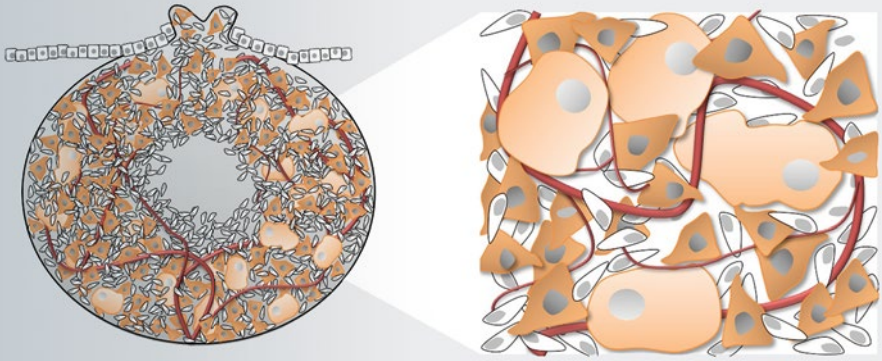

Rina Meidan *Editor*

The Life Cycle of the Corpus Luteum

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Preface

In this book, *The Life Cycle of the Corpus Luteum*, we try to provide state-of-the-art knowledge of the corpus luteum, throughout its lifespan, in different species.

The corpus luteum is a fascinating endocrine organ that is essential for fertility in mammals. Recent developments in understanding the lifespan of the corpus luteum provide new insights for reproductive biologists and also provide insights into tissue dynamics that translate to other research disciplines (e.g., developmental biology, vascular development, metabolic disorders, cancer). More research on the corpus luteum is needed to provide clinicians, veterinarians, researchers, and livestock producers with the information they require to successfully intervene in human, and other mammalian, fertility outcomes.

The ovarian corpora lutea (yellow bodies) were first named by Marcello Malpighi and then described by Regnier de Graaf in the late 1600s. Two centuries later, Prenant suggested that the corpus luteum may serve as a gland that produces substances which regulate pregnancy. This observation was confirmed rapidly by several groups in the early 1900s, and the biologically active substance progesterone was crystallized and characterized nearly simultaneously in 1934 by four independent groups. The corpus luteum is a temporary endocrine structure that forms within the ovary after ovulation and is essential to the establishment and early maintenance of pregnancy in most mammals, including humans, primates, livestock, rodents, canines, and felines. The ephemeral corpus luteum is generally considered to have three phases during its life cycle: formation, maintenance, regression, and a fourth potential phase: rescue and sustained function during pregnancy. Each stage of the corpus luteum life cycle has unique regulatory and signaling events that differentiate each stage from another. The chapters in this book review current research advances into each phase of the life cycle of the corpus luteum.

Enormous structural reorganization occurs as the postovulatory follicle transitions to a highly vascularized corpus luteum. Based on its size when fully functional, the blood supply to the corpus luteum exceeds that of most other organs. Much interest has been focused on factors and the cellular mechanisms that contribute to the development of new blood vessels in the corpus luteum and their importance to the function of the gland. Immune cells and factors released from these cells

contribute to tissue remodeling and new blood vessel development. As the process of angiogenesis is important in cardiovascular disease, inflammatory responses, and cancer biology, understanding how the vascular supply to the corpus luteum is regulated may provide unique insights that translate to other research disciplines. The chapters provided by Robert S. Robinson (Chap. 1), Kiyoshi Okuda, and Akio Miyamoto (Chaps. 2 and 6, respectively) provide new insight into the process and regulation of angiogenesis and immune cell infiltration in the corpus luteum.

Luteinizing hormone (LH) surge is responsible for initiating the differentiation of the somatic cells of the ovarian follicle (theca and granulosa cells) into the small and large steroidogenic cells of the corpus luteum. The newly formed corpus luteum is an extremely active gland that produces enormous amounts of the hormone progesterone, which provides an intrauterine environment that supports implantation, placentation, and fetal–placental growth and development. Insufficient progesterone secretion early in the first trimester is associated with pregnancy loss and is attributed to premature loss of luteal function. To further highlight the significance of progesterone to fertility research, studies indicate that progesterone acting locally via its nuclear receptor acts to promote ovulation and serves as a luteal cell survival factor. Therefore, understanding the control steroidogenesis is crucial for control of fertility in mammals. The chapters by Holly A. LaVoie (Chap. 3) and John S. Davis (Chap. 4) focus on understanding the control of steroidogenic processes and ovarian metabolic events and their potential for controlling progesterone synthesis. The chapter by Jan Kotwica et al. (Chap. 5) discusses the impact of steroid receptors and orphan nuclear hormone receptors on luteal function. Reproductive strategies vary considerably among species; these are especially evident with regard to the ovarian cycle and luteal function and lifespan. The chapter by Marta Tesone et al. (Chap. 7) reviews the rodent corpus luteum, and the chapter by Mariusz Pawel Kowalewski (Chap. 8) reviews the canine and feline corpus luteum, describing unique features of corpus luteum development and regression.

In the absence of pregnancy, the corpus luteum will regress so the next reproductive cycle can begin. The process of luteolysis is associated with a marked reduction in progesterone production and intense tissue remodeling, resulting in the loss of steroidogenic cells and the blood supply; and an increase the deposition of fibrotic connective tissue, forming the so-called corpus albicans (white body). Luteolysis is accompanied by the influx of immune cells and the activation of inflammatory signaling pathways that act in concert with luteolytic factors to inhibit progesterone and remodel the corpus luteum. Luteal regression in ruminants is covered in the chapter by Rina Meidan et al. (Chap. 9), and luteal regression in pigs is discussed in the chapter provided by Adam J. Ziecik (Chap. 12). Understanding how pregnancy hormones act to block corpus luteum regression gives us insights into the prevention of fibrotic processes observed in other tissues during inflammation and disease states and may provide insight into mechanisms responsible for tissue repair and regeneration. The impact of the corpus luteum in women's health is explored in the chapter by W. Colin Duncan (Chap. 13). If pregnancy occurs, a hormone released from the developing conceptus (embryo and its associated membranes) blocks or rescues corpus luteum structure, function, and blood supply. The interruption of

luteolysis allows the corpus luteum to support the pregnancy: in women, this hormone is hCG. The chapter by Richard Stouffer and Jon D. Hennebold (Chap. 10) reviews corpus luteum rescue from luteolysis in primates. In cows and sheep this factor is interferon tau, which acts to prevent uterine production of PGF2α and possibly acts by direct actions on the corpus luteum. In Chap. 11, Thomas R. Hansen discusses corpus luteum maintenance during early pregnancy of ruminants, and Adam J. Ziecik (Chap. 12) presents a chapter devoted to maintenance of the corpus luteum in early pregnancy in pigs.

I thank the authors for contributing their time, effort, and expertise to this book and hope the information presented will be a valuable source of the current state of knowledge for experts as well as beginners who wish to pursue future research in this exciting area. I thank John Davis and Heather Talbott (University of Nebraska Medical Center) for their help in composing these introductory notes.

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Chapter 1
Luteal Angiogenesis

Robert S. Robinson and Kathryn J. Woad

Abstract The structure and function of the corpus luteum (CL) is dependent on the development of an intricate vasculature via the process of angiogenesis. The establishment of the luteal vascular network begins in the preovulatory follicle and is ultimately stimulated by the luteinizing hormone (LH) surge. Following ovulation, the corpus luteum undergoes extremely rapid growth and intense angiogenesis that is tightly regulated by a balance achieved between pro-angiogenic and anti-angiogenic factors. This review summarizes what is known about the critical control of luteal angiogenesis and the complex interplay between numerous factors, the functions of which are only just beginning to be elucidated.

Keywords Corpus luteum • Luteal • Angiogenesis • Vasculature • FGF2 • VEGFA • Endothelial • Pericyte

1.1 Critical Importance of Luteal Angiogenesis

The principal function of the corpus luteum (CL) is to produce vast quantities of progesterone, which is absolutely essential for the establishment and maintenance of mammalian pregnancy. Groundbreaking work by the research labs of Hamish Fraser and Dick Stouffer in the early 2000s elegantly demonstrated the wholly crucial role of angiogenesis and the extensive vascularization required for the development and function of the CL. Namely, the inhibition of vascular endothelial growth factor-A

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(VEGFA, pro-angiogenic factor) with targeted antibody or soluble VEGF receptor isoforms completely prevented luteal vascularization and progesterone production [1–3] in primates. Similarly, in cows, the local neutralization of either VEGFA or fibroblast growth factor-2 (FGF2) also suppressed luteal development and progesterone production, albeit to a lesser extent [4]. Additional evidence of the crucial role of luteal angiogenesis for CL functions has been demonstrated using transgenic, knockout or knock-in mice models in which angiogenesis has been directly or indirectly targeted. Table 1.1 summarizes such studies where ovulation appeared normal but CL development and function was disrupted. Overall, compromised plasma progesterone levels were associated with disrupted luteal vascularization and reduced

Table 1.1 Summary of knockout mouse studies in which ovulation occurred but corpus luteum (CL) function was disrupted

Gene	Plasma P4 levels	Steroidogenic enzyme and/or StAR expression	Luteal vascularization	Fertility	Reference
Brain and muscle ARNT-like protein 1 (Bmal1)	↓	↓ ↑	↑ VEGFA	Infertile	[115]
Cyclin-dependent kinase 4 (cdk4)	↓	Unaffected	n.d.	Infertile	[116]
Endothelial nitric oxide synthase (eNOS)	↑	n.d.	n.d.	n.d.	[117]
Fibroblast growth factor-2 (FGF2)	n.d.	n.d.	n.d.	Normal	[117]
Frizzled4 (Fzd4)	↓	↓	Fragmented and punctate vascular network ↓ VEGFA	Infertile	[118]
Neat1	↓	↓	↓ VEGFA	Subfertile	[119]
Nr5a2 (Lrh1)	↓	↓	↓ VEGFA	Infertile	[120, 121]
Plasminogen	↓	Unaffected	Unaffected	n.d.	[122]
Prolactin	↓		↓ VE-cadherin	Infertile	[123, 124]
Scavenger receptor-B1 (SCARB1)	↓	n.d.	No effect	n.d.	[125]
Superoxide dismutase (SOD1)	↓	↓	↓ Vasculature	Infertile	[126]
Tissue inhibitor of metalloproteinase 1 (TIMP1)	↓	n.d.	n.d.	n.d.	[127]
Transforming growth factor-B1 (TGFB1)	↓	↓	n.d.	Infertile	[128]

n.d. not determined

fertility, providing further evidence for the close relationship between luteal angiogenesis and function.

The formation of the CL is a truly remarkable biological process with numerous and integrated events occurring in a relatively short timeframe, including (1) the luteinization/differentiation of granulosa and theca cells into luteal cells; (2) a steroidogenesis shift from estradiol to progesterone production that is accompanied with a massive upregulation in steroidogenic output (up to 1000 fold in the cow); (3) extensive remodeling, intermingling of cells (particularly in ruminants), and proliferation of the follicular tissue into a fully developed CL [5]. The bovine CL undergoes rapid growth (Fig. 1.1a), developing from less than 5 mm in size at ovulation to more than 20 mm within 10 days; this equates to a 60- to 100-fold increase in cellular volume, and the luteal growth rate is only matched by the fastest growing tumors [6]. Intriguingly, in the cow the postovulatory rise in progesterone lags behind the growth of the CL by 2–3 days (Fig. 1.1a). It has been speculated that the relatively slower rise in progesterone in ruminants compared to primates might be related to differences in luteal tissue remodeling and vascularization processes [7]. All these processes are totally dependent on intense angiogenesis, or formation of new blood vessels. This intensity is exemplified by the 15-fold increase in the total luteal volume of endothelial cells (EC) during formation of a mature bovine CL (Fig. 1.1b). Not surprisingly, the majority of the proliferating cells in the early CL are of vascular origin, with proliferation indices greater than 25 % [8, 9].

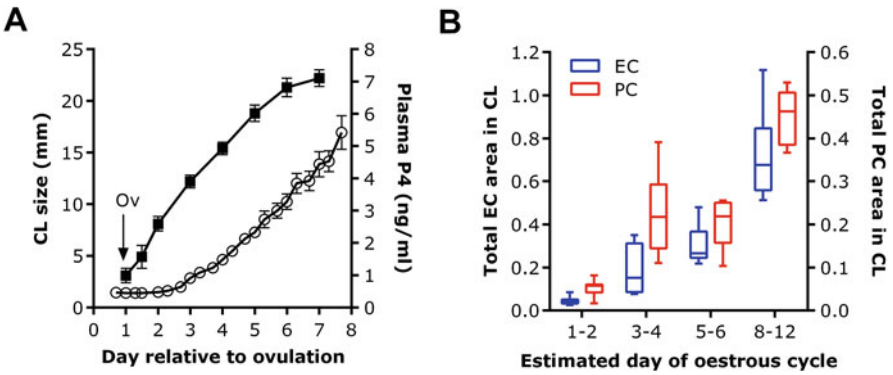

Fig. 1.1 Relationship between corpus luteum (CL) growth, plasma progesterone concentrations, and vasculature in the developing bovine corpus luteum. (**A**) Rapid increase in CL size after ovulation as determined by transrectal ultrasonography ($n = 15$). There is a similar increase in plasma progesterone concentrations, but this is initiated 2–3 days later compared to CL growth. (**B**) Tenfold increase in total luteal vasculature (EC; von Willebrand factor immunohistochemistry) during the formation of the CL ($P < 0.01$). There is a similar pattern of increase in the total area covered by pericytes (PC: smooth muscle actin immunohistochemistry). Data adapted from references [8, 114]

1.2 Follicular Programming of Luteal Angiogenesis

1.2.1 Preovulatory Follicular Vascular "Seed"

The structural and functional framework for CL development is provided by the preovulatory follicle, which leads to the concept of follicular programming of luteal function [5, 10, 11]. Although less is known about the role of follicular vascularization in this programming, it is entirely feasible that the degree of vascularization within the theca layer of the follicle could determine the rate of subsequent luteal vascularization. It has been proposed that during the follicle–luteal transition endothelial cells form 'vascular initiation points.' These points then create a scaffold from which endothelial cells can migrate and proliferate during the reconstruction of the vascular bed [7, 12]. As technologies for measuring microvascular blood flow develop, it will be important to determine the association between follicular vascularization and subsequent luteal function.

The second likely role of the preovulatory follicle in luteal angiogenesis is the active accumulation during folliculogenesis of various pro-angiogenic growth factors [e.g., fibroblast growth factor-2 (FGF2) and vascular endothelial growth factor-A (VEGFA)]. Typically, concentrations of these factors in follicular fluid reach 1 ng/ml for FGF2 [13] and 2–5 ng/ml for VEGFA [14, 15]. It is further likely that these factors are sequestered (e.g., by perlecan) within the follicular basement membrane through their heparin-binding properties [16] and are released following the LH surge-induced activation of proteases during ovulation [17]. Indeed, addition of human follicular fluid to human umbilical vein endothelial cells (HUVECs) dose-dependently increased EC proliferation [18]. Intriguingly, however, heat inactivation or immuno-neutralization of FGF2/VEGFA failed to block this stimulation. Further experiments revealed that the mediator of follicular fluid-induced angiogenesis was the lipid molecule sphingosine-1-phosphate [18]. An alternative candidate is the biologically active phospholipid, lysophosphatidic acid (LPA), which is produced by granulosa cells and is present in greater concentration in follicular fluid (up to 25 μM) compared to serum (~0.6 μM) [19]. Moreover, 10 μM LPA-treated granulosa-lutein-conditioned media stimulated migration, permeability, and proliferation of HUVECs, and this was mediated through increased interleukin (IL)-6 and -8 production [20]. LPA has also been shown to increase bovine aortic endothelial cell proliferation as well as progesterone production by bovine luteal cells [21].

1.2.2 Initiation by the LH Surge

The LH surge, as well as stimulating the ovulatory process, is also crucial in the upregulation of a plethora of genes, many of which are involved in regulating angiogenesis. Indeed, the size of the endogenous LH surge was positively associated with the degree of luteal vascularization in the developing CL [5]. Hence, the LH surge is often considered to initiate luteal angiogenesis [5]. For example, follicular FGF2 mRNA and protein concentrations are dramatically increased shortly after the LH surge in cows

[13, 22, 23]. However, VEGFA expression appears to be less affected by the LH surge [13, 24]. It is still unclear as to the exact effect of the LH surge on VEGFA expression in luteinizing granulosa cells of other species, with some reports of significant stimulation [25, 26], whereas others reported decreased expression [27, 28].

The development of next-generation sequencing has enabled detailed profiling of the effect of the LH surge on the follicular transcriptome. Ingenuity pathway analysis has revealed that the expression of a number of angiogenesis-related genes are regulated by the LH surge: a summary of these genes is shown in Table 1.2. This approach has confirmed several factors already known to stimulate the angiogenic process and/or endothelial cell function (e.g., FGF2, fibronectin, and ephrin B2). It has also

Table 1.2 Identification of key angiogenesis-related genes by transcriptomic analyses that are affected by luteinizing hormone (LH) surge/gonadotropin stimulation

Gene	Gene name	Fold change	Function	Reference
ADAM10	ADAM metallopeptidase domain 10	↑ 2.5-fold (b; GC/TC)	Cleaves VE-cadherin, enabling EC migration and increasing vasculature permeability	[129]
ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif; 1	↑ (m) ↑ 9-fold (b)	Cleaves versican and aggrecan, enabling EC invasion	[52, 78]
ANGPT1	Angiopoietin 1	↑ 3-fold (h)	Promotes vascular stabilization	[28]
CD24	CD24 molecule	↑ 80-fold (h)	Pro-angiogenic, and promotes cell invasion	[28]
CD36	CD36 molecule	↑ 3-fold	Thrombospondin receptor	[129]
EFNB2	Ephrin B2	↑ 16-fold (h)	Regulates cell adhesion and migration during angiogenesis	[28]
FGF2	Fibroblast growth factor 2	↑ 22-fold (b)	Promotes EC proliferation, migration, and sprouting	[22]
FN1	Fibronectin 1	↑ 5-fold (m) ↑ 3-fold (b) ↑ 5-fold (h)	Component of ECM that promotes EC migration and proliferation	[28, 52, 78]
ITGA5	Integrin, alpha 5	↑ 10-fold (h)	Adhesion molecule; acts as the fibronectin receptor	[28]
IGFB1	Integrin, beta 1	↑ 6-fold (m)	Adhesion molecule; acts as fibronectin receptor	[52]
PTX3	Pentraxin 3	↑ 700-fold (b)	Inhibitor of angiogenesis	[78]
PDGFBB	Platelet-derived growth factor BB	Upstream regulator	Expressed in EC tip cells and stimulates pericyte recruitment	[130]
SEMA3A/C	Semaphorin 3A and 3C	↑ 16-fold (h)	Anti-angiogenic and induces EC apoptosis	[28]

(continued)

Table 1.2 (continued)

Gene	Gene name	Fold change	Function	Reference
SPARC	Secreted acidic cysteine-rich glycoprotein	↑ 7-fold (m)	Pro- and anti-angiogenic properties	[52]
THBS2	Thrombospondin 2	↑ 10-fold (b, GC) ↑ 5-fold (b, TC)	Potent inhibitor of angiogenesis/VEGFA; inhibits EC proliferation and migration; Induces EC apoptosis	[78]
TGFB1	Transforming growth factor-B1	↑ 6-fold (h) Upstream regulator	Pro- and anti-angiogenic properties	[28]
VEGFA	Vascular endothelial growth factor-A	↓ 2-fold (h)	Pro-angiogenic	[28]

It should be noted that the different studies have collected tissue at different times relative to the LH surge, ranging from 1 to 36 h depending on the study design. Species: *b* bovine, *h* human, *m* mouse; cell type: *GC* granulosa cell, *TC* theca cell, *EC* endothelial cell

revealed several genes associated with tissue remodeling that also affect angiogenesis (e.g., secreted acidic cysteine-rich glycoprotein and transforming growth factor-β1), discussed later. The final and surprising observation was the identity of several upregulated factors classically considered as anti-angiogenic factors (e.g., pentraxin 3, semaphorin 3A/C, thrombospondin 1). It is feasible that this reflects a suppression of angiogenesis in the immediate post-LH surge period that is important until after ovulation has occurred.

Ovulation requires the production of prostaglandins (PG), and treatment with cyclooxygenase-2 (COX-2) inhibitors impairs ovulation [29]. The synthesis of prostaglandins from the granulosa cells is upregulated by the LH surge, reaching peak concentrations just before ovulation [30, 31], and PGE2 has been identified as the key ovulatory prostaglandin, at least in primates [32]. There is increasing evidence that PGE2 is not only crucial for ovulation but that it is also an important stimulator of luteal angiogenesis. Sakurai et al. [33] showed that treatment of rats with the COX-2 inhibitor NS-398 suppressed the formation of luteal vasculature in the newly formed CL as well as decreasing progesterone production. Furthermore, the coadministration of PGE2 reversed this effect. It is likely that PGE2 is acting through the PGE type 2 receptor (PTGER2), because the PTGER2 antagonist AH6809 suppressed luteal EC tube formation in rats [34]. The PTGER1-4 co-localized with EC within the luteinising granulosa layer 36 h after hCG treatment in primates [32]. Furthermore, intrafollicular injection of PTGER1 and PTGER2 agonists promoted vascularization of the luteinizing granulosa layer, via stimulating branching angiogenesis [32]. An additional complexity is the potential interplay between PGE2 and the key pro-angiogenic growth factors FGF2 [35] and VEGFA [34]; this concept warrants further investigation.

1.3 Control of Luteal Angiogenesis

1.3.1 Local Regulation of Luteal Angiogenesis

1.3.1.1 Fibroblast Growth Factor-2 and Vascular Endothelial Growth Factor-A

Luteal angiogenesis requires the highly coordinated and orchestrated interplay between endothelial and steroidogenic cells as well as fibroblasts and pericytes to create an extensive and complex vascular network that is absolutely essential for luteal function. The best characterized pro-angiogenic regulators of luteal angiogenesis are FGF2 and VEGFA, which are both potent mitogens of vascular endothelial cells (EC) as well as stimulating EC migration and survival. FGF2 is a more potent stimulator of luteal EC proliferation than VEGFA [36], whereas VEGFA also induces vascular permeability [37, 38]. The critical importance of VEGFA for luteal angiogenesis is emphasized in studies where inhibition of VEGFA action massively reduced luteal vascularization and progesterone production in primates [2].

In the developing CL, VEGFA protein is localized predominantly to steroidogenic cells in cows [39] and humans [40], and this is thought to direct angiogenesis toward the hormone-producing cell [41]. Intriguingly, in the very early CL, there are transiently very high levels of FGF2, which return within a few days to basal levels [13, 36]. At the same time, FGF2 protein localization shifts from EC to steroidogenic cells and back again [23], which led us to speculate that FGF2 has a dynamic role in the initiation of luteal angiogenesis in the cow [5].

We have further dissected the function of FGF2 and VEGFA in regulating bovine luteal angiogenesis using a physiologically relevant in vitro system in which multiple luteal cell types (steroidogenic cells, ECs, fibroblasts, pericytes) isolated from a recently ovulated CL are co-cultured in a specialized EC media [42]. Importantly, in this system EC form an intricate network, and the degree of network formation is highly responsive to angiogenic stimuli [42, 43]. Image analysis of these EC networks revealed that there are multiple branch points and interconnections that develop with time in culture [44, 45]. Simultaneously, progesterone production increases over time, and this is responsive to an LH challenge.

The formation of EC networks was suppressed with the addition of specific VEGF receptor (VEGFR) and FGF receptor (FGFR) inhibitors, with EC appearing most sensitive to FGFR inhibition [43]. Using a different approach, the treatment of bovine luteal cells with either FGF2 or VEGFA antibody more or less completely inhibited the formation of EC networks, even when the other angiogenic factor was present (Fig. 1.2). Similarly, FGF2-induced EC proliferation and migration were inhibited by treatment of bovine luteal cells with small molecule 27 (a fragment of thrombospondin that sequesters FGF2) [36]. Further investigation revealed that EC were most sensitive to FGFR inhibition during the time in which islands of EC are starting to sprout/branch [45]. More importantly, FGF2 promoted the precocious transition of undeveloped EC islands into organized EC networks, which was associated with an increased number of EC branch points [44]. The crucial process for

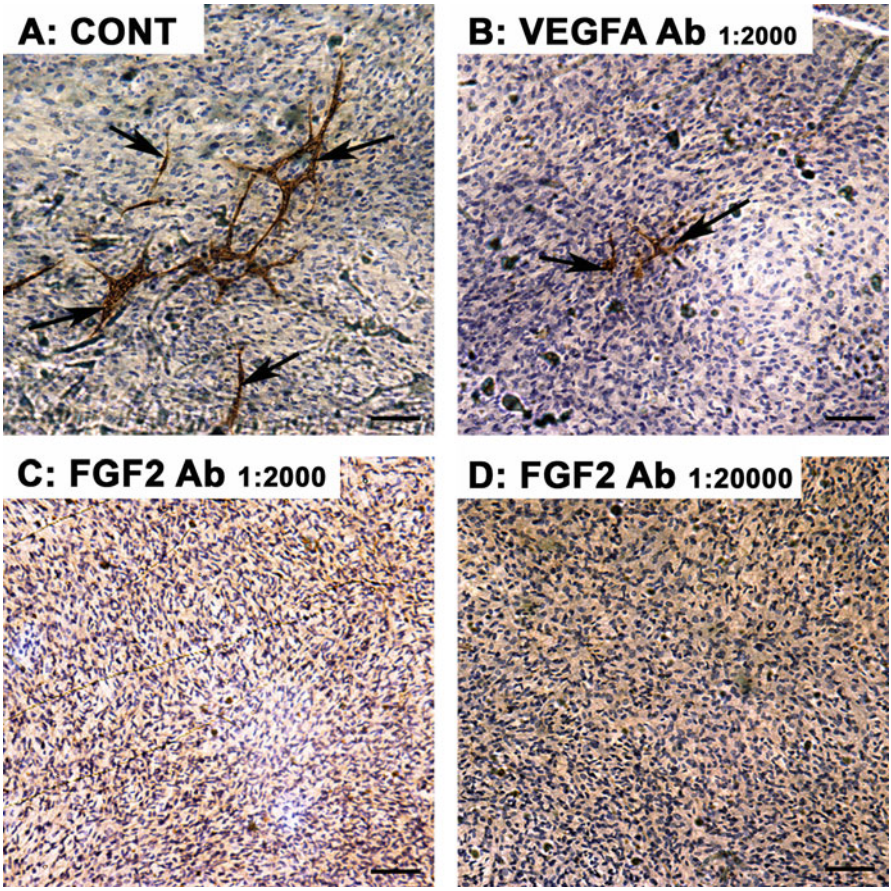

Fig. 1.2 Effect of immuno-neutralization of FGF2 and VEGFA on luteal endothelial cell (EC) networks in a physiologically relevant culture system that mimics luteal bovine angiogenesis and function. Dispersed bovine luteal cells were cultured on fibronectin-coated wells in specialized EC media for 9 days. Immunohistochemistry for von Willebrand factor was performed to identify the EC. (**A**) Bovine luteal cells treated with control showed extensive EC networks/islands (brown staining, *arrows*) that resembled a capillary bed. (**B**) Bovine luteal cells treated with VEGFA antibody at (Ab) 1:2000 dilution. Some reduced EC networks are present. Bovine luteal cells treated with FGF2 antibody at 1:2000 (**C**) and 1:20,000 (**D**) had no EC networks. However, there was extensive proliferation of other cell types. *Bars* 100 μm

EC sprouting is the formation of a specialized EC tip cell within the established vasculature that is capable of migrating toward the angiogenic stimulus. Thus, it is likely that FGFR signaling is crucial for endothelial tip cell formation and vascular sprouting [46]. However, knowledge is still limited about the way FGF2 induces EC tip cells. The effects of intraluteal infusion of FGF2 post ovulation on luteal vasculature and function in vivo certainly warrant investigation.

The regulation of vascular permeability is a key function of the endothelial cell and is important in the supply of nutrients/hormones to the luteal tissue. As mentioned earlier, VEGFA is a potent stimulator of EC permeability, which is controlled by adherens (e.g., VE-cadherin) and tight junctions (e.g., claudins). Herr et al. [47] showed that hCG induced a VEGFA-dependent downregulation of VE-cadherin and claudin 5 expression, which was associated with increased endothelial cell permeability in a human granulosa-endothelial co-culture system. Additionally, treatment of marmoset monkeys with VEGFA Trap during the mid-luteal phase increased the degree of claudin 5 staining in the CL [48].

1.3.1.2 Interleukins

Interleukin 8 (IL-8) is a neutrophil-specific chemoattractant and pro-angiogenic cytokine that is highly expressed in the early CL, coincident with the abundance of polymorphonuclear neutrophils (PMNs). Moreover, conditioned media from culture of early luteal cells, but not those from a mid-luteal phase CL, stimulated PMN migration in vitro that was blocked with an IL-8 antibody. Importantly, recombinant bovine IL-8 as well as PMN supernatant stimulated luteal EC proliferation and tube formation in vitro [49]. However, Talbott et al. [50] found that IL-8 had no effect on purified luteal endothelial cells derived from early pregnant CL. It is feasible that there is interplay with lysophosphatidic acid (LPA) and IL-8 as LPA induced IL-8 expression in granulosa-lutein cells [20].

1.3.1.3 Secreted Protein Acidic Rich in Cysteine (SPARC)

SPARC is a matrix-associated glycoprotein that regulates cell differentiation, migration, and cell–cell communication. Importantly, the function of SPARC is changed by targeted proteolytic degradation, with the mature SPARC protein being anti-angiogenic and its proteolytic fragments generally being pro-angiogenic [51]. Transcriptomic analysis of hCG-regulated genes in murine granulosa cells revealed that SPARC mRNA was upregulated sevenfold by hCG [52]. The precocious expression of SPARC was dose-dependently induced by transforming growth factor-beta (TGFβ) and fibronectin in bovine luteinizing granulosa cells [53]. Furthermore, SPARC protein expression was abundantly present in luteal and endothelial cells of the developing bovine CL [13, 54]. Functionally, a plasmin proteolytic fragment of SPARC (KGHK) increased EC network formation in vitro and also stimulated progesterone production (to a greater extent than LH) in bovine luteal cells [53]. Thus, SPARC or KGHK-containing peptides could be novel targets for the treatment of luteal inadequacy.

1.3.1.4 Hypoxia

A key driver of angiogenesis during tumor development is the hypoxia-induced upregulation of VEGFA. Cells respond to hypoxia through the activation of hypoxia-inducible factor 1 (HIF1), which is a transcription factor that binds to hypoxia

response elements (HRE) in the promoter region of hypoxia-regulated genes. HIF1 is a heterodimer consisting of the oxygen-regulated HIF1A and the constitutively expressed HIF1B [55]. Oxygen concentrations in follicular fluid decrease in the latter stages of antral follicle development [56]. The remodeling of vasculature at ovulation and during early luteal development lowers tissue oxygen concentrations and therefore higher HIF1A would be expected. Indeed, HIF1A is upregulated around the ovulatory period in pigs [57], humans [58], cows [59], and mice [60]. Moreover, echinomycin, a small molecular inhibitor of HIF1 binding to HRE, blocked ovulation in mice [26]. Culturing luteal cells in hypoxic conditions (2–3 % O_2) or chemically induced hypoxia increases HIF1A expression and VEGFA [59, 60]. There is also some evidence that hCG/LH directly upregulates HIF1A in granulosa-lutein cells [25, 60]. The relationship between hypoxia and FGF2 expression remains largely unknown. In HUVECs, hypoxic conditions increased FGF2-induced proliferation and tube formation in comparison to normoxia [55]. The exact function of hypoxia in regulating luteal angiogenesis and EC network formation remains to be elucidated.

1.3.1.5 Notch System

The Notch signaling pathway plays an integral role in EC tip formation with the membrane-bound delta-like ligand 4 (DLL4) being expressed in tip cells [61]. When DLL4 binds to its receptor, Notch, on adjoining EC, it converts them into stalk cells [62]. DLL4 and Notch1–4 have been detected in endothelial and steroidogenic cells of developing CL in mice [63, 64]. In mice, treatment with a γ-secretase inhibitor (to block downstream Notch signaling) impaired preovulatory follicle development: this was associated with the theca layer having a disorganized EC framework and an increased vascular smooth muscle cell density. Furthermore, plasma estradiol concentrations were nearly threefold lower [65]. In the same model, treatment with a DLL4-blocking antibody had minimal effects on follicular appearance, although in marmosets, inhibition of DLL4 during the periovulatory period caused the CL to be hypervascularized and decreased progesterone production [66]. This EC patterning has several similarities to that induced by high concentrations of FGF2 in bovine luteal cells [44]. Recent observations showed that the treatment of luteal cells with a γ-secretase inhibitor reduced progesterone concentrations in rats [67]. The potential interplay between FGF2, VEGFA, and the Notch system warrants investigation.

