Regulation of the porcine corpus luteum during pregnancy

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Abstract

The new corpora lutea (CLs) in pigs are formed from the preovulatory follicles after the luteinizing hormone (LH) surge. However, total autonomy and independence of CLs from LH up to Day 12 of cycle has recently been questioned. Transformation of estrous cycle CL to CL of pregnancy initiated by embryonic signals requires not only the cessation of prostaglandin F₂ (PGF_{2α}) supply to the luteal tissue but also needs the CL to overcome luteolytic acquisition and/or changing its sensitivity to PGF_{2α} during Days 12–14 of pregnancy. The luteolytic cascade is prevented by inhibition of lymphocyte infiltration and leucocyte recruitment, limitation of cell apoptosis, upregulation of pregnancy-associated genes and an enhanced antiluteolytic role of PGE₂. Our ‘two-signal switch hypothesis’ highlights the importance of *post* PGF_{2α} and PGE₂ receptor signaling pathways activation in CLs during luteolysis and rescue. The ‘luteolytic switch’ involves increased expression of many regression mediators and activation of the *post* PTGFR signaling pathway. The ‘rescue switch’ initiated by embryonic signals – estradiol 17β and PGE₂ – induces *post* PTGER2/4 pathway, turning the ‘luteolytic switch’ off and triggering activity of genes responsible for CL maintenance. In mid and late pregnancy, CLs are maintained by LH and the synergistic action of metabolic hormones. This paper provides an outline of recent views on CL regression, rescue and maintenance during pregnancy in pigs that conflict with previous paradigms and highlights new findings regarding the actions of prostaglandins, role of microRNAs (miRNA) and immune system and signaling pathways governing the life cycle of porcine CL.

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Introduction

The life cycle of the corpus luteum (CL) during the estrous cycle includes three phases: formation, maintenance and regression. However, the presence of embryo maintains the CL and instead of regression, CL undergoes rescue and the sustained maintenance during the 114 days long pregnancy in pigs. The presence of functional CLs is necessary not only for establishment but also for continuation of pregnancy until parturition. After Day 12 of each estrous cycle, when embryos are not present in the porcine uterus, CLs undergo regression that is crucial to prepare the ovaries for the next ovulation and to allow oocytes to be released and fertilized in the next cycle. This review is focused on the rescue and maintenance of porcine CLs during pregnancy, but some aspects of CL regression during the estrous cycle are also discussed.

The paradigmatic theories of maternal recognition of pregnancy, i.e. ‘endocrine vs exocrine’ (Bazer & Thatcher 1977) and/or ‘retrograde’ (Krzymowski & Stefanczyk-Krzymowska 2004) transfer of prostaglandin F_{2α} (PGF_{2α}) in the porcine reproductive tract were focused on the redirection of PGF_{2α} towards the ovary during the luteal phase or on PGF_{2α} accumulation in the uterus during early pregnancy respectively. Fortunately, the ‘omics era’ brought a handful of evidence for crucial roles of

several other factors in CL rescue, thereby broadening our knowledge beyond a simple role of circulating PGF_{2α} in CL regression (Østrup *et al.* 2010, Franczak *et al.* 2013, Zhang *et al.* 2013, Kiewisz *et al.* 2014). During pregnancy, CL rescue is possible due to the paracrine action of conceptuses on both the endometrium and luteal tissue (for review see: Waclawik *et al.* 2017). For many years, estrogen was recognized as the most important secretion of embryonic origin involved in the maintenance of luteal function during pregnancy; however, our studies clearly indicated a strong role of conceptuses and uterine PGE₂ in the rescue of porcine CLs (Waclawik & Ziecik 2007, Przygodzka *et al.* 2016). Here, we present our updated ‘two signal-switch hypothesis’ on the role of both PGF_{2α} and PGE₂ signaling pathways in CL regression and rescue during the maternal recognition of pregnancy in the pig.

The lack of global profiling studies on pregnancy-associated genes involved in rescue of the porcine CL during pregnancy forced us recently to evaluate the expression of over 50 genes potentially involved in the process of porcine CL maintenance (Przygodzka *et al.* 2015, 2016). Moreover, due to increasing understanding of the regulatory role of non-coding RNAs and, on the other hand, the lack of evidence for their involvement

in luteal function maintenance, we decided to identify miRNAs in porcine CLs to target genes associated with luteal function maintenance (unpublished). The suggested roles of the most important genes and miRNA–mRNA interactions are discussed in this review and contrasted with the available scientific literature.

Finally, a role of potential pituitary stimuli (LH and prolactin) affecting luteal function during the estrous cycle and pregnancy is summarized and known controversies are discussed in the light of new evidence.

CL function during the estrous cycle

Formation, early maintenance and role of LH

During the last stages of follicular maturation under the influence of LH, the theca and granulosa cells of the ovulatory follicle undergo luteinization and differentiation into luteal cells. After luteinization and formation of new CLs, both the granulosa- and theca-derived cells can utilize cholesterol for *de novo* steroidogenesis and production of progesterone, the major functional steroid and product of CLs (LaVoie 2017). The high production of progesterone in pigs is necessary to prepare the uterus for implantation and early conceptus development. The presence of CLs in pigs is required during the entire pregnancy.

