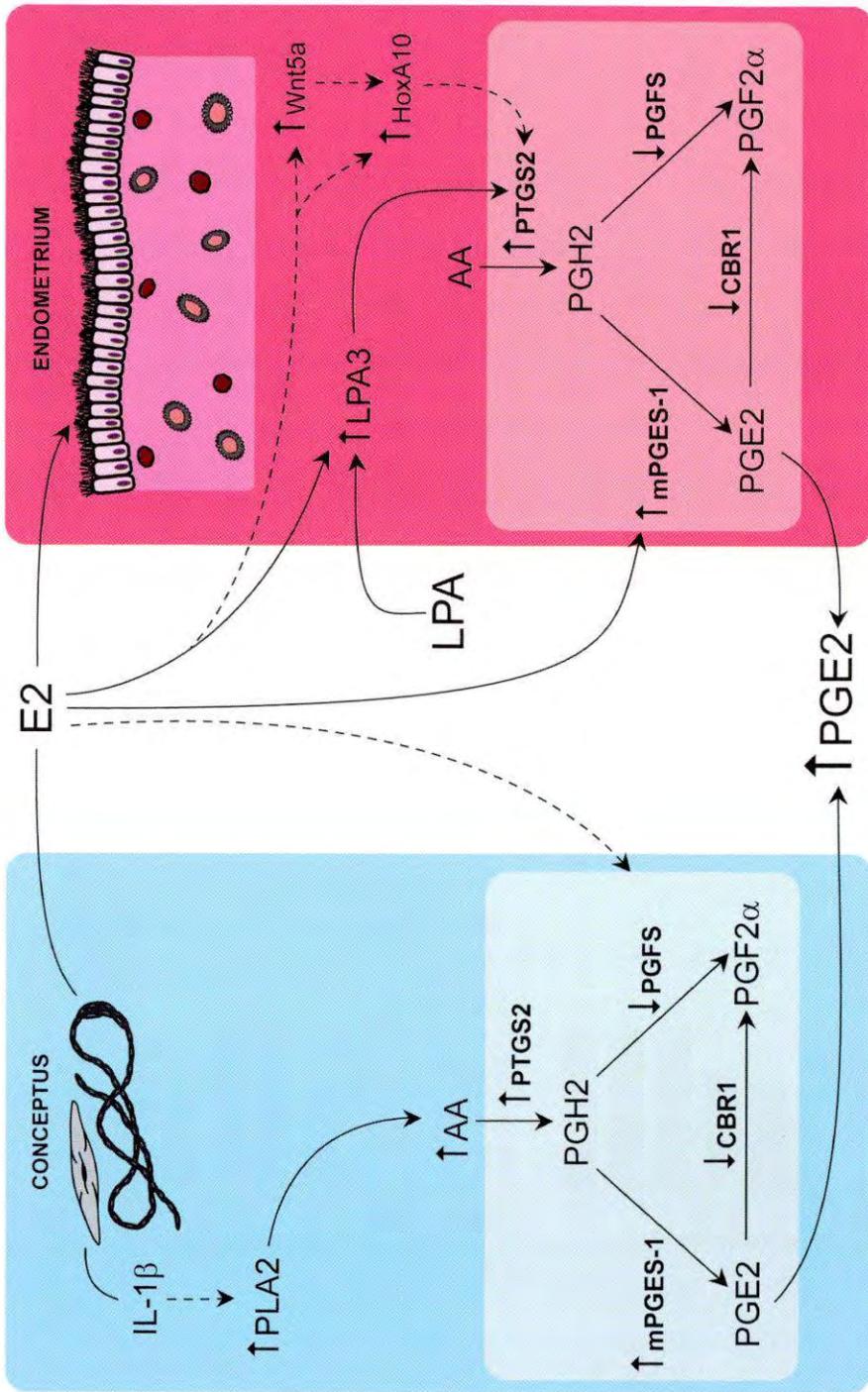
Fig. 2 Proposed concept of the role of the conceptus in the increase of PGE2 level and the PGE2/PGF2α ratio during the maternal recognition of pregnancy in the pig. Antagonistic changes in the expression of PGE synthase (mPGES-1) and the enzymes involved in PGF2α synthesis - PGFS and CBR1 in the endometrium or/and conceptus on days 10–13 of pregnancy may be a result of conceptus estrogen. LPA present in the uterus, activates PTGS2 expression via LPA3 receptors which are up-regulated by estrogen during maternal recognition of pregnancy. Abundant levels of IL-1β secreted by the conceptus induce phopholipase A2 (PLA2) and increase arachidonic acid (AA) release from cell membrane. Additionally, up-regulation of Wnt5a and HoxA10 in pregnancy may contribute to increase of PTGS2. PGH2 – prostaglandin endoperoxide H2.
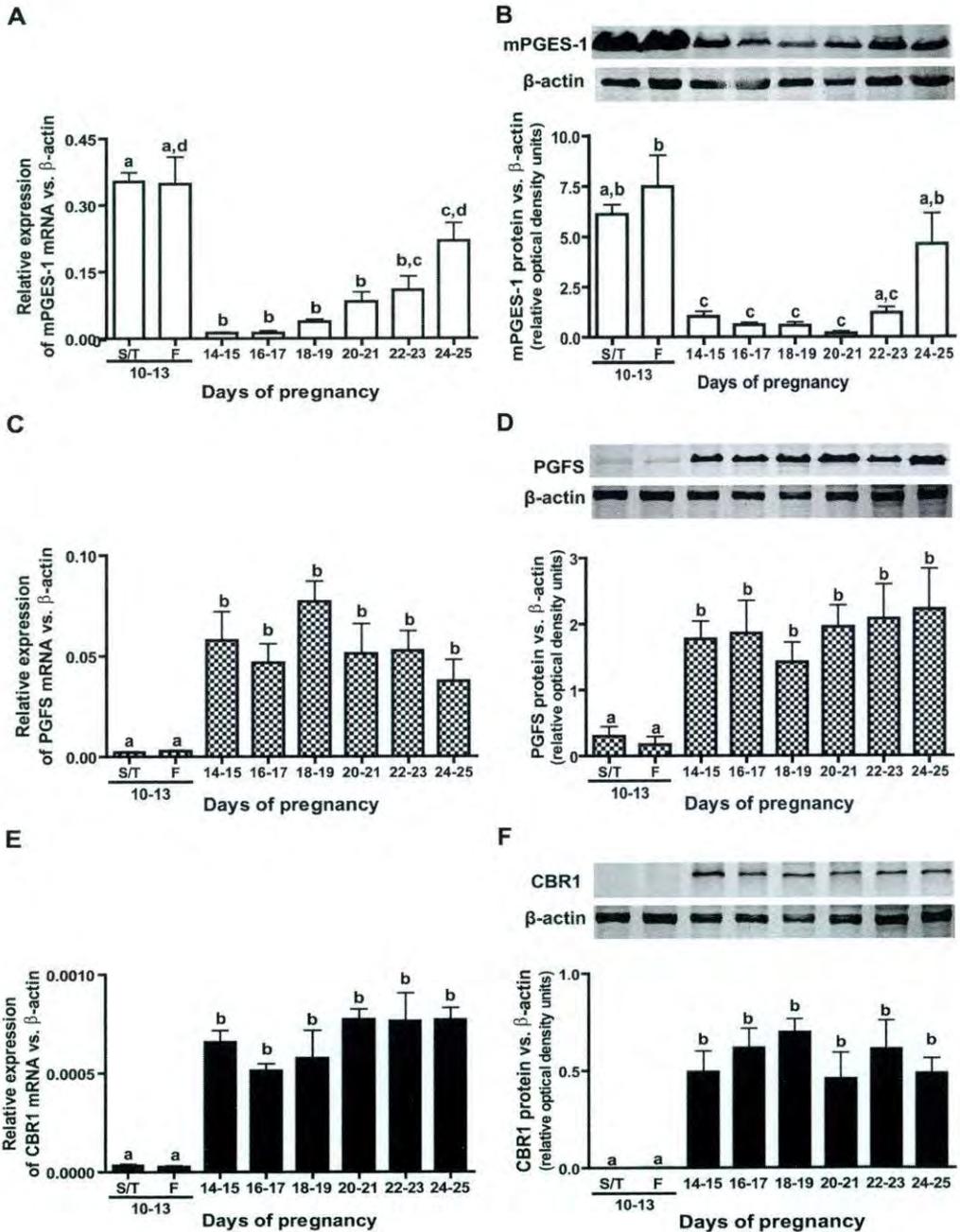
Fig. 3 Expression of PGE synthase (**A, B**), PGF synthase (**C, D**) and prostaglandin 9-ketoreductase/carbonyl reductase (**E, F**) in the porcine conceptus/trophoblast during early pregnancy (Waclawik & Ziecik 2007).