1.3.2 Role of Anti-Angiogenic Factors

1.3.2.1 Thrombospondin (THBS)

Thrombospondin-1 and -2 are large glycoproteins secreted by several cell types that bind to the extracellular matrix (ECM). THBS, acting through its receptor CD36, regulates several processes in EC including migration, adhesion, and apoptosis. Contrary to expectation, the expression of THBS1, THBS2, and CD36 was greatest

during the early luteal phase in rats [68, 69]. FGF2 and THBS1 had opposing actions on bovine luteal EC in vitro, with THBS1 reducing cell numbers by inducing EC apoptosis [70]. Furthermore, THBS1 inhibited FGF2-induced luteal EC migration and proliferation [36] and THBS/FGF2 downregulate the opposing gene [70]. These in vitro observations are in agreement with the effect of the thrombospondin-mimetic peptide, ABT898, on follicular angiogenesis in marmoset monkeys [71]. However, in the same study ABT898 had no effect on ovulation or plasma progesterone concentrations [71].

1.3.2.2 Vascular Endothelial Growth Factor A: 'b' Isoform

The alternative splicing of exon 8 in the VEGFA gene yields two different families of VEGFA isoforms. The first family is the classic pro-angiogenic isoform with exon 8a, and the other is the anti-angiogenic isoform with exon 8b present. This isoform is termed $VEGFA_{xxx}b$ [72]. Relatively little is known about this additional complexity, and most VEGFA antibodies do not distinguish between these two different isoform families. Recently, it was reported that the $VEGFA_{120}b$ isoform was expressed in parallel to $VEGFA_{120}a$, with expression increasing with CL age, whereas $VEGFA_{164}b$ was not detected in the ovine CL [73]. Importantly, mice overexpressing $VEGFA_{164}b$ had reduced fertility, CL number, and degree of vascularization within the CL [74], further emphasizing the importance of the balance between pro- and anti-angiogenic growth factors during luteal development.

1.3.2.3 Vasohibin 1

Vasohibin 1 (VASH1) and IGF-binding protein 7 (IGFBP7) are another two recently identified negative feedback regulators of vascularization. VASH1 is constantly expressed throughout bovine luteal development in luteal endothelial cells, and its expression is upregulated by VEGFA [75]. In the cow, VASH1 suppressed VEGFA-induced luteal EC tube formation [75], suggesting that it could prevent overstimulation of angiogenesis. The high-affinity insulin, low-affinity IGF1-binding protein, IGFBP7, regulates cell proliferation, adhesion, and angiogenesis. It has been detected in the follicular fluid and corpora lutea of rats [76], but to date has not been reported in other species. Recently, IGFBP7 (at 160 ng/ml) was shown to reduce VEGFA- and LH-stimulated luteal EC tube formation, but it had no effect under basal conditions [77]. Lower concentrations of IGFBP7 had no effect [77], and the physiological role of IGFBP7 in regulating luteal angiogenesis remains to be determined.

1.3.2.4 Pentraxin 3

Pentraxin 3 (PTX3) is a 45-kDa glycosylated protein that is produced by endothelial cells and activated phagocytes. It is known to bind FGF2 with a high affinity and thus prevents FGF2 from binding to FGFR, blocking its angiogenic actions [37].

The massive upregulation of PTX3 in bovine granulosa cells following the LH surge [78] shortly before a period of intense angiogenesis seems counterintuitive, especially because PTX3 inhibited FGF2 action on bovine luteal ECs [36]. Interestingly, PTX3 expression is greatly increased in the mature CL following PGF2α administration, suggesting that PTX3 has an anti-angiogenic function in luteolysis [36].

1.4 Establishment of Luteal Vasculature

1.4.1 VE-Cadherin

VE-cadherin is an endothelial-specific molecule that forms adherens junctions between adjacent endothelial cells. VE-cadherin not only maintains vascular integrity but also regulates cellular processes such as EC proliferation, apoptosis, and VEGFR function [79]. It was recently discovered that Notch/VEGFR signaling alters the dynamics of VE-cadherin junctions that drive endothelial rearrangements during sprouting [80]. The immuno-neutralization of VE-cadherin with E4G10 antibody in mice reduced CL development, degree of vascularization, and plasma progesterone concentrations [81], highlighting the critical importance of VE-cadherin for luteal angiogenesis. Treatment of bovine CLENDO cells with TGFB1 caused the loss of VE-cadherin from cell junctions, reduced cell–cell contacts, and increased EC permeability [82]. Conversely, FGF2 signaling promotes VE-cadherin expression and maintains vascular integrity [83].

1.4.2 Pericytes

Pericytes (mural cells) form intimate contacts with endothelial cells and are an integral component of the microvasculature. Classically, pericytes are considered to be involved in the latter stages of angiogenesis by stabilizing newly formed EC tubes. It is increasingly evident that pericytes are also active in the early stages of angiogenesis [84]. The induction of platelet-derived growth factor receptor-B (PDGFRB) expression activates pericytes and stimulates their recruitment to endothelial cells. Indeed, intraovarian PDGFR blockade reduced the number of CL and progesterone production [85, 86] as well reducing the microvessel EC density by nearly 50 %, as well as pericyte coverage of those vessels [86]. It has been observed that pericytes appear to migrate into the luteinizing-granulosa layer "ahead" of EC in the developing sheep [87] and cattle CL [7, 88]. Furthermore, in the developing CL, pericytes are in abundance (Fig. 1.1b) and form a large proportion of proliferating cells [87]. Pericytes could have several functions to support the intense luteal angiogenesis, including (1) laying down fibronectin strands along which EC can migrate [7]; (2) increased migratory phenotype through the suppression of contractile vascular smooth muscle cell [89]; and (3) making multiple contacts between EC that assist with their stabilization

and thus co-ordinating the luteal vascularization. This idea is supported by the observation that smooth muscle actin (SMA)-positive mural cells are often localized in close proximity to EC islands and are often an integral component of these islands [5]. Furthermore, one particular mural phenotype has several finger-like projections that connect to EC and other mural cells [5]. Importantly, Woad et al. [43] demonstrated that in vitro PDGFRB blockade with a receptor tyrosine kinase inhibitor greatly attenuated the ability of EC to develop into networks with the earliest stages most sensitive to PDGFRB inhibition.

1.4.3 Insulin-Like Growth Factor (IGF) System

Insulin-like growth factor-1 (IGF1) is a well-established endocrine and paracrine growth factor that is known to be important in regulating follicular and luteal function. IGF1 is locally expressed in the CL [90] and has a stimulatory effect on progesterone secretion [91]. It is also known to have pro-angiogenic properties [92, 93], particularly in respect to developmental angiogenesis and neovascularization [94]. Recent evidence has shown that IGF1 promotes angiogenesis by stabilizing endothelial cell tubes and nascent blood vessels in the retina in response to VEGFA [95]. The latter might be particularly important for luteal angiogenesis because the neovascularization of the CL initially involves the destabilization of the vasculature within the theca layer. Knowledge is limited about the role of IGF1 in regulating ovarian angiogenesis; however, IGF1 decreased thrombospondin 1 expression in porcine granulosa cells [96].

1.5 Clinical Opportunities

Appropriate vascularization is critical to normal ovarian function. Dysregulated vascular growth has been implicated in the origin or development of several ovarian pathologies and is therefore a promising target for the treatment of disease.

1.5.1 Ovarian Cancer

Ovarian cancer, primarily epithelial in type, is a leading cause of female cancer death and the most aggressive of the gynecological cancers. More than 200,000 women develop epithelial ovarian cancer worldwide each year, with most cases diagnosed in women over 55 years of age. It is characterized by a high death rate, largely attributed to the late presentation of many cases and hence associated metastatic disease. Most cases of advanced disease also recur, with disease-free intervals becoming progressively shorter [97]. Standard clinical management is by surgery

and platinum-based cytotoxic chemotherapy; however, new therapeutic strategies are emerging, including anti-angiogenic treatment [98].

Tumor blood vessels exhibit multiple abnormalities of structure and function and are especially dynamic [99]. Tumor growth and eventual metastasis require active angiogenesis, resulting from both the upregulation of pro-angiogenic factors and downregulation of endogenous inhibitors, thereby presenting multiple opportunities for therapeutic intervention.

Advanced epithelial ovarian carcinoma is associated with raised serum VEGFA levels [100], and VEGFA has been the primary therapeutic target to date: approaches include using antibodies against VEGFA or VEGFR, soluble decoy receptors (VEGF Trap), or VEGFR tyrosine kinase inhibitors. Phase 3 trials have demonstrated increased progression-free survival in women with ovarian cancer treated with VEGF inhibition (bevacizumab) in addition to standard chemotherapy [101, 102]. The benefit of anti-VEGFA therapy was evident as first-line treatment, maintenance therapy, or at recurrence, although the impact on overall survival is less clear. Further advances are expected in the use of multi-angiokinase inhibitors that target tumor angiogenesis at multiple levels, for example, via combined VEGF, FGF, and PDGF signaling inhibition [103, 104].

Another promising target is the angiopoietin/Tie2 pathway [105, 106]. Treatment with Trebananib (AMG 386), which binds Angpt1 and -2, thereby preventing Tie2 activation, prolonged progression-free survival in women with ovarian cancer [107]. Opportunities to exploit the endogenous inhibition of angiogenesis are also being explored. For example, thrombospondin-mediated inhibition of angiogenesis enhanced the clinical effectiveness of chemotherapy in a mouse model of ovarian cancer by altering vascular morphology, facilitating drug uptake, and increasing apoptotic cell death [108].

1.5.2 Ovarian Hyperstimulation Syndrome

Ovarian hyperstimulation syndrome (OHSS) is a rare but potentially life-threatening complication of ovarian stimulation for fertility treatment. It occurs during the luteal phase or early pregnancy [109] and is characterized by a systemic increase in vascular permeability, thought to result from the ovarian secretion of vasoactive peptides such as VEGFA. VEGFA is implicated because of an association of OHSS with raised luteal and follicular fluid VEGFA expression and bioavailability [110] and the stimulation of vascular permeability in response to VEGFA [111]. Despite the potential importance of VEGFA in mediating OHSS, targeting VEGFA directly led to undesirable side effects in animal models, and hence most preventative treatments have targeted the stimulation protocol itself, such as reducing the duration of the LH surge. An attractive alternative clinical strategy to reduce the incidence and severity of OHSS is the use of dopamine agonists acting as VEGF inhibitors [112]. Treatment of high-risk women with dopamine agonists lowered the incidence of OHSS without detrimental effects on implantation or pregnancy outcome following assisted reproduction [113], despite the clear need for ovarian and endometrial angiogenesis.

1.6 Conclusion

Angiogenesis is critical to support the dramatic growth and development of the CL. Although VEGFA and FGF2 may be seen as the primary regulators of luteal angiogenesis, it is increasingly evident that numerous other factors are expressed by several luteal cell types and that these also have important modulatory functions. Transcriptomic studies are shedding new light on the complexities of luteal angiogenesis at the molecular level, and a better understanding of the control of luteal angiogenesis will also highlight potential new therapeutic opportunities to tackle angiogenesis-dependant ovarian disease.

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Chapter 2
Roles of Hypoxia in Corpus Luteum Formation

Kiyoshi Okuda and Ryo Nishimura

Abstract The corpus luteum (CL) is an organ that is formed and regressed during the female reproductive cycle. The structural and functional changes from follicle to CL after ovulation occur in association with rapid angiogenesis. Angiogenesis is known to be stimulated by a variety of growth factors, one of the strongest of which is vascular endothelial growth factor (VEGF). VEGF also has a function in the angiogenesis of newly formed CL and is strongly induced by a transcription factor hypoxia-inducible factor-1 (HIF1). HIF1 is a heterodimeric transcription factor and strongly induces a variety of genes under hypoxic conditions. Luteal formation has been suggested to progress under hypoxic conditions, because of bleeding in the ruptured follicle and because the vasculature is scant and immature. This chapter describes the diverse phenomena caused by hypoxic conditions on functional and structural changes in the ovary immediately before and after ovulation.

Keywords Luteal formation • Hypoxia • Hypoxia-inducible factors • HIF1 • Luteinization • Angiogenesis • Ovulation • Cell proliferation • VEGF • Blood flow • Steroidogenesis

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2.1 Introduction

During the mammalian ovulatory cycle, follicles mature and rupture following the LH surge, and the corpus luteum (CL) is formed after ovulation. The CL is formed by the differentiated follicular granulosa cells and theca cells. Follicular granulosa and theca cells, which produce estradiol-17β, rapidly differentiate into luteal cells that mainly produce progesterone (P4) after the LH surge. The structural and functional changes from follicle to CL occur in association with angiogenesis [1–4]. Oxygen concentration in follicular fluid decreases concomitant with the growing follicles [5, 6]. Thus, the maturation of follicles, ovulation, and luteal formation seem to progress under low oxygen conditions [5–11]. CL is composed of large and small luteal cells (derived from granulosa and theca cells, respectively), endothelial cells, pericytes, and a few stromal cells [1–4]. This chapter describes luteal formation and its related phenomena under hypoxic conditions, including the drastic changes of the function and structure of ovarian organs immediately before and after ovulation.

2.2 Ovarian Blood Flow During Luteal Formation

Cyclic changes in ovarian arterial blood flow were demonstrated in several studies in the 1970s, in which ovarian blood flow was monitored with electromagnetic probes around the ovarian artery in sheep and cattle [12–15]. These studies showed that blood flow remained at high levels during the luteal phase, decreased during CL regression, remained at low levels during the periovulatory period until 5–6 days after ovulation, and then increased toward the luteal phase (Fig. 2.1).

Recently, intraovarian blood flow was monitored using color Doppler ultrasonography [16–18]. The blood flow area and velocity (time-averaged maximum velocity, TAMXV) in the preovulatory follicular wall were elevated temporally (a few hours) just after the LH surge and were correlated with an increase in plasma concentration of estradiol-17β and the LH surge [17, 18]. Inducing ovulation by injection of GnRH caused blood flow area and velocity to increase for 120 h, accompanied by an increase in plasma P4 concentration [16, 18]. Similar changes in blood flow have been observed in the mare [19]. The correlation between the increase of

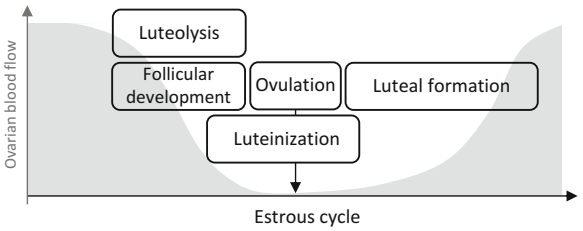

Fig. 2.1 Changes in ovarian blood flow (modified based on [15]) and ovarian processes during the estrous cycle in cattle

blood flow and plasma P4 concentration was similar to the findings of the classical studies that used electromagnetic flow probes [12, 13, 15], so that the blood supply to the ovary and P4 synthesis in the ovary appear to be strongly related. Because the blood supply affects oxygen levels, these changes also strongly suggest a relationship between oxygen supply and steroidogenesis. This relationship is further discussed in Sect. 2.4.

The low levels of ovarian blood flow at the time from luteal regression to luteal formation [12–15], which includes luteinization of follicular granulosa and theca cells before and after ovulation, are thought to be the basis of the decreased oxygen supply to the ovary during the period. The oxygen concentration in bovine arterial blood does not change significantly during the estrous cycle, but tends to be at high levels during the functional luteal phase and then decreases before ovulation [15]. Furthermore, blood supply to the ovary is significantly lower during the several days around ovulation in cows [15]. These findings support the idea that oxygen conditions inside the ovary are low around ovulation, and that the ovarian events in this period, including end-stage maturation of follicles, ovulation, and luteal formation, are considered to progress under low oxygen conditions [6–11].

2.3 Cellular Responses to Hypoxic Conditions

Mammals have cellular mechanisms to adapt to hypoxic conditions. These mechanisms are conserved and expressed in almost every mammalian cell type [20]. The transcription factors activated specifically under hypoxic conditions are called hypoxia-inducible factors (HIFs), which are heterodimeric transcription factors consisting of two subunits, HIF-α and an aryl hydrocarbon receptor nuclear translocator [ARNT; also called HIF1β (HIF1B)] [21]. Both subunits contain basic helix-loop-helix (bHLH)-Per Arnt-Sim (PAS) domains that mediate heterodimerization and DNA binding [22]. HIF1B is constitutively expressed whereas the activity and expression of HIF1A depends on cellular oxygen concentrations [23–25]. Mammalian cells have three HIF-α genes (HIF1A, 2A, 3A) [23–25]. Each gene contains an oxygen-dependent degradation domain (ODD) [26], which interacts with the von Hippel–Lindau (pVHL) E3 ubiquitin ligase complex [27–31] that targets HIF-α for proteasomal degradation under normoxia [26, 32–35]. HIF1A is expressed ubiquitously, whereas the expression of HIF2A and HIF3A is more restricted [21]. HIF1A and HIF2A dimerize with HIF1B, forming HIF1 and HIF2, both of which activate key transcription factors [21, 22]. HIF3A is found in three isoforms [HIF3A, neonatal and embryonic PAS (NEPAS), and inhibitory PAS protein (IPAS)] [21, 22]. HIF3A isoforms dimerize with HIF1B, forming HIF3 and HIF3NEPAS [21, 22]. In general, HIFs bind to hypoxia-response elements (HREs) on DNA, which leads to the regulation of some 200 genes, of which 70 have been studied in detail [21, 22]. HIF1 induces the transcription of homeostasis-related genes such as vascular endothelial growth factor (VEGF) and erythropoietin (EPO), whereas HIF2 and HIF3 have more specialized and tissue-specific regulatory roles

[21, 22, 36, 37]. HIF1 initiates the defense against hypoxia by a variety of mechanisms. In kidney and liver, hypoxia induces the synthesis of EPO [38, 39], which stimulates erythropoiesis, thereby increasing the O_2 capacity of the blood [40]. In virtually all tissues, hypoxia induces the synthesis of proteins controlling local blood flow, such as VEGF [41, 42], endothelial nitric oxide synthase (eNOS) [43], and heme oxygenase-1 (HOX-1) [44]. VEGF stimulates angiogenesis and increases the permeability of blood vessels [45]. eNOS and HOX-1 generate NO and carbon monoxide, which are potent vasodilatory substances that augment perfusion of the hypoxic tissue. At the cellular level, hypoxia induces the expression of virtually all glycolytic enzymes, including phosphoglycerate kinase-1 (PGK-1), enolase 1, and lactate dehydrogenase-1 [46, 47]. Furthermore, the expression of membranous glucose transporters (primarily GLUT-1) is increased under hypoxic conditions, thereby increasing glucose uptake for glycolysis [48, 49]. To promote gene expression of all these proteins, HIF1 binds to HREs present in the promoter and enhancer regions [24]. HIF1 also induces transcription of an apoptosis regulatory gene, 19-kDa interacting protein-3 (BNIP3) [50]. Apoptosis is important in ovarian physiology, especially during follicular atresia [51] and luteal regression [52]. In cultured bovine luteal cells, the expression of HIF1 and BNIP3 increases under hypoxic conditions [53, 54], suggesting that hypoxia-induced BNIP3 is related to apoptosis during luteolysis. Recently, BNIP3 has been reported to function in mitochondrial autophagy, resulting in suppression of superoxide generation and protection of cells under hypoxic conditions [55, 56]. We found that the expressions of HIF1A [57] and BNIP3 proteins (unpublished data) in bovine CL are significantly higher at the early luteal stage than at other stages. This finding suggests that (1) the HIF1-BNIP3 signal is more active in luteal formation than in luteal regression during the estrous cycle, and (2) apoptosis or mitochondrial autophagy controlled by active BNIP3 under hypoxia has some part in luteal formation. Hypoxic signals mainly controlled by HIFs are now becoming known to regulate ovarian function. These topics are discussed in the following sections.

2.4 Hypoxia Before Ovulation

The involvement of hypoxia as well as HIF1 in the regulation of steroidogenic gene expression has been previously suggested by a number of studies. Hypoxic conditions (1–3 % O_2) significantly reduced P4 production in rat [58] and porcine [5] granulosa cells and in bovine luteal cells [53, 59] Culturing bovine mid-luteal cells, culture under 3 %, 5 %, and 10 % O_2 for 24 h decreased P4 synthesis and 3 % O_2 decreased CYP11A1 (also known as P450scc) mRNA expression and activity [53]. Exposure to cobalt chloride ($CoCl_2$, a chemical inducer of HIF1) decreases CYP11A1 mRNA expression and hence P4 production in testicular Leydig cells [60]. HIF1 is also suggested to bind to and activate the promoter of the HSD3B gene in Leydig cells [61] and the gene for CYP19A1 (also known as aromatase) in breast adenocarcinoma cells [62]. In other cell types, hypoxia has been found to decrease

aromatase expression and activity [60, 63]. In human trophoblastic cells, HIF1 decreases aromatase expression via estrogen-related receptor-alpha (EERa), which is an orphan receptor (a receptor whose function is not known) known as an oxygen-dependent transcription factor [60]. HIF1 also inhibits CYP19A1 mRNA expression by activating a micro-RNA (miR-98) in H295R cells [63]. However, it is unclear whether hypoxia also inhibits aromatase activity in ovarian granulosa cells.

The lower pO_2 in the FF in the large follicles than in the small follicles has been suggested to promote VEGF production via HIF1 in granulosa cells [5]. On the other hand, in the primate ovary, nuclear immunostaining of HIF1A is mostly absent in growing preantral and antral follicles and is upregulated in the granulosa cells at ovulation [64]. In the periovulatory period, follicles rapidly change their functions, and the CL is formed after ovulation with active angiogenesis [3]. The HIF1-VEGF-induced angiogenesis system may be involved in the later period of follicular development and in the beginning of luteal formation immediately after ovulation.

Regulation of HIF1A expression has been found by the interaction between cAMP and hypoxia in bovine luteinized granulosa cells and human granulosa cells [65]. HIF1A protein expression was increased by chemical hypoxia, and the increased expression was further augmented by LH and cAMP. These results suggest that HIF1A is induced transcriptionally and post-transcriptionally, the first enhanced transcription by LH, which cannot be manifested in higher HIF1A protein levels unless the protein is stabilized under hypoxic conditions.

During the later period of follicular development before ovulation, oxygen levels seem to participate in follicular function by regulating of multiple phenomena, such as steroidogenesis and angiogenesis [66]. This regulation is only a part of the whole system of luteinization and ovulation, and the roles of hypoxia in ovarian function during this period are still largely unknown. Although the levels of oxygen concentration in newly forming luteal tissue immediately after ovulation are not known, the signal generated by hypoxic conditions has been suggested to be a key in luteinization and luteal formation [67, 68].

2.5 Hypoxia in Ovulation

At the time of ovulation, mammalian ovaries express high levels of chemokines, such as interleukin 8 (IL-8), monocyte chemoattractant protein-1 (MCP-1), growth-regulated oncogene-α (GROα), chemokine CCL5 (also known as RANTES: regulated on activation normal T cell expressed and secreted), and thymus-expressed chemokine (TECK) [69–71]. One of the CXC chemokine families, stromal cell-derived factor-1 (SDF-1) and its receptor CXCR4, have been recently suggested to regulate follicular function before ovulation [72, 73]. SDF-1, first isolated from bone marrow-derived stromal cells, is a natural ligand for CXCR4 and was found to be expressed in several tissues and organs [74]. CXCR4 mRNA is also expressed in bovine ovarian granulosa cells, and the levels are high in the preovulatory follicles [73]. Because CXCR4 mRNA expressions have been found to increase under

hypoxic conditions in vitro, the hypoxia-SDF-1/CXCR4 system has been suggested to be involved in ovulation [73].

P4 is required for ovulation and acts via its nuclear receptor progesterone receptor (PGR) [75]. PGR also regulates the genes for HIF1A, HIF2A, and HIF1B in mice [76]. In addition, the expressions of HIF1A, HIF2A, and HIF1B in *pgr*-null mice after induction of superovulation by gonadotropin were lower than those in wild-type mice, and inhibiting the transcription of HIF1 by echinomycin reduced the expressions of three ovulation-related genes (ADAMTS1, VEGFA, EDN2) [76]. These findings support the idea that P4, PGR, HIF1, and HIF2 signals are operating during ovulation.

Although the importance of hypoxia and the signals that it generates in ovulation have been getting more attention recently, further studies are needed to understand the crosstalk between hypoxic signals and ovulatory signals to clarify the function of hypoxia in ovulation. After ovulation, the tissues of the ruptured follicle immediately form a CL with functional and structural changes. These changes are supported by rapid angiogenesis [1–4], which has been found to be induced by hypoxia [57, 73–75]. These topics are described in the following section.

2.6 Hypoxia After Ovulation

After ovulation, the ruptured follicle is thought to be under hypoxic state because of bleeding, immature vasculature, and cell proliferation without matching blood supply [41] (Fig. 2.2). HIF1A protein expression, a well-known indicator of hypoxic conditions, is high in the newly forming CL 2 days after ovulation in the primate ovary [64], and is also significantly higher at the early and developing luteal tissue (2–6 days after ovulation) than at other stages of the estrous cycle in the bovine ovary [57, 59]. Although the oxygen concentrations in CL tissues have not been determined in any species, these findings about the protein expression of HIF1A [57, 64] strongly support the idea that newly forming CL tissue is under hypoxic conditions.

Angiogenesis during luteal formation was first investigated in the early 1990s [1] and has been the subject of several reviews [2–4, 77, 78]. VEGF, a potent angiogenic factor, was first identified in 1989 by Ferrarra and Henzel [79], and was found to promote angiogenesis during luteal formation in cows [80] and in women [81]. HIF1, soon after its discovery in 1995 [82], was found to be the most potent transcription factor for VEGF [41]. The early luteal tissue just after ovulation is thought to be under hypoxic conditions because of the destruction of the vasculature by ovulation as an explanation to the hypoxic condition and because the intensive cell proliferation in early CL is not matched initially by number of blood vessels. In bovine luteal endothelial cells, the mRNA expressions of HIF1A and VEGF were not significantly different in normoxic (20 % O_2) and hypoxic (1 % O_2) culture [83]. On the other hand, the mRNA expression of HIF1A in porcine CL was found to be high at the early luteal stage, which suggested that HIF1 assists in luteal formation [84]. To confirm the participation of HIF1A in luteal formation, analyses of HIF1A protein

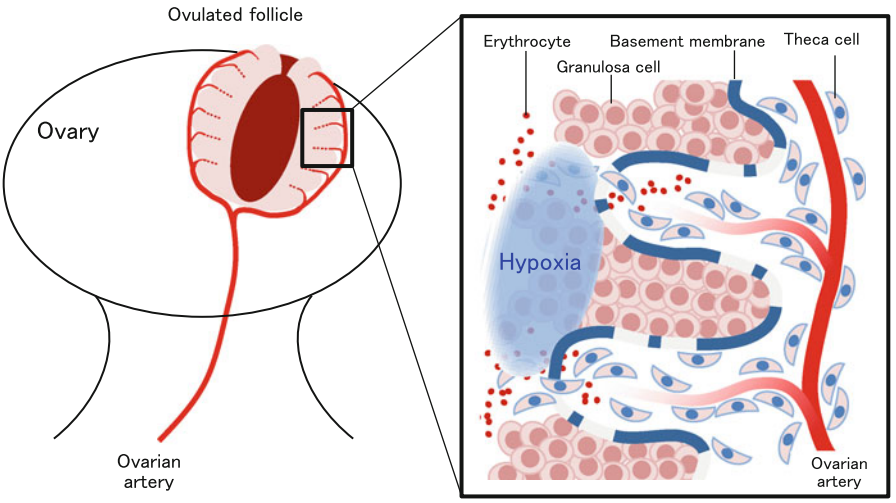

Fig. 2.2 Schematic of an ovary and its microenvironment after ovulation. The ruptured follicle is thought to be under hypoxic conditions because of bleeding and because the vasculature is immature

are crucial as HIF1 is mainly regulated by protein hydroxylation, as detailed in Sect. 2.3 [32]. Under normoxic conditions, the HIF1A subunit is rapidly degraded by the ubiquitin-proteasome pathway, whereas under hypoxic conditions, it becomes concentrated through downregulation of its degradation, and becomes functional after it dimerizes with the other subunit HIF1B [32]. In primates, immunostaining showed that the nuclear localization of HIF1A protein is found in the early CL [64]. In addition, HIF1A protein expression in the bovine CL is higher at the early and developing luteal stages than at the other luteal stages [57, 59]. Hypoxia also induced the expression of HIF1α protein, VEGF mRNA, and protein in cultured developing bovine luteal cells [57]. In luteinizing bovine granulosa cells and human granulosa cells, chemical hypoxia (cobalt chloride) induced HIF1α protein and VEGF mRNA expressions, and LH augmented both of these [65].

In ovarian steroidogenic cells, hypoxia also increased the expressions of several other proteins, including EG-VEGF (prokineticin-1, another type of VEGF) and its receptor PK-R2 [77, 85–87], a vasoactive peptide endothelin-2 (EDN2) [77, 88] and fibroblast growth factor 2 (FGF2) [77], suggesting that these factors also have roles in the hypoxic signals required for luteal formation. EDN2, for instance, induced changes that characterize the developing CL: cell proliferation as well as upregulation of VEGF and cyclooxygenase-2 [88]. The chemokine IL-8, which is a proangiogenic factor [88–94], was also found to be increased by hypoxia in human granulosa cells [95]. Furthermore, expression of IL-8 is higher at the early luteal stage than at other stages of the estrous cycle in the bovine ovary [96]. Thus, the angiogenesis induced by the hypoxia IL-8 system seems to assist luteal formation. Together, these findings suggest that hypoxic conditions generate several signals mainly via HIFs, which are essential for angiogenesis in luteal formation. A schema illustrating these ideas is shown in Fig. 2.3.

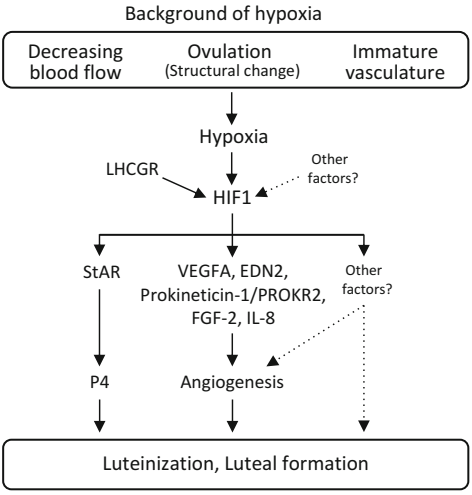

Fig. 2.3 Possible hypoxia-related signaling during luteal formation. *HIF1* hypoxia-inducible factor-1, *LHCGR* luteinizing hormone/chorionic gonadotropin receptor, *STAR* steroidogenic acute regulatory protein, *P4* progesterone, *VEGFA* vascular endothelial growth factor A, *PROKR2* prokineticin receptor 2, *FGF2* fibroblast growth factor 2, *IL-8* interleukin 8

Hypoxia is considered to stimulate the proliferation of luteal endothelial cells during luteal formation [97]. Luteal steroidogenic cells also proliferate during luteal development in cattle as shown by the co-expression of proliferation marker Ki-67 and steroidogenic marker HSD3B [98]. However, rapid growth of CL after ovulation is believed to be mainly the result of an increase in size of steroidogenic cells (hypertrophy) rather than an increase in their number [99–101]. Luteal steroidogenic cells have been suggested to express VEGF in response to the stimulation of hypoxia, resulting in the proliferation of endothelial cells for angiogenesis during luteal formation [57, 64, 77, 88]. Recently, porcine luteal endothelial cells have also been shown to proliferate in response to hypoxic conditions [97]. However, how hypoxia induces steroidogenic cell proliferation remains unclear. In cultured bovine luteinized granulosa cells, severe hypoxia (1 % O_2) decreases a proliferation marker, proliferating cell nuclear antigen (PCNA), whereas chemical hypoxia (cobalt chloride) increases the marker as well as HIF1A protein expression [59], implying that chemically induced HIF1 promotes steroidogenic cell proliferation, although the reason why severe hypoxia inhibits luteal cell proliferation is also unclear. To clarify how hypoxia regulates cell proliferation in CL, further studies are needed to determine how hypoxia-induced signals differ, and how their relationships differ, among different cell types, such as endothelial cells, luteinizing granulosa cells, and luteal steroidogenic cells.