The early reports of Du Mesnil Buisson and Leglise (1963) and Anderson *et al.* (1967) suggested that after the initial preovulatory LH surge, the porcine CL can act autonomously up to Day 12 of the estrous cycle, so neither hypophysectomy nor decreased LH levels by progesterone administration (Woody *et al.* 1967) cause immediate regression of this endocrine gland.

The application of specific anti-LH antibodies produced in pigs (Szafranska & Ziecik 1989) significantly decreased progesterone concentrations in both non-pregnant gilts on Days 8–14 of the estrous cycle and pregnant animals on Days 42–46. Also, inhibition of LH secretion with a gonadoliberein (GnRH) antagonist during the early luteal phase reduced CL development and progesterone production (Brussow *et al.* 2001), indicating that the porcine CL appears to be not as autonomous during the estrous cycle as was believed earlier (Bazer *et al.* 1982). This was further supported by *in vitro* studies showing LH-induced secretion of progesterone by cultured large and small luteal cells (Buhr 1987) as well as slices of CLs collected at the mid-luteal phase (Przygrodzka *et al.* 2014).

The amplitude of LH pulses in the mid-luteal phase of the estrous cycle and early gestation reaches 50% of the magnitude of the preovulatory LH surge (Ziecik *et al.* 1983), while their duration is 60–70 min with frequencies of 4–10 during 12 h (Hoover & Young 1979). Although LH is thought to be main luteotropic hormone in pigs, there is no correlation between pulses of LH and progesterone in their blood plasma (Brinkley 1981,

Van de Wiel *et al.* 1981), except during late pregnancy (Parvizi *et al.* 1976).

Measurements of the luteal LH receptor (LHCGR) content in porcine CLs revealed dramatic changes during the cycle and early pregnancy. During the estrous cycle, its concentration peaks on Days 10 and 12 before declining as the CLs regress (Ziecik *et al.* 1980). Interestingly, administration of estrogen upregulates luteal LHCGR concentrations in non-pregnant animals (Garverick *et al.* 1982).

Factors responsible for luteal regression during the luteal phase

In the absence of embryonic signal(s) at the end of the luteal cycle, regression process in the pig is initiated; CL regression is mediated by pulsatile endometrial secretions of PGF_{2α} on Days 12–13 and 15–16 of the estrous cycle (Moeljono *et al.* 1977). The biological rationale for CL regression is preparation of the ovary for the next ovulation and to allow oocytes to be released and fertilized in the next cycle. The actions of PGF_{2α} include the reduction of steroidogenic enzyme activity and progesterone production within luteal tissue (Niswender 2000). In a number of studies, oxytocin (Carnahan *et al.* 1996), tumor necrosis factor α (TNFα; Wutke *et al.* 1998) and LH (Blitek & Ziecik 2005) were suggested as potential modulators of endometrial prostaglandin production within luteal tissue.

The role of LH in luteolysis was, however, reported to be limited to the late luteal phase of the estrous cycle (Ziecik *et al.* 2001). Interestingly, both components of the porcine uterus, endometrium and myometrium, also possess LHCGR (Ziecik *et al.* 1986). The appearance of relatively high amounts of LHCGR in the endometrium coincides with the elevation of PGF_{2α} secretion (Moeljono *et al.* 1977) and perhaps with decreased expression of progesterone receptors (PGRs). After the beginning of luteolytic PGF_{2α} release on Days 14–16, the amount of endometrial LHCGR declines. It was found that LH upregulates prostaglandin-endoperoxide synthase 2 (PTGS2) protein expression and PGF_{2α} secretion from endometrial cells *in vitro* (Stepien *et al.* 1999). Furthermore, intravenous administration (Ziecik *et al.* 2001) or intramuscular challenge with human chorionic gonadotropin (hCG; Guthrie & Bolt 1983) induces uterine PGF_{2α} release *in vivo*. It seems that a window of endometrial sensitivity to LH *in vivo* decreases during Days 15–17 of the estrous cycle. Additionally, there is a much higher correlation between peaks of LH and PGF_{2α} metabolite (PGFM; 75.5%; Ziecik *et al.* 2001) than between oxytocin and PGFM (30%; Kotwica *et al.* 1999). This means that the majority of the 'luteolytic' action of LH *via* its endometrial receptors contributes to destruction of unnecessary cyclic CLs to allow the next ovulation and another chance for successful conception. The luteal regression occurring on Days 13–14 of the

estrous cycle is also thought to be caused by an increase of luteolytic sensitivity (LS) by CLs to endometrial $\text{PGF}_{2\alpha}$.

The mechanisms responsible for luteal regression are complex and still not well understood in pigs. There are older and some recent reviews summarizing the roles of several molecules involved in luteolysis (Ziecik 2002, Wacławik *et al.* 2017, Ziecik *et al.* 2017). Many of the identified factors associated with luteal regression, such as endothelin 1 (*EDN1*), hypoxia-inducible factor 1 α (*HIF1A*), estrogen receptor 2 (*ESR2*) and *PTGS2*, undergo profound changes in their expression levels after Day 12 of the estrous cycle when CLs have acquired LS. Simultaneously, decreased expression of genes involved in lipid cleavage was observed (Przygodzka *et al.* 2016; Fig. 1).