Although actions of luteal PGE2 in autoregulation of CL function cannot be excluded, synthesis of PGE2 in the conceptus and the endometrium likely contributes to the process of maternal recognition of pregnancy (Waclawik *et al.* 2008, Wasielak *et al.* 2008, Ziecik *et al.* 2008).

Lysophosphatidic acid (LPA)

LPA influences PG synthesis by increasing endometrial PTGS2 expression (Seo *et al.* 2008). Content and type of this phospholipid-derived mediator in uterine lumen differs between day 12 of the estrous cycle and pregnancy. LPA acts through specific G-protein coupled receptors LPA1-LPA4 and LPA3 is the dominant receptor in the porcine endometrium. Ye *et al.* (2005) indicated a critical role of LPA3 mediated signaling in mouse conceptus implantation. LPA3 is localized to LE and GE and its expression is elevated by estrogen in the pig (Seo *et al.* 2008). Endometrial LPA3 expression is increased during early pregnancy with the highest levels on days 11-12 and its mRNA content is higher in endometrium from the gravid compared to the non-gravid uterine horn (Kaminska *et al.* 2008).

HoxA10

Hox (for human HOX) genes are vertebrate homologs of *D. melanogaster* homeotic genes, that determine the identity of specific body segments. Recently, HoxA10 gene has attracted more attention, because it plays a role in uterine development. In mice, targeted disruption of expression of this transcription factor results in implantation failure (Benson *et al.* 1996). Moreover, this gene was implicated in the control of endometrial PTGS2 expression and PG synthesis in mice (Paria *et al.* 2002). In human, ovarian steroid-regulated HOXA10 expression in endometrium and peak expression of HOXA10 during the window of implantation suggests an important role for this gene in controlling uterine receptivity (Daftary & Taylor 2006). Our latest studies showed up-regulation of endometrial HoxA10 during implantation in the pig (Kaczmarek *et al.* 2009a) and the stimulatory effect of estradiol-17β on its expression in endometrium (A Blitek, unpublished).

Wnt

HoxA10 gene expression may be regulated by Wnt (Bartol *et al.* 2006). Wnt may mediate trophoblast-epithelial interactions critical for uterine receptivity to implantation (Hayashi *et al.* 2007). Our recent research indicates that Wnt4 mRNA expression does not change significantly in the endometrium during the estrous cycle and early gestation whereas level of Wnt5a is higher during early pregnancy (Kiewisz *et al.* 2009). Moreover, expression of Wnt4 is down-regulated and Wnt5a and E-cadherin up-regulated in the CL during early pregnancy (J Kiewisz, unpublished).

Luteinizing hormone receptors

LH is able to increase PGE2 secretion and PTGS2 expression in the endometrium (Blitek *et al.* 2007). Additionally, estrogen administration between days 11-15 after estrus results in an increase of LH-induced PGE2 release from endometrium *in vitro* (G Bodek, A Ziecik, unpublished). Thus, it is possible that conceptus estrogen maintains endometrial LHR expression ensuring a higher output of PGE2. However, only simultaneous action of estradiol-17β and

progesterone results in increased PGE2 secretion in response to LH in stromal cells (Blitek *et al.* 2007). Therefore, the endometrial expression of LHR during early pregnancy may also be involved in the luteoprotective action of LH.

Uterine receptivity and the establishment of pregnancy

Pigs have a true epitheliochorial placenta in which LE remains intact throughout pregnancy. Hormonally regulated signals from the ovary induce essential changes manifested by proliferation, differentiation, and spatiotemporal expression of specific biological molecules, leading to a temporary state of uterine receptivity for implantation. This state may be further enhanced by additional factors secreted by conceptus. However, some changes are independent of conceptus presence and controlled mainly by progesterone (for review, see Bowen & Burghardt 2000). Sustained stimulation of the endometrium by progesterone over 7-8 days causes a loss of progesterone receptors (PR) from LE and GE by day 10 of the estrous cycle and pregnancy, which is highly associated with the development of endometrial receptivity for conceptus implantation (for review, see Geisert *et al.* 2006). Since PR are maintained in stroma and myometrium, the effects of ovarian progesterone on expression of many factors in LE may be mediated indirectly by either progesterone-induced progestamedins produced by PR-positive stromal cells or by induction of molecules in LE that causes loss of PR to regulate expression of endometrial genes (Geisert *et al.* 2006, Ka *et al.* 2007).