2.7 Summary and Future Aspects

The discovery of HIF1 [82] elucidated responses to hypoxia in numerous cell types: these responses are related to pathological (cancer progression) as well as to physiological (female reproductive system) tissue growth [20–25, 102, 103]. HIFs are

expressed in a variety of organs, and some of their functions have been determined, which suggests that the cells in such organs have the ability to respond to hypoxic conditions. Hypoxia is an important signal in reproductive physiology [20–22, 24, 25, 102]: hypoxia-generated responses have crucial functions in luteal formation in all species examined thus far [57, 64, 67, 68, 77, 78, 85–88, 97, 104]. Yet, some regulatory mechanisms of hypoxic effects on luteal formation remain unclear. HIF1A is regulated by hormones such as human chorionic gonadotropin (hCG) [104, 105] and LH [65] under both hypoxic and normoxic conditions. Understanding of the cross-talk between hypoxia-generated signals and hormone-induced signals could help to clarify the roles of hypoxia in luteal formation as well as ovarian physiology.

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Chapter 3
Luteal Steroidogenesis

Holly A. LaVoie

Abstract Progesterone is the major functional steroid end product of the corpus luteum. In contrast to the ovarian follicle where mainly the theca cell layer could utilize cholesterol for de novo steroidogenesis, in the corpus luteum both the granulosa- and theca-derived luteal cells have this ability. This increased capacity for de novo steroidogenesis allows greater production of progesterone by the corpus luteum compared to the follicle. Luteinization, particularly of the follicular granulosa cells, is accompanied by a dramatic increase in the expression of genes and their corresponding proteins that mediate progesterone synthesis. The proteins include those involved in cholesterol transport, delivery of cholesterol into the inner mitochondria by steroidogenic acute regulatory protein, conversion of cholesterol to pregnenolone by the cytochrome P450 cholesterol side-chain cleavage complex, and conversion of pregnenolone to progesterone by 3-beta-hydroxysteroid dehydrogenase. Estrogen synthesis capability is lost in the corpora lutea of many species, but in some species such as primates and the pregnant rodent estrogen synthesis is reinitiated in luteal cells through renewed expression of aromatase. Androgen synthesis occurs in luteal cells of species where the corpus luteum makes estrogen and involves the enzymes cytochrome P450 17-alpha-hydroxylase/17, 20 lyase, and 17-beta-hydroxysteroid dehydrogenase. This chapter provides an overview of the hormonal and transcriptional regulation of the genes and proteins involved in luteal steroidogenesis.

Keywords Steroidogenesis • Progesterone • Pregnenolone • Steroidogenic acute regulatory protein (STARD1) • Cytochrome P450 cholesterol side-chain cleavage enzyme (CYP11A1) • Cholesterol • Low density lipoprotein • High density lipoprotein • 3-Beta-hydroxysteroid dehydrogenase (HSD3B) • Cytochrome P450 17-alpha-hydroxylase/17 • 20 lyase (CYP17A1) • 17-Beta-hydroxysteroid dehydrogenase (HSD17B) • Aromatase (CYP19A1) • Granulosa • Theca • Transcription factors • Gene promoters • Luteinizing hormone • Human chorionic gonadotropin START domain proteins

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Steroidogenesis is the primary function of the corpus luteum, with progesterone being the major functional hormone. In some species estradiol is also produced by the corpus luteum. The high level of progesterone production is needed to maintain the uterine lining for implantation and conceptus development. Several species, including mice, rats, pigs, goats, and cows, require the corpus luteum to produce progesterone for most of (cow) or the entire pregnancy, whereas others such as primates (including humans) and sheep only require the corpus luteum for early pregnancy until the placenta is sufficiently developed to produce adequate steroid [1, 2].

During the last stages of follicular maturation, under the influence of the luteinizing hormone (LH) surge, and even before ovulation, the theca and granulosa cells of the ovulatory follicle start terminal differentiation or luteinization into luteal cells [3]. Before the LH surge, steroidogenesis in the dominant follicle(s) had the ultimate goal of making the steroid hormone estradiol. For most mammals, it is widely accepted that the theca cells utilize cholesterol for de novo synthesis of pregnenolone, which is then stepwise enzymatically converted into androgens. The androgens cross the basement membrane into the granulosa cell layer, where they are converted to estrogens by the actions of aromatase (CYP19A1) [4]. The LH surge shuts off this production of estrogen by decreasing aromatase. In most species aromatase production remains off for the luteal lifespan, whereas in other species such as primates and pregnant rodents, the corpus luteum reacquires the ability to make estrogen [5, 6]. A major event that happens during luteinization is that granulosa cells gain the ability for massive de novo steroidogenesis by upregulating the protein machinery for the delivery of cholesterol substrate into the mitochondria, and by upregulating the components of the cytochrome P450 cholesterol side-chain cleavage enzyme (P450scc, CYP11A1) complex, which converts cholesterol into the first steroid hormone pregnenolone [7]. Luteinized thecal cells retain these functions. The P450scc enzyme dictates the initial enzymatic control point for the conversion of cholesterol into pregnenolone. P450scc complexes with electron transfer proteins adrenodoxin and adrenodoxin reductase to carry out the complete conversion of cholesterol to pregnenolone, and thus appropriate levels of these P450scc partners must be present. 3-Beta-hydroxysteroid dehydrogenase (3beta-HSD/HSD3B) abundance must be increased above follicular levels to convert the augmented pregnenolone produced into progesterone. The transfer of cholesterol from the outer to the inner mitochondrial membrane, where the P450scc complex resides, is widely accepted to be the critical rate-limiting step in de novo steroidogenesis, and in ovarian follicles and luteal cells this task is carried out by steroidogenic acute regulatory protein (StAR or STARD1) [8, 9].

3.1 Cholesterol Substrate for De Novo Steroidogenesis

Unesterified cholesterol is the substrate for de novo steroidogenesis. For the rapid increase in progesterone production observed in the early corpus luteum to occur, this substrate must be abundant. Cholesterol for steroidogenesis can be obtained in several ways: de novo synthesis from acetate through the HMG CoA synthase/reductase

pathway, from exogenous plasma low density lipoproteins (LDLs) and high density lipoproteins (HDLs), and existing intracellular stores [10]. Cholesteryl esters stored in lipid droplets serve as a readily available source for the steroidogenic pathway, and the removal of the ester group to yield free cholesterol by hormone-sensitive lipase (also known as cholesterol ester hydrolase) is increased upon luteinization [11]. Cholesterol within the plasma membrane may be used when other sources are not readily available. De novo cholesterol synthesis is energetically expensive to cells and thus may only be significant when intracellular stores are depleted. Lipoproteins from plasma are accepted to be the primary source of cholesterol for steroidogenesis. Low density lipoproteins are a major source of sterol in larger mammals [12], and HDL serves as the major source in rodents [13]. Cholesterol esters from HDL enter the cell via the scavenger receptor type B class 1 (SR-B1) encoded by the SCARB1 gene. LDL binds its surface receptor (LDLR) and the complex is internalized, where cholesterol is ultimately liberated from the LDL particle in lysosomes. Recent work indicates that cholesterol esters associated with the LDL particle can be delivered inside the cell by SR-B1 also [14]. Both SR-B1 and LDL receptors are increased by gonadotropin signals, and both genes are increased by intracellular cholesterol depletion through activation of SREBP transcription factors [15, 16]. Cholesterol from these external sources can be stored as cholesteryl esters or move into the steroidogenic pathway. Data indicate that levels of SR-B1 and LDL receptor mRNA drop in the regressive corpus luteum compared to the functional corpus luteum, suggesting cholesterol uptake is diminished with luteal regression [17, 18].

Transport of cholesterol through the cytoplasm of the steroidogenic cell is still poorly understood. Vesicular and nonvesicular transport mechanisms exist, yet little is known about these modes of transport in ovarian cells [10]. A few cholesterol transport proteins have been hypothesized to move cholesterol through the cytoplasm to the outer mitochondria, but their individual contributions are not clear and even controversial. Nonspecific cholesterol transporters sterol carrier 2 (SCP2) and specific StAR-related lipid transfer (START) domain proteins may serve this purpose. START domain proteins possess a lipid-binding pocket similar to STARD1 [19]. In addition to STARD1, START domain protein 4 (STARD4) and 6 (STARD6) are the only other cholesterol-binding START domain family members localized to steroidogenic cells of the ovary including luteinized granulosa and/or luteal cells [20, 21]. As does SCP-2, STARD4 and STARD6 possess cholesterol transport properties and promote steroidogenesis in model cells [22–24], but whether this occurs naturally in ovarian steroidogenic cells has not been proven. SCP-2 is regulated by LH and estradiol in rodents [25, 26], whereas the regulation of STARD4 and STARD6 in ovarian cells is not yet known. As these proteins lack a mitochondrial targeting sequence they likely transport cholesterol between organelles randomly or by interacting with other proteins [19]. A requirement for SCP2, STARD4, or STARD6 in ovarian cholesterol transport has yet to be demonstrated experimentally. Moreover, the presence of multiple cytoplasmic cholesterol transporters suggests possibly functional redundancy.

Figure 3.1 summarizes the major steps and molecules participating in luteal cell steroidogenesis.

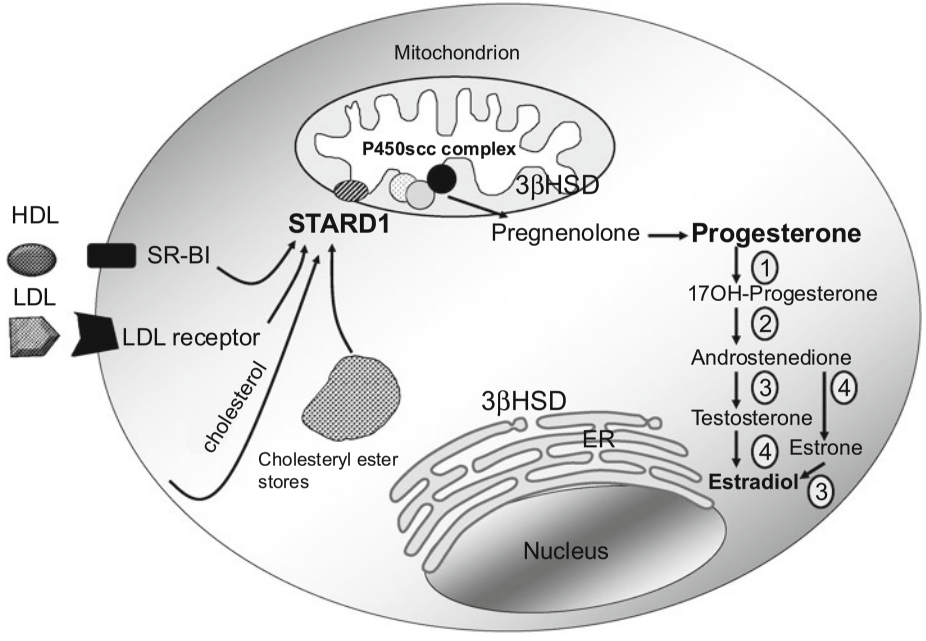

Fig. 3.1 Steroidogenesis in luteal cells. Cholesterol can be taken up from extracellular lipoprotein through the actions of the low density lipoprotein (LDL) receptor (LDL only) and scavenger receptor B1 (SR-B1, cholesteryl esters from HDL and LDL). Cholesterol can also be synthesized by the cell. Intracellular- and extracellular-derived cholesterol can be stored as cholesteryl esters. Liberated free cholesterol is transported to the outer mitochondrial membrane potentially by sterol carrier protein 2 (SCP2) and/or START domain proteins 4 and 6 (not shown). Steroidogenic acute regulatory protein (STARD1) sits on the outer mitochondrial membrane and serves to transfer cholesterol to the inner mitochondrial membrane, where the cytochrome P450 cholesterol side-chain (P450scc) cleavage complex resides. The P450scc enzyme partners with electron transfer proteins adrenodoxin and adrenodoxin reductase to convert cholesterol to the first steroid hormone pregnenolone. Pregnenolone is converted to progesterone by the actions of 3-beta-hydroxysteroid dehydrogenase (3βHSD), which has been localized in both mitochondria and the endoplasmic reticulum (ER). In species with luteal estradiol synthesis, steps 1–4 are present. The enzymes mediating these reactions needed for estradiol synthesis are *1* and *2*, CYP17A1 (17-alpha-hydroxylase/17,20-lyase); *3*, HSD17B (17-beta-hydroxysteroid dehydrogenase); *4*, CYP19A1 (aromatase)

3.2 Regulation of the Genes Involved in Steroidogenesis

In vivo the main stimulus for the induction genes encoding the machinery needed for de novo synthesis of luteal steroids, namely, STARD1, CYP11A1, and HSD3B, is the mid-cycle LH surge [27]. Both mural granulosa and theca cells of the ovulatory follicle possess LH receptors. Activation of the seven-transmembrane G protein-coupled LH receptor by surge levels of the gonadotropin activates adenylate cyclase-generating cyclic AMP (cAMP), thereby activating protein kinase A (PKA), the major regulator of transcription factors targeting the genes governing the steroidogenic pathway [28, 29]. In addition, high levels of LH have also been shown to increase intracellular calcium levels, although the full implications of activating this secondary pathway on luteal steroidogenesis are unclear [30, 31]. Exogenous human chorionic gonadotropin (hCG) can also bind and activate the LH receptor [32], and it is frequently used in immature PMSG (pregnant mare serum gonadotropin)-primed animals to mimic the LH surge. Similarly, exogenous hGC is frequently used in assisted reproduction protocols for final follicle maturation. Downstream of PKA signaling there is activation of ERK signaling [29, 33] that can contribute to regulation of transcription of these genes as well. Protein kinase A typically activates cyclic AMP response-element-binding protein (CREB), and although CREB activation via its phosphorylation by PKA may be a major mechanism for activating genes involved in steroidogenesis in the follicle [34], its importance in the corpus luteum is reduced and other transcription factors tend to mediate PKA effects on the genes of the steroidogenic pathway. Among the major transcription factors involved in mediating PKA signaling are members of the NR5A, NR4A, GATA, Sp1, Activator Protein (AP), and CCAAT/enhancer binding protein (C/EBP) families [7].

Much of what we know about the transcriptional regulation of these steroidogenic genes comes from studies with primary cultures of luteinizing granulosa cells, with fewer studies being actually performed with luteal cell preparations. Next we summarize the hormonal regulation of the mRNA and protein levels and the transcriptional regulation of the major steps in steroidogenesis in luteal cells.

3.3 Regulation of StAR/STARD1

In most mammals examined, STARD1 mRNA and its protein are expressed in theca cells but are not significantly expressed in granulosa of healthy follicles before the LH surge [35–38]. Exogenous hGC given in lieu of LH to PMSG-primed follicles can mimic the LH surge, inducing ovulation and STARD1 mRNA and protein [36, 39]. In luteinizing granulosa cell cultures, STARD1 mRNA and protein can be increased by follicle-stimulating hormone (FSH) through PKA [40–42], although the relevance of this to corpus luteum function is uncertain because FSH receptors are typically down-regulated with luteinization in vivo [43, 44]. Several other hormones and growth factors have been shown to inhibit or stimulate STARD1 mRNA and/or protein levels, most often by modulating the cellular response to gonadotropin or PKA signaling. Of those relevant to luteal function known to be stimulatory are estradiol, insulin-like

growth factors (IGFs), insulin, and prostaglandin E2 (PGE2) [40, 42, 45–47]. Bone morphogenetic proteins (BMPs) tend to be inhibitory to STARD1 expression, as are tumor necrosis factor-alpha (TNFα), and activin A [48–53]. Prostaglandin F2-alpha (PGF2α) is inhibitory and may serve to reduce STARD1 at the onset of luteal regression [54–56]. Factors such as transforming growth factor-beta (TGFβ) and leptin have stimulatory or inhibitory actions depending on the context of the granulosa/luteal cell [57–60]. Active STARD1 exists as a phosphoprotein with PKA mediating phosphorylation [61]. There is evidence in ovine large luteal cells that STARD1 is phosphorylated by the high basal PKA activity in these cells [62]. The mechanism by which STARD1 works is not fully understood, but data support the model that STARD1 protein sits on the outer mitochondrial membrane to transfer cholesterol to the inner membrane [63]. Internalization of STARD1 protein into the mitochondria inactivates its activity [64].

The proximal 5′-flanking DNA of the STARD1 gene serves as the main promoter region and has transcription factor-binding sites that are highly conserved across species. Although many different types of transcription factors, including NR5A, GATA, NR4A, AP-1, Sp1/3, SREBP, Kruppel-like factors (KLFs), and forkhead proteins, have been shown to regulate the promoter [7], presented here are only those factors relevant to the corpus luteum.

Several different NR5A response elements that have the ability to bind steroidogenic factor 1 (SF-1/NR5A1) and liver receptor homologue 1 (LRH-1/NR5A2) reside in both the proximal STARD1 promoter region (first −150 bp) with others lying more distal between −900 and −3400 bp upstream of the transcriptional start site [65–67]. One of the proximal NR5A sites was shown to be specifically recruited in rodent luteal cells, although it was not utilized in less differentiated granulosa cells [66]. NR5A sites participate in both basal and cAMP-stimulated transcriptional activity [7]. In bovine luteal cells, beta-catenin interacts with LRH-1 to promote STARD1 transcription [68]. A member of the NR4A family Nur77 can also recognize the same NR5A response elements, and Nur77 reduction lowers STARD1 mRNA levels in theca cells [69]. It is unknown if Nur77 impacts STARD1 gene expression in luteal cells.

A conserved GATA consensus site (TTATCT) is located within the −70- to −55-bp region of the proximal STARD1 promoter of numerous species, and mutational analyses have demonstrated its importance to basal and/or cAMP-stimulated transactivity [7]. The transcription factors GATA4 and GATA6 are present in follicular cells and luteal cells, and both factors have been demonstrated to bind to the site [70]. There is some debate about the importance of GATA4 versus GATA6 to STARD1 promoter activity. Although both factors bind the STARD1 promoter GATA element in gel shift assays and overexpression of both recombinant factors can promote transactivation [71–73], most evidence indicates GATA4 as the major regulator of the promoter. This observation is complicated by the fact that GATA4 levels are typically lower with luteinization (especially in rodents) where GATA6 is strongly expressed [71, 74, 75]. To help clarify this point, one study in luteinizing pig granulosa cells showed that lowering GATA4 levels by RNAi actually increased STARD1 mRNA levels but not when GATA6 was also reduced, inferring that GATA6 has the potential to drive STARD1 gene expression when GATA4 levels drop [76]. These data support the idea that the ratio of GATA6 to GATA4 influences the contribution of GATA factors to STARD1 transcription in the corpus luteum.

Several C/EBP-binding sites exist in the proximal STARD1 promoter. Two of the sites are highly conserved whereas the sequence and location of other binding sites vary by location between species [7]. There is also overlap between C/EBP sites and CREB half-sites. Although CREB can associate with the promoter, studies in rodents showed that an ovulatory stimulus of hCG causes rapid C/EBPβ association with the promoter region, whereas CREB associates more slowly [77]. In addition, studies with several species of luteinized granulosa cells indicate C/EBP elements are needed for basal and cAMP responsiveness of the promoter [66, 72]. Further emphasizing the need for C/EBPs, cultured mouse granulosa with depleted C/EBPα and C/EBPβ have reduced induction of STARD1 mRNA in culture [78]. In rodent luteal cells, AP-1 family member Fra-2 displaces the CREB bound to the promoter of less mature granulosa [66]. In sum, although CREB can transactivate the promoter, in luteal cells C/EBPβ and Fra-2 occupy potential CREB-binding regions.

Other ovarian transcriptional regulators influence luteal STARD1 expression in a less clear manner. Forkhead transcription factor FOXO1 may act to repress the expression of STARD1 in granulosa cells before luteinization induced by the LH surge [79]. KLF4, -9, and -13 overexpression in luteinizing porcine granulosa cells reduced LH stimulated STARD1 promoter activity, yet KLF13 overexpression increased STARD1 mRNA levels [80]. There are no data yet as to whether KLF factors interact with the STARD1 promoter region in the context of the luteal cell.

3.4 Regulation of P450scc/CYP11A1

In many respects the regulation of CYP11A1 shares similarities to the regulation of STARD1. CYP11A1 mRNA and protein are present in follicular theca and some granulosa cells before the ovulatory LH surge; levels increase in granulosa cells during luteinization, are maintained at high levels in the functional corpus luteum, and drop off with regression [27]. The cow has a transient downregulation of CYP11A1 mRNA in the late preovulatory follicle [81]. CYP11A1 is constitutively expressed in the rodent corpus luteum once pregnancy is established [82]. Similar to STARD1, LH or exogenous hCG increases CYP11A1 mRNA and protein [27]. In luteinizing granulosa cell cultures, FSH, insulin, IGFs, epidermal growth factor, progesterone, PGE2, and prolactin act alone or in concert with gonadotropin to increase CYP11A1 mRNA [47, 83–88]. Repressors of CYP11A1 mRNA levels include TNFα, some BMPs, and luteolytic PGF2α [50, 51, 89–92]. TGFβ and activin A have mixed effects on CYP11A1 mRNA levels [57, 58].

The timing and abundance of CYP11A1 appears to be important for normal luteal function as transgenic mice overexpressing CYP11A1 have reduced progesterone production by the early corpus luteum [93]. In these transgenic mice, corpus luteum function is able to normalize by mid-pregnancy, suggesting a delayed luteinization of follicles.

There is fairly high homology between species within the first −100 bp upstream of the transcriptional start site of the CYP11A1 gene [7]. A proximal NR5A site in the cow promoter is active in basal and cAMP-driven activity in luteal cells [94, 95]. Similarly, rodent granulosa demonstrate NR5A site importance [96], and targeted

reduction of LRH-1 in mice express reduced CYP11A1 mRNA in granulosa when given an ovulatory stimulus of hCG [97].

Overexpression of GATA can drive the promoter in nonovarian cells. and a GATA site at −475 to −470 of the rat promoter helps confer granulosa responsiveness to FSH [98]. However, in luteinizing pig granulosa cells there is little impact of reduction of GATA4/6 factors on CYP11A1 mRNA expression, suggesting GATA may not be important to CYP11A1 expression in luteal cells [76].

At least one functional Sp1 site exists in the proximal promoter region [7]. Bovine luteal extracts exhibit Sp1 and/or Sp3 binding, and this site is important for basal and cAMP-stimulated activity [95]. In pig luteinizing granulosa cells, the Sp1 site confers responsiveness to both IGF1 and cAMP stimuli [99].

CYP11A1 promoter activity is also influenced by the differentiation status of the cell. Demonstrating this, CREB binds to a CRE half-site in less differentiated rodent granulosa cells and is replaced by Fra-2 upon luteinization [98].

Similar to STARD1, forkhead factors and KLF factors may influence luteal expression. FOXO1 may serve to repress the expression of CYP11A1 in granulosa cells before luteinization [100]. KLF4, -9, and -13 overexpression in luteinizing porcine granulosa cells reduced CYP11A1 promoter activity and KLF13 overexpression decreased CYP11A1 mRNA levels as well [80].

3.5 Regulation of 3-Beta-HSD/HSD3B (1 or 2)

Depending on the species, one or more HSD3B genes for 3-beta-hydroxysteroid dehydrogenase/delta5 delta4-isomerase exist, and the numbering system between species varies. For example the human has two genes (HSD3B1 and HSD3B2), the mouse has six genes (hsd3b1-6), and the pig has one gene (HSD3B1) [101]. In humans the main ovarian gene expressed that mediates steroidogenesis is HSD3B2 and in other species HSD3B1 (hsd3b1 in rodent). In rat luteal cells, using electron microscopy immunoreactive HSD3B protein has been localized to both the smooth ER and the mitochondria [102]. The mRNA for the ovarian form of HSD3B is expressed in theca cells of developing follicles, appears in the granulosa cells of growing follicles even before the ovulatory period, and is widely distributed in functional luteal cells, falling off with luteal regression [27]. Similar to STARD1 and CYP11A1, LH (or exogenous hCG) is a potent inducer of HSD3B transcripts in granulosa cells during the periovulatory period [27]. Other positive regulators of ovarian HSD3B expression include FSH and IGF1 or PGE2 either alone or in combination with gonadotropin [42, 47]. TGFβ and prolactin affect HSD3B expression either positively or negatively depending on the cellular setting [57, 58, 88]. Several BMPs and luteolytic PGF2α are inhibitory to HSD3B mRNA expression [47, 48, 90, 92].

Most cellular studies of HSD3B promoter regulation have been performed in cells lines, and it is unclear how these studies relate to periovulatory and luteal transcriptional events. However, there have been a few studies with luteinized granulosa and luteal cells. NR5A sites have been identified in the human HSD3B gene 5′-flanking DNA [7]. Overexpression of SF-1 can drive HSD3B promoter activity

in human granulosa tumor cells [103]. LRH-1 has been shown to bind two NR5A sites in luteinized granulosa cell extracts, and both sites contribute to transactivation with the more proximal site at −309 bp being most critical [104]. In contrast, in mice with granulosa cell-targeted loss of LRH-1, hsd3b1 mRNA levels following an ovulatory hCG stimulus are not affected [97]; however, there is always the possibility that SF-1 may substitute for LRH-1 in this setting.

There are at least four potential GATA-binding sites in the 5′-flanking DNA of the human HSD3B2 gene [7]. The GATA site at −196 bp relative to the transcriptional start site has been shown to stimulate promoter transactivation in nonovarian cells [105]. Studies in luteinizing pig granulosa cells indicate that a loss of GATA4 suppresses basal expression of HSD3B1 mRNA [76].

Nur77 may contribute to HSD3B gene expression in human theca cells [69], but the relevance of this factor to luteal cells is unknown.

3.6 Luteal Estrogen Synthesis

3.6.1 Regulation of Luteal Aromatase/CYP19A1

The CYP19A1 gene encodes aromatase, whose activity is required to convert androgens into estrogens. In the developing follicle, mural granulosa cells are the primary site for aromatase expression, which is driven mainly by FSH and cAMP/PKA signaling [6]. The LH surge downregulates granulosa aromatase [106]. In most large mammals, the CYP19A1 gene stays quiescent for the remainder of the corpus luteum lifespan. In the cow, downregulation of CYP19A1 by the LH surge is associated with silencing DNA methylation in the promoter 2 region in luteal cells [107]. This finding may extend to other species that lack luteal aromatase. In comparison, CYP19A1 is expressed robustly in the rodent corpus luteum of pregnancy and the primate corpus luteum, enabling luteal estradiol synthesis [106, 108, 109]. During the follicular phase, estradiol production and granulosa cell aromatase expression increase to reach a peak in the dominant follicle(s) [6]. In the human, mid- and late-luteal phase corpora lutea have the highest CYP19A1 mRNA expression levels compared to other follicular/luteal stages [109]. Granulosa cells utilize the ovarian-specific CYP19A1 promoter region to drive transcription in response to cAMP/PKA signals [110]. In the rat this region is located within −300 bp upstream of the gene and involves a cAMP-response element-like (CLS) sequence regulated by CREB, two NR5A sites that can bind SF-1 or LRH-1, and a GATA-4-binding site [110, 111]. In the luteal cell, the rat CLS site loses its transcription factor-binding ability and an AP3 site becomes recruited [111].

The human CYP19A1 promoter also has a functional CLS and NR5A region. When human CYPA19A1 promoter constructs are transfected into bovine luteal cells, both regions confer reporter gene activity [112]. The primate corpus luteum requires pituitary LH to maintain steroidogenesis during the luteal phase. In human cultured granulosa-lutein cells, LH increases aromatase mRNA and enzyme activity [113, 114], which infers that the human CYP19A1 promoter likely has some continued dependence on PKA signaling during the luteal phase.

A comparison of the CYP19A1 promoters of several species showed that cow and goat promoter constructs had minimal responsiveness to PKA activation in luteinizing granulosa cells, whereas the human and rat promoters were activated [115]. In addition, the responsiveness to overexpressed transcription factors LRH-1 and FOXL2 varied, providing a possible explanation for the species differences in luteal aromatase expression.

3.6.2 Regulation of Luteal P450c17/CYP17A1

In the follicle cytochrome P450 17-alpha-hydroxylase/17, 20 lyase (P450c17 or CYP17A1) is expressed in the thecal cell layer where it converts pregnenolone and progesterone-derived intermediates into androgens. This enzyme becomes downregulated with luteinization [116]. In the primate corpus luteum, theca lutein cells later reexpress this enzyme to provide the androstenedione and testosterone for estrogen synthesis [108]. Rodent CYP17A1 expression is increased by hGC treatment of luteal cells [117]. In the rodent, luteal androgen synthesis occurs in early pregnancy but the placenta serves as the main source of androgens for estradiol synthesis as pregnancy progresses [118].

3.6.3 Regulation of Luteal 17-Beta-HSD/HSD17B

More than a dozen types of 17-beta-hydroxysteroid dehydrogenases have been identified with various substrate specificities [119]. The main roles of ovarian HSD17B enzymes are to interconvert androgen forms and interconvert estrogen forms. In luteal cells, androstenedione is aromatized to estrone and then can be converted to estradiol by HSD17B. Alternatively, androstenedione can first be converted to testosterone by HSD17B and then aromatized to estradiol. HSD17B1 and HSD17B2 have been localized to luteal cells in humans [120]. HSD17B type 1 activity, which converts estrone to estradiol, predominates in human granulosa-luteal cells [121]. Mice null for hsd17b1 have increased estrone:estradiol and androstenedione:testosterone ratios, reduced progesterone, structural changes in corpora lutea, and are subfertile [122]. HSD17B7 serves to convert estrone to estradiol and is found in the corpora lutea of all mammalian species examined including rodents [123]. Expression of hsd17b1 disappears in the rodent corpus luteum, where hsd17b7 is strongly expressed [124]. Additionally, in rodents the hsd17b1 gene is upregulated by LH, whereas the hsd17b7 gene is increased by prolactin and repressed by LH/hCG. The promoter for rat hsd17b7 has a functional Sp1 site that regulates basal promoter activity and a NF-Y-binding site that mediates its inhibition by PKA [125]. Of note, HSD17B7 has also been demonstrated to mediate a reaction in cholesterol biosynthesis [119]. Deletion of hsd17B7 in mice is embryonic lethal because of deficiencies in cholesterol synthesis [126, 127]. These data infer that the HSD17B7 enzyme may have a dual purpose in the corpus luteum: cholesterol synthesis and estradiol synthesis.

3.7 Conclusions

Luteal progesterone biosynthesis depends on the coordinated upregulation and maintenance of the gene products of STARD1, CYP11A1 and its mitochondrial partner proteins, and HSD3B. Luteal estradiol synthesis by the corpus luteum occurs in a species-specific manner and involves CYP17A1, HSD17B, and CYP19A1. Most of the transcriptional studies performed have utilized various stages of luteinized granulosa cells in culture to study of upregulation of these genes during the periovulatory luteinization period. Comparison of the transcriptional activity of STARD1, CYP11A1, and CYP19A1 in granulosa cells and luteal cells of the rodent has demonstrated that the extent of cellular luteinization is critical for the recruitment of specific transcription factors. Figure 3.2 depicts

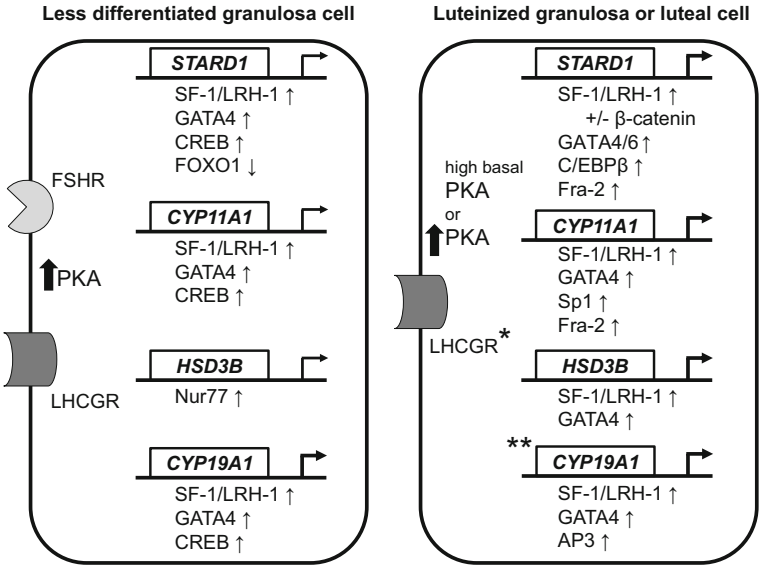

Fig. 3.2 Summary of the major transcription factors shown to regulate the STARD1, CYP11A1, HSD3B, and CYP19A1 genes in less differentiated granulosa cells or luteinized granulosa cells and luteal cells. * indicates that the presence of luteinizing hormone receptors (LHCGR) on luteal cells varies by steroidogenic luteal cell type and by species. ** indicates that the expression of CYP19A1 occurs in primate and pregnant rodent corpora lutea. *Thin arrows* indicate the transcription factor increases (↑) or decreases (↓) transactivation of the gene immediately above it in one or more species. *Thick arrows* indicate protein kinase A (PKA) is increased by activation of the follicle-stimulating hormone receptor (FSHR) and LHCGR by their respective ligands. In most cases PKA leads to increased transcriptional activity of the factors shown. Less differentiated granulosa cells refers to those that have not been luteinized in vivo by a gonadotropin surge or by culture conditions in vitro. STARD1 is likely repressed in vivo by FOXO1 or other unknown factor before the LH surge, as its mRNA is minimally expressed in the pre-surge maturing follicles of most species

a summary of putative regulators of STARD1, CYP11A1, HSD3B, and CYP19A1 by cellular differentiation status. Even though LH activates the genes for the major steroidogenic pathway molecules in the early corpus luteum, how the expression of these genes is maintained in the mature corpus luteum and that of pregnancy is an area that requires much more research.