Data on the role of inflammatory cytokines and immune cells in the control of luteolysis are also limited in pigs. Our recent results, however, have revealed that genes associated with infiltration of lymphocytes are induced during the beginning of functional regression in the pig (Przygodzka *et al.* 2016). The role of macrophages in luteal regression is well established in

pigs (Zhao *et al.* 1998, Chang *et al.* 2017). Macrophages are present in all phases of the porcine CL lifespan and their function depends on the luteal microenvironment and phase of the estrous cycle. However, their numbers are highest during the late luteal phase. Macrophages are the primary source of $\text{TNF}\alpha$, an inflammatory cytokine involved in the regression process during the late luteal phase (Zhao *et al.* 1998). The LS to $\text{PGF}_{2\alpha}$ is also known to be associated with infiltration of macrophages into the porcine CL (Chang *et al.* 2017). Using flow cytometry, we showed that, whereas $\gamma\delta$ (T-null) cells might have a role in CL regression, Treg (regulatory T cells) percentages were higher in CLs obtained from pregnant animals on Days 13–14. Although only a small proportion of T cells were $\gamma\delta$, their percentage was significantly higher on Day 14 of the estrous cycle compared to Day 9 (Andronowska A, Jalali BM, Likso P & Witek K unpublished data). Leukocyte-derived cytokines such as $\text{TNF}\alpha$, interferon γ ($\text{IFN}\gamma$) and interleukins (IL-1, IL-4 and IL-6) are present in the CL at different stages of the luteal phase (Sakamoto *et al.* 2006, Zmijewska *et al.* 2012). Both $\text{TNF}\alpha$ and $\text{IFN}\gamma$ are known to interact with $\text{PGF}_{2\alpha}$ leading to a decline in progesterone synthesis (Sakamoto & Okuda 2004). $\text{TNF}\alpha$ is also involved in structural luteolysis through its role in apoptosis (Okano *et al.* 2006). Sensitization of porcine CLs to the luteolytic action of $\text{PGF}_{2\alpha}$ involves upregulation of $\text{TNF}\alpha$ observed on Days 12–14 of the estrous cycle (Przygodzka *et al.* 2014). The action of $\text{TNF}\alpha$ is mediated through two different receptors (TNFR1 and TNFR2) in porcine luteal and endothelial cells. TNFR1 mediates an apoptotic function because it possesses a death domain. On the other hand, TNFR2 is rather involved in the anti-apoptotic effects of $\text{TNF}\alpha$. Indeed, a contribution of the $\text{TNF}\alpha$ pathway to survival, development and differentiation of porcine CLs during early gestation has been shown by Przygodzka *et al.* (2015). In pigs, an increased presence of both TNFR1 mRNA and protein was found on Day 12 of the estrous cycle, but whether the luteolytic action of $\text{TNF}\alpha$ is dependent on the level of $\text{TNF}\alpha$ or the abundance of TNFR1 as proposed for bovine CLs (Skarzynski *et al.* 2003) needs further investigation.

Structural luteolysis in the porcine CL has been reported to be associated with apoptosis (Zorrilla *et al.* 2013, Przygodzka *et al.* 2015). However, no change has been observed in the expression of apoptotic markers such as BAX and BCL2 or in BAX/BCL2 ratio during luteal regression (Przygodzka *et al.* 2015). Similarly, the expression of caspase 3 and caspase 8 is also maintained at steady levels during the mid and late luteal phases of the estrous cycle (Zorrilla *et al.* 2013). However, mRNA expression of factors associated with extrinsic apoptosis (*TNFA*, *TNFRSF1A*, *FAS* and *IFNG*) are already elevated on Day 12 of the estrous cycle compared to the same day of pregnancy, while *TNRSF1A* showed higher abundance in luteal

Figure 1 Schematic presentation of mRNA expression of 21 genes potentially involved in the function of porcine CL on Day 14 of the estrous cycle and pregnancy. Arrows indicate decrease (↓) or increase (↑) of transcript when compared to Day 12 of the estrous cycle/pregnancy. *CYP19A1*, cytochrome P450 family 19 subfamily A member 1; *ESR2*, estrogen receptor 2; *HSD3B1*, 3 beta hydroxysteroid delta-isomerase 1; *LHCGR*, lutropin-choriogonadotropic hormone receptor; *NR5A1*, nuclear receptor subfamily 5, group A, member 1; *PGRMC1*, progesterone receptor membrane components 1; *PTGS2*, prostaglandin-endoperoxide synthase 2; *PTGER4*, prostaglandin E_2 receptor 4; *PTGFR*, prostaglandin F receptor; *EDN1*, endothelin 1; *HIF1A*, hypoxia-inducible factor 1 α ; *IFNG*, interferon gamma; *SCARB1*, scavenger receptor class B, member 1; *STAR*, steroidogenic acute regulatory protein; 1 *TNFA*, tumor necrosis factor alpha; *TNFRSF1A* and *TNFRSF1B*, tumor necrosis factor receptor superfamily member 1A and 1B; *VEGFA*, vascular endothelial growth factor A.

tissue of pregnant gilts on Day 14 (Fig. 1). Two factors involved in apoptotic pathways (*FOS*, *JUN*) showed distinct upregulation on Day 14 of the estrous cycle compared to earlier days (Przygodzka *et al.* 2015; Fig. 1). Our proteomic data showed higher abundance of N-ethylmaleimide-sensitive factor (NSFL1) and heat shock protein b6 (HSPb6) and a decrease in eukaryotic elongation factor 1 (eEF1) and lysosomal adaptor MTOR activator (LAMTOR3) on Day 16 of the estrous cycle relative to the corresponding day of gestation. Since NSFL1, HSPb6 and LAMTOR3 are involved in autophagy and eEF1 is a known modulator of apoptotic proteins, there is a possibility that luteolysis in pigs is associated with autophagy and apoptosis (Jalali BM, Likso P & Skarzynski DJ 2017 unpublished observations).