Progesterone induces down-regulation of PR from LE and GE resulting in the decrease in mucin-1 from the apical surface of LE and exposure of integrins for trophoblast attachment (Bowen *et al.* 1996). Expression of $\alpha 4$, $\alpha 5$, $\beta 1$ integrin subunits increases on days 11–15 in both cyclic and pregnant gilts and is regulated by progesterone alone or together with estrogen (Bowen *et al.* 1996). Secreted phosphoprotein 1 (SPP1), induced by estrogen in LE during apposition phase of implantation, is a potent adhesion protein mediating attachment between trophoblast and LE (White *et al.* 2005).

Cytokines

Among cytokines, interleukin-1β (IL-1β) seems to play a pivotal role during maternal recognition of pregnancy, and may be involved in cell adhesion and other cytokine stimulation. Abundant levels of IL-1β secreted by the conceptus are associated with rapid trophoblast elongation (Ross *et al.* 2003). Moreover, Geisert *et al.* (2006) suggested that IL-1β, an inducer of phospholipase A2, regulates arachidonic acid release from cell membrane, thus ensuring the membrane fluidity necessary for trophoblast remodeling during conceptus elongation and contributing to PG synthesis (Fig. 2). Interferon γ (INFγ) and interferon δ (INFδ) of conceptus origin, IL-6 of conceptus and endometrial origin and leukaemia inhibitory factor (LIF) produced by endometrium (Modric *et al.* 2000, Joyce *et al.* 2007) may also serve as conceptus-maternal signaling molecules involved in the proliferation, differentiation and cell survival that is associated with conceptus growth and implantation.

Growth factors

Growth factors are also implicated in conceptus development and successful establishment of pregnancy since endometrial expression of fibroblast growth factor-7 (FGF-7), transforming growth factor β (TGFβ), vascular endothelial growth factor (VEGF) and uterine lumen content of insulin-like growth factor-I (IGF-I) are elevated on days 12-13 of pregnancy and may be