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Chapter 4
Lipid Droplets and Metabolic Pathways Regulate Steroidogenesis in the Corpus Luteum

Heather Talbott and John S. Davis

Abstract This review focuses on recent advances in the understanding of metabolic processes used by the corpus luteum to control steroidogenesis and other cellular functions. The corpus luteum has abundant lipid droplets that are believed to store cholesteryl esters and triglycerides. Recent studies in other tissues indicate that cytoplasmic lipid droplets serve as platforms for cell signaling and interactions with other organelles. Lipid droplets are also critical organelles for controlling cellular metabolism. Emerging evidence demonstrates that LH via activation of the cAMP and the protein kinase A (PKA) signaling pathway stimulates the phosphorylation and activation of hormone-sensitive lipase (HSL), an enzyme that hydrolyzes cholesteryl esters stored in lipid droplets to provide cholesterol for steroidogenesis and fatty acids for utilization by mitochondria for energy production. The energy sensor AMP-activated protein kinase (AMPK) can inhibit steroidogenesis by interrupting metabolic pathways that provide cholesterol to the mitochondria or the expression of genes required for steroidogenesis. In addition to lipid droplets, autophagy also contributes to the regulation of the metabolic balance of the cell by eliminating damaged organelles and providing cells with essential nutrients during starvation. Autophagy in luteal cells is regulated by signaling pathways that impact AMPK activity and lipid droplet homeostasis. In summary, a number of signaling pathways converge on luteal lipid droplets to regulate steroidogenesis and metabolism. Knowledge of metabolic pathways in luteal cells is fundamental to understanding events that control the function and lifespan of the corpus luteum.

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4.1 Introduction

Recent research has provided great insight into mechanisms contributing to corpus luteum formation, function, and regression. Many of these studies have focused on changes in gene expression and protein expression and activity. The availability of new techniques for metabolomics, lipidomics, and proteomics has renewed interest in determining how cellular metabolic events control steroidogenesis. Specifically, there is an interest in understanding how lipids are stored and utilized during the lifespan of the corpus luteum. One of the notable features observed during luteal development is the acquisition of cytoplasmic lipid droplets (LDs). These unique organelles are surrounded by a phospholipid monolayer that coats a core of neutral lipids including cholesteryl esters and triglycerides. Lipid droplets have been most extensively studied in adipocytes and preadipocytes for their pivotal role in energy conservation and homeostasis [1, 2]; however, LDs have been observed in nearly all cell types, from prokaryotes [3] to hepatocytes [4], cardiac myocytes [5], macrophages [6], and steroid-secreting cells [7, 8]. In many of these cells, LDs are a sign of pathological stress because of an overabundance of environmental lipids (e.g., the foamy macrophage seen in atherosclerotic lesions [6]). However, LD formation and presence in steroidogenic tissues such as the ovarian follicle and corpus luteum appear to be nonpathological and required for healthy, fully functional steroidogenic ovarian cells.

4.2 Lipid Droplets

Recent reviews point to cytoplasmic LDs as critical mediators of metabolic health and disease [1, 9, 10]. Intracellular LDs store triglycerides and cholesteryl esters as reservoirs for energetic substrates (fatty acids) or cholesterol for membrane biosynthesis or sterol production [11, 12]. They also serve to protect cells from lipotoxicity [13]. Key to understanding LD size and activity is the presence or absence of specific LD coat proteins [14]. The family of perilipin (PLIN) proteins serves as LD coat proteins and organizing centers for enzymes and transporters in lipid metabolism [15–17]. The PLIN family of proteins is composed of PLIN1 (perilipin), PLIN2 (adipophilin or ADRP), PLIN3 (previously Tip47), PLIN4 (previously S3-12), and PLIN5 (previously OXPAT). PLIN1 and PLIN4 are highly expressed in white adipose [16] whereas PLIN2, PLIN3, and PLIN4 are widely expressed; although PLIN2 is abundant in liver and PLIN5 is found in oxidative tissues such as heart and

brown adipose [18]. *Plin1*-null mice have a distinct phenotype of reduced fat mass, increased lipolysis, and increased β-oxidation [19]. *Plin2*-null mice are resistant to high-fat diet-induced obesity [20], and *Plin3* compensates for the loss of *Plin2* in these mice [21]. Inactivation of *Plin4* downregulates *Plin5* and reduces cardiac lipid accumulation in mice [22]. It seems, therefore, that the level of PLIN proteins in specific cell types regulates lipolysis in target tissues. Reports in the monkey [23] and mouse [24] indicate that the ovary expresses PLIN2, a LD coat protein associated with cholesteryl ester storage [25]. We have found that the bovine corpus luteum predominately expresses *PLIN2* and *PLIN3 mRNA* with low levels of *PLIN1*, a different pattern of PLINs when compared to adipose tissue (Fig. 4.1a). Bovine large and small luteal cells express comparable levels of *PLIN2* and *PLIN3* mRNA but different levels of *PLIN1* and *PLIN4* mRNA (Talbott, Krauss, and Davis, unpublished data). Exactly how the LD-associated PLINs impact luteal LDs and steroidogenesis are subjects of current investigation.

Hormone-sensitive lipase (HSL) is a key cytosolic enzyme in the regulation of lipid stores in adipocytes that translocates to the LD in response to catecholamine stimulation [26–28]. A current view of the mechanisms regulating lipolysis in adipose tissue suggests that the LD-associated PLIN1 coats the LD and functions as a scaffold in the regulation of lipolysis [16, 29, 30]. Under basal conditions, PLIN1 acts as a barrier to the hydrolysis of lipids within the LD by preventing access of adipocyte triglyceride lipase (ATGL) and HSL, the major lipases in adipose cells. Following β-adrenergic stimulation of cAMP and protein kinase A (PKA) signaling, PLIN1 and HSL are phosphorylated, which leads to the movement of HSL from the cytosol to the LD [31]. The phosphorylation of HSL facilitates its association with the LD and with lipid substrates [32], permitting lipid hydrolysis to proceed. Phosphorylation of HSL by PKA occurs on multiple sites, including Ser-563 and Ser-660, which stimulate catalytic activity and translocation of HSL to LDs [33–36]. Phosphorylation of HSL also occurs at Ser-565, a non-PKA site, which is a negative regulator of HSL activity and is believed to be mutually exclusive with phosphorylation on the Ser-563 site [37]. Thus, hormonal cues that signal for elevations in systemic energy stimulate PKA to phosphorylate HSL, which contributes to lipolysis to maintain energy homeostasis.

The presence of both PLIN coat proteins [38] and HSL [39] in the ovary suggests that LH via a cAMP/PKA signaling pathway may regulate the phosphorylation of PLINs and HSL to hydrolyze cholesteryl esters stored in luteal LDs to produce substrate for progesterone synthesis. Studies with HSL-null mice revealed that knockout of HSL resulted in decreased steroidogenesis in the adrenals and inhibited sperm production in the testis [40, 41]. These findings suggest that HSL is involved in the intracellular processing and availability of cholesterol for adrenal and gonadal steroidogenesis. Manna et al. [42] recently reported that activation of the PKA pathway in MA-10 mouse Leydig cells enhanced expression of HSL and its phosphorylation at Ser-563 and Ser-660. Inhibition of HSL activity suppressed cAMP-induced progesterone synthesis and resulted in increased cholesteryl ester levels in MA-10 cells. Also of interest is a report [43] demonstrating an interaction between StAR and HSL in the rat adrenal following treatment with ACTH. Furthermore, the coexpression of

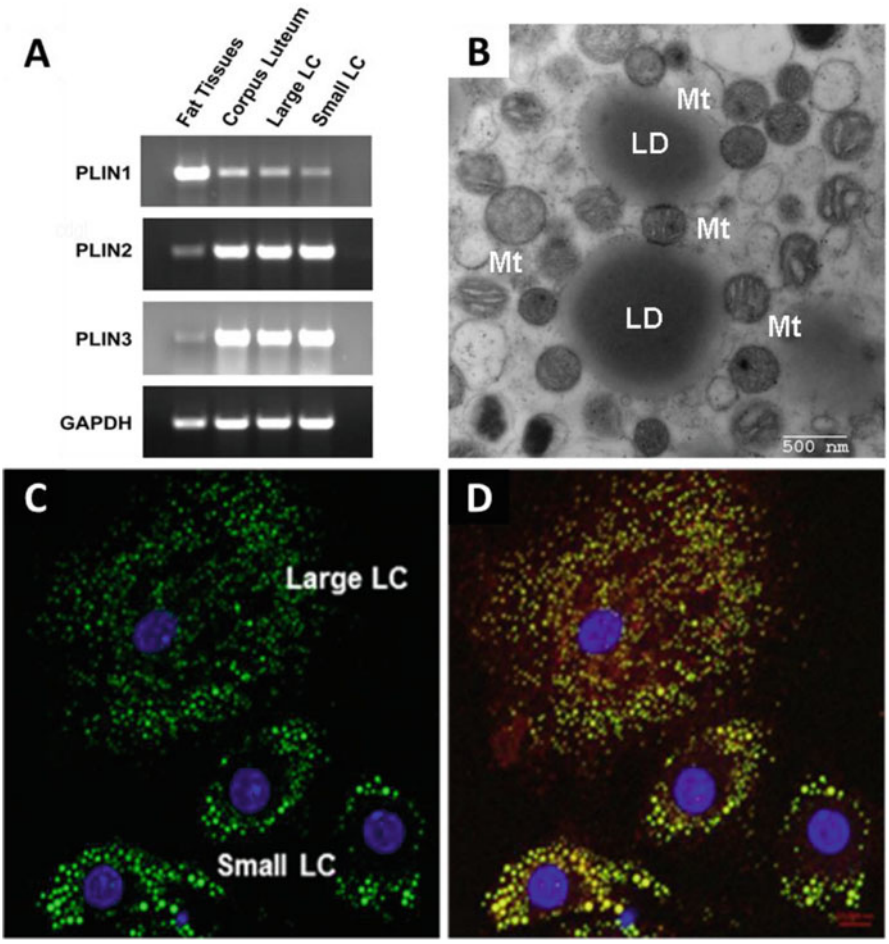

Fig. 4.1 Large and small bovine luteal cells express lipid droplet (LD) coat proteins and have unique LDs. Panel **a**: Expression of the PLIN family of LD coat proteins in bovine white adipose tissue, corpus luteum, and centrifugal elutriation-enriched large and small luteal cells (LC). Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of mRNA isolated from bovine fat and luteal tissue. Panel **b**: Electron microscopy of lipid droplets (LD) and mitochondria (Mt) in a bovine luteal cell. Panels **c** and **d**: Small and large luteal cells were stained with Bodipy 493/503 (Molecular Probes, 10 μg/ml) to detect neutral lipids (*green*). Nuclei: DAPI (*blue*). Cells in Panel **d** were were also immuno-labeled with with adipocyte triglyceride lipase (*red*) showing colocalization with the LDs and the difference in LD morphology between small and large luteal cells

StAR and HSL resulted in elevated HSL activity and mitochondrial cholesterol content. These observations suggest that the proteins that produce and transport cholesterol may colocalize in LDs and mitochondria. Furthermore, we have observed that mitochondria are closely associated with cytoplasmic LDs in bovine luteal cells (Fig. 4.1b) indicating that luteal LDs and mitochondria may interact to facilitate steroidogenesis. Although the evidence points to an important role for HSL in ste-

roidogenesis, there is little information concerning the LD and the events that control these early steps in ovarian steroidogenesis [44].

Despite the renewed interest in cytoplasmic LD as platforms for cell signaling, interactions with other organelles, and metabolic control [45, 46], few studies have characterized the protein and lipid composition of the LD. The LD proteome has been characterized to varying degrees in a few mammalian tissues or cell lines (mouse mammary epithelial cells [47] and 3T3-L1 adipocytes [48, 49], rat liver and mouse muscle tissue [50, 51], and human cell lines [52–54]). Khor et al. [55] compared the proteome of LDs from rat granulosa cells treated in vitro with either high density lipoproteins or fatty acids to enrich cytoplasmic LDs with cholesteryl esters or triacylglycerides, respectively. When comparing the LD proteomes 278 proteins were common to the LDs prepared from either treatment. These proteins included PLIN2 and were similar to other studies on LD proteomes. They also identified 61 proteins unique to the cholesteryl ester-rich LDs and 40 unique proteins unique to triacylglycerol-rich LDs. Notably, they identified Hsd3b1, vimentin, and voltage-dependent anion channel (Vdac1) proteins enriched in the cholesteryl ester-rich LDs. Recent reports on the proteomic analysis of LD isolated from the mouse Leydig tumor cell line MLTC-1 [56] and mouse testes [57] also revealed the presence of PLIN family proteins and enzymes involved in the synthesis of steroid hormones. Despite the recent work on characterization of the LD proteome in various tissues, there is still a lack of information about the protein composition of luteal LDs and the effects of hormones or metabolic alterations on luteal LD properties. In our studies (Talbott, Krauss, and Davis, unpublished) the LDs isolated from bovine luteal tissue predominantly contain PLIN2 and PLIN3 coat proteins, as well as HSL, HSD3B1, CYP11A1, and StAR. Collectively, these studies indicate that the LD may serve as a novel hormonally responsive platform that is essential for steroidogenesis.

Comprehensive analysis of the lipid composition of LDs in other tissues is just beginning to be evaluated [58]. The protein composition of LDs, particularly the PLIN family of LD coat proteins, is believed to influence the type of lipids stored in LDs and metabolic activity of tissues [1, 59]. The lipid composition of ovarian LDs and the effects of hormones on the lipids contained therein are currently unknown. Our preliminary studies indicate that compared to granulosa and theca cells, the total lipid content of luteal cells is increased. Several studies reported the types and changes of lipids in the intact corpus luteum of rats [60], pigs [61, 62], sheep [63], and humans [64].These studies reported that cholesteryl esters and free fatty acids remain relatively constant during the functional phases of the luteal lifespan whereas triglycerides accumulated in the regressing corpus luteum. The increased lipid content of luteal cells is likely to be stored exclusively within the LDs; however, this remains to be shown experimentally. Additional studies are needed to determine the role and fate of lipids in LDs during both function and regression of the corpus luteum.

Bovine and ovine corpora lutea have two distinct steroidogenic cells, large and small luteal cells, with different abilities to produce progesterone [65–67]. The small luteal cells respond to LH with robust increases in progesterone secretion whereas the large luteal cells have a high basal rate of progesterone secretion and respond to LH with a comparatively modest fold increase in progesterone secretion. The luteal tissue of women, monkeys, pigs, and rodents also possess large and small luteal

cells, although the basal and LH-stimulated progesterone secretion differ from the bovine corpus luteum [68]. Our preliminary data indicate that bovine large and small luteal cells have LDs with distinctive morphology. As indicated by BODIPY 493/503 staining of neutral lipids (green) and the LD protein adipocyte triglyceride lipase (ATGL), small luteal cells have large LDs, whereas large cells have abundant dispersed small LDs (Fig. 4.1c, d). Whether and how the LDs in either cell type contribute to the ability to respond to LH or to the basal rate of progesterone secretion is currently unknown. Studies in other tissues indicate that PKA-dependent phosphorylation of PLIN1 induces dispersion of clustered LDs in HEK293 cells, fibroblasts, and 3T3L1 adipocytes [69, 70]. Based on these findings it seems possible that the dispersed LDs observed in bovine large luteal cells may be the result of constitutive PKA activity reported to be present in large luteal cells [71].

Fatty acids (either synthesized de novo or provided by the hydrolysis of stored cholesteryl esters, triglycerides, or phospholipids) are essential for energy production and the synthesis of most lipids, including those found in membranes and lipids involved in cellular signaling. Despite their fundamental physiological importance, an oversupply of nonesterified fatty acids can be detrimental to cellular function [10]. Fatty acids are transported across the outer mitochondrial membrane by carnitine palmitoyltransferase I (CPT1A), the rate-limiting step in fatty acid oxidation. Fatty acids are consumed by mitochondria through β-oxidation to produce acetyl-CoA, nicotinamide adenine dinucleotide (NADH), and flavin adenine dinucleotide ($FADH_2$) for use in the electron transport chain to produce ATP [72]. The hydrolysis

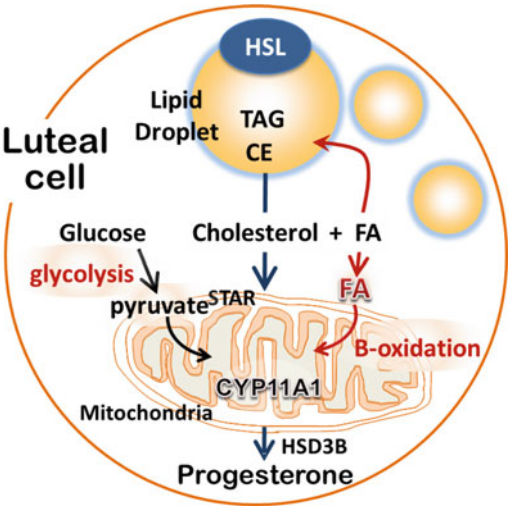

Fig. 4.2 Hormone-sensitive lipase (HSL) stimulates the hydrolysis of cholesteryl esters (CE) stored in lipid droplets to liberate cholesterol and fatty acids. The cholesterol is converted to pregnenolone by the cytochrome p450 side-chain cleavage enzyme (CYP11A1) in the mitochondria and subsequently converted by the enzyme 3β-hydroxy-steroid dehydrogenase (HSD3B) to progesterone. The released fatty acids (FA) are re-esterified and stored in the lipid droplets or used for energy production by mitochondrial β-oxidation

of cholesteryl esters by HSL liberates cholesterol and fatty acids (Fig. 4.2). The fatty acids are either re-esterified and stored in LDs or membranes or used for β-oxidation, producing reducing equivalents and acetyl-CoA for the citric acid cycle [72]. Although little is known about the role of fatty acid β-oxidation in luteal cells, recent studies indicate that fatty acid β-oxidation is a key in cumulus oocyte complex metabolism and oocyte maturation [73, 74]. These studies found that promoting β-oxidation with *L*-carnitine improved embryo development and that pharmacological inhibition of fatty acid β-oxidation with etomoxir, a CPT1A inhibitor, impaired oocyte maturation and embryo development. Steroidogenic tissues use glycolysis to support steroidogenesis [75]; however, it seems likely that the production of large quantities of progesterone by luteal cells would also require β-oxidation of fatty acids to provide the energy needed for optimal steroidogenesis under basal conditions, but this remains to be critically evaluated. It seems likely that large and small luteal cells may have different energy-processing requirements, based on the pronounced differences in the ability of large and small luteal cells to produce progesterone under basal and stimulated steroidogenesis. Our preliminary studies indicate that *CPT1A* mRNA expression in large luteal cells is 5.6 fold greater than in granulosa cells, whereas no difference in *CPT1A* mRNA expression was observed between theca and small luteal cells. These data support our idea that β-oxidation may be important in the metabolic regulation of large luteal cells. Given the intense interest in pathologies that result in lipid accumulation and conditions (i.e., obesity, diabetes, metabolic syndrome) that elevate free fatty acids and alter metabolism, understanding how LDs, glycolysis, and β-oxidation are regulated in the corpus luteum may provide clues for improving ovarian function, treating ovarian disorders, and enhancing fertility.

4.3 AMP-Activated Protein Kinase

The AMP-activated protein kinase (AMPK) is a master regulator of cellular metabolism [72, 76]. The AMPK complex is a heterotrimer consisting of an α-catalytic subunit and noncatalytic β- and γ-regulatory subunits [77]. Studies from a number of investigators demonstrate that AMPK is present in the oocyte, granulosa and theca cells of the follicle, as well as luteal cells (reviewed by Bertoldo et al. [78]). As its name suggests, AMPK is allosterically activated by adenosine monophosphate, AMP. The enzyme is activated by increases in AMP:ATP or ADP:ATP ratios, which occur when cellular energy status has been compromised by metabolic stresses that either interfere with ATP production or accelerate ATP consumption [79]. AMPK acts to restore energy homeostasis by activating alternate catabolic processes generating ATP while inhibiting energy-consuming processes, such as protein, carbohydrate, and lipid biosynthesis, as well as cell growth and proliferation (Fig. 4.3). AMPK acts via direct phosphorylation of metabolic enzymes and by longer-term effects via phosphorylation of transcription regulators [80, 81].

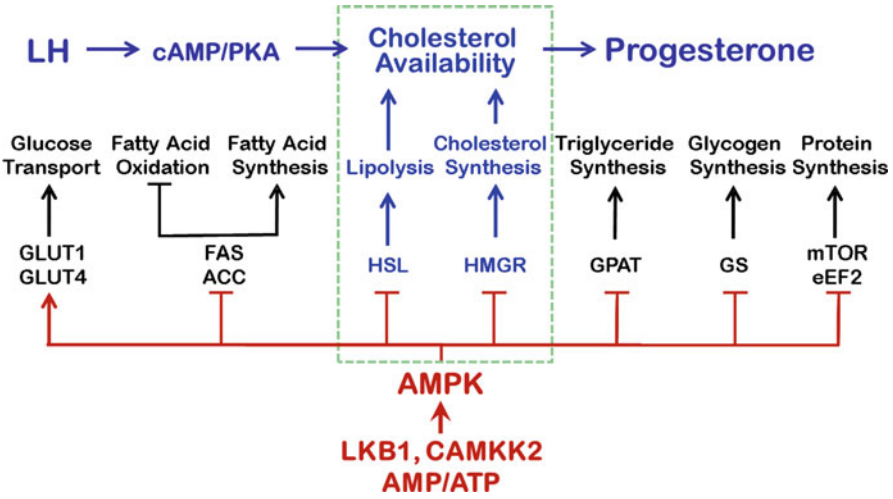

Fig. 4.3 Luteinizing hormone (LH) stimulates cAMP and protein kinase A (PKA) to activate proteins that will supply cholesterol for progesterone synthesis. The master metabolic regulator AMP-activated protein kinase (AMPK) is a highly conserved metabolic fuel gauge and can influence progesterone secretion by luteal cells. Elevations in AMP to ATP ratios stimulate AMPK to restore energy homeostasis by activating alternate catabolic processes generating ATP while inhibiting energy-consuming processes, that is, protein, carbohydrate, and lipid biosynthesis, as well as cell growth and proliferation. Activation of AMPK can disrupt steroidogenesis by phosphorylating and inhibiting hormone-sensitive lipase (HSL) and blocking HMGCR (3-hydroxy-3-methylglutaryl-coenzyme A reductase), the rate-controlling enzyme of the pathway that produces cholesterol. AMPK can be activated by the tumor suppressor kinase liver kinase B1 (LKB1) and the Ca^{2+}/calmodulin activated protein kinase CaMKK2, which is activated when intracellular Ca^{2+} is increased by hormones such as PGF2α

AMPK can be activated by a number of synthetic allosteric effectors (A-769662, 991, MT 63–78) identified by Abbott Laboratories using high-throughput screens for AMPK. Other allosteric effectors are salicylate, the major breakdown product of aspirin, and pro-drugs: AICAR (5-amino-imidazole-4-carboxamide riboside) and C13, which are converted into AMP analogues following cellular uptake. For example, AICAR, a widely used AMPK activator, is taken into cells and then converted to the monophosphorylated derivative ZMP, which mimics the effect of AMP on both the allosteric activation of the kinase and inhibition of the dephosphorylation of Thr-172 on AMPK. Pharmacological AMPK activators (e.g., metformin, berberine, resveratrol, hydrogen peroxide) are typically viewed as metabolic poisons that inhibit ATP synthesis and stimulate AMPK indirectly by increasing cellular AMP levels [79]. Activation of AMPK by upstream kinases occurs by phosphorylation of a conserved threonine within the 'activation loop' of the kinase domain (Thr-172). The primary upstream kinases that phosphorylate Thr-172 are the tumor suppressor liver kinase B1 (LKB1) (also known as serine and threonine kinase 11 or STK11), and the calcium/calmodulin-dependent protein kinase kinase 2, CAMKK2. The latter is activated when intracellular Ca^{2+} is increased by the action of hormones.

AMPK likely controls multiple aspects of metabolism in ovarian cells. AMPK phosphorylates and inactivates acetyl-CoA carboxylase (ACC) and 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), key enzymes involved in regulating de novo biosynthesis of fatty acids and cholesterol (Fig. 4.2). Activation of AMPK also blocks the activation of the mechanistic target of rapamycin (MTOR) and protein synthesis by phosphorylating the key regulatory proteins, raptor and tuberous sclerosis proteins [81]. Another immediate consequence of enhanced AMPK activity is the phosphorylation of HSL at Ser-565, which precludes activation of HSL by PKA [82]. Conversely, conditions that stimulate PKA-induced phosphorylation of HSL at Ser-660 and Ser-563 suppress the phosphorylation of HSL at the AMPK site Ser-565. In vitro kinase assays using purified PKA and AMPK support the notion that phosphorylation of HSL at Ser-563 and Ser-565 is mutually exclusive. Thus, in steroidogenic tissues, activation of AMPK can inhibit HSL-mediated hydrolysis of cholesteryl esters and prevent the release of free cholesterol for steroidogenesis [83]. The observation that HSL is a key enzyme in adipocytes and steroidogenic cells strategically positions AMPK to control the expression of genes required for steroidogenesis and the availability of cholesterol for ovarian progesterone synthesis (Fig. 4.4).

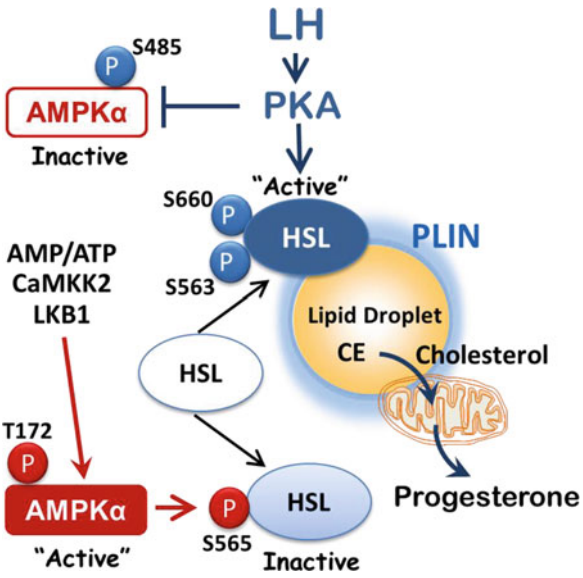

Fig. 4.4 Luteinizing hormone (LH) stimulates protein kinase A (PKA)-dependent phosphorylation on Ser-563 and Ser-660, resulting in activation of hormone-sensitive lipase (HSL), which hydrolyzes cholesteryl esters (CE) stored in lipid droplets (LD) to release cholesterol and fatty acids (FA). AMP-activated protein kinase (AMPK) suppresses the activation of HSL by phosphorylation of HSL on Ser-565. LH also inactivates AMPK by increasing AMPK phosphorylation on Ser-485 and reducing phosphorylation on Thr-172. The ability of LH to suppress AMPK and activate HSL ensures adequate cholesterol availability for progesterone synthesis

Reports from the DuPont Laboratory [84, 85] demonstrate that AMPK activators metformin and AICAR inhibit the secretion of progesterone or estradiol by granulosa cells in a manner dependent on the state of cellular differentiation and the species investigated [78, 84, 86]. In rat and bovine granulosa cells, AMPK activation induced by metformin reduced the expression of mRNA for key enzymes required for progesterone synthesis, *HSD3B1, CYP11A1*, and *StAR* [85, 87]. In the human KGN granulosa cell line (L. Huang, X. Hou and J.S. Davis, unpublished data), treatment with the AMPK activator metformin inhibited *StAR* expression and progesterone synthesis. In general, the studies in granulosa cells suggest that the reduction in steroidogenesis was a result of a reduction in the transcription of genes in the steroidogenic pathway. Other studies showed that metformin impairs proliferation of bovine granulosa cells and rat theca cells via mechanisms involving AMPK-mediated inhibition of MTOR signaling and protein synthesis [88–90].

Bowdridge et al. recently reported increases in the expression of AMPKα-, β-, and γ-subunits during the maturation of the bovine corpus luteum, with the exception of AMPKγ1- and γ2-subunits [91]. Other studies from the Flores Laboratory provide evidence for increased expression of genes encoding distinct protein kinase C isoforms and genes participating in Ca^{2+} homeostasis during luteal maturation [92]. Goravanahally et al. [93] reported that *CAMKK2*, a downstream target of Ca^{2+} and upstream regulator of AMPK, is also more highly expressed in mature bovine corpus luteum than in newly formed luteal tissue. It should be noted that two important physiological processes occur during this developmental period: (1) the corpus luteum develops maximal capacity for progesterone secretion and (2) the corpus luteum develops the capacity to undergo luteolysis in response to PGF2α. Based on the high rate of progesterone production during the mid-luteal phase and pregnancy, it seems likely that any factors that influence metabolic activity in steroidogenic cells would increase or decrease AMPK activity and impact steroid secretion. Hou et al. [94] reported that treatment of primary cultures of bovine luteal cells with AICAR rapidly increased AMPK activity and significantly reduced LH-stimulated MTOR activity and progesterone secretion. Additional findings in this report indicated that the response to AICAR was independent of MTOR since other experiments showed that inhibition of MTOR with rapamycin did not contribute to the reduction in LH-stimulated progesterone secretion. More recently, Bowdridge et al. [91] observed that treatment of bovine luteal tissue slices with either metformin or AICAR acutely reduced basal progesterone secretion. These results indicate that AMPK activators acutely inhibit luteal progesterone synthesis, suggesting that the energy status of luteal cells is an important regulator of steroidogenesis.

4.4 LH Inhibits AMPK

The C-terminal domains of AMPKα-subunit isoforms in vertebrates contain a serine/threonine-rich insert of 50–60 amino acids, the so-called ST loop [95]. Phosphorylation of the ST loop serves as a means for negative regulation of AMPK. The amino acid residues defining the ends of this loop are close to the

Thr-172 residue and contain a number of regulatory phosphorylation sites. The best characterized of these sites is Ser-485 on the AMPKα1-subunit. The Ser-485 site is phosphorylated by the cyclic AMP-dependent protein kinase, PKA [96], or by Akt [97], which subsequently inhibits the phosphorylation of the AMPKα-subunit Thr-172 residue by upstream kinases, LKB1 or CaMKK2 [95]. The AMPK-α2 subunit contains a similar conserved ST loop, and phosphorylation of Ser-491 is likely to exert the same inhibitory effect, although Ser-491 is a poor substrate for Akt and appears to be modified by autophosphorylation [95]. Additionally, PKA can phosphorylate the Ser-173 residue (adjacent to Thr-172 within the activation loop), which can inhibit Thr-172 phosphorylation [98]. In a study using primary cultures of bovine luteal cells, Hou et al. reported that treatment with LH rapidly inhibited AMPK activity as evidenced by reduced AMPK Thr-172 phosphorylation and reduced phosphorylation of the AMPK substrate acetyl-CoA carboxylase [94]. Treatment with LH also increased phosphorylation of AMPK on Ser-485, which is associated with inhibition of AMPK activity [94].

In contrast to granulosa cells, bovine luteal cells contain the required steroidogenic machinery including HSL, which enables luteal cells to respond to LH or cAMP with rapid increases in progesterone synthesis. The increases in progesterone occur within 10–30 min [99–101] and precede the LH-induced increase in STAR expression, which is typically observed 2–4 h after treatment [102]. These changes are associated with reduced phosphorylation of HSL at the inhibitory AMPK phosphorylation site Ser-565 and increased phosphorylation of HSL at Ser-563 and -660, residues that are required for HSL activity (Krause, Talbott, Hou, and Davis, unpublished). Thus, the ability of LH to reduce AMPK activity may allow optimal LH- and PKA-dependent activation of HSL and provide cholesterol for the already existing steroidogenic machinery. An experimental model of the proposed interaction among PKA and AMPK regulation of HSL is shown in Fig. 4.4. Physiological conditions that increase the activity of AMPK require phosphorylation of the AMPK α-subunit on Thr-172 residues [103], leadings to the phosphorylation of the AMPK substrates ACC (Ser-79) and HSL (Ser-565), which could reduce the ability of luteal cells to provide cholesterol substrate in response to a pulse of LH. LH or PKA activators attenuate AMPK activity through modulation of at least two AMPK α-subunit phosphorylation sites, Thr-172 (reduced), and Ser-485 (increased). Reduced HSL phosphorylation by AMPK allows PKA to phosphorylate HSL on Ser-563 and Ser-660 resulting in increased HSL activity, which presumptively provides cholesterol for progesterone synthesis.