Unlike in other species such as humans and cattle, the level of vascular endothelial growth factor A (*VEGFA*) mRNA is maintained almost constant until Day 15 of the estrous cycle in pigs (Kaczmarek *et al.* 2007, Przygodzka *et al.* 2016). There is a decrease in the expression of *VEGFA* and its receptors only during the late estrous cycle (Days 16–17; Kaczmarek *et al.* 2007). Luteal expression of *VEGFA* during pregnancy (Days 16–25) is similar to Days 5–12 of the estrous cycle. Other angiogenic factors such as angiopoietins (*ANGPT1*, *ANGPT2*) do not seem to be associated with luteal regression in pigs (Przygodzka *et al.* 2016).

Rescue and maintenance of CLs for pregnancy

Old theories and new aspects of maternal recognition of pregnancy

The luteolytic effect of $\text{PGF}_{2\alpha}$ on structural and functional luteolysis needs to be prevented during early pregnancy in order to rescue the CLs, since the maintenance of luteal function ensures continued release of progesterone needed for the secretion of specific endometrial products and constituents of the histotroph required for blastocyst growth and development. It is believed that at least 4 ng/mL progesterone in the circulation is critical to maintain pregnancy in the pig (Ellicott & Dziuk 1973) and removal of CLs during pregnancy results in its termination (Belt *et al.* 1971).

According to the original theory of maternal recognition of pregnancy in pigs created by Bazer and Thatcher (1977), the main role in the process of CL rescue is played by conceptus-driven redirection of endometrial $\text{PGF}_{2\alpha}$ secretion after Day 12 *post coitum* (p.c.) from endocrine to exocrine, i.e., into the uterine lumen. Thus, the threshold level of $\text{PGF}_{2\alpha}$ concentration in the peripheral blood reaching the luteal tissues is too low to begin luteolysis. An alternative antiluteolytic mechanism was proposed by Krzymowski and Stefanczyk-Krzymowska (2004), based on the retrograde transfer of $\text{PGF}_{2\alpha}$ from the uterine venous blood and

lymph into the uterus, causing $\text{PGF}_{2\alpha}$ accumulation in the veins and arterial walls of the uterus instead of reaching the late luteal CLs.

A closer look into CL characteristics helped us to understand that the prolonged insensitivity to the luteolytic action of $\text{PGF}_{2\alpha}$ during early pregnancy was also explained by decreased numbers of $\text{PGF}_{2\alpha}$ -binding sites in freshly isolated luteal cells obtained from porcine CL on Day 14 of pregnancy in comparison to Day 14 of the estrous cycle (Gadsby *et al.* 1990, 1993). However, more recent *ex situ* studies performed on whole porcine luteal tissue even showed an increase of PTGFR protein and mRNA in CL on Days 12 and 14 of pregnancy relative to the same days of the estrous cycle (Przygodzka *et al.* 2016). A similar abundance of *PTGFR* mRNA was reported in cycling and early pregnant sheep (Wiepz *et al.* 1992). Davis and Rueda (2002) also suggested the presence of alternatively spliced *PTGFR* in ovine CLs, leading to either their reinforcement or destruction.

This discrepancy between the earlier pioneering work of Gadsby *et al.* (1990, 1993) and our recent results (Przygodzka *et al.* 2014, 2016) could be due to at least two reasons: application of completely different methods, i.e., receptor binding sites vs Western blot and/or PCR, and use of isolated luteal cells instead of whole luteal tissue. Worth mentioning is the presence of PTGFR not only on luteal but also on endothelial cells (Zannoni *et al.* 2007) responsible for building vascular network of the CL. Interestingly, Przygodzka *et al.* (2016) found an elevated intraluteal content of $\text{PGF}_{2\alpha}$ on Day 14 of the estrous cycle vs the corresponding day of gestation, confirming earlier suggestions of possible $\text{PGF}_{2\alpha}$ production in CLs with acquired LS (Diaz *et al.* 2000, Wasielek *et al.* 2008). It seems that protein expression of PTGFR is decreased in CLs on Day 14 of the estrous cycle due to a negative correlation between high levels of luteal $\text{PGF}_{2\alpha}$ and PTGFR abundance (Diaz *et al.* 2000). In contrast to reports by Gadsby *et al.* (1990, 1993), the above-mentioned recent observations and earlier report by Zorrilla *et al.* (2009) suggest that induction of different *post PTGFR* signaling networks including specific kinase C (PKC) expression is more important than PTGFR abundance during the course of luteolysis.