regulated by estrogen and/or progesterone (for review, see Ziecik *et al.* 2006). TGFβ stimulates expression of extracellular matrix molecules and integrins as well as proteases and protease inhibitors to facilitate conceptus-uterus connections during implantation and to limit trophoblast invasiveness (Burghardt *et al.* 2002). Additionally, IGF-I increases steroidogenesis by stimulation of P450 aromatase expression in filamentous conceptuses (Green *et al.* 1995). Transition from spherical to filamentous trophoblast is associated with the decrease of IGF binding proteins (IGFBP) in the uterine lumen that regulates IGF bioavailability (Geisert *et al.* 2006). Both IGF-I and PGE2 stimulate VEGF expression in stromal cells during maternal recognition of pregnancy, which may result in growth and remodeling of endometrial vasculature (Kaczmarek *et al.* 2008). Additionally, conceptus VEGF164 expression increases gradually with conceptus development until day 16 of pregnancy (Kaczmarek *et al.* 2009b) suggesting that VEGF164 may induce endometrial vascular permeability and edema during implantation.
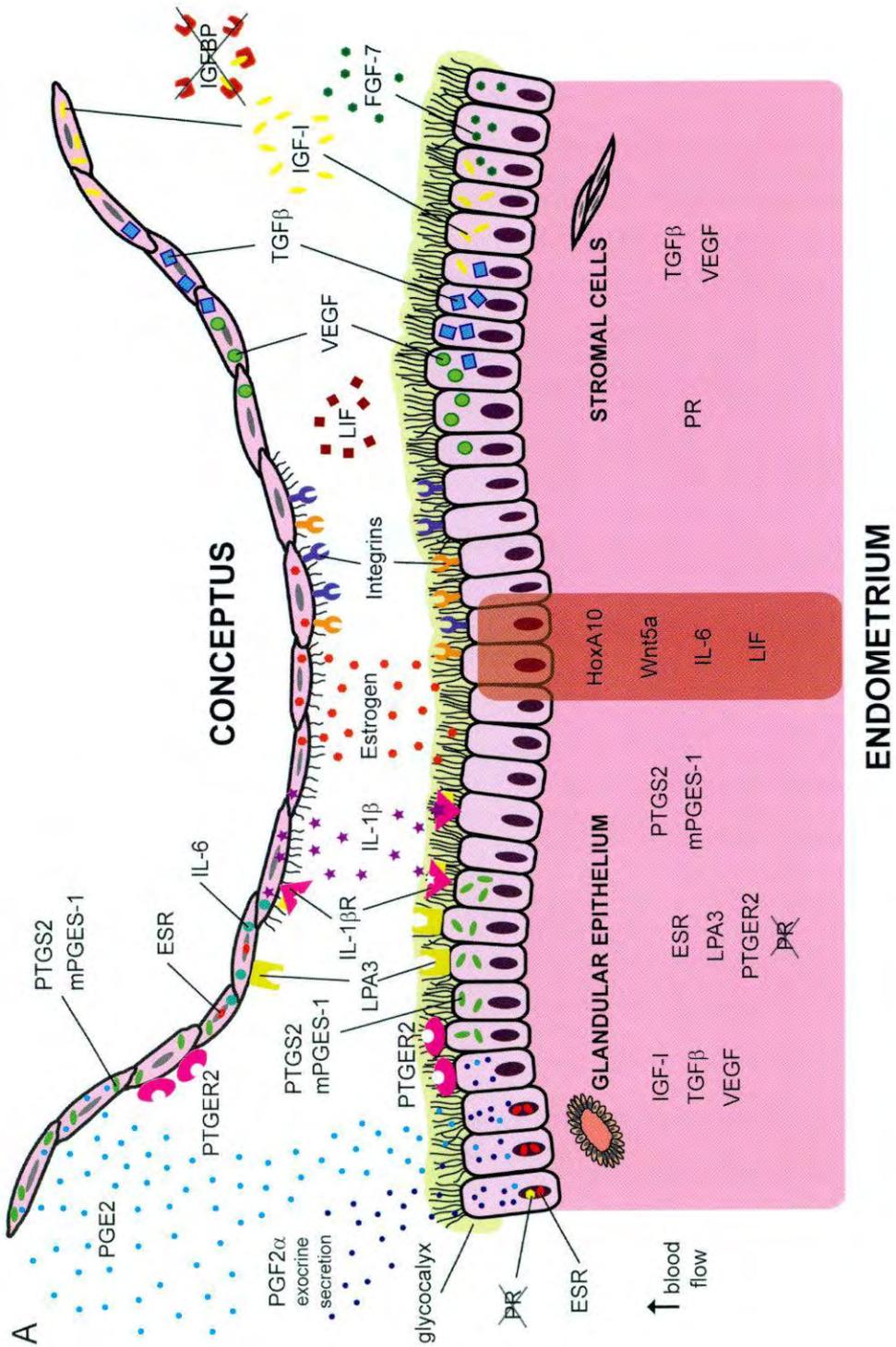
The VEGF ligand-receptor system is involved in maintenance and stabilization of the vascular bed in CL during pregnancy. PGE2-stimulated VEGF secretion by luteal cells on days 10-12 of pregnancy suggest that the luteoprotective actions of PGE2 may be partially mediated by luteal VEGF (Kowalczyk *et al.* 2008). Interestingly, VEGF soluble receptor (sVEGFR-1) mRNA levels are lower in the CL on day 12 of pregnancy compared to the estrous cycle (Kaczmarek *et al.* 2009b). Down-regulation of sVEGFR-1 (strong endogenous antagonist of VEGF) and the consequent elevation of bioavailable VEGF may sustain progesterone production by increasing luteal capillary permeability, which may aid transport of PGs from the circulation and delivery of cholesterol to the luteal cells.
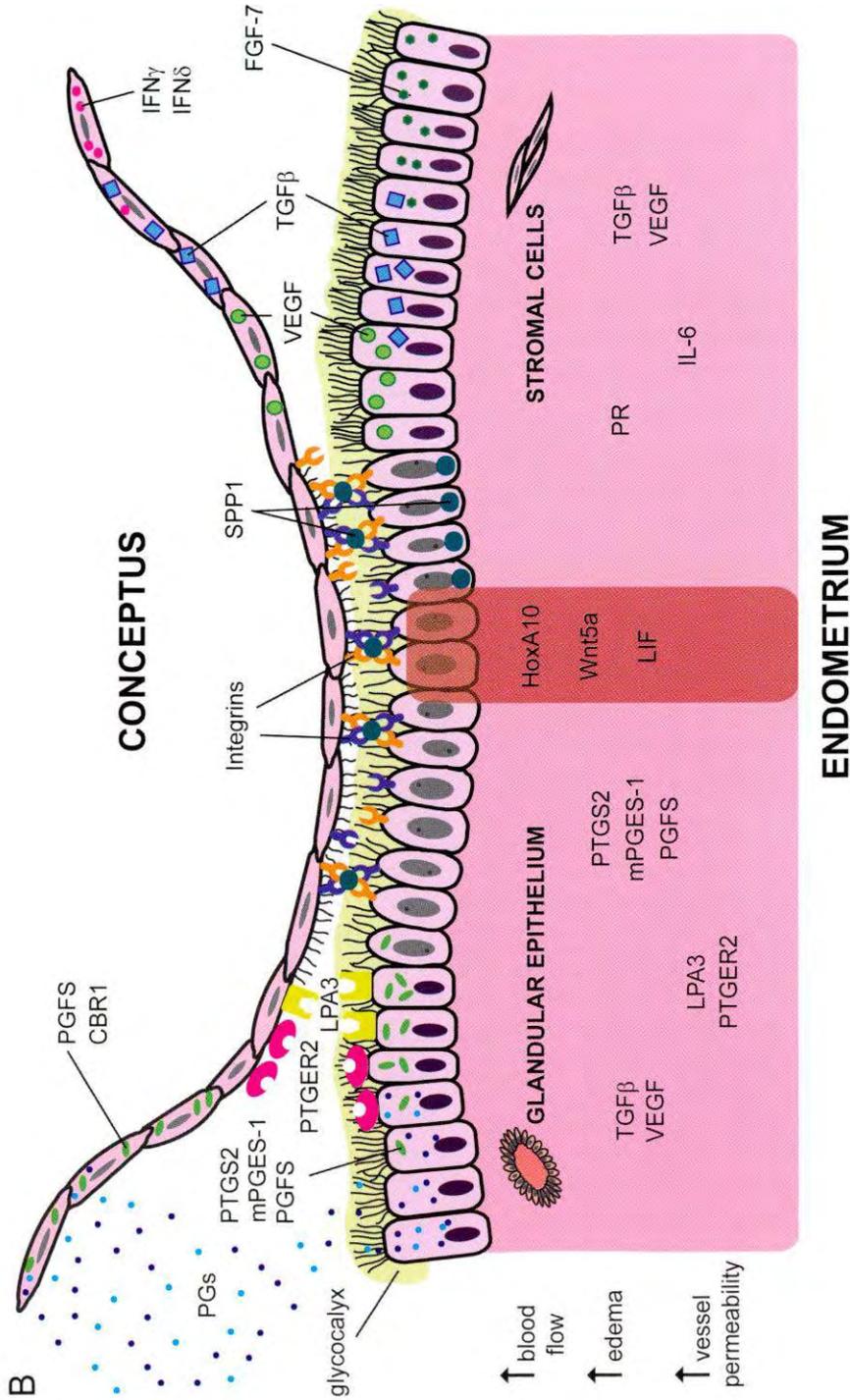
Conclusions