4.5 PGF2α Activates AMPK

Early studies established that PGF2α binds to and activates its cognate G_q protein-coupled receptor, the prostaglandin F receptor, PTGFR. This initial event provokes the rapid activation of phospholipase C, which leads to increases in both cytoplasmic Ca^{2+} and activation of protein kinase C. These early events contribute to the activation of additional protein kinase cascades such as the mitogen-activated protein kinases

(ERK1/2, p38, and JNK) [104] that contribute to the induction of early-response genes such as FOS, JUN, EGR1, and ATF3 [105–108]. Although these early-response genes have been implicated in the luteolytic response to PGF2α, it is not clear how or whether they impact metabolic events in luteal cells. The developmental-specific expression of protein kinase C and CAMKK2 isoforms, proteins involved in Ca^{2+} homeostasis, and AMPK have been implicated in the cellular mechanisms of acquisition of luteolytic capacity by bovine corpus luteum [92, 93, 109]. Based on these observations it seems reasonable to predict that PGF2α could activate Ca^{2+}/CAMKK2 pathways leading to the activation and phosphorylation of AMPK on Thr-172.

Bowdridge et al. [91] recently reported that PGF2α rapidly (2 min) and transiently stimulated the phosphorylation of AMPK on the Ser-485 site in dispersed bovine luteal cells. The response was prevented by treatment with STO-609, a CAMKK2 inhibitor. Treatment with STO-609 also prevented the modest inhibitory effect of PGF2α on progesterone synthesis in overnight incubations of dispersed luteal cells [91]. In recent studies using bovine large luteal cells, we have observed that PGF2α rapidly stimulates the phosphorylation of AMPK on the stimulatory Thr-172 residue as well as the inhibitory Ser-485 residue (Hou, Zhang, Talbott, and Davis, unpublished data). The phosphorylation of AMPK was coupled to the phosphorylation of the AMPK target ACC, indicating that AMPK was activated by PGF2α. The observation that PGF2α can target multiple sites on AMPK is consistent with findings that PGF2α activates multiple protein kinase pathways in luteal

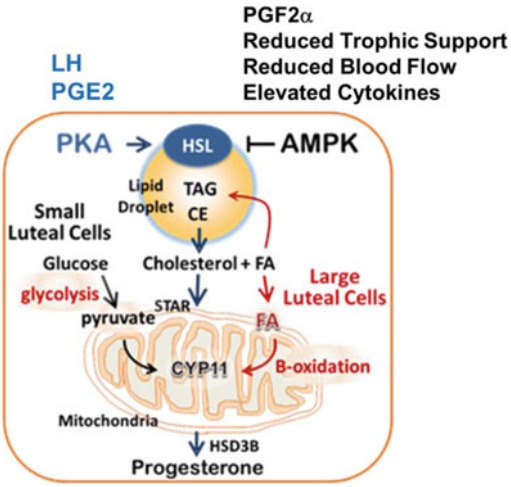

Fig. 4.5 LH and PGF2α have opposite effects on AMP-activated protein kinase (AMPK). LH-dependent activation of protein kinase A (PKA) activates hormone-sensitive lipase (HSL). In contrast, activation of AMPK blocks activation of HSL. LH-dependent stimulation of cellular metabolism regulates the use of glucose and fatty acids (FA) for optimal progesterone synthesis. Conditions that activate AMPK (hormones, cytokines, reduced nutrients, reduced blood flow, hypoxia, drugs, and environmental insults) reduce the ability of LH to provide cholesterol for progesterone synthesis

cells: pathways linked to calcium signaling, protein kinase C, mitogen-activated protein kinases, and MTOR signaling [110]. Although additional studies are needed to determine exactly how PGF2α regulates AMPK in luteal cells, it seems clear that activation of AMPK with pharmacological tools disrupts luteal progesterone synthesis (Fig. 4.5). Studies are also needed to determine whether AMPK is activated *in vivo* during natural and PGF2α-induced luteolysis. It is conceivable that changes in luteal blood flow, hypoxia, and the presence of inflammatory mediators all contribute to altering the metabolic status of steroidogenic luteal cells, resulting in the activation of AMPK and disrupting progesterone synthesis.

4.6 Autophagy

Autophagy plays an important role in cellular and tissue physiology [111–113]. The main function of autophagy is to protect cells against starvation by allowing cells to salvage nutrients by digesting organelles and macromolecules at times of nutrient scarcity as well as to ensure cell homeostasis by eliminating damaged organelles and misfolded proteins. Three different types of autophagy (macroautophagy, microautophagy, and chaperone-mediated autophagy) have been described, based largely on the processes by which cargo is delivered to the lysosomes. In general, autophagy can be induced by limitations in amino acids, growth factors, energy, and oxygen. The formation of autophagosomes requires the activation of a number of protein complexes: the autophagy-related 1 (Atg1)–Unc-51-like kinase complex, which is a key signaling intermediate that is regulated by MTOR and AMPK; the autophagy-specific class III phosphatidylinositol 3-kinase Vps34 complex (consisting of Vsp34, Beclin 1, Vsp15, and Atg14L), which produce a pool of phosphatidylinositol-3-phosphate that is necessary for autophagosome formation; and a complex of ubiquitin-like proteins: Atg12, Atg5, Atg16, and LC3-I (Atg8) and their conjugation machinery, which leads to the lipidation of microtubule-associated protein light chain 3 (LC3) with phosphatidylethanolamine, a process required for autophagosome formation and closure. The presence of LC3-II, an LC3 cleavage product, inside the mature autophagosome is generally used as a marker of autophagy.

Autophagy has been shown to occur in oocytes, granulosa cells, and luteal cells and is often associated with apoptosis. Genetic mouse models demonstrate that *Atg7*$^{(-/-)}$ ovaries [114] or germ cell-specific deletion of *Atg7* [115] compromised autophagy in the perinatal mouse ovary, resulting in the early loss of female germ cells. Loss of Beclin 1 (*Becn1*), which has a central role in the regulation of autophagy through activation of the Vps34 complex, also resulted in a significant loss of germ cells at birth [114]. These findings indicate that autophagy may promote survival of germ cells during ovarian development. Other studies provide evidence for the presence of autophagosomes in the granulosa cells of atretic follicles of several species [116, 117]. Studies in the rat support the idea that activation of the AKT/MTOR signaling pathway suppresses autophagy as assessed by levels of LC3-II in granulosa cells [116].

The presence of lysosomes and autophagosomes in the corpus luteum was described more than 45 years ago [118–121]. Recent studies have documented the presence of autophagy-related proteins: Beclin 1 and LC3 in luteal tissue of rodents, cows, and humans [122–125]. However, in luteal cells, it remains unclear whether autophagy promotes cell survival versus cell death. In the rat, LC3-II-positive autophagosomes were identified during the late luteal phase and were correlated with luteal cell apoptosis [125, 126]. Furthermore, treatment of rat luteal cells with PGF2α under serum-free conditions increased autophagosomes, LC3-II protein, and luteal cell apoptosis, suggesting that autophagy may be involved in luteal cell death. Choi et al. [126] observed that although PGF2α increased both ERK1/2 and MTOR activity in rat luteal cells, autophagy could be prevented by inhibition of ERK1/2 signaling and appeared to be independent of phosphatidylinositol 3-kinase/AKT/MTOR activity. It will be important to understand the sequence of events in vivo and to determine whether the stimulatory effects of PGF2α on AMPK activation are linked in some way to autophagy in the corpus luteum.

Gawriluk et al. reported that *Becn1* deficiency in the mouse ovary resulted in a reduction of progesterone production and preterm labor [122]. To avoid the loss of germ cells associated the *Becn1* knockout animal, this group targeted *Becn1* deletion to the granulosa cells and as a result they were able to follow luteal function throughout pregnancy. Although ovulation, implantation, and progesterone levels during early pregnancy were not affected by *Becn1* ablation, they found that *Becn1* abrogation resulted in a reduction of circulating progesterone in mid- to late pregnancy. The reduction in progesterone resulted in early parturition, which was reversed by treatment with exogenous progesterone. Of relevance to luteal metabolism were the findings that the numbers of LDs were reduced and the mitochondria were smaller in the *Becn1*-deficient ovaries compared to controls. These changes were not accompanied by changes in the expression of genes important for the synthesis of progesterone. Exactly how the reduction in LDs and reduced autophagy contributed to reduced progesterone synthesis remains to be firmly established, but it could be a consequence of impaired lipid transport mechanisms and reduced expression of key receptors on the luteal cells [122]. Studies in other systems indicate that Becn1 expression and activity is controlled via transcriptional regulation, miR-30a, and by posttranslational modifications (reviewed in [127]). Recent studies in cardiac tissue showed that the transcription factor ATF3 binds to the ATF/cAMP response element of the Becn1 promoter and that ATF3 is capable of reducing autophagy via suppression of the Becn1-dependent autophagy pathway [128]. As PGF2α rapidly increases activation of mitogen-activated protein kinases (ERK1/2, p38, and JNK) and ATF3 expression in bovine and rat luteal cells in vivo and in vitro [104, 107], it is important to determine whether Becn1 expression or activity impacts autophagy during luteal regression.

It should also be appreciated that Becn1 directly interacts with B-cell lymphoma 2 (Bcl2) family proteins (Bcl2 and Bcl2/X_L) in a manner that negatively regulates autophagy. To complicate matters, a variety of ligands that regulate intracellular protein kinases, including Dapk, Rock1, Mst1, and Mapk8 (death-associated protein kinase 1, rho-associated coiled-coil containing protein kinase 1, macrophage

stimulating 1, mitogen-activating protein kinase 8, respectively), can positively or negatively regulate Becn1/Bcl2 effects on autophagy [127]. Beclin 1 can also secondarily affect apoptosis through regulation of anti-apoptotic and pro-apoptotic BH3 domain-containing proteins. In addition to the Bcl2 family, the VDAC (voltage-dependent anion channel) family is also involved in ovarian apoptosis and autophagy regulation [129]. Vdac2 directly interacts with Bcl2-antagonist/killer 1 (Bak1) to inhibit its oligomerization, thus suppressing cell apoptosis. Yuan et al. [129] recently reported that Vdac2 inhibits autophagy in the developing ovary by interacting with Becn1 and Bcl2L1 to stabilize the Becn1 and Bcl2L1 complex. Recent work by several groups have found a close relationship between autophagy and LDs [130–132]. In particular, LC3 [131], and ATG2 [133], ATG7 [130], and several VDAC [56, 57] proteins are often associated with LDs and appear to have important roles in LD formation and function, suggesting that events associated with autophagy may also impact the formation and function of ovarian LDs. Further work is needed to understand how LDs and autophagosome components influence both autophagy and apoptosis and thereby affect luteal function and lifespan.

4.7 Summary

Metabolic processes in the corpus luteum are tightly controlled by luteotropic and luteolytic factors. Signaling cascades involving LD homeostasis, PKA, AMPK, and autophagy are clearly important in the control of steroidogenesis. It remains to be determined how these cellular events are integrated into a physiologic context over the lifespan of the corpus luteum. Understanding the complex interplay of metabolic and hormonal clues underpinning steroidogenesis is essential to understanding and developing new therapies for infertility, particularly in the setting of increasing prevalence of obesity and metabolic diseases such as diabetes and polycystic ovary syndrome.

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Chapter 5
Steroid Hormone Receptors in the Corpus Luteum

Robert Rekawiecki, Magdalena K. Kowalik, and Jan Kotwica

Abstract The function of the corpus luteum (CL) is to produce progesterone (P4), which is the main regulator of estrous cycle duration and creates suitable conditions for embryo implantation and development. The CL also synthesizes moderate amounts of estradiol (E2). The action of these steroid hormones on target cells are evoked by specific nuclear receptors that belong to the family of receptor-dependent transcription factors. The physiological effect of P4 upon target cells is mediated through interaction of this hormone with nuclear progesterone receptor (PGR) isoforms A (PGRA) and B (PGRB) and that of E2 through the alpha (ERα) and beta (ERβ) receptors. Steroids may also affect cells through a nongenomic mechanism, which involves the membrane steroid-binding proteins such as the progesterone receptor membrane component (PGRMC) 1 and 2 and the membrane progestin receptors (mPR) alpha (mPRα), beta (mPRβ), and gamma (mPRγ), and the G protein-coupled estrogen receptor (GPR30). These proteins rapidly activate the appropriate intracellular signal transduction pathways, and subsequently they can initiate specific cell responses or modulate genomic cell responses. The diversity of nuclear and membrane steroid hormone receptors enhances their regulatory influence on the CL function.

Keywords Corpus luteum • Progesterone receptor • Estradiol receptor • Steroid receptor isoforms

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5.1 Introduction

The corpus luteum (CL) is a transient endocrine gland formed from the secretory cells of the ovarian follicle following ovulation. The main function of the CL is the production of progesterone (P4), which has a key role in many processes that regulate female fertility; however, the CL also synthesizes a moderate amount of estradiol (E2). The action of these steroid hormones is carried out by specific nuclear receptors that belong to the family of receptor-dependent transcription factors, which affect the regulation of specific target gene expression after their activation. It was also found that P4 and E2 affect cells by a nongenomic mechanism because the effect of hormone action occurs in a few minutes or even seconds after application and the effect is not inhibited by inhibitors of transcription and translation.

5.2 Structure of the Progesterone and Estradiol Receptors

The classical, well-studied mechanism by which steroids influence cells is via nuclear receptors. Progesterone works mainly by two distinct isoforms of the receptor: A (PGRA) and B (PGRB), which are encoded by the same gene but are transcribed under the influence of two different promoters. The bovine PGR gene consists of eight exons and is located on chromosome 15 [1]. The specific element that differentiates PGRB from PGRA is an additional section located at the N-terminal end of the protein. The length of this section ranges from 128 amino acids in chickens [2] to approximately 164 amino acids in humans [3]. The receptor protein is composed of a number of different regions, which are responsible for different functions of the receptor. Starting from the N-terminus part of the PGR, there are two domains: AF-1 and AF-3 (Fig. 5.1), which bind transcriptional factors that are responsible for the activation of the appropriate promoter and turn on transcription of the isoforms. The AF-1 domain is present in both isoforms of PGR, but AF-3 is only found in isoform B. The AF-1 domain is located upstream in the inhibitor domain (IF), which includes approximately 140 amino acids. The antagonist receptor is connected to this domain and thereby inhibits receptor activity. AF-3 contributes to PGRB transcriptional activity by suppression of the IF domain activity, which is contained within the sequence common to PGRA and PGRB [4]. The most conserved part of the receptor isoforms is the DNA-binding domain (DBD), adjacent to the AF-1 domain. It contains approximately 66 to 68 amino acids that form two zinc fingers; these are responsible for the interaction of a hormone–receptor complex with the appropriate regulatory sequences within the promoter of the target gene and therefore regulate transcription [4]. A ligand-binding domain (LBD) is located on the C-terminal domain of the DBD. An additional AF-2 domain is found in this part of the receptor, which is responsible for the activation of the receptor by connecting transcription factors. Moreover, the AF-2 domain binds an inactive receptor with heat shock proteins (HSPs), and it is also responsible for receptor dimerization [3]. PGRA and

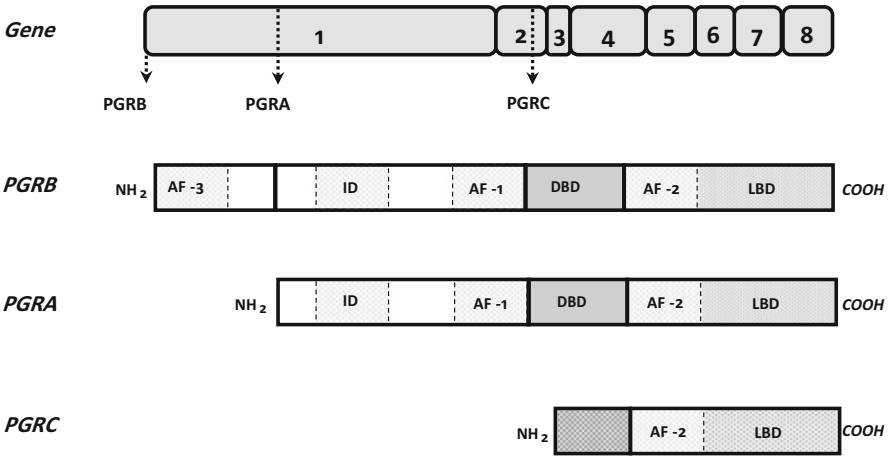

Fig. 5.1 Schematic representation of the human progesterone receptor gene and protein domains of progesterone receptor (PGR) B (PGRB), progesterone receptor A (PGRA), and progesterone receptor C (PGRC) isoforms. In humans, the progesterone receptor gene consists of eight exons. All receptor isoforms are transcribed from the same gene but are under the influence of different promoters. *DBD* DNA-binding domain, *LBD* ligand-binding domain, *AF1–AF3* activation domains, *ID* inhibitory domain

PGRB affect the target genes in a different manner. PGRB is a potent activator of progesterone-dependent genes in different cells. When both PGR isoforms are activated in the cell, PGRA acts as a potent inhibitor of PGRB and decreases the effect of P4 on target cells [5].

Moreover, in human breast cancer cell lines, but not in the CL, isoform C (PGRC) was found, which does not have one of the zinc fingers in the DBD domain [6] and therefore shows no transcriptional activity. The sequence of PGRC is limited to the full ligand-binding domain (LBD), and the sequence responsible for the dimerization and receptor localization is in the nucleus. PGRC exhibits the ability to bind P4 and its antagonists with the same affinity as PGRA and PGRB. The action of PGRC is not yet fully understood, but it has been claimed it can form heterodimers with the isoforms PGRA and PGRB, thus controlling their transcription properties [7].

In the CL, there are two types of estradiol receptors (ERs): alpha (ERα) and beta (ERβ) [8, 9], which are encoded by two separate genes (ESR1 and ESR2) [10]. Both receptors have a modular structure and contain all the domains typical for the construction of nuclear receptors.

5.3 Activation of the Steroid Receptor

Receptor activation involves the conversion of the biologically inactive form of the receptor to the active form that is capable of binding to genes and regulating their transcription. The inactive form of the receptor is associated with a complex of

chaperone proteins including HSP 90, HSP 70, p23, and immunophilins [11]. Formation of this intermediate complex requires energy released from ATP breakdown, which suggests involvement of phosphorylation processes. P4 binding initiates activation of the receptor, which entails a change in the conformation of the receptor and disconnection of the chaperone proteins, leading to unveiling of the DBD and nuclear translocation (Fig. 5.2). This process also requires energy from ATP breakdown [12]. Phosphorylation of the receptor causes a change in electric charge, which causes further changes that enable receptor dimerization. Both PGR

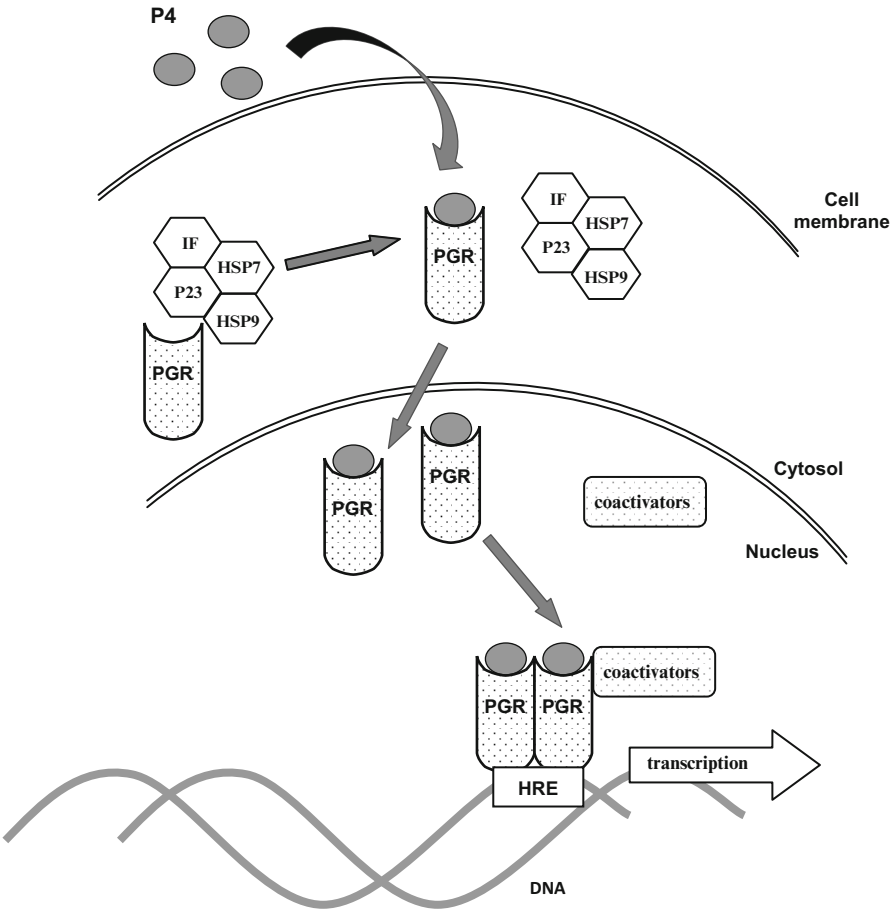

Fig. 5.2 Schematic illustration of PGR receptor action. The inactive form of the receptor is located in the cytoplasm and is associated with a complex of chaperone proteins. Progesterone penetrates the cell membrane and connects to the LBD of the receptor. Aggregation of the hormone causes disconnection of the associated chaperone protein complex and dislocation of the receptor to the nucleus where it undergoes dimerization. Receptor dimers connect to the hormone response element (HRE) located within the specific gene promoter. After activation of the receptor dimer by the receptor coactivators, the transcription process begins. *P4* progesterone, *IF* immunophilins, *HSP* heat shock protein

isoforms, PGRA and PGRB, can bind as a homodimer A:A, a homodimer B:B, and a heterodimer A:B. Dimerization consequently modulates the transcriptional activities of PGR and determines the diversity of physiological responses associated with P4 action [3]. After translocation to the nucleus, receptors bind (as a dimer) to a hormone response element (HRE), which is located in the promoter of a target gene. The next step is connection of coregulators to the receptor dimer, and then, the transcription process of the target gene is initiated or inhibited [13]. The activation of ERs is also followed by a classical pattern of nuclear receptor activation [10].

5.4 Regulation of Steroid Receptor Transcriptional Activity

Coregulators are a large group of transcription factors that regulate gene transcription activated by P4. They interact with the AF-2 domain of the receptor without binding to the DNA of the target gene sequence [14]. There are two groups of coregulators: coactivators that enhance the transcription of genes, and corepressors, which are proteins that inhibit the transcription of genes. The main PGR coactivators are representative of steroid receptor coactivators, which contain SRC-1, SRC-2, SRC-3, and the CREB-binding protein (also described as CREBBP or CBP), which includes CBP and p300 protein [15] and also the P300/CBP-associated factor (p/CAF) (also described as K(lysine) acetyltransferase 2B; KAT2B) [16]. Moreover, there are proteins that do not belong to any of these groups of coactivators, including L7/SPA, RIP140, TIF1, ARA70, HMG-1/2E6-AP, and RPF-1 [17]. The coactivators interact with PGR through the highly conserved motif known as the "NR box," which consists of three leucine amino acids and two unspecified amino acids (Leu-X-X-Leu-Leu motif) [18, 19]. Coactivators also have the activity of histone acetyltransferase (HAT), which transfers an acetyl group from acetyl CoA to lysine amino acids on histone proteins, leading to their acetylation and causing loosening of chromatin and, consequently, greater availability of transcription factors and polymerase to the appropriate gene sequence. This process is also referred to as transformation of heterochromatin to euchromatin [20]. Another group of coregulators are corepressors, which include two main proteins: nuclear receptor corepressor (N-CoR) and silencing mediator for retinoid and thyroid hormone receptor (SMRT) [19]. Corepressors also have a conservative sequence containing additional amino acids compared to the "NR box" forming the following sequence: Leu-Leu-X-X-X-Ile-X-X-X-Leu. This motif is defined as a CoRNR ('corner') box and is responsible for the interaction of the corepressor with the PGR receptor [21]. Corepressor proteins are connected with histone deacetylases (HDACs), which, in contrast to the HAT, remove an acetyl group from the lysine amino acid on a histone; this results in an increase in chromatin condensation and transcription of the target genes not being initialized [22]. We recently found that mRNA expression of the coactivator P300/CBP-associated factor (PCAF) and nuclear receptor corepressor (NCoR) are positively correlated with luteal level of P4 and negatively correlated with mRNA expression for both PGR isoforms in the CL during the estrous cycle in cows

(unpublished data). This finding indicated that coregulator involvement is an important step in the regulation of PGR isoform action in the CL. Transcriptional activity of ERs is also regulated by the same groups of coregulators [10, 23].

Other factors involved in the regulation of PGR activity are their antagonists. These compounds negatively regulate receptor interaction with HRE and weakly bind or prevent proper binding of agonists to the receptor, which impairs the activation of the receptor. One of the PGR antagonists is mifepristone (RU 486), which competes with greater affinity than P4 for the LBD [24]. The removal of 42 amino acids from the C-terminus of the receptor abolished P4 binding to the LBD but had no effect on RU 486 binding [25]. However, a single substitution of Gly-Cys amino acids at position 722 of the LBD inhibited binding of the antagonist to this domain and did not affect the binding of P4 to the LBD [26, 27]. The inhibition of PGR may occur in different ways. Antagonists modify the C-terminus segment of the receptor, which is followed by the blockade of coactivators binding to the AF-2 domain, leading to a lack of receptor activation [28]. Full activity of PGR requires interactions between the C- and N-terminus parts of the receptor. RU 486 causes conformational changes in PGR that inhibit this interaction, and as a result, none of the coactivators can be attached to the receptor [29]. Receptor antagonists may also act indirectly, by interaction of PGR with another transcriptional factor, as this happens when HRE of the receptor is partially overlapping with the transcription factor-binding site. For example, RU486 induced inhibition of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) activity associated with blocking of PGR receptor activity. Moreover, Rothchild [30] suggested that RU486 after binding to PGRA may act as an inhibitor of the receptor; however, after connection to the PGRB isoform, it may be a highly active agonist of the receptor, as was found earlier [31, 32]. A similar effect of PGR antagonists was observed in bovine endometrial cells. Both ZK299 and RU486 appeared to affect the mRNA and protein expression levels of the PGRA and PGRB isoforms. Thus, the final physiological effect evoked by an antagonist depends on the PGR isoform that is bound to it [33].

5.5 Progesterone and Estradiol Receptor Isoforms in the CL

The variable expression of isoforms PGRA and PGRB during the estrous cycle has been observed in the CL. The profile of their expression is similar; however, PGRB mRNA levels are minor than those of PGRA mRNA in human (100–1000 times) [34] and bovine CL (500–2000 times) [35]. The highest level of PGRA and PGRB mRNA in the human [36] and bovine [35] CL is at the beginning of the ovarian cycle, and thereafter it is gradually decreased (Fig. 5.3). Sakumoto et al. [37] suggested that, in the newly formed CL, high PGR mRNA and protein expression appeared to be related to the increase in the number of blood vessels that occur in the CL. Additionally, an increase in PGR mRNA expression may also be induced by the LH surge in granulosa cells of preovulatory follicles in cattle [38], and in this way PGR signaling pathways may help mediate the effects of the preovulatory LH surge

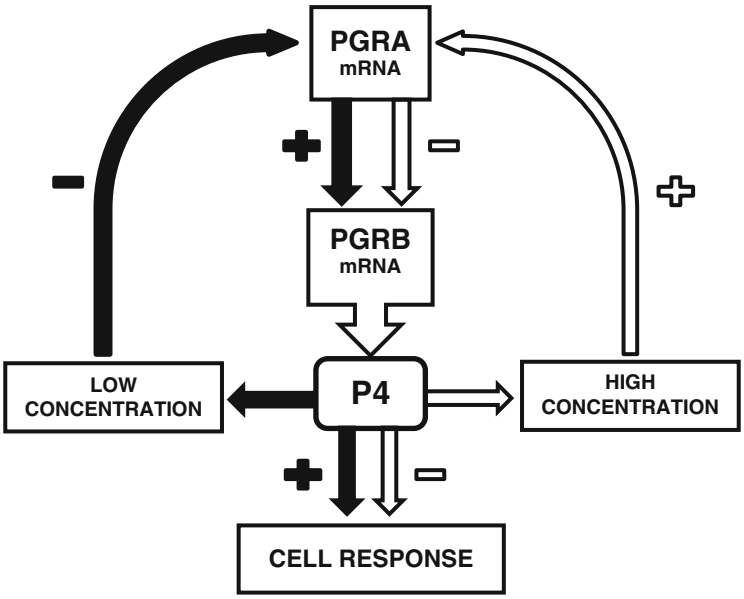

Fig. 5.3 Graphic demonstration of the influence of P4 on the mRNA level of PGRA and PGRB isoforms. A high concentration of P4 within luteal cells induces the expression of PGRA mRNA expression, which results in the repression of PGRB mRNA transcription and finally reduces the cell response. A low level of P4 may decrease the expression of PGRA mRNA followed by an increase in PGRB mRNA transcription. This will induce PGR action and increase the cell response to P4. *White arrows* impact of high P4 concentrations; *black arrows* impact of low P4 concentrations [36]

on follicle rupture in cattle. Moreover, the LH surge increases the oxytocin (OT) level and mRNA expression for the oxytocin receptor in the newly formed CL. Because OT is involved in the regulation of P4 production in the bovine luteal cells, it thus forms a positive feedback loop with P4 [38, 39].

Earlier studies by Misao et al. [36] on human CL suggested that a high concentration of P4 within luteal cells induces the expression of PGRA mRNA expression, which results in repression of the transcription of PGRB mRNA, and the effects of P4 in the luteal gland are suppressed [36]. On the other hand, a low level of P4 may decrease the expression of PGRA mRNA, which is followed by an increase in PGRB mRNA transcription: this will induce PGR action and increase the effects of P4 in target cells (Fig. 5.3). However our recent studies in cows revealed the expression of mRNA for PGRA and PGRB decreases from day 6 of the estrous cycle in the CL [35] (Fig. 5.4) along with an increasing concentration of P4 in the CL. So, it is possible there are essential differences between species in regulation of PGR isoform expression. Progesterone was also found to enhance its own effect by increasing the activity of 3-beta-hydroxysteroid dehydrogenase (3β-HSD) on days 6–10 of the estrous cycle in cows [40] and to stimulate gene expression of 3β-HSD, steroidogenic acute regulatory protein (StAR), and cytochrome P450scc [41] without affecting the level of

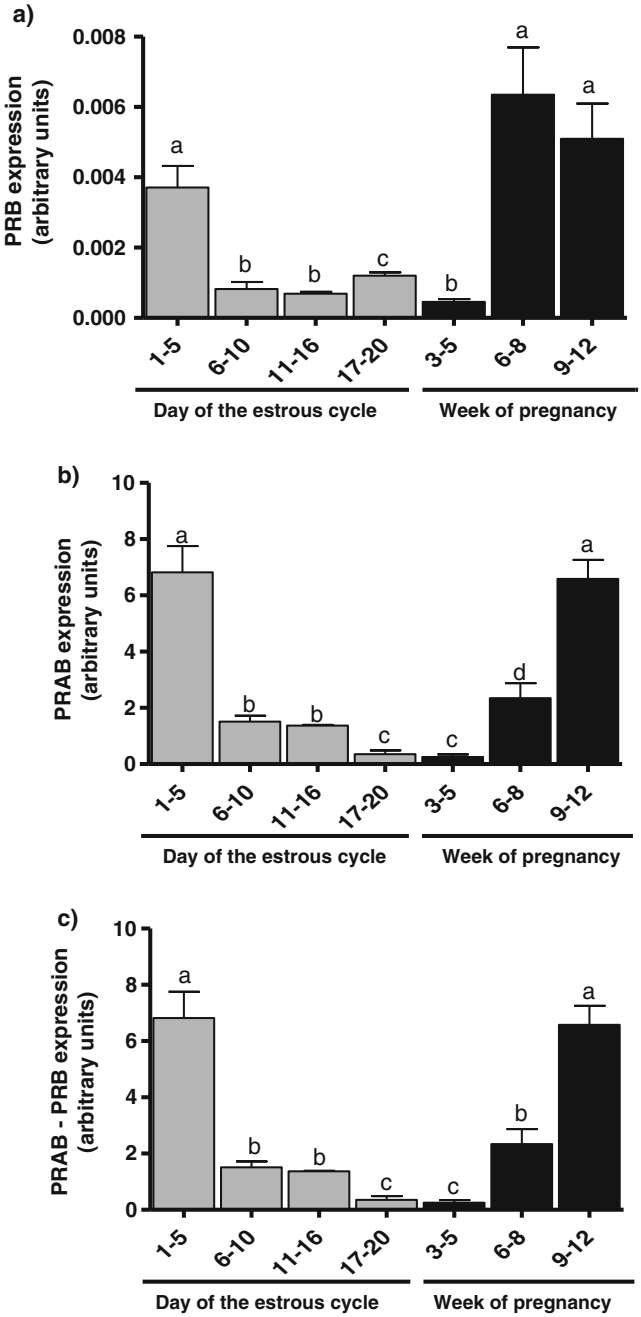

Fig. 5.4 Progesterone receptor isoform mRNA (mean ± SEM; $n = 4$ per stage) levels in bovine corpora lutea collected on days 1–5, 6–10, 11–16, and 17–20 of the estrous cycle and 3–5, 6–8, and 9–12 weeks of early pregnancy. The probe and primers for the PGRA isoform were designed against the sequence common to both isoforms; therefore, the mRNA expression determined was the total mRNA expression for both isoforms A and B (PGRAB). The mRNA level for PGRA was obtained after subtracting the mRNA level of PGRB from the mRNA level of PGRAB. (**a**) PGRB mRNA levels; (**b**) PGRAB progesterone receptor mRNA levels; (**c**) PGRA mRNA. Values with different superscripts are significantly different ($P < 0.05$). Reproduced with permission from [35]

mRNA for total PGR in the CL of the cycling cows [42, 43]. Furthermore, luteotrophic factors (e.g., LH, E2, and PGE2) affect the expression level of mRNA in contrast to the NO donor (NONate) and inhibitor of cytochrome P450scc (aminoglutethimide), and in this way, they modify the action of P4, as shown in the endometrial cells from cycling cows [44].