Besides the well-known and described role of conceptus estrogens in the rescue of porcine CLs from luteolysis during establishment of pregnancy (Ford *et al.* 1982), PGE_2 started to be recognized as another or second conceptus signal in the pig (Waclawik *et al.* 2017). The antiluteolytic role of PGE_2 in the pig was known for decades, ever since an increased ratio of $\text{PGE}_2/\text{PGF}_{2\alpha}$ content in the uterus was observed during the period of maternal recognition of pregnancy (Christenson *et al.* 1994). The expression of microsomal PGE_2 synthase-1 (mPGES-1) is observed in spherical conceptuses since Day 10 p.c. and dramatically increases in tubular conceptuses on Day 13

(Waclawik & Ziecik 2007). The production of PGE₂ in embryos and the endometrium seems to be involved in the escape of porcine CLs from luteolysis due to (1) a much higher luteal concentration of PGE₂ on Day 14 of pregnancy than on the same day of the estrous cycle (Przygodzka *et al.* 2016) and (2) higher PGE₂ content only in CLs ipsilateral to the gravid horn of unilaterally pregnant gilts (Wasielak *et al.* 2008), not accompanied by increased *mPGES-1* expression. In addition, a local transfer of PGE₂ via blood and lymph vessels from the uterus to the ovary (Stefanczyk-Krzyszowska *et al.* 2006) can aid this process. The porcine CL possesses at least two forms of PGE₂ receptors: PGE₂ receptor 2 (PTGER2) and PGE₂ receptor 4 (PTGER4), both upregulated on Day 14 of pregnancy and participating in the production of progesterone in cultured luteal slices through a cAMP-mediated pathway (Waclawik *et al.* 2010). Our recent study revealed an increased phosphorylation of protein kinase A (PKA) at the position of Threonine 197 (Thr197), cAMP response element-binding protein (CREB) at the position of Serine 133 (Ser133) and protein kinase B (PKB) at the position of Serine 473 (Ser473) in porcine CL on Day 14 of pregnancy compared to the same day of the estrous cycle. Simultaneously, we observed an increase in levels of PGE₂ and elevated content of steroidogenic acute regulatory protein (STAR), aromatase CYP19A1 (cytochrome P450 family 19 subfamily A member 1) and VEGFA in luteal tissue slices on Day 14 of pregnancy (E Przygodzka, A Waclawik & A J Ziecik, unpublished observations). These results suggest that binding of PGE₂ to PTGER2/4 may activate PKA and PKB signaling pathways as well as enhance the expression of proteins involved in processes determining maintenance of luteal function during early pregnancy in pigs. Moreover, PGE₂ significantly stimulated VEGFA secretion by luteal cells on Days 10–12 of gestation (Kowalczyk *et al.* 2008). Additionally, downregulation of endogenous antagonist of VEGFA-soluble receptor (sFLT1) in porcine CLs during early pregnancy may serve to enhance their content of bioavailable VEGFA. Consequently, prolonged progesterone secretion appears to be possible due to increased permeability of luteal small blood vessels and supply of cholesterol to the cells as well as easier transfer of prostaglandins from the peripheral circulation (Kaczmarek *et al.* 2009).

The evidence that PGF_{2α} may stimulate progesterone secretion in early CLs of the estrous cycle and during early gestation or inhibit it in CLs approaching luteolysis was documented in early *in vivo* experiments employing a microdialysis system (Wuttke *et al.* 1998). Also the 'luteolytic' PGF_{2α} elevated production of cAMP previously stimulated via both increased Ca²⁺ release and PKC activation in bovine CLs (Mamluk *et al.* 1999). Recently, it was shown that PGF_{2α} increased the content of CREB in CL slices of early pregnant pigs and progesterone secretion in mid-luteal phase CLs, but decreased progesterone production by luteal

slices from CLs with acquired LS at the late-luteal phase (Przygodzka *et al.* 2014, 2016).

The aforementioned studies show the dual and opposing roles of PGF_{2α} in the porcine CL, depending on the phase of the estrous cycle and pregnancy, i.e., its luteotropic role in CLs without acquired LS (early CLs during the estrous cycle and CLs of early pregnancy) and its luteolytic role in CLs with acquired LS (the late luteal phase of the estrous cycle, approaching luteolysis).

Lessons learned from gene expression studies

Unfortunately, so far, there is a lack of global profiling studies on pregnancy-associated genes contributing to the antiluteolytic mechanisms in the porcine CL. Such data are available for ovine CLs (Romero *et al.* 2013) and revealed 734 genes differentially expressed on Day 14 of pregnancy. On the other hand, early stages of luteolysis (Days 12–14) in the sheep were associated with altered expression of 682 genes in the luteal tissue. However, different embryo signaling systems for CL rescue in ruminants (IFNT) and pigs (mainly E₂ and PGE₂) make it difficult to find genes universal for antiluteolytic mechanisms in these animal species.