The conceptus affects the lipid signaling system (prostaglandin and LPA) as well as HoxA10 and Wnt (factors which may be involved in PG synthesis) in the porcine endometrium in order to inhibit luteolysis and/or to create the appropriate uterine environment for conceptus development and implantation (Fig. 2). Conceptus estrogen may prevent luteolysis through modification of expression of the enzymes involved in PG synthesis, reducing release of PGF2α and favoring PGE2 release on days 10-13 of pregnancy. Down-regulation of luteal sVEGFR-1 may be supportive of CL function during maternal recognition of pregnancy. Although it is evident that estrogen and progesterone induce essential proliferative and differentiative changes in the endometrium leading to a temporary state of uterine receptivity, a number of growth factors, cytokines and extracellular matrix and adhesion molecules are required to establish the necessary dialog between the conceptus and endometrium (Fig. 4).

Acknowledgements

A

CONCEPTUS

ENDOMETRIUM

IGF-BP

FGF-7

IGF-I

TGFβ

VEGF

LIF

Integrins

Estrogen

IL-1β

IL-6

ESR

IL-1βR

LPA3

PTGS2
mPGES-1

PTGS2
mPGES-1

PTGER2

ESR

PTGER2

PGE2

PGF2α
exocrine
secretion

glycocalyx

PR

ESR

↑ blood
flow

STROMAL CELLS

TGFβ
VEGF

PR

HoxA10

Wnt5a

IL-6

LIF

GLANDULAR EPITHELIUM

PTGS2
mPGES-1

ESR
LPA3
PTGER2

PR

IGF-I
TGFβ
VEGF

Fig. 4 Maternal recognition of pregnancy on days 11-12 (**A**) and the initial attachment of conceptus to the endometrium on days 14-16 (**B**) is dependent on various biological molecules produced by the endometrium and conceptus. Factors for which localization of expression in specific endometrial cells remains undetermined, are indicated in a red box.

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