Toward the end of the estrous cycle, P4 production is markedly reduced, which entails a decrease in the mRNA and protein concentration of PGRA and PBRB to their lowest levels [35]: this is the effect of luteolysis initiation and intensification of luteal cells apoptosis, characterized by changes in the nucleus structure, chromatin condensation, and the DNA cutting by endonucleases [45]. Thus, it is possible that the decrease in the level of mRNA and protein expression of both PGR isoforms is a part of the luteolytic events.

During the initial period of pregnancy, the expression of mRNA and protein for both receptor isoforms is low, but it increases as pregnancy progresses [35] (Fig. 5.4). At that time, relatively intense secretion of P4 also occurs, which is followed by a decrease in the transcription of both PGR isoforms. At approximately 45 days of pregnancy, the placenta becomes an additional source of P4 [46]. Thus, the local countercurrent transfer of P4 from the uterus to the ovary [47] may increase the impact of P4 on the CL. As a result, the increased impact of P4 on luteal cells can lead to higher mRNA and protein expression levels for each isoform. Additionally, the ratio of PGRA to PGRB may indicate predominance of isoform B over isoform A at the beginning of pregnancy, which is a crucial period for embryo development. However, secretion of P4 from the placenta may account for the alteration of the PGRA:PGRB ratio and may lead to a modification of cell responsiveness to P4 [48].

A different response of P4 action during the estrous cycle in the CL of monkeys has been reported. Although the P4 profile is similar to that of humans [49], the level of PGRB protein expression in the luteal tissue predominates, and this persists for the duration of the estrous cycle. However, the protein expression of PGRA decreases from its highest level at the early phase of the estrous cycle to its lowest level at the end of the cycle [50]. These data indicated that PGRA is not the dominant isoform in all species and suggests different regulation of PGR isoform expression in luteal cells of different species.

The highest mRNA expression for ERα was detected in the bovine CL during the early luteal phase, followed by a significant decrease to the end of the estrous cycle. In contrast, ERβ mRNA expression is relatively high during the early stage, decreases during the mid-stage, and increases significantly again during the late luteal phase and after CL regression [8]. These data suggested that both isoforms of ERs are involved in CL formation, but that ERβ may also take part in luteolysis.

5.5.1 Nongenomic Effects of Steroid Action Hormones in Cells

Steroids can also affect cells by a nongenomic mechanism in which the effects of the hormone are observed after a very short time following its application (i.e., several seconds or minutes) and are not diminished by inhibitors of transcription and

translation [51–53]. These nongenomic actions of P4 and E2 have been demonstrated in several tissues from the female reproductive tract in different species [54–58], including that of cows [59–62]. The mechanism of this steroid action is not fully understood. It has been proposed that the cytoplasmic fractions of nuclear PGR, mainly isoform B, may participate in the nongenomic signaling pathway of P4 [63]. However, this rapid action of P4 has also been observed in cells lacking PGRs, and several studies have shown that it is initiated at the cell membrane [52, 56–58]. Hence, the following putative mechanisms have been suggested: (a) P4 modulates other membrane receptors or impairs the binding of these receptors with their ligands, as has been demonstrated for the OT receptor [51, 59]; (b) P4, as a lipophilic substance, may modify the fluidity of the cell membrane and thus alter the affinity of other membrane receptors for their ligands [64]; and (c) P4 could interact with specific proteins that function as a membrane progesterone receptor [52, 56, 57]. This group consists of membrane progesterone-binding proteins such as the progesterone receptor membrane component (PGRMC) 1 and 2 and the membrane progestin receptors (mPR) alpha (mPRα), beta (mPRβ), and gamma (mPRγ) (Fig. 5.5). Similarly, estradiol may also affect the cell in a nongenomic manner via ERα and ERβ and the G protein-coupled estrogen receptor (GPR30 or GPER1) localized in the cell membranes [65, 66].

The presence of membrane proteins that bind steroids ensures the generation of a more rapid cellular response compared with the genomic responses to the steroid. This mechanism allows the target cells to respond quickly to changes in the hormonal milieu and modulate the cell response elicited by the signals that activate the genomic mode of action. Therefore, it is possible that P4 and other steroids can activate the synthesis of new proteins within cells, and at the same time, they can initiate a series of changes at the level of the cell membrane. This effect of steroid hormones can essentially affect cell sensitivity to P4 and to other hormonal factors.

5.5.2 PGRMC1 and PGRMC2 Structure, Expression, and Function

PGRMC1 and PGRMC2 belong to a family of membrane-associated progesterone receptor (MAPR) proteins [53, 56, 57]. PGRMC1 protein was isolated for the first time from porcine vascular smooth muscle cells [54]. This protein is composed of 194 amino acids [54] with a molecular weight of approximately 25–28 kDa in different species [52, 57, 67]. It contains a short N-terminal extracellular domain, a single transmembrane domain, and a cytoplasmic domain with a sequence that binds cytochrome b5 and steroids, and it also contains three Src homology domains that are involved in ligand-dependent signal transduction [52, 57, 67]. PGRMC1 protein is localized mainly to the cell membrane [52, 68–70] but is also found in the endoplasmic reticulum and the Golgi apparatus of rodents and humans [52, 54]. The expression of PGRMC1 mRNA/protein was detected in human [71], mouse [72], rat [73], and cow [74, 75] granulosa and luteal cells.

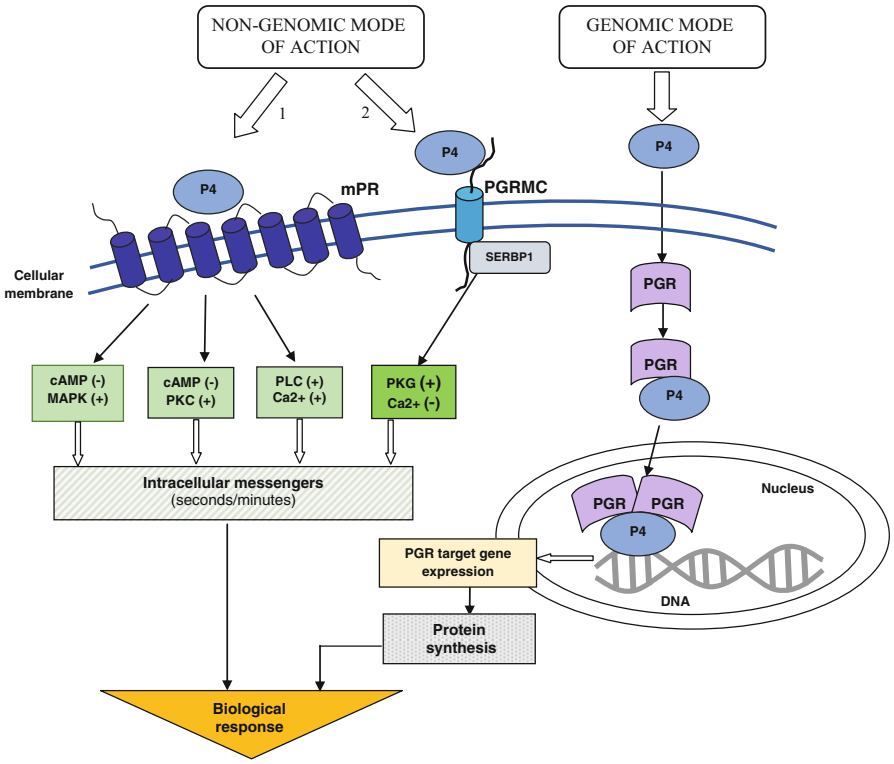

Fig. 5.5 Hypothetic model of progesterone (P4) action in the cell. In the genomic pathway, P4 binds to the nuclear progesterone receptor (PGR) and activates PGR gene expression, which stimulates or inhibits the cellular synthesis of proteins. This pathway requires a long time from hormonal activation to induction of a biological effect. In the nongenomic pathway (*1*), P4 can bind to the membrane progestin receptor (mPR) and activate the mitogen-activated protein (MAP) kinase pathway by decreasing cyclic AMP (cAMP). Moreover, mPR can stimulate protein kinase C (PKC) and phospholipase C (PLC), leading to an increase in the mobilization of Ca^{2+} in the cell. (*2*) P4 can also activate the progesterone receptor membrane component (PGRMC), which may form a membrane complex with the serpine mRNA-binding protein 1 (SERBP1) and activate protein kinase G (PKG) to decrease the levels of Ca^{2+} in the cell. Stimulation of second messengers allows for the target cells to quickly respond to the changes in the hormonal milieu but can also modulate the genomic pathway, leading to the synthesis of new proteins

It has been found that PGRMC1 is involved in the regulation of cholesterol metabolism, [76], steroidogenesis [77], myometrium contractility [78], oocyte maturation [79], and survival of normal and cancerous ovarian cells in vitro [80, 81]. It was assumed that PGRMC1 can associate with another polypeptide, serpin mRNA-binding protein 1 (SERBP1), and these proteins form a membrane receptor complex that binds P4 [53, 80, 81]. This membrane complex may mediate the anti-apoptotic effect of P4 in ovarian cells via activation of protein kinase G and reduction of the calcium levels [53, 80, 82].

There are fewer data available on PGRMC2. This protein has high homology with PGRMC1, but the sequences of these two proteins differ in the N-terminal and transmembrane domains, suggesting that these two receptors can potentially interact with different proteins [83]. The expression of PGRMC2 mRNA or protein was detected in the endometrium of mice [70] and monkeys [84] and in the endometrium, myometrium [85, 86], oviduct [87], and CL [75] of cattle. Moreover, this protein has been proposed to be involved in oviduct function [87] and preterm labor [88].

5.5.3 Membrane Progestin Receptor (mPR) Structure, Expression, and Function

The nongenomic effects of P4 on target cells may also be mediated by its binding to membrane receptors (i.e., mPRs), belonging to the progestin and adipoQ receptor family (PAQR) family of proteins [58, 89]. These receptors were initially isolated from the spotted sea trout ovary [89] and were subsequently identified in other species, including the female reproductive tract tissues in humans [90, 91], pigs [92], mice [93], sheep [94, 95], rats [73], and cows (M.K. Kowalik, unpublished data). Three isoforms of the receptor encoded by different genes, mPRα, mPRβ, and mPRγ, also called PAQR7, PAQR8, and PAQR5, respectively, have been detected in humans and other vertebrates [57, 58]. The mPRa receptor is a protein composed of 352 amino acids with a mass of approximately 40 kDa in humans [89]. This protein and other mPR isoforms contain an extracellular N-terminal domain, seven transmembrane domains, and a cytoplasmic domain [57, 89], and are localized mainly in the cell membrane and endoplasmic reticulum [89, 90].

It was also found that the mPR isoforms were involved in the maturation [92] and transport of oocytes [93], as well as preparation of the uterus for implantation [92, 94, 95], pregnancy [90], and labor [91].

5.5.4 The Hypothetical Role of Membrane Progesterone Receptors in the CL

A number of studies have demonstrated nongenomic effects of P4 in the CL in different species [52, 71, 73, 82] including cows [59, 75, 96]. Data obtained from cattle confirmed the existence of transcription-independent effects of P4 on the luteal cells because the administration of actinomycin D, which is an inhibitor of transcription, did not change the effect evoked by P4 on PGE2 secretion from bovine luteal cells [59, 97]. Moreover, P4 decreased the mobilization of intracellular calcium within seconds in PGE2-stimulated luteal cells in vitro [96, 97]. These findings suggested that the effect of P4 on the secretion or production of PGE2 in luteal cells takes place through nongenomic action, probably by membrane P4 receptors, because the mRNA and protein expression of PGRMC1, PGRMC2, mPRα, mPRβ, and mPRγ were all found in the CL [74, 75, 98] (Fig. 5.6; Kowalik, unpublished data). Moreover, the

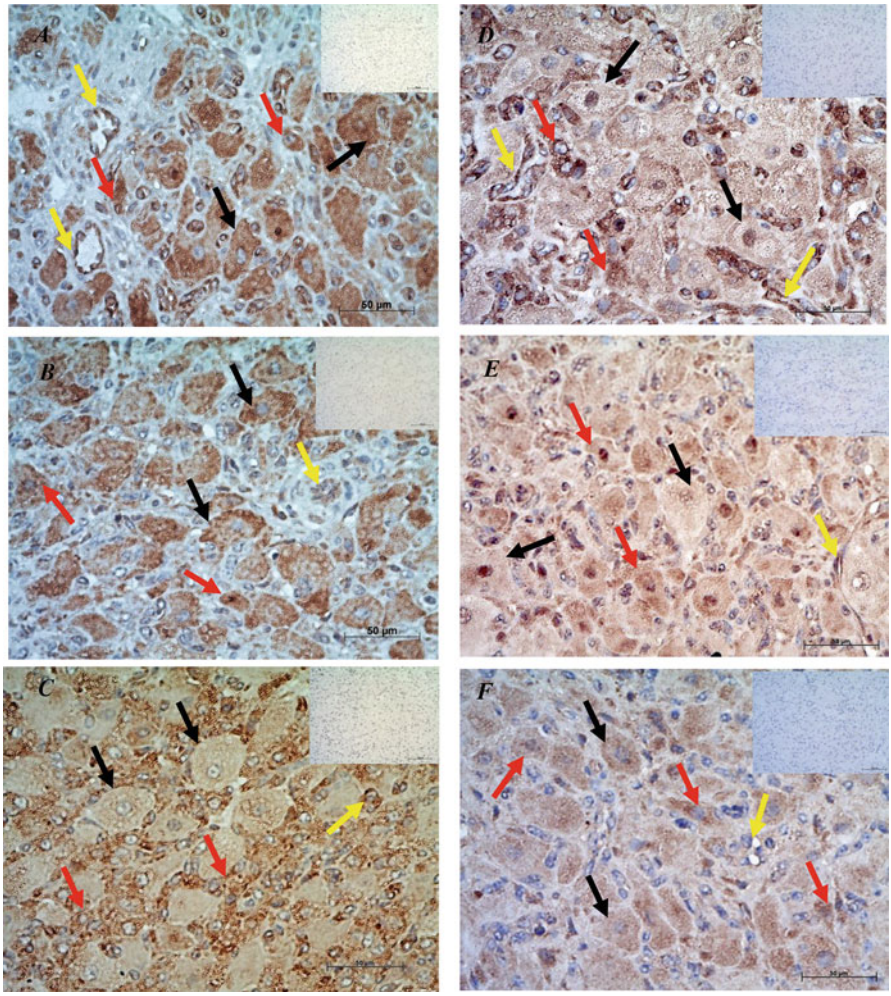

Fig. 5.6 Cellular localization of PGRMC1 (**a**), PGRMC2 (**b**), SERBP1 (**c**), mPRα (**d**), mPRβ (**e**), and mPRγ (**f**) in the bovine CL on days 11–16 of the estrous cycle. Control immunohistochemistry was performed without primary antibodies (*inserts*). *Black arrows* large luteal cells; *red arrows* small luteal cells; *yellow arrows* endothelial cells of blood vessels. *Bars* 50 µm

expression of mRNA or protein for these membrane receptors in the bovine CL changed during the estrous cycle and first trimester of pregnancy, and PGRMC1 and PGRMC2 expression was positively correlated with P4 concentrations in luteal tissue [74, 75, 98]. These findings suggested that membrane progesterone receptors participate in signaling in the bovine CL during the estrous cycle and pregnancy. However, how P4 influences these processes via membrane P4 receptors or which P4 receptors are involved remains unclear. The highest expression of PGRMC1 and PGRMC2 mRNA is observed on days 6–16 and days 11–16, respectively, during the estrous cycle and the first trimester of the pregnancy in cows [75]. Similarly, expression of

mPRα and mPRβ mRNA is high during the second half of the estrous cycle, but the mPRγ mRNA level is the highest on days 17–20 of the estrous cycle (Kowalik, unpublished data). Moreover, PGRMC1 and PGRMC2 protein expression occurs mainly in large luteal cells (Fig. 5.6), which are the major source of P4 in the bovine CL, but also in small luteal cells, which produce P4 in response to LH stimulation [45]. However, mPR proteins are mainly present in small luteal cells but also in large luteal cells (Fig. 5.6) (Kowalik, unpublished data). Therefore, it is possible that the membrane P4 receptor genes and their protein products present in middle and late CL stages may participate in luteal steroidogenesis and, in this way, protect the CL before premature luteolysis. This suggestion is further supported by data on the participation of PGRMC1 in steroidogenesis in adrenocortical cells [99] and in rat granulosa cells [52, 80]. Moreover, it was found that PGRMC1 can bind to cytochrome P450 [77, 100] and to form complexes with SCAP (SREBP cleavage activation protein) and Insig1 (insulin-induced gene) proteins [76, 101], which are involved in cholesterol biosynthesis. These results support the notion that the expression and function of PGRMC1 and PGRMC2 may be associated with the synthesis of P4 in the CL.

Localization of membrane P4 receptors in the endothelium of blood vessels in the bovine CL (Fig. 5.6) and uterus [75, 85] indicated that these receptors may contribute to the nongenomic effects of P4 in blood vessels in the female reproductive tract and, in this way, participate in the processes promoting the development and maintenance of pregnancy, such as cell differentiation, regulation of cell apoptosis, steroidogenesis, and contractility of the uterus [70, 102] and uterine blood flow. Expression of membrane P4 receptors in the endothelial cells of blood vessels suggested that they can participate in the fast, nongenomic effects of P4 on blood flow and make it an important regulator of reproductive system function.

The presence of P4 membrane receptors, except those of the nuclear receptor isoforms, illustrates the different ways hormones influence cellular processes. Selective blockade of these receptors by means of specific blockers or silencing genes using siRNA on one hand or receptors stimulation with agonists on the other hand may demonstrate the physiological importance of these receptors. This method of selective stimulation or inhibition of P4 receptors can also be a convenient tool to modify intracellular processes and subsequently to obtain a diverse cell response.

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Chapter 6
Immune Cells and Their Effects on the Bovine Corpus Luteum

Koumei Shirasuna and Akio Miyamoto

Abstract In the past two decades, accumulating evidence has indicated that various types of immune cells (T cells, macrophages, neutrophils, eosinophils, and dendritic cells) exist within the CL and regulate luteal function. These immune cells accumulate during luteal development and support angiogenesis and progesterone production. PGF2α stimulates the production of inflammatory cytokines and chemokines in the mature CL; these factors recruit immune cells into the CL to enhance luteolytic cascades through inflammatory responses. When pregnancy is established, the embryo secretes interferon-tau (IFNT) as a pregnancy recognition signal; this indirectly maintains the CL by inhibiting luteolysis. In addition to its uterine function, IFNT regulates immune cell function and is associated with the transformation of the cyclic CL into the pregnancy CL. This review describes the current state of research on the effect of immune cells on the bovine CL, which is essential for a better understanding of reproductive physiology.

Keywords Corpus luteum development • Luteolysis • Immune cells

6.1 Introduction

Lobel and Levy [1] first described the presence of white blood cells in the bovine corpus luteum (CL). Later on, experimentally induced lymphopenia caused luteal dysfunction in cattle [2]. During the past two decades, accumulating evidence indicated that various types of immune cells such as T lymphocytes, macrophages, neutrophils, eosinophils, and dendritic cells exist within the CL, and plays key roles to regulate luteal function throughout the lifespan of the CL.

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6.2 Immune Cells in the CL: Where Do They Come From?

The immune system is known to contribute to the regulation of ovarian function including ovulation, the development of the CL and luteolysis [3]. Leukocytes present within the ovary are potential regulators of ovarian function by local secretion of modulating cytokines.

T lymphocytes that mature in the thymus are central immune cells to regulate cell-mediated immunity. Within the bovine CL, several types of T lymphocytes include T-helper cells (Th cells, $CD4^+$ T cells), cytotoxic T cells ($CD8^+$ T cells), regulatory T cells (suppressor T cells, $Foxp3^+$ T cells), and $\gamma\delta^+$ T cells [4, 5]. All T cells originate from hematopoietic stem cells in the bone marrow. Hematopoietic progenitor cells populate the thymus; these expand by cell division to generate immature thymocytes. Mature thymocytes are released from the thymus to peripheral tissues. In mice, intact thymic function has been found to be necessary in the maintenance of normal ovarian function; congenitally athymic or postnatally thymectomized animals are infertile and exhibit abnormal ovarian function [6].

Cells of the innate immune system, such as macrophages, neutrophils, eosinophils, and dendritic cells, originate from stem cells in the bone marrow. These cells are recruited into the ovary, where they are critical in ovulation and luteal function. Kizuka et al. [7] used a mouse bone marrow transplantation model to demonstrate that transplanted green fluorescent protein $(GFP)^+$ bone marrow cells were recruited into the developing CL; these GFP^+ cells were F4/80 (marker of macrophages)$^+$, indicating that macrophages are recruited to the ovary from the bone marrow during CL formation. The spleen is another immune cell reservoir for the ovary. Swirski et al. [8] demonstrated that the spleen is a site for the storage and rapid deployment of monocytes and identified splenic monocytes as a resource that the body exploits to regulate inflammation. Importantly, Oakley et al. [9] showed that a strong inverse relationship exists between the quantity of leukocytes in the ovary and that in the spleen. They also showed a significant reduction in the leukocyte infiltration of ovaries of splenectomized rats, indicating that the spleen may serve as an immediate reservoir of leukocytes for the ovary [9]. Furthermore, the number of leukocytes as well as progesterone concentration significantly decreased in splenectomized pseudo-pregnant rabbits [10].

6.3 Corpus Luteum Development and Maintenance

6.3.1 Role of T Lymphocytes Within the Functional CL

The phenotypes of T lymphocytes present in the bovine CL and the number of $CD4^+$ and $CD8^+$ T cells does not vary from the early to late luteal phase [11]. Poole and Pate [12] observed the proportion of T lymphocytes within the bovine CL to be that 25 % of the T lymphocytes were T-helper cells ($CD4^+$), 45 % were cytotoxic T cells ($CD8^+$), and 30 % were $\gamma\delta^+$ T cells. It is well understood that peripheral blood mononuclear cells (PBMC), including T lymphocytes and monocytes, clearly have a function for steroidogenic cells. Indeed, Hashi et al. [13] reported that PBMC

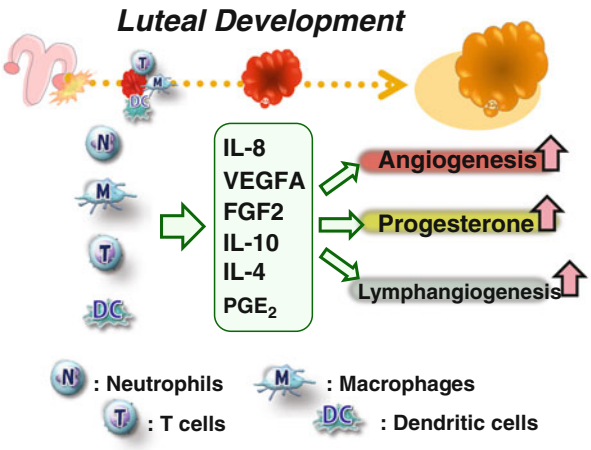

Fig. 6.1 Proposed model of inflammation-like luteal development. The mechanism of luteal development is considered as an inflammation-like response as many types of immune cells such as neutrophils, macrophage, T cells, and dendritic cells are recruited within the CL. They participate in luteal development via increases in angiogenesis, progesterone production, and lymphangiogenesis

from women could stimulate progesterone production from luteal cells in vitro and that Th-2 cytokines such as IL-10 and IL-4 are candidates for these positive effects of PBMC. Walusimbi and Pate [5] indicated that luteal cells from the mid-luteal phase predominantly induced proliferation of $\gamma\delta^{+}WC1^{-}$ T cells together with increased IL-10 expression, but not IFNG expression, on its cell surface. Therefore, it is suggested that a functional CL has an immunosuppressive role to suppress proliferation of resident T lymphocytes by the production of immunosuppressive cytokine IL-10 (Fig. 6.1) [14]. High concentrations of progesterone from a functional CL may be one of the candidates as a communication tool between luteal cells and T lymphocytes. Indeed, progesterone induces IL-10 synthesis and other Th2-associated cytokines in murine $\gamma\delta^{+}$ T cells via progesterone-induced blocking factor [15]. Interestingly, luteal cells regulate proliferation of T lymphocytes devoid of the nuclear progesterone receptor [16, 17]. Alternatively, bovine T lymphocytes express membrane progesterone receptor; therefore, progesterone can induce specific and rapid functional effects on T lymphocytes [18].

6.3.2 Role of Monocytes and Macrophages Within the Functional CL

Macrophages exhibit a high level of phenotypic plasticity and participate in diverse physiological processes, including the innate immune system, host defense together with lymphocytes against external pathogens, removal of apoptotic cells, and

angiogenesis. Monocytes originate from bone marrow progenitor cells and travel via the circulation to specific tissues where they differentiate to macrophages depending on the tissue microenvironment.

The number of macrophages increases in the early developing CL in cows [11] and humans [19, 20], as well as in the newly luteinized CL in pigs [21]. Turner et al. [22] reported the importance of macrophages in maintaining vascular integrity in the CL. Progressive macrophage elimination was associated with ovarian hemorrhage, which affected luteal tissue as a result of significant endothelial cell depletion and increased erythrocytes [22]. Additionally, Care et al. [23] convincingly demonstrated that macrophage depletion after conception caused embryo implantation arrest associated with decreased plasma progesterone because of disruption of the luteal microvascular network. In fact, peripheral blood macrophages co-cultured with granulosa cells exerted a luteotropic effect [24–26]. In humans, co-culture of monocytes and granulosa-lutein cells increased interleukin-8 (IL-8) release [27]. Additionally, monocytes have interleukin-8 (IL-8) receptors including CXCR1 and CXCR2, and a granulosa-lutein cell-conditioned medium stimulated monocyte migration via IL-8 [28]. Thus, an initial interaction between granulosa-lutein cells and monocytes may contribute to increased chemokine release and leukocyte recruitment to the forming CL [27]. Therefore, macrophages play a central role in maintaining the integrity of the ovarian vasculature (Fig. 6.1).

6.3.3 Role of Polymorphonuclear Cells Within the Functional CL

Polymorphonuclear leukocytes (PMNs) such as neutrophils and eosinophils are detected in the CL during the estrous cycle in certain species [29–31]. Neutrophils are important in the primary, unspecific stages of acute inflammatory reaction. In humans, neutrophils account for 60 % of circulating leukocytes, and 90 % of these PMNs are neutrophils.

A considerable number of neutrophils and a high concentration of IL-8 (a neutrophil-specific chemoattractant) are present in the bovine CL during the early luteal phase [31]. Similarly, neutrophils have been detected in large numbers in the human CL [32], and found at relatively high density in the rat CL in the early phases of pregnancy [33]. The formation of the early CL induced PMN migration in vitro using IL-8 and the supernatant of activated PMNs, and IL-8 stimulated the formation of capillary-like structures of CL-derived endothelial cells [31]. Importantly, IL-8 effectively stimulates progesterone production in bovine luteinizing granulosa cells [34] as much as vascular epithelial growth factor (VEGF)A and fibroblast growth factor (FGF)2 in vitro. These findings indicate that IL-8 and neutrophils may function to stimulate the developing CL (Fig. 6.1). However, Talbott et al. [35] reported that in co-culture of neutrophils with luteal cells, neither IL-8 nor activated neutrophils altered luteal cell progesterone synthesis, suggesting the multiple functions of neutrophils depending on its condition or activation.

Neutrophils are thought to be important in physiological and pathophysiological angiogenesis [36]. PMNs and IL-8 could induce angiogenesis in vivo [37, 38] and in vitro [39, 40], indicating that PMNs and IL-8 may function not only in the induction of tissue inflammation and wound healing, but also in the regulation of angiogenesis in the developing CL. In mice, neutrophils expressing VEGFA were detected in the microvessels of the endometrium [41]. Moreover, the proliferation of endothelial cells was significantly reduced in neutrophil-depleted mice compared with control mice [42]. Interestingly, similar to IL-8, FGF2 and VEGFA (10 and 100 ng/ml) stimulated PMN migration in vitro (Shirasuna et al., unpublished observations), and FGF2 and VEGFA are also expressed at high levels within the developing CL. Ancelin et al. [43] demonstrated that VEGFA was chemotactic for neutrophils in humans and that neutralization with anti-VEGFA antibody blocked this effect. Indeed, FGF2 also enhanced recruitment of neutrophils in rats [44]. Therefore, it is speculated that IL-8, VEGFA, and FGF2 act synergistically as stimulators of PMN recruitment in the early CL in cows.

6.3.4 Role of Dendritic Cells Within the Functional CL

Dendritic cells (DCs) are specialized antigen-presenting cells that prime T cells and are thus essential to both innate and adaptive immunity. Increasing evidence suggests that DCs are involved in both the classical induction of immunity against infectious agents and immune tolerance to innocuous antigens. Indeed, a transient ablation of DCs on embryonic day 4.5 leads to complete embryo resorption, which suggests that DCs are pivotal in fetal immune tolerance [45]. Although the presence of DCs within the bovine CL has not been confirmed, Spanel-Borowski [46] suggests that cytokeratin-positive cells isolated from the bovine CL are promising candidates to mature into DCs. Recently, Cohen-Fredarow et al. [47] reported that, in mice, DCs are present in the ovary and accumulate in the newly formed CL. In addition, the conditional depletion of $CD11c^{+}$ DCs blocks hCG-induced ovulation, interferes with the development of lymphatic vessels, and significantly inhibits progesterone secretion. Hence, they conclude that in the early luteal phase, the DCs localized in the newly formed CL facilitate progesterone production as well as lymphangiogenesis (Fig. 6.1).

6.3.5 Role of Other Blood Cells Within the Functional CL

Platelets are blood cells that have a pivotal role in coagulant systems. They have been reported to produce chemoattractants that are capable of inducing endothelial cell migration and have a role in wound healing and tissue remodeling. Furukawa et al. [48] clearly reported that platelets localize within the human CL in the course of neovascularization. Furthermore, platelets are involved in the development of the CL to upregulate progesterone production [48].

Eosinophils are known to be involved in the immune response against parasitic infection, asthma, and allergic conditions. Eosinophils infiltrate into the CL shortly after ovulation in cows [49], sheep [50], and humans [30], and they appear to be recruited into the developing CL by the expression of P-selectin on endothelial cells [30]. Human granulosa cells express RANTES (eosinophil-attracting chemokines) at the very early stage of development, indicative of their importance in the angiogenic and steroidogenic regulation of CL growth [30]. In cattle, a decrease in the quantity of eosinophils induced by the administration of dexamethasone results in lower progesterone concentrations [51]. However, in pigs, the quantity of eosinophils decreases shortly after ovulation [21].

6.4 Corpus Luteum Regression

6.4.1 Role of T Cells and Macrophages During Luteolysis

Uterine-derived or exogenous prostaglandin F2α (PGF2α) initiates luteolysis and rapidly reduces progesterone secretion by the CL [52]. During luteolysis, leukocytes, particularly macrophages and T lymphocytes, increase significantly in number in cows [53], humans [19], pigs [21], mares [54], and mice [55, 56], suggesting an active role of both cell types in luteolysis. Moreover, inflammatory cytokines such as tumor necrosis factor (TNF)α, IL1β, and IFNG and chemokines such as monocyte chemoattractant protein 1 (CCL2; recruitment of macrophages) are involved in luteal regression [6, 57–61]. These cytokines and chemokines may recruit macrophages and T lymphocytes within the CL to enhance luteolytic cascades through inflammatory and immune responses.