Nevertheless, an examination of over 50 genes potentially involved in the process of porcine CL maintenance during early pregnancy helped to identify 14 genes with increased expression in CLs on Day 14 of pregnancy involved in steroidogenesis (i.e. scavenger receptor class B, member 1 (*SCARB1*); *STAR*; hydroxy-delta-5-steroid dehydrogenase, 3 beta and steroid delta-isomerase 1 (*HSD3B1*); *LHCGR*; nuclear receptor subfamily 5, group A, member 1 (*NR5A1*); estrogen receptor 1 (*ESR1*); *PGR*; *PGR* membrane components 1/2 (*PGRMC1/2*) and in angiogenesis (i.e. angiopoietin 2 (*ANGPT2*); prostaglandin F synthase (*PTGFS*); hydroxyprostaglandin dehydrogenase 15-(NAP) (*HPGD*); kinase insert domain receptor (*KDR*); *VEGFR2*; low density lipoprotein receptor (*LDLR*); nuclear factor of kappa light polypeptide gene enhancer in B cells (*NFKB1*) and pentraxin 3 (*PTX3*)). Downregulated genes on Day 14 of pregnancy included *EDN1*, *CYP19A1*, *ESR2*, *PTGS2*, *JUN* and *FOS* (Fig. 1; Przygodzka *et al.* 2015, 2016).

Factors involved in progesterone synthesis and metabolism, i.e., *LHCGR*, *SCARB1*, *STAR* and *HSD3B1* were augmented on Day 14 vs Day 12 of gestation and the respective day of the estrous cycle. Also, expression of *NR5A1*, a transcription activator of steroidogenic genes important for maintenance of progesterone secretion in gilts, was upregulated in CLs on Day 14 of gestation. These changes together with elevated abundance of *PGRMC1* on the same day of pregnancy seem to be essential to support an anti-apoptotic mechanism mediated by progesterone in luteal tissue (Kowalik & Kotwica 2008, Diaz *et al.* 2011).

The observed relatively low expression of genes related to metabolism and action of E_2 (*CYP19A1* and *ESR2*) in CLs during early pregnancy is due to their paradoxical high expression in porcine CLs with acquired LS during induced (Diaz & Wiltbank 2005) or spontaneous luteolysis (Przygrodzka *et al.* 2016). However, the abundance of *CYP19A1* protein, sufficient to increase intraluteal content of E_2 and enhance conversion of testosterone to E_2 on Days 12 and 14 of gestation, suggest that luteal tissue of early pregnant pigs can produce E_2 .

Although increased *EDN1* (Fig. 1) levels on Day 14 of the estrous cycle were suggested to play a role in development of LS in the pig (Zorrilla *et al.* 2010), a high abundance of *HIF1A* (Fig. 1) seems to act in the opposite way. Our recent *in vitro* study on the involvement of hypoxia and *HIF1A* in maintenance of porcine CL function revealed that $CoCl_2$, a *HIF1A* activator, stimulated *HIF1A*, *VEGFA* and *STAR* expression only in CLs of cycling gilts. In addition, *in silico* analysis revealed the presence of *HIF1A* transcription-binding sites within the promoters of *VEGFA* and *STAR* genes. Other genes associated with luteal function maintenance (e.g., *PGRMC1*, *EDN1*) were also found to be potential targets of *HIF1A*. Thus, it seems likely that *HIF1A* can be another modulator of luteal function maintenance (E Przygrodzka, K Myszczyński & AJ Ziecik, unpublished observations).

MicroRNAs as new players in CL maintenance

Since it was found that almost two-thirds of the genomic DNA is transcribed in eukaryotes, but less than 2% is translated into proteins, appreciation of the role of ncRNAs in regulating processes occurring in eukaryotes has dramatically increased (Rinn & Chang 2012). However, the role of ncRNAs in luteal function maintenance remains poorly investigated. Among various types of ncRNAs the most studied are the miRNAs defined as short ~22-nt long RNA molecules that alter transcription and/or translation of mRNA molecules by binding mainly to its 3' untranslated region (3'UTR). The general role of miRNAs in ovarian functions was stressed in studies performed on mice with deletion of *DICER*, an endonuclease responsible for cleavage of pre-miRNA to mature miRNA. This genetic modification led to inhibited follicle growth and reduced ovulation rate as well as faulty oocyte development and oviductal and uterine defects (Tang *et al.* 2007, Hong *et al.* 2008, Nagaraja *et al.* 2008). Global profiling of miRNAs during bovine and ovine CL development (Fiedler *et al.* 2008, McBride *et al.* 2012) and CL regression and rescue during pregnancy in cows (Ma *et al.* 2011, Maalouf *et al.* 2014) showed a number of differentially expressed miRNAs, but the specific roles of selected miRNAs have rarely been demonstrated. For instance, miR-378 was found to regulate apoptosis in bovine CLs (Ma *et al.* 2011),