In cows and mares, large numbers of $CD4^+$ and $CD8^+$ T cells were found in the regressing CL [53, 54], suggesting that both cell types play an active role in luteolysis. In vitro, activated PBMC (mainly T cells) clearly inhibit LH-stimulated progesterone secretion from luteal cells [35]. Cannon et al. [17] reported that the stimulation of T cell proliferation was greater in luteal cells isolated after PGF2α administration than in luteal cells isolated before PGF2α administration. Furthermore, exogenous progesterone inhibits T cell proliferation [17]. $\gamma\delta^+$ T cells represent a major proportion of circulating T cells in ruminants; $\gamma\delta^+$ T cells account for 35 % of the T cells in the bovine CL [12]. Recently, Walusimbi and Pate [14] observed that luteal cells from the regressing CL predominantly induced proliferation of $\gamma\delta^+$ $WC1^+$ T cells, whereas luteal cells from the functional CL preferentially induced proliferation of $\gamma\delta^+$ $WC1^-$ T cells. Interestingly, luteal cells from the functional CL increased the proportion of $\gamma\delta^+$ T cells expressing IL-10, an antiinflammatory cytokine, and decreased the proportion of $\gamma\delta^+$ T cells expressing IFNG, a major luteolytic factor. Therefore, the environment in a functional CL suppresses the activity of resident T cells, whereas the environment in a regressing CL restores T-cell activity to promote the structural demise of luteal tissue [14]. Regulatory T cells ($CD4^+$ $CD25^+$ $Foxp3^+$ T cells) are known to be essential in the regulation of immune tolerance during implantation [62]. Although $CD4^+$

Foxp3$^+$ T cells are more prevalent in the bovine CL than in peripheral blood, PGF2α administration drastically decreases the quantity of Foxp3$^+$ T cells within the regressing CL [12]. These findings suggest that Foxp3$^+$ T cells regulate the function of resident T cells to prevent the initiation of luteolysis; therefore, a decline in the quantity of Foxp3$^+$ T cells is directly associated with luteal regression.

In the regressing CL in the cow, 70 % of proliferating cells are CD14$^+$ macrophages [53]. Macrophages are essential in functionally healthy CL, whereas abundant macrophages exist in the CL during functional regression (early stage of regression) [56]. In rabbits, functional and structural luteolysis correlated with an increased number of X4$^+$ and CD68$^+$ macrophages [63]. Moreover, inflammatory cytokines, such as TNFα, IL1β, and IFNG, and chemokines, such as CCL2 are involved in luteal regression [6, 57–61, 64–67]. These cytokines and chemokines may induce the accumulation of macrophages and T lymphocytes within the CL to support luteolytic cascades such as inflammatory and immune response (Fig. 6.2). Thereafter, the CL regresses primarily through the loss of cells by apoptosis [68, 69], and apoptotic luteal cells are phagocytosed by macrophages in rats [70].

In general, macrophages and T lymphocytes have the potential to produce multiple cytokines such as IL-1s, IL-2, IL-4, IL-6, TNFα, IFNA, IFNG, and prostaglandins to increase the immune inflammatory response and to communicate with peripheral resident cells depending on the stimulation conditions. There have been numerous studies on the relationships between luteal regression, leukocytes, particularly macrophages and T lymphocytes, and these cytokines. For example, TNFα protein exists in large and small luteal cells, and endothelial cells, as well as immune cells in the bovine CL [71]. TNFα inhibits progesterone secretion and induces IFNG and Fas-mediated apoptotic cell death in bovine luteal and endothelial cells by increasing caspase-3 activity [72, 73]. IFNG inhibits LH-stimulated progesterone production, increases prostaglandin synthesis, and induces cell death [74, 75].

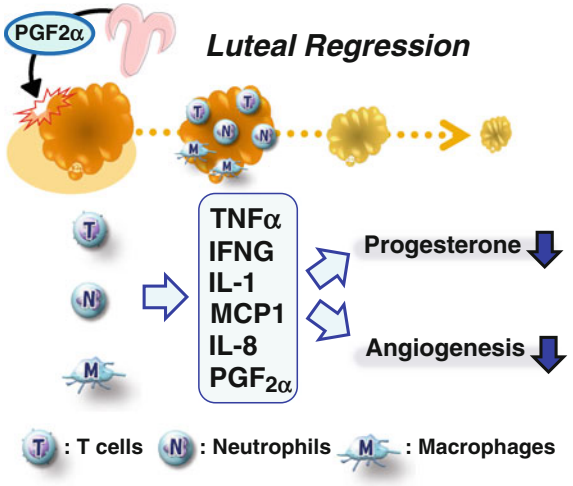

Fig. 6.2 Proposed model of the inflammation-like luteolytic response. The luteolytic cascade is similar to general acute inflammation with respect to the infiltration of immune cells (neutrophils, macrophages, and T cells). The luteolytic cascade is signaled by PGF2α secreted by the uterus and is a type of acute inflammatory immune response that promotes CL regression

6.4.2 *Role of Neutrophils During Luteolysis*

Neutrophils are the first cells recruited to inflammatory sites, providing cytokines and proteolytic enzymes [76]. During luteolysis, PGF2α rapidly induces the accumulation of neutrophils within the bovine CL at 5 min after administration [77]. Also, neutrophils accumulate in the equine CL after PGF2α administration [54]. To occur this rapid response, PGF2α directly stimulates P-selectin expression and enhanced neutrophil adhesion in luteal endothelial cells via P-selectin [77]. Generally, an acute inflammation is characterized by the infiltration of neutrophils within a few minutes and continuous occurrence of T lymphocyte and macrophage migration. Neutrophils can produce various types of inflammatory cytokines recruiting T lymphocytes and macrophages such as IL-8, TNFα, and IFNG [40, 76, 78, 79]. Also, a large number of T lymphocytes and macrophages were observed within the bovine CL at 6–24 h after PGF2α administration, and these immune cells are considered to be essential for a rapid demise of the CL tissue [11, 61, 65, 80]. Therefore, it is suggested that luteolytic cascade by PGF2α involves an acute inflammatory-like response in response to acute migrated neutrophils in cows, and these neutrophils may have a potential to recruit other immune cells in the regressing CL (Fig. 6.2). Indeed, a pretreatment with antibody against CD18 (leukocyte integrin) significantly inhibited not only PGF2α-induced neutrophil accumulation but also the decrease in serum progesterone concentrations [81]. In rats, co-incubation of luteal cells with activated neutrophils by *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) reduced LH-stimulated cAMP accumulation and progesterone secretion, which was dependent upon the number of neutrophils [82]. However, Talbott et al. [35] reported that treatment of neutrophils with IL-8 and PMA to activate neutrophils did not reduce progesterone secretion under co-culture condition with the bovine luteal cells. Therefore, neutrophils infiltrated within the bovine CL may also play a role as an initiating factor, not directly inducing factor of functional luteolysis.

6.5 Neutrophil and Macrophage Polarization: A New Concept for Luteal Function

6.5.1 *Polarization of Neutrophils: "N1" Versus "N2" Neutrophils*

As described previously, the CL closely resembles "transitory tumors" because development of the CL is associated with angiogenesis and infiltration of leukocytes. Interestingly, Fridlender et al. [83] demonstrated N1 (antitumoral) and N2 (protumoral) tumor-associated neutrophils. The antitumor activities of N1 neutrophils include increased expression of immune-activating cytokines and

chemokines and enhanced killing of tumor. Blockade of TGFβ signaling favors the accumulation of N1 neutrophils, suggesting that TGFβ is a major proximal cytokine within tumors that defines the neutrophil phenotype and inclines differentiation toward the N2 protumorigenic neutrophil phenotype [83]. Indeed, TGFβ can inhibit neutrophil activity and cytotoxicity [84]. N2 neutrophils do not produce high levels of pro-inflammatory cytokines (TNFα, IL-12, and GM-CSF), whereas tumor-associated N2 neutrophils express higher levels of MMP-9, VEGFA, and CCL2 to stimulate tumor angiogenesis [85]. On the basis of this novel concept of neutrophil polarization, the developing CL may differentiate infiltrated neutrophils to "N2 neutrophil phenotype" through the action of TGFβ, VEGFA, and MMP-9 (Fig. 6.3). On the other hand, N2 neutrophil depletion increased the activation status of $CD8^+$ T cells, whereas N1 neutrophil depletion decreased the activation status of intratumoral $CD8^+$ T cells, suggesting that N2 neutrophils act in an immunosuppressive fashion and N1 neutrophils are an immunostimulatory type [83]. This new concept for neutrophils raises a number of important intellectual prospects when considering luteal development and regression. Therefore, the existence and properties of "N1" versus "N2" neutrophils in the bovine CL should be carefully investigated.

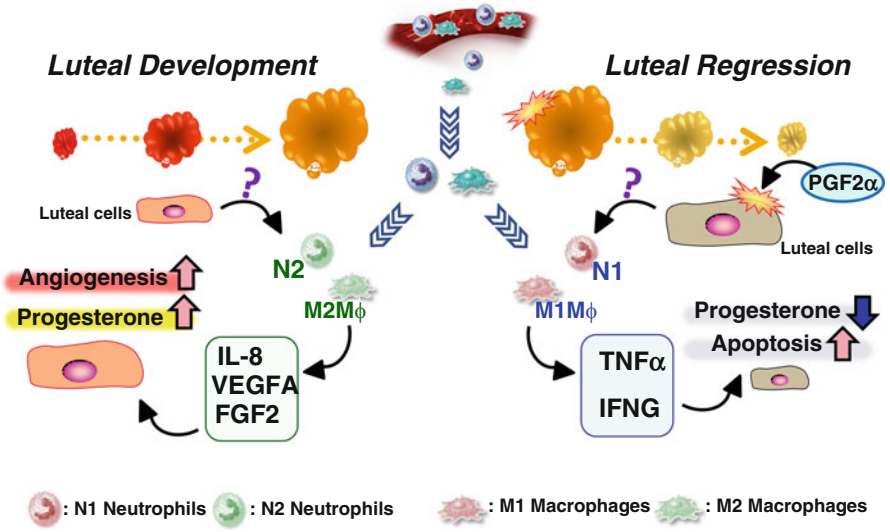

Fig. 6.3 Proposed model of the polarization mechanism of neutrophils (N1 vs. N2) and macrophages (M1 vs. M2), depending on the microenvironment of CL. The polarization of neutrophils and macrophages may be regulated by the luteal microenvironment. Luteotropic and angiogenic factors such as IL-8, VEGFA, and FGF2 facilitate differentiation into N2-neutrophils and M2-macrophages whereas luteolytic factors such as TNFα and IFNG facilitate differentiation into N1-neutrophils and M1-macrophages

6.5.2 *Multiple Roles of Macrophages: "M1" Versus "M2" Macrophages*

Polarization of macrophages is well understood compared with that of neutrophils [86, 87]. Macrophages activated by pro-inflammatory cytokines (TNFα and IFNG) and microbial products (LPS) are termed M1-type macrophages. M1 macrophages are characterized by high production of pro-inflammatory cytokines including TNFα, IFNG, and IL-12. M1 macrophages promote the differentiation of naïve $CD4^+$ T cells into Th1 effector cells and Th17 cells and secrete high levels of nitric oxide, and thus are a key cell type in the progression of inflammation [86]. In contrast, M2-macrophage polarization is activated by Th2-type cytokines, such as IL-4, IL-10, and IL-13, and stimulates differentiation to $CD4^+$ Th2 cells and regulatory T cells, indicating involvement in regulation of the inflammatory response, Th2 immunity, and tissue remodeling and repair [88].

Development of the bovine CL (angiogenesis and tissue remodeling) contrasts markedly with regression of the CL (angiolysis and tissue disruption); regardless, high numbers of macrophages are observed in both the developing and regressing CL [11]. Therefore, we postulate that the characteristics of these macrophages to differ depending on luteal environment. We investigated the mRNA expression of CD40 (as a marker of the M1 type) and CD163 (as a marker of the M2 type) in the bovine CL. M2-type macrophages were predominant in the developing CL, whereas M1-type macrophages were predominant in the regressing CL (Shirasuna et al., unpublished observations). Thus, the bovine CL has the potential to recruit and regulate macrophages by secretion of cytokines and chemokines, and macrophage function may be closely regulated by the luteal microenvironment (Fig. 6.3).

6.6 The Corpus Luteum in Early Pregnancy

6.6.1 *Lymphatic Systems of the CL of Early Pregnancy*

The lymphatic vascular system is considered the body's second circulation system for maintaining interstitial fluid pressure equilibrium and transporting tissue fluid, proteins, and cells [89]. The lymphatic system is also crucial during the immune response to infectious agents, as lymphatic vessels are the route by which dendritic cells, macrophages, and neutrophils migrate to the lymph nodes and lymphoid organs to present antigens to T cells. Two VEGF family members, VEGFC and VEGFD, regulate the lymphatic endothelial cells via their receptor VEGFR-3 [90–92]. Xu and Stouffer [93] have reported that the VEGFC/VEGFD-VEGFR3 system regulates lymphangiogenesis as well as luteal structure and function in the primate CL. Importantly, an injection of soluble VEGFR3 (which acts as an anti-VEGFR3 antibody) into the preovulatory follicle inhibited follicle rupture and ovulation and suppressed progesterone production in the monkey CL [93]. Interestingly, it has

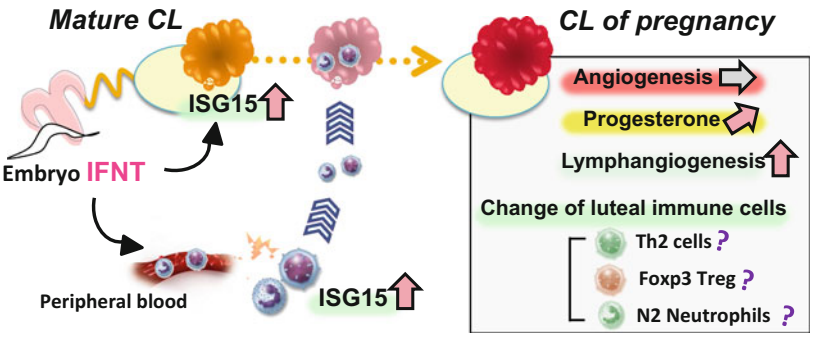

Fig. 6.4 Proposed model of the conversion of the cyclic CL into the pregnancy CL, and the possible role of IFNT as an immune regulator. IFNT produced by the embryo is released from the uterus into peripheral blood, and directly affects immune cells and luteal tissue; this results in the recruitment of immune cells to the CL. IFNT and the recruited immune cells stimulate progesterone secretion and lymphangiogenesis to convert the cyclic CL into the pregnancy CL

been reported that expressions of lymphatic vessel endothelial hyaluronan receptor (LYVE1, a marker of lymphatic vessel) and VEGFC were increased within the bovine CL of pregnancy [94]. In addition, interferon-τ (IFNT), a well-known pregnancy recognition signal for maintenance of CL in ruminants [95] secreted by embryonic trophoblast cells, stimulates lymphatic endothelial cell proliferation and formation of capillary-like tubes in vitro [94]. Hein et al. [96] reported that the concentration of progesterone was higher in ovarian lymph vessels than in uterine lymph or ovarian vein plasma during all stages of pregnancy in cows. These findings suggest that the lymphatic system of the bovine CL may function during early pregnancy [94] (Fig. 6.4).

6.6.2 *Immune System of the CL in Early Pregnancy*

In addition to its intrauterine function, IFNT produced by the conceptus passes through the uterine lumen and enters the uterine vein [97]. Compared to other cycling animals, IFNT upregulates the expression of IFN-stimulated gene 15 (*ISG15*) mRNA in both the endometrium and the CL as well as peripheral immune cells of pregnant ewes and cows [98–100]. These findings indicate that IFNT has a crucial role in transformation of the cyclic CL into the pregnancy CL using the systemic immune system in ruminants (Fig. 6.4). Moreover, ISG15 mRNA levels in bovine PBMC and neutrophils were higher in pregnant cows than in nonpregnant cows after artificial insemination [98, 99, 101]; this suggests the transmission of IFNT signals from the uterus to peripheral immune cells. In addition, IFNT treatment regulates the in vitro expression of ISG15 and IL10 mRNA in PBMCs and PMNs [102]. Therefore, IFNT might cause these changes via ISG responses and the regulation of Th1/Th2 cytokines in cows. We recently reported that compared to the

CL of nonpregnant animals, the CL of pregnant animals (day 16 after insemination) had a higher number of neutrophils and a greater expression of IL-8 and ISG15 [103]. Interestingly, IFNT stimulated IL-8 expression in luteal cells; this resulted in the increased migration of IFNT-activated neutrophils [103]. These results suggest that IFNT causes an increase in the number of neutrophils and upregulates their function via IL-8 expression in luteal cells in the early pregnancy CL, and that both neutrophils and IL-8, when stimulated by IFNT, are associated with the maternal recognition in cows (Fig. 6.4).

Fujiwara [104] proposed a new hypothesis that in humans, PBMCs contribute to embryonic–maternal cross-talk through the systemic circulation to transmit pregnancy status to the CL [13]. Indeed, PBMCs from pregnant woman stimulated the progesterone secretion of luteal cells from both pregnant and nonpregnant women [13]. The production levels of Th-2 cytokines such as IL-4 and IL-10 were increased in the co-culture of PBMCs and luteal cells derived from pregnant women, and these cytokines promoted progesterone production in vitro [13]. This hypothesis could be the basis for "systemic maternal recognition of pregnancy in ruminants" in addition to the local interaction between the uterus and embryo.

6.7 Conclusion

The physiological function of the CL is to produce a large amount of progesterone, thereby playing a vital role in the fate of the embryo. After ovulation, immune cells including neutrophils, macrophages, T cells, and dendritic cells are recruited into the developing CL by chemokines and cytokines produced by the luteal cells (Fig. 6.1). During the developing luteal phase, these immune cells can potentially increase progesterone secretion by releasing IL-8, PGE2, VEGFA, and FGF2 within the early CL (Fig. 6.1). In addition, these cytokines released from recruited immune cells and luteal cells stimulate angiogenesis and lymphangiogenesis. We hypothesize that the recruited neutrophils and macrophages may differentiate into "N2-type neutrophils" and "M2-type macrophages," respectively, in the microenvironment in the developing CL and produce potent promoters of angiogenesis (Fig. 6.3).

Uterine PGF2α, which functions as a start signal of luteolysis, drastically increases the recruitment of various immune cells including macrophages, T lymphocytes, eosinophils, and neutrophils into the CL (Fig. 6.2). Moreover, PGF2α stimulates the production of various types of inflammatory cytokines such as TNFα, IL-1, and IFNG, and chemokines such as CCL2 and IL-8. These cytokines and chemokines recruit macrophages, neutrophils, and T cells into the CL to enhance luteolytic cascades through inflammatory and immune responses (Fig. 6.2). In addition, the inhibition of angiogenesis and induction of vasoconstriction induce an inflammatory immune response, which leads to decreased progesterone production, cell apoptosis, and phagocytosis within the regressing CL. We hypothesize that, in contrast to those in the developing CL, recruited neutrophils and macrophages in the microenvironment of the regressing CL may differentiate into

"N1-type neutrophils" and "M1-type macrophages," respectively, and produce potent promoters of luteolytic cascades, such as TNFα and IFNG (Fig. 6.3).

In cows, maternal progesterone concentrations have a marked influence on the development of the embryo and on its ability to produce IFNT. The maternal immune system should be able to accept the conceptus as a semi-allograft not only in the local interaction between the embryo and the uterus but also in the peripheral interactions between the conceptus and the maternal organ systems, including immune cells and the CL (Fig. 6.4). A proposed hypothesis is that in response to embryo recognition, peripheral immune cells, especially T cells and neutrophils, transmit signals (detected by ISG15 expression) to various organs in the whole body, including the uterus and maternal vessels, to prepare for and maintain embryo implantation. The immune cells that are activated by IFNT may accumulate within the CL and participate in the establishment of the pregnancy CL, which includes an increase in progesterone concentration and lymphangiogenesis (Fig. 6.4). Furthermore, we speculate that IFNT (and/or ISGs stimulated by IFNT) in peripheral blood regulates immune tolerance to stimulate the differentiation of Th2 cells, forkhead/winged helix transcription factor $(Foxp3)^+$ Treg cells, and N2-type neutrophils, which support the acceptance of the conceptus as a semi-allograft.

Further investigations of the regulatory mechanisms of luteal function involving angiology and immunology are essential for better understanding of reproductive physiology.

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Chapter 7
The Rodent Corpus Luteum

Paula Accialini, Silvia F. Hernandez, Dalhia Abramovich, and Marta Tesone

Abstract The corpus luteum (CL) is a tissue having great differences among species. Rodents have particular features in CL formation, function, and regression. The different types of mammalian corpora lutea can be classified in CL of pregnancy, cyclic CL, CL of lactation, and CL of pseudo-pregnancy. Among mammals, only rodents present the four types of corpora lutea.

Rodents are an excellent model to study reproductive physiology. Advantages of this animal model include their small size, their high reproductive rate, and the possibility to obtain inbred strains. Transgenic technologies developed in mice are also a helpful strategy to study gene function. Knowing the similarities and differences among mammalian species is crucial to translate the findings described in rodents to other species.

In the present chapter, we review the regulation of luteinization, the multiple factors involved in this process, the structure and function of the CL, including remodeling, development, and the mechanisms involved in the survival and regression of CL. In particular, we describe the role of the Wnt and Notch signaling pathways in CL function.

Keywords Ovary • Ovulation • Steroidogenesis • LH • PGF2α • PRL • Angiogenesis • VEGF • Notch

7.1 Introduction

In the wild, rats and mice not only have a short lifespan but also poor offspring survival. For these reasons, rodents have evolved a reproductive system that is able to maximize the number of offspring per time period. To achieve this goal, rodents have developed particular strategies in both follicular development and corpus luteum (CL) physiology, which are discussed throughout this chapter. The CL represents the largest differences among species, and rodents have particular features

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in CL formation, function, and regression that make them unique and different from other groups of mammals [1].

Based on the CL lifespan and steroidogenic capacity, the different types of mammalian CL can be classified into four groups: CL of pregnancy, cyclic CL, CL of lactation, and CL of pseudo-pregnancy. The CL of pregnancy is the only one that is present in all species, but with variable duration. Not only do they have four types of CL [2], rodents have an ultrashort CL lifespan [3]. The estrous cycle in these mammals lasts 4–5 days and lacks a true luteal phase. The follicular cells that remain in the ovary after ovulation exhibit some of the features of luteal cells. However, the resulting CL is not well developed and is considered not functional because it is not able to secrete sufficient quantities of progesterone to stimulate a uterine response to implantation signals [1]. Progesterone is rapidly converted to its inactive analogue 20α-hydroxyprogesterone, another unique feature of this group of animals. Lack of a fully active CL allows the occurrence of follicular recruitment and ovulation within a few days [4]. Cervical stimulation during estrus without mating results in a condition called pseudo-pregnancy that in fact does not exist in nature because, in wild conditions, mating usually results in pregnancy [3]. Pseudo-pregnancy is characterized by the formation of a true CL that secretes progesterone for 12–14 days. In the case of pregnancy, the CL secreting progesterone is required throughout the whole gestation. At the end of gestation, the CL regresses and the drop in progesterone levels is an important signal that triggers parturition [2]. In addition, ovulation in pregnant rats occurs immediately after delivery, and two different types of CL are, therefore, present in the postpartum ovary: one is the CL of pregnancy and the other is a newly formed CL [5]. Usually, the CL of pregnancy undergoes regression. Conversely, the new CL is sensitive to environmental signals, such as prolactin (PRL), whose serum levels are increased by pup suckling stimulation in lactating rats [5]. PRL signaling regulates the survival of the CL in these rats, and the CL is called CL of lactation.

It is worth pointing out that rodents are an excellent tool to study mammalian physiology and pathology. Despite species differences, rodents have several advantages: their small size, high reproductive rate, and the ease to obtain inbred strains. Transgenic technologies developed in mice have emerged as a useful and valuable strategy to study gene and protein function. For these reasons, knowledge of similarities and differences among mammalian species is indispensable to translate the findings among species and to correctly interpret the results obtained.

7.2 Regulation of the Luteinization Process

7.2.1 Luteinization and Cell Division

Luteinization represents the exit from the cell cycle and terminal differentiation of granulosa cells (GCs) in the CL. In mice, most GCs from preovulatory follicles stop synthesizing DNA and luteal steroidogenic cells arrest at the G_0–G_1 stage, 7 h after the luteinizing stimulus of ovulatory doses of luteinizing hormone (LH) [6–8].

Cyclin-dependent kinases (CDKs) act in concert to coordinate growth- and differentiation-related intracellular processes. Several proteins that either stimulate or inhibit their activities regulate the G_1 phase of the cell cycle, governing the transition between proliferation and quiescence [9]. Cessation of cell proliferation during luteinization is associated with a progressive loss of positive cell-cycle regulators: downregulation of cyclin D2 (detected early after the LH surge) and cyclin E (detected after 20 h of the LH surge) and upregulation of the CDK inhibitors $p21^{cip1}$ and $p27^{kip1}$. $P21^{cip1}$ has also been involved in luteinization, based on its induction in the incipient CL of hypophysectomized rats after administration of an ovulatory dose of LH. The Cip/Kip family of kinase inhibitors regulates cyclin D complexes. Cyclin D2 is necessary for GC proliferation, because its targeted deletion impairs both normal and gonadotropin-induced GC mitosis. Cyclin D2 expression at mRNA and protein levels is downregulated within 4 h in GCs undergoing luteinization, which suggests that the LH surge arrests mitosis by concurrent inhibition of cyclin D2 and upregulation of $p27^{kip1}$ and $p21^{cip1}$ [10–12]. In mice, deletion of $p21^{cip1}$ causes no detectable effect on the proliferation of luteinized cells or fertility [9], whereas targeted deletion of the CDK inhibitor $p27^{Kip1}$ results in infertility, attributed to a failure of the CL to develop [13].

7.2.2 Molecular Factors Involved in Luteinization

Gene expression profiling has allowed recognizing genes in the ovary that are differentially expressed before and after the LH surge [14]. In rodents, the most studied receptors involved in this process are those of follicle-stimulating hormone (FSH), LH, PRL, estrogen, and progesterone. The LH surge leads to changes in the expression of these receptors, causing the silencing of the FSH receptor and a transitory decline in the LH receptor (LH-R). Also, it stimulates PRL receptor expression, induces a quick and short-term increase in progesterone receptor, and changes the expression of estrogen receptor-β (ERβ) to ERα [9].

7.2.3 PRL and PRL Receptor

In rodents, in addition to LH signaling, there are other key factors that regulate CL formation and maintenance [9]. PRL is a well-known stimulator of LH-R expression in GCs [15]. In the rat, PRL-R is expressed in two variant forms that result from differential splicing of a single gene: long (PRLR-L) and short (PRLR-S). Both these receptors increase during luteinization. During CL formation, PRL upregulates LH-R both in vivo and in vitro.

The main pathway activated by PRL binding to PRLR-L in the ovary is the JAK2/STAT pathway, and activation of STAT5 proteins is essential for CL formation [16] and function [9]. In luteal cells, 20α-hydroxysteroid dehydrogenase (20αHSD) is one of the most important regulators of progesterone levels in rodents.

PRL represses the expression of the 20αHSD gene, preventing the catabolism of progesterone into the inactive progestin 20α-dihydroprogesterone (20αDHP) [9].

Semi-circadian surges of PRL secretion are induced by cervical stimuli and are believed to be responsible for the conversion of CL into CL of pregnancy, leading to an increase in the lifespan of this gland and its capacity to secrete a sufficient amount of progesterone to maintain pregnancy [17, 18]. This conversion is thought to involve distinct luteotropic effects of PRL: upregulation of LH-R expression as well as progesterone secretion and repression of 20αHSD [19].

The role of PRL in CL development has also been studied in PRL-R knockout mice. PRL-R−/− females are infertile because of implantation failure [20]. In the absence of PRL-R, luteinization and CL formation are delayed, occurring 2 days after mating. In addition, PRL-R knockout mice show luteal regression caused by apoptosis and associated with reduced vascularization, and decreased levels of $p27^{kip1}$ and steroidogenic enzymes [21]. Progesterone administration is able to rescue preimplantation egg development and embryo implantation in PRL-R-deficient females [21]. In conclusion, PRL triggers an early signal that induces the survival, vascularization, and steroidogenic capacity of the CL. Therefore, PRL-R is a key element in the regulation of the luteal function.

7.2.4 Estrogen Receptor (ER)

Both ERα and ERβ are expressed in the rodent ovary but they differ in their levels of expression and localization: ERβ is abundantly expressed in the GCs of the follicle, and ERα is found predominately in the CL [22, 23]. During pro-estrus, ERβ expression decreases significantly in association with the LH surge. Both ERβ and ERα remain expressed throughout the lifespan of the CL during pregnancy, mostly because of PRL stimulation [9].

GCs of ERβ knockout mice do not differentiate properly in response to FSH, exhibiting reduced aromatase activity, estradiol synthesis, and LH-R expression [24]. These alterations lead to a reduced response to LH stimulus and a lower rate of follicle rupture [24]. In contrast, ERα knockout female mice show minimal alterations in GC differentiation and ovulation. Therefore, FSH-induced differentiation of GCs depends on the effects of estradiol acting through its receptor ERβ and, in the absence of this receptor, ovulation and CL formation are halted [24].

7.2.5 Progesterone Receptor (PGR)

Progesterone nuclear receptor, similar to ER, functions as a ligand-activated transcription factor regulating long-term effects on gene expression. With this, progesterone stimulates rapid physiological effects that are independent of transcription. This pathway, termed nongenomic, is mediated by a membrane PGR.

Rat and mouse granulosa and luteal cells do not express nuclear PGR but are able to bind progesterone (reviewed in [25, 26]). Incubation of rat luteal cells with R5020 (a synthetic progestin) increases the production of progesterone in a dose-dependent manner and downregulates the expression of 20αHSD. Furthermore, progesterone administration to rats prevents CL apoptosis [27, 28]. These observations suggested that rat granulosa and luteal cells express a progesterone-binding protein that is not the classic PGR and may function as a mediator of progesterone actions. This mediator of progesterone action is the progesterone receptor membrane component 1 (PGRMC1). Rat granulosa and luteal cells express high levels of PGRMC1 [29]. PGRMC1 is expressed in different ovarian cells, such as thecal cells, stromal cells, ovarian surface epithelial cells, and oocytes, and its expression is independent of gonadotropins. Furthermore, PGRMC1 expression is induced by gonadotropins as part of the mechanism of differentiation of GCs into luteal cells [29].

7.2.6 Other Factors

7.2.6.1 Kisspeptin

Kisspeptin mRNA is expressed in rat and mouse GCs, and the LH surge has been demonstrated to directly stimulate its expression [30]. Recently, it has been postulated that the presence of kisspeptin in GCs is important for the integrity of ovarian follicles [31, 32]. Kisspeptin has also been proposed as a factor directly involved in tissue remodeling during luteinization, allowing CL formation [30]. Moreover, ovarian intrabursal administration of a kisspeptin antagonist in rats induces distortional changes in the morphology of the CL, suggesting that, during luteinization, kisspeptin is involved in tissue remodeling and CL formation [30].

Some of these topics are illustrated in Fig. 7.1.

7.3 Structure and Function of the CL

7.3.1 Tissue Remodeling

The extracellular matrix (ECM) is a complex system composed of a network of collagens associated with proteoglycans and glycoproteins, components providing the architecture to hold cells together. Further, matrix metalloproteinases (MMPs: collagenases, gelatinases, stromelysins, membrane-type MMPs) cleave specific components of the ECM and are inhibited by tissue inhibitors of metalloproteinases (TIMPs) [9]. The ECM has a profound effect on cellular functions and has an important part in the processes of follicular development and atresia, ovulation, and in the maintenance and regression of corpora lutea.