miR-17-5p and miR-7p can regulate luteal angiogenesis in mice (Otsuka *et al.* 2008), while miR-96 was associated with luteal survival and steroidogenesis in human CLs (Mohammed *et al.* 2017). Our latest studies showed that miRNAs can also be involved in molecular and consequently hormonal changes in the porcine CL. Using microarrays, we identified several differentially expressed miRNAs in porcine CLs collected during the period of luteolysis (Day 14 of the estrous cycle) and maternal recognition of pregnancy (Day 14 of pregnancy). Among predicted targets of miRNAs upregulated during pregnancy, we found genes previously described as markers of luteolysis, e.g., *EDN1*, *FOS*, *JUN*, *PTGS2*, *FAS*, *ESR2*. In contrast, miRNAs upregulated in CLs during luteolysis were found to target genes associated with luteal function maintenance, e.g., *PGR*, *PGRMC2*, *IGF2*, *FLT1*, *CREB*, *PTGER2* (Przygrodzka & Kaczmarek 2016 unpublished data). Considering results of global studies performed in other animal species and with porcine CLs, it is likely that some miRNAs can have a supportive role in the luteolytic or luteotropic actions of primary factors regulating luteal function. That conclusion is further supported by our *in vitro* studies on luteal tissue slices, showing E_2 , a luteotropic conceptus-derived signal in pigs, to be a factor able to upregulate the expression of miRNAs targeting genes involved in progesterone metabolism (Przygrodzka & Kaczmarek 2017 unpublished data). These results shed new light on the role of miRNAs in a novel molecular mechanism supporting the luteotropic action of conceptus-derived signals during early pregnancy.

Two-signal switch hypothesis

Recently, we proposed the 'two-signal switch hypothesis' to highlight the importance of *post* $PGF_{2\alpha}$ and PGE_2 receptor signaling pathways activation in CLs during luteolysis and CL rescue during maternal recognition of pregnancy in the pig (Ziecik *et al.* 2017). The updated version of the hypothesis is presented in Fig. 2. After acquisition of LS by CLs on Days 12–14 of the estrous cycle due to the influence of cytokines, e.g., $TNF\alpha$, $IFN\gamma$ and *EDN1* (forming the 'luteolytic switch'), luteal cells begin to be sensitive to the luteolytic activity of $PGF_{2\alpha}$. As a result, the *post* $PTGFR$ signaling pathway activates two phospholipase C (PLC) pathways through: 1) diacylglycerol (DAG) and PKC, leading to inhibition of cAMP production and 2) inositol 1,4,5-triphosphate (IP_3) and Ca^{2+} activating proto-oncogene protein-serine threonine kinase (RAF1) and the downstream RAF/MEK/ERK signaling cascade in the cytoplasm. Phosphorylated ERK1/2 proteins are translocated into the nucleus and induce both *FOS* and *JUN* and other proto-oncogenes, contributing to AP1 transcription factor dimers formation. Furthermore, this leads to transcription activation of genes involved in functional and structural luteolysis. Before the 'luteolytic switch' is triggered, the porcine

Figure 2 The updated version of the 'two signal switch' hypothesis of $\text{PGF}_{2\alpha}$ and PGE_2 involvement in regression or rescue of porcine CL. Before the luteolytic sensitivity (LS) of luteal tissue is reached about Day 12 of estrous cycle $\text{PGF}_{2\alpha}$ through its specific transmembrane G-protein coupled receptor (PTGFR) amplifies LH – stimulated cAMP accumulation (dotted green arrows). After acquisition of LS by mediators of luteal regression the 'luteolytic switch' is turned on (red pathway) changing the post-PTGFR sequence of events leading to inhibition of cAMP accumulation and luteolysis through DAG-PKC and IP_3 -ERK1/2 subpathways respectively. During early pregnancy, however, the embryo signals (estradiol 17β and PGE_2) of conceptuses and endometrial origin turn the 'rescue switch' on (green solid arrows) leading to induction of the post-PTGER2/4 pathway and activation of both protein kinases PKA, PKB and activation of CREB for maintenance of steroidogenesis, angiogenesis and cell survival. Simultaneously PKA inhibits RAF, and turns the 'luteolytic switch' off. AC, adenyl cyclase; cAMP, cyclic adenosine monophosphate; CREB, cAMP response element-binding protein; DAG, diacylglycerol; IP_3 , inositol triphosphate; ERK1/2, extracellular signal-regulated kinase $1/2$; PKC, protein kinase C; PLC, phospholipase C; RAF, proto-oncogene serine/threonine-protein kinase (figure modified from Ziecik *et al.* 2017).

CL is supported by synergistic luteotropic actions of LH and $\text{PGF}_{2\alpha}$ on cAMP accumulation. However, a lack of embryo signals and LS acquisition unavoidably leads to regression of the luteal tissue.

In contrast, during maternal recognition and establishment of pregnancy, embryo signals, estradiol (E_2) and PGE_2 ('rescue switch') through its PGE_2 receptors (PTGER2/4), activate PKA and inhibit (1) AMPK (AMP-activated protein kinase) and hormone-sensitive lipase,

reducing cholesterol availability for progesterone synthesis (Talbot & Davis 2017) and (2) RAF1 (Bos *et al.* 2004). Together, LH and PGE_2 increase the availability of cholesterol for progesterone synthesis. At the same time, phosphorylation of PKA, PKB and CREB by PGE_2 supports maintenance of angiogenesis, steroidogenesis and cell survival in the CL. Interestingly, the results of recent report on mechanisms for rescue of bovine CL during early pregnancy (Ochoa *et al.* 2018) also strongly support a luteoprotective role of PGE_2 .

CL function during pregnancy

Role of LH and prolactin

It was suggested that later in pregnancy, prolactin can maintain luteal function in hypophysectomized gilts (Du Mesnil du Buisson & Denamur 1968, Li *et al.* 1989). However, it is not clear whether the influence of LH on CL function is ever lost during pregnancy, since temporal interrelationships between pulses in circulating LH and progesterone levels have been demonstrated as late as on Day 90 (Parvizi *et al.* 1976).

The first reports on LH concentrations during various periods of pregnancy in pigs were fragmentary and contradictory (Tilson *et al.* 1970, Guthrie *et al.* 1972, Parvizi *et al.* 1976) until the profile of plasma LH during the course of pregnancy in the pig was described in detail (Ziecik *et al.* 1983). It was shown that from the first 6–7 days of gestation until parturition, LH release showed rhythmic pulses throughout pregnancy with decreasing amplitudes from Days 12 to 24 until parturition. The levels and patterns of LH secretion in early and mid-gestation were similar to the mid-luteal phase of the estrous cycle.

The plasma LH fluctuated in a pulsatile manner throughout gestation with declining amplitude toward parturition and a significant correlation between the decrease in LH concentrations and the day of gestation was determined: $y = 1.758 - 0.0088x$, where variable 'x' is a day of gestation (Ziecik *et al.* 1983). It is possible that increasing levels of estrogens during pregnancy (Robertson & King 1974) can negatively influence the basal LH level and the magnitude of its fluctuations. The reduction in LH levels may cause a simultaneous decrease in the concentration of progesterone (Guthrie *et al.* 1972).

The onset of prolactin dependency is supposed to be associated with an increased content of prolactin receptors in CLs during early pregnancy (Jammes *et al.* 1985). Our results indicated that decreasing the concentration of blood plasma prolactin by treatment with bromocriptine (inhibitor of prolactin secretion) is not mandatory for the weakening of CL steroidogenesis during mid-pregnancy (Szafranska & Ziecik 1990a) and in the second half of pregnancy in pigs (Szafranska & Ziecik 1990b). It is possible that pregnant gilts have a

specific substitute mechanism of compensation at the level of the hypothalamus and/or pituitary, developed to maintain CL function during pregnancy if LH or prolactin deficiency occurs. It seems likely that pregnancy stage has no effect on prolactin concentrations until shortly before term, when a dramatic surge of prolactin occurs presumably due to lactogenesis (Dusza & Krzymowska 1981). Prolactin together with other metabolic hormones, growth factor (GH) and insulin-like growth factor 1 (IGF1), may act synergistically to increase progesterone secretion by porcine luteal cells (Yuan & Lucy 1996).

Strategies to support enhanced CL function during pregnancy

The majority of studies concerning CL function during pregnancy were focused on the period of maternal recognition of pregnancy and implantation, i.e., Days 12–18 of pregnancy. Some reports extended observations to Day 30 of pregnancy, since the highest embryonic mortality (20–30%) in pigs happens between Days 12 and 30 of pregnancy (Lambert *et al.* 1991). According to Jindal *et al.* (1997), progesterone is important in mediating the supply of nutrients influencing embryonic survival in gilts. Thus, to date, many attempts have been undertaken to stimulate luteal function in order to reduce early embryo mortality by application of various hormones that enhance luteal function in pigs. However, multiple injections of hCG, a known analog of LH, during the first 8 days of gestation (Stone *et al.* 1987) or a single administration of hCG after Day 12 (Tilton *et al.* 1989, Bolzan *et al.* 2013) did not affect the level of progesterone in the blood plasma of pigs. A single injection of hCG on Day 12 of pregnancy elevated E_2 concentration and increased expression of STAR, accompanied by reduced levels of early apoptotic luteal cells and elevated percentages of live luteal cells in CLs on Day 30 of pregnancy (Bolzan *et al.* 2013). It is worth mentioning that resorption of all fetuses on Day 30 of pregnancy did not affect the level of progesterone in blood until Day 60 of pregnancy, which may suggest that fetus-derived signals are not necessary to maintain CL function between Day 30 and 60 of pregnancy in pigs (Webel *et al.* 1975).

Concluding remarks

The earlier fundamental theories of maternal recognition of pregnancy based on redirection and cessation of $PGF_{2\alpha}$ supply to CLs have not considered the overcoming of LS acquisition during the process of CL rescue after Day 12 *p.c.* This complex process initiated by embryo signals, mainly E_2 and PGE_2 , depends on cooperation between many pleiotropic factors determining luteal function maintenance. Overcoming of LS to $PGF_{2\alpha}$ also relies on the change of the 'luteolytic' to the 'luteotropic' role of

this prostaglandin to support CL function, besides the proven and already known actions of LH and PGE_2 during early pregnancy.

Our 'two signal switch' hypothesis concerning participation of $PGF_{2\alpha}$ and PGE_2 and their *post* receptor signaling systems, activated in CLs during luteolysis and during the process of luteal rescue, is based on available knowledge and our recent studies at the molecular level. It also shows the complexity of the interactions driving luteal control during the most sensitive periods of the life cycle of CLs: regression and rescue.

Complete understanding of the mechanisms driving luteal function in pigs and other species requires establishment of a sequence hierarchy and particular timing of molecular interrelationship between many mediators of the CL life span (development, regression, rescue and maintenance) as well as their endocrine and paracrine actions at the systemic, local and subcellular levels.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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