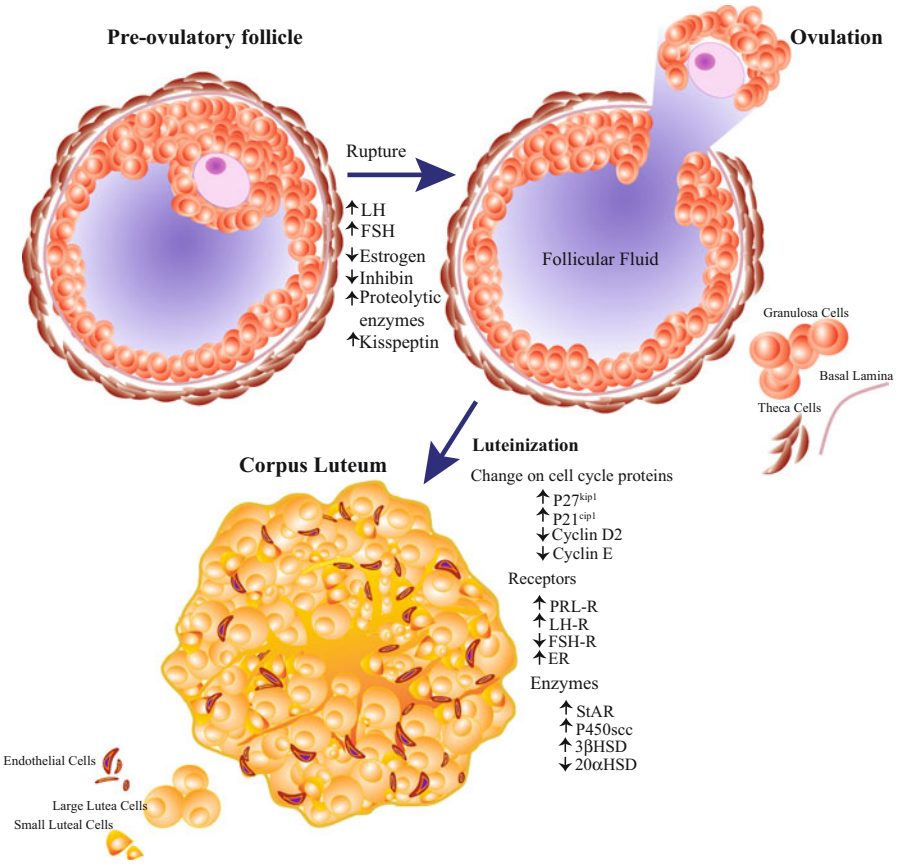

Fig. 7.1 Schematic representation of the changes that take place during the luteinization process in rodents

The development of the CL is accompanied by extensive remodeling of the ECM, and luteal differentiation is associated with construction of the ECM. There is increasing evidence that ECM components enhance luteinization, whereas loss of ECM results in luteal cell death [33]. In the rat CL, laminin and collagen type IV (ligand of integrin α2) are detected in GCs 6 h before ovulation. The LH surge induces expression of several MMPs and TIMPs, whereas PRL stimulates the expression of α2-macroglobulin, a protease inhibitor [34, 35]. The increase in collagen type IV in GCs at the moment of ovulation persists through all the luteinization process. Integrin α6β1 is also present in early CLs and interacts with laminin and CD9, both involved in cell adhesion and migration. The temporal and differentiation-dependent expression of adhesion molecules confirms their involvement in CL induction. In the rat ovary, MMP-2 is present in granulosa, theca, and luteal cells, whereas MMP-9 is located only in the plasma membrane of luteal cells [36]. The regulation of genes involved in ECM remodeling varies among species. LH induces the expression of genes that act in several species, such as MMP-1 or

MMP-10 [1], but also of genes that are present in rodents but not in primates, such as protease serine 35 [37]. Furthermore, there are genes that are expressed in different species but whose temporal pattern and function are different, such as disintegrin and MMP with thrombospondin-like repeats-1 (ADAMTS-1) [1]. In this regard, it is important to point out that ADAMTS-1 has an important role in ovarian ECM remodeling. It has been demonstrated that ADAMTS-1 null mice show impaired development of growing follicles and ovulation [38]. In addition, LH and PR regulate ADAMTS-1 gene expression in rat GCs, with PR acting as an inducible coregulator of the ADAMTS-1 gene [39]. However, as previously mentioned, there are functional divergences of ADAMTS-1 function among species. In rodents, for example, this protease regulates follicular rupture, whereas in primates it is involved in early luteal development [1].

7.3.2 Types of Luteal Cells in Rodents

During luteal development, the growth of the CL is the result of a huge increase in the size of large luteal cells, whose number remains constant, and an increase in the number of small luteal and endothelial cells.

In the rat CL, small luteal cells have a large oval nucleus, few lipid droplets, and a stellate shape. In contrast, large luteal cells have a smaller spherical nucleus and high lipid content [40]. Both luteal cell types express enzymes involved in steroidogenesis and, despite the greater expression of LH-R in large luteal cells, both small and large luteal cells respond to LH with similar increase in progesterone production, with large cells producing 2- to 40-fold more progesterone than small cells, largely because of the difference in their size rather than their origin in the follicle [9, 40]. Both luteal cells have similar pituitary hormone receptors and steroidogenic protein profile. However, it has been reported that the expression of PRL-R is greater in large luteal cells than in small luteal cells [9].

Macrophages are important regulators of luteal function. The corpora lutea of macrophage-depleted mice produce substantially less progesterone, have disrupted blood vasculature, and exhibit changes in the local expression of genes encoding angiogenic regulators [41].

7.4 Role of LH-Induced Local Factors

The activation of LH-R leads to the induction of several signaling pathways that participate in the process of luteinization and maintenance of luteal function [9]. One of these pathways is the Wnt/Frizzled, which is stimulated by LH-R activation. This pathway is a highly conserved system composed of secreted glycoprotein that acts locally, regulating key processes related to homeostasis, development, proliferation, and cell death in many cell types [42]. In the rodent ovary, Wnt-4 performs critical functions during early ovarian development [43]. Wnt-4 expression increases

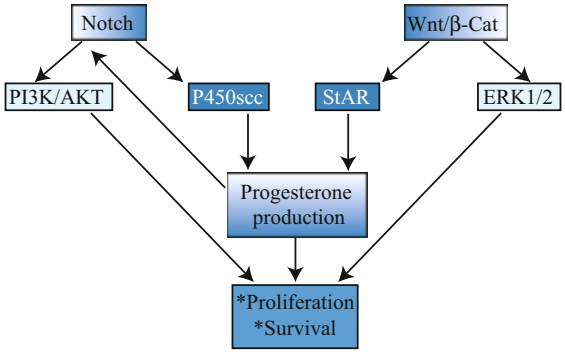

Fig. 7.2 Role of the Notch and Wnt/β-catenin signaling pathway in CL survival and function of superovulated rats. The Notch pathway induces in vitro production of luteal progesterone through an increase in P450scc synthesis, increases cell proliferation, and decreases apoptosis-mediated cell death. Then, progesterone could regulate the intracellular active Notch domain. There would also exist an association between the antiapoptotic action of progesterone and Notch/PI3K/AKT signaling, suggesting that this pathway might be interacting with progesterone, intensifying the survival role of this hormone in luteal cells. Wnt/β-catenin signaling controls in vivo progesterone production through an increase in StAR levels, a decrease in apoptosis-mediated cell death, and an increase in cell proliferation, likely through the ERK signaling pathway

after human chorionic gonadotropin (hCG) treatment and remains increased in the CL during pregnancy [44]. Luteal cells express Disheveled and β-catenin [44], which are components of the Wnt-4 intracellular signaling pathway. Studies carried out in our laboratory to determine the role of the canonical Wnt/β-catenin transduction pathway in luteal function in the rat have shown that in vivo blockade of Wnt signaling leads to inhibition of luteinization. In addition, there is a decrease in progesterone serum levels associated with a decrease in StAR levels, and an increase in apoptotic parameters (e.g., increase in the expression of active caspase 3 and an imbalance between pro- and antiapoptotic proteins) (Fig. 7.2).

The phosphotidylinositol-3 kinase (PI3K)/AKT/ERK1/2 interaction mediates relevant pathways involved in the promotion of cell survival or apoptosis inhibition [45, 46]. In the rat ovary, AKT is constitutively expressed in GCs and luteal cells [47]. FSH and LH mediate luteinization by inducing a complex pattern of gene expression in ovarian cells that is regulated by the coordinate input from different signaling cascades such as the cAMP/protein kinase A, PI3K/AKT, and ERK1/2 cascades [48, 49]. In addition, in luteal cells, the luteolytic hormone PGF2α also regulates the ERK1/2 pathway [50].

7.5 Mechanisms Involved in CL Survival

Despite the crucial role of LH in the luteinization process, autocrine and paracrine factors are able to modulate LH action [9]. In addition, components of the Notch system are able to regulate this process.

The Notch system is an evolutionarily conserved pathway involved in cell fate decisions, including proliferation, differentiation, and apoptosis. In mammals, the Notch family of proteins consists of four receptors (NOTCH 1–4) and five ligands [JAGGED 1–2, DELTA-like 1, 3, and 4 (DLL4)] expressed on the cell surface. When Notch signaling is initiated, the receptors expose a cleavage site and the active intracellular domain (NICD) is released, translocates to the nucleus [51], and exerts pleiotropic effects by initiating a transcriptional cascade [52]. Notch proteins and ligands have been localized in granulosa, luteal, and vascular cells of the rodent ovary [53, 54]. Recently, it has been demonstrated that the in vivo inhibition of the Notch signaling pathway in mice impairs folliculogenesis and induces disruption of gonadotropin-stimulated angiogenesis [55]. In addition, we have demonstrated that Notch 1, Notch 4, and DLL4 are expressed in small and large luteal cells of CL from pregnant rats, and evidence has shown that Notch signaling promotes both luteal cell viability and steroidogenesis [56]. Thus, we have described a luteotropic role for Notch signaling in promoting both luteal cell viability and steroidogenesis in CL. In addition, intraovarian Notch inhibition decreases circulating progesterone levels, confirming that Notch has a direct action on luteal function [56]. Moreover, our group [57] has demonstrated the existence of an interaction between the Notch signaling pathway and progesterone, which maintains the functionality of the rat CL [57]. These studies, performed in rat CL cultures, have shown that Notch inhibition causes an increase in apoptotic parameters and a decrease in AKT phosphorylation, whereas progesterone inhibition decreases the NICD active Notch levels. These data provided the first evidence of a crosstalk between the Notch system and progesterone, which upregulates the survival of luteal cells. One mechanism of Notch action is the increase in CYP11A1 (P450scc) synthesis and, in turn, progesterone could regulate the intracellular active Notch domain. This finding supports that Notch induces progesterone production in vitro through the activation of CYP11A1 and decreases apoptotic cell death [57] (Fig. 7.2).

7.6 Mechanisms Involved in CL Regression

After each estrous cycle, when pregnancy does not occur, or when progesterone is no longer required for the maintenance of pregnancy, the CL ceases to produce progesterone and regresses in a process called luteolysis [58]. In rodents, the process of luteolysis involves two phases: the first one, termed functional regression, is associated with a marked decrease in progesterone content caused by catabolism to its biologically inactive form 20αDHP [59]. The second phase is known as structural regression and occurs after the initial decline in progesterone output. During this phase, the CL loses its vascular integrity and luteal cells die through programmed cell death (apoptosis) [9].

McCracken et al. [4] have postulated the concept of a central oxytocin (OT) pulse generator that functions as a pacemaker for luteolysis. According to this concept, the uterus transduces hypothalamic signals in the form of episodic OT secretion into luteolytic pulses of uterine PGF2α. Experiments with transgenic mice

suggest that OT acts as a luteotropic hormone opposing the luteolytic action of PGF2α. Thus, to initiate labor, it might be essential to generate sufficient PGF2α to overcome the luteotropic action of OT in late gestation [60]. At this point, it is worth noting that many of the descriptions of OT action come from experiments performed in transgenic mice. However, there is great diversity of effects depending on the species studied, and most of the effects regarding the action of OT on CL have been described in primates, cattle, sheep, and pigs.

Several factors including PGF2α and LH have been involved in shutting off luteal progesterone production [9]. PGF2α is essential for the inhibition of progesterone synthesis in the CL [61, 62]. This PGF2α effect is mediated by the induction of 20αHSD [63]. At the end of pregnancy in the rat, progesterone production decreases as a consequence of 20αHSD increased activity and a concomitant increase in the concentration of 20αDHP. This decline in progesterone secretion at the end of pregnancy has an even more important function because the levels of circulating progesterone must fall to allow parturition [9]. PGF2α action in the process of luteolysis was discovered in mice lacking the PGF2α receptor. These PGF2α receptor-deficient mice do not show the usual decline of serum progesterone concentrations that precede parturition. As a consequence, parturition fails to occur, indicating that it is initiated when PGF2α interacts with its receptor in mouse luteal cells to induce luteolysis. In addition, these mice do not respond to exogenous OT because of the lack of induction of OT receptor (a proposed triggering event in parturition) [64]. PGF2α also reduces cholesterol transport in the ovary through a decrease in SCP-2 [65] and StAR [66] expression. PGF2α prevents both LH and PRL stimulation of progesterone biosynthesis [67]. This anti-LH action involves the blockage of LH-induced cAMP accumulation and the inhibition of luteal cells to respond to cAMP [67, 68]. PGF2α inhibits PRL-R expression and PRL signaling through the JAK/STAT pathway, which prevents PRL-induced Stat5 activation [69]. PGF2α also reduces progesterone synthesis through inhibition of luteal aromatase, which in turn suppresses estradiol production [70].

Structural luteolysis is characterized by a reduction in CL size and weight. Apoptosis of luteal and vascular cells is one key event that defines structural regression and is associated with CL regression in many species [71]. Particularly in rats, caspases play an important role during the early stage of luteolysis in CL of the estrous cycle [71]. We have demonstrated that an increase in the activity of caspase-2, -8, -9, and -3 is associated with the early events of natural luteolysis at the end of pregnancy, and that PGF2α regulates members of the caspase family in the rat CL [72]. In addition, Notch signaling is involved in the apoptosis of luteal cells associated with CL regression in pregnant rats [56]. Even more, gene expression of some Notch members is dynamically regulated during PGF2α-induced luteolysis. Our results demonstrate that the expression of Notch family members declines after PGF2α administration, suggesting that PGF2α might act in part by reducing the expression of some of the Notch pathway components (Fig. 7.3a). Furthermore, intraovarian inhibition of Notch signaling by a gamma secretase inhibitor (DAPT) in pregnant rats increases proapoptotic proteins such as active caspase-3 and BAX and decreases the antiapoptotic protein BCL2 and also serum progesterone (Fig. 7.3b) [56]. These results support the hypothesis of a luteotropic role for Notch signaling in pregnant rats.

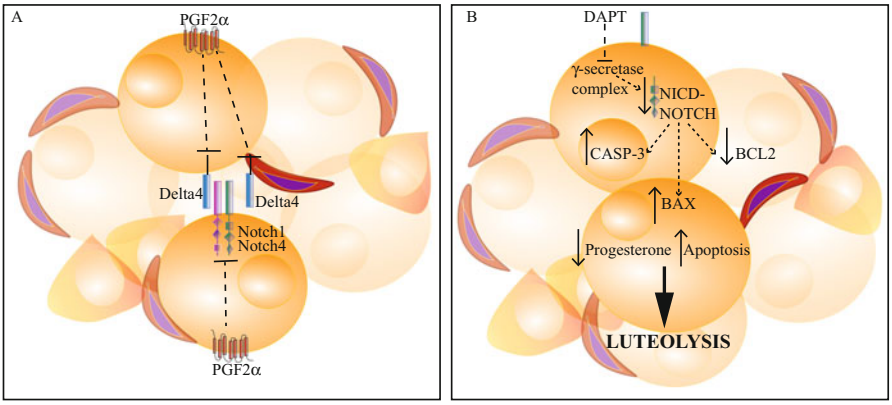

Fig. 7.3 Role of the Dll4-Notch system in PGF2α-induced luteolysis in the pregnant rat. (**a**) The expression of Notch family members declines after PGF2α administration, suggesting that PGF2α might act in part by reducing the expression of some of the Notch pathway components. (**b**) Intraovarian inhibition of Notch signaling by a gamma secretase inhibitor (DAPT) in pregnant rats increases proapoptotic proteins such as active caspase-3 and BAX. In addition, DAPT administration decreases the antiapoptotic protein BCL2 and also serum progesterone, sustaining a luteotropic role for Notch signaling during pregnancy

7.7 Future Perspectives

Although luteal function has been studied for several decades, its regulation and the processes involved in CL function are incompletely understood. Rodent models have been very useful in mimicking processes occurring in other species, including humans. Thus, studies of translational medicine are new challenges where researchers have to apply their knowledge in clinical syndromes. Knowledge of the mechanisms regulating the progress and regression of CL could contribute to the understanding and treatment of luteal dysfunction associated with infertility. In humans, a short luteal phase or low levels of progesterone characterize the inadequate luteal phase syndrome. The lack of a clear etiology of this syndrome has led to multiple treatments, but few therapies have solved the infertility associated with this syndrome in most of the patients. Similarly, an understanding of the mechanism causing regression of the CL could lead to new ovary-based approaches to contraception.

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Chapter 8
Regulation of Corpus Luteum Function in the Domestic Dog (*Canis familiaris*) and Comparative Aspects of Luteal Function in the Domestic Cat (*Felis catus*)

Mariusz Pawel Kowalewski

Abstract The domestic dog (*Canis familiaris*) and the cat (*Felis catus*), although sharing the same goal of ensuring maximal fertility, have developed different reproductive strategies. Significant differences can be found in the mechanisms regulating luteal function. In the dog, the lack of an acute luteolytic mechanism in the absence of pregnancy results in prolonged regression of the corpus luteum (CL), extended luteal progesterone secretion, and CL lifespan, features that are similar in pregnant and nonpregnant bitches until the acute prepartum luteolysis. This observation emphasizes the differences between pregnant and nonpregnant dogs in mechanisms regulating the termination of CL function and further highlights the interspecies differences. In the domestic cat, successful mating results in pregnancy and a luteal lifespan that extends until parturition, and after a nonfertile mating ovulation is followed by pseudo-pregnancy. However, differing from the dog, the duration of pseudo-pregnancy is approximately half the gestation length observed during pregnancy. The persistence of luteal function in pregnant queens over the duration of pseudo-pregnancy is, most probably, caused by the supportive role of placental steroidogenesis, which is lacking in the dog. Interestingly, in both species luteal function, at least in the absence of pregnancy, is independent of a uterine luteolysin, as it remains unaffected by hysterectomy. Consequently, in both species the luteal regression/luteolysis during pseudo-pregnancy appears to be a passive degenerative process in the absence of a luteolytic principle of uterine origin; however, the inherent luteal lifespan is much shorter in the feline than in the canine species, facilitating and hastening reproduction in cats.

Keywords Domestic dog (*Canis familiaris*) • Domestic cat (*Felis catus*) • Corpus luteum function • Pregnancy • Pseudo-pregnancy • Luteal regression • Luteolysis

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8.1 Introduction

Among the domestic animal species, in dogs ovarian function appears to have only minimally albeit successfully evolved, representing a basic model of mammalian reproduction. Thus, dogs are classified as "aseasonal monoestrous," that is, ovulating only once per breeding season; they are polytocous and spontaneous ovulators. The periods of sexual activity are separated from each other by obligatory periods of sexual inactivity, referred to as "anestrus," which can last as long as 36 weeks [1, 2]. Contrasting with this is the "seasonally polyestrous" reproductive pattern of domestic cats, providing them with increased opportunities for facilitating fecundity and production of offspring. Cats are polytocous, predominantly induced ovulators repeating estrus until mating (or ovulation). Consequently, several regulatory mechanisms governing reproductive function in both species are species specific.

Indisputably, both species represent the most important pets, while also serving as laboratory animals. Dogs are accepted as one of the best models for studying multifactorial human diseases [3], and investigations on cats serve for better understanding of reproductive function in endangered felids, for example, lynxes [4–6]. Consequently, following increased scientific interest, knowledge concerning canine reproductive function in particular has greatly expanded during the past few years. This expansion relates mostly to the mechanisms regulating maintenance of CL function by luteotropic mechanisms. The physiological processes associated with luteal regression or luteolysis in dogs are still not fully understood. Even less is known about CL regulation in domestic cats, with most knowledge derived from clinical and endocrinological observations; little attention has been paid so far to the underlying molecular regulatory mechanisms.

Based on the available reports and reviews, including those from our own laboratory [2, 6–16], this chapter presents our current understanding of the endocrine and molecular mechanisms regulating CL function in pregnant and nonpregnant dogs, with some comparative data from studies with cats. Although some of the data generated still remain to be published, they are discussed here to provide new insights and ideas for prospective research directions. A historical perspective is provided by presenting the earliest observations published by Bischoff [17] in his pioneering work, dated 1845, regarding canine reproduction. Finally, interesting but largely neglected comparative aspects of feline and canine luteal functions are presented.

8.2 Shining a Spotlight: Species-Specific Peculiarities of Canine Luteal Function

Taking into account the indispensable role of progesterone (P4) during the establishment and maintenance of mammalian gestation, one of the most interesting peculiarities of canine reproduction is the lack of placental steroidogenesis [18, 19]. This characteristic is unique among domestic animal species, as even in those species

with gestation dependent on luteal P4, such as pigs or goats, the placenta is capable of producing steroids [20–22]. Consequently, the dog is virtually the only domestic animal species devoid of placental steroids, which further underlines the central role of the CL in regulating canine fertility. Moreover, the lack of an extra- or intraluteal luteolytic principle leads to a somewhat reverse relationship between the duration of the luteal lifespan during pregnancy, determining the length of gestation, and the extended luteal phase in nonpregnant cyclic bitches, referred to as pseudo-pregnancy, which frequently exceeds the length of normal gestation [18, 23, 24]. Therefore, as discussed later, different mechanisms must have evolved to regulate the gradual luteal regression observed in nonpregnant dogs and the acute prepartum luteolysis. Importantly, however, the similar progesterone profiles observed in these situations, that is, in pregnant and nonpregnant dogs, preclude P4 as a reliable marker for pregnancy determination in this species. Consequently, relaxin of fetal placental origin is the only marker of canine gestation identified so far [25]. There are no other markers available in dogs allowing for early pregnancy detection, i.e., preimplantation. Compared with other species, in which ovarian cyclicity is maintained by periodic uterine PGF2α production, the dog exhibits a more primitive form of CL control where there is no relationship between the uterus and control of the CL. This difference is further expressed in the lack of an embryo-derived anti-luteolytic signal; such a relationship is a more evolutionarily advanced system. Thus, in the dog there is no classical maternal recognition of pregnancy, and both during pregnancy and pseudo-pregnancy the canine genital tract is exposed to a high P4 milieu of luteal origin [26]. Concerning luteotropic support, one of the most interesting species-specific peculiarities is the fact that, although especially during the second half of the luteal phase PRL and LH act as luteotropic factors, luteal regression/luteolysis take place despite their increased availability (discussed later). Finally, regarding the CL as being the only source of circulating steroids during canine pregnancy, there is no pregnancy- and/or parturition-specific increase in estrogens [18, 23, 27].

8.3 Periovulatory Endocrine Events

The hormonal changes characterizing the periovulatory endocrine milieu in the dog are shown in Fig. 8.1. Thus, pro-estrus is the phase when the bitch is under the influence of increasing estradiol (E2) levels secreted from ovarian follicles. E2 concentrations increase continuously from levels of about 5–15 pg/ml at the beginning of pro-estrus to average levels of 70 pg/ml (40–120 pg/ml) at the peak, 1 or 2 days before the onset of estrus. Estrogens alone are, however, not responsible for the breeding activity, which is normally associated with decreasing E2 levels occurring concomitantly with rising P4 concentrations [28]. The latter, being designated as follicular luteinization, sets in before the first significant LH increase (LH surge), which indicates the final maturation of ovarian follicles. During this time, circulating P4 rises slowly from basal levels of 0.2–0.4 ng/ml to 0.6–1.0 ng/ml [29]. E2 starts to decrease progressively toward its intermediate estrous values of 10–20 pg/ml [2].

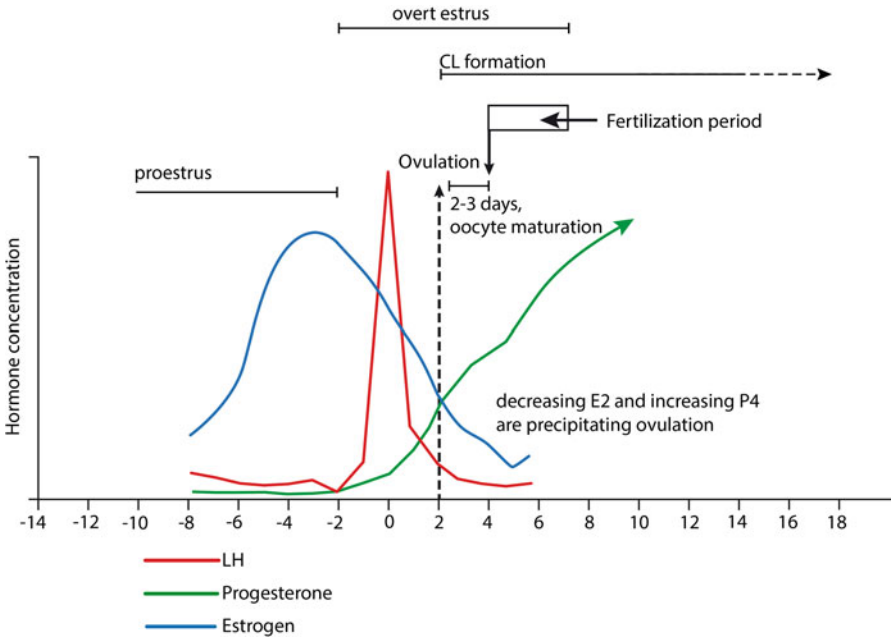

Fig. 8.1 Schematic representation of the most important endocrine patterns before and after ovulation, leading to establishment of the canine CL. A detailed explanation is provided in the text. Ovulation takes place accompanied by relatively high circulating progesterone (P4) levels, >5 ng/ml, and is precipitated by decreasing estradiol (E2) and increasing P4. The structural formation of CL begins before cessation of clinical estrus signs (overt estrus). (Modified from [26])

The LH surge is the result of increasing P4 and decreasing E2 levels, providing a strong positive feedback on the hypothalamus and hypophysis, also leading to enhanced follicle-stimulating hormone (FSH) production. Thus, hormonally, estrus that lasts on average 9 days is characterized by declining estrogen and rising P4 concentrations, strongly stimulating LH secretion and precipitating ovulation. The LH surge takes place 0.5–3 days (average, 1 or 2 days) after the E2 peak. It has been defined more accurately by Concannon [2] as the first detectable rise >200 % of preceding mean concentrations of LH and >50 % of its peak concentrations (i.e., the first significant LH increase). This abrupt surge of gonadotropins at the end of proestrus results in a 1- to 3-day elevation of LH (average, 2 days, usually peaking in the first 12–18 h) and a 1- to 4-day elevation of FSH, leading to ovulation at 48–60 h (2–3 days) after the LH surge [2, 24, 26]. It is noteworthy, and unique among the domestic animal species, that canine oocytes are ovulated at the stage of primary oocytes and that their maturation and completion of the first meiotic division are delayed, taking place in the oviducts 2–3 days after ovulation (i.e., 4–5 days after the LH surge) [24].

The time period from the LH surge to ovulation is characterized by rapid proliferation of follicular theca cells and emergence of a vascular network supplying them. Consequently, luteinizing follicular cells are capable of producing amounts of

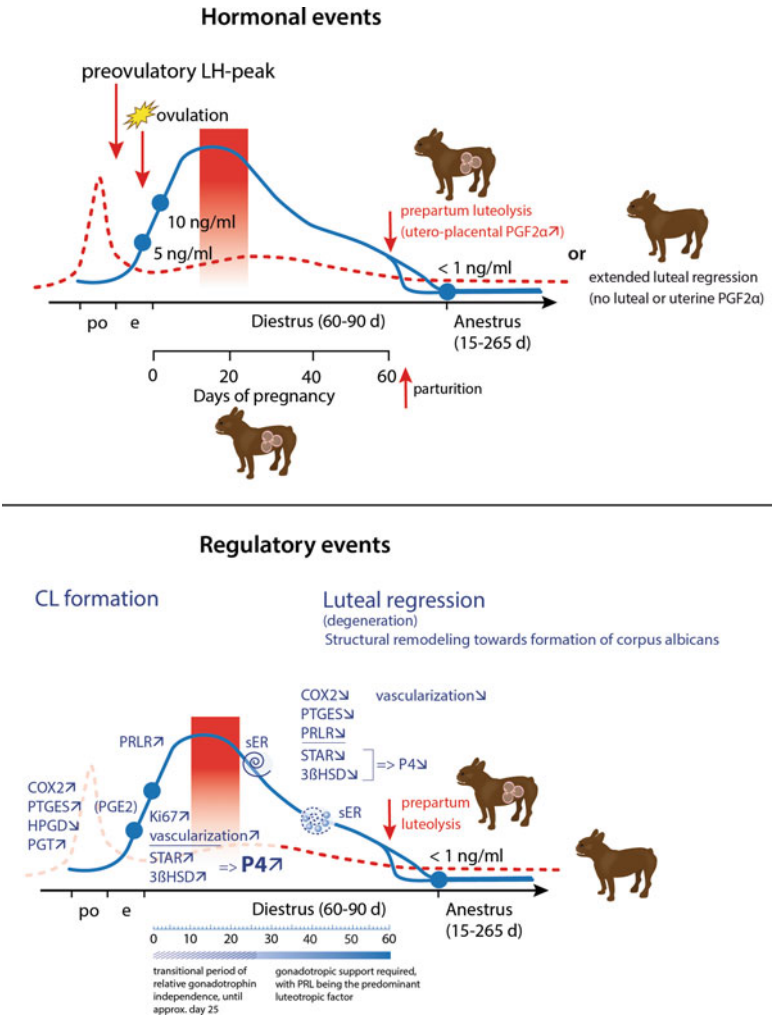

Fig. 8.2 Diagrammatic representation of the most important hormonal mechanisms regulating luteal function during pregnancy and pseudo-pregnancy in dogs. A detailed explanation is provided in the text. *COX2* cyclooxygenase 2 (PTGS2), *PTGES* PGE2-synthase, *Ki67* proliferation marker, *PRLR* prolactin receptor, *LH* luteinizing hormone, *STAR* steroidogenic acute regulatory protein, *3βHSD (HSD3B2)* 3β-hydroxysteroid-dehydrogenase, *PGT* prostaglandin transporter, *HPGD* 15-prostaglandin dehydrogenase, *sER* smooth endoplasmic reticulum, an organelle in which microsomal enzymes such as 3βHSD, CYP19arom, 17αHSD, or inducible PTGES isoform are being synthesized (ongoing degenerative processes characterized by whorl-like structures are indicated). (Modified from [8])

P4 considerably exceeding basal values, reaching levels of about 5 ng/ml at the time of ovulation (Figs. 8.1 and 8.2) [24]. The morphological changes associated with this phenomenon were described for the first time by Bischoff [17], who found

luteal-like structures and strong proliferation and folding areas in canine preovulatory follicles. Recently, these changes have been associated with high local concentrations of prostaglandin (PG) E2 and PGF2α in the newly forming CL [30], indicating the involvement of both PGs in the process of ovulation, similar to events described in other species. The structural formation of CL continues immediately following ovulation and before cessation of the clinical estrus, defined as male acceptance (overt estrus). Thus, already at this time, both functionally and morphologically, ovarian structures enter the stage of luteal dominance, commonly referred to as diestrus. Endocrinologically, estrus ends when plasma E2 concentrations decrease below 15 pg/ml, which is associated with cytological and clinical or behavioral signs of progesterone domination [28].

8.4 Luteal Steroidogenic Activity During Pregnant and Nonpregnant Cycles

The biology of canine CL has been extensively studied and thoroughly discussed recently, covering broad aspects of luteal physiology including growth and maintenance, as well as divergent patterns of slow regression and luteolysis in pregnant and pseudo-pregnant bitches [2, 7–11, 31]. A cumulative schematic representation of the most important regulatory mechanisms is shown in Fig. 8.2.

Corpora lutea develop within the ruptured follicular cavities, which release cumulus oocyte complexes within 12–96 h [32]. In dogs, the number of ovulating follicles varies and, at least to some extent, depends upon the breed and animal size, with smaller breeds ovulating fewer oocytes (2 to 10) and larger breeds ovulating more, 5–15 oocytes [28]. Those follicles not mature enough to ovulate undergo atresia. The ovulated follicles reorganize and luteinize quickly, resulting in a strongly increasing steroidogenic capacity during the early luteal phase, manifested in rapidly rising peripheral P4 levels that reach their highest levels usually within 15–30 days after ovulation [23]. At that time, the average circulating P4 concentrations range between 30 and 35 ng/ml (although sometimes displaying values of 80–90 ng/ml or higher) [2, 24]. Afterward, the steroidogenic capacity of the CL starts to decrease gradually, indicating the turning point in its functional lifespan when the slowly ongoing and considerably extended luteal regression sets in. Because canine CL function remains unaffected by hysterectomy [23, 33], this reveals an inherent lifespan, which in nonpregnant dogs is independent of any acute (luteolytic) regulatory mechanism. It can even last as long as 1–3 months until the peripheral P4 levels reach the baseline limit of <1 ng/ml, indicating, per definition, the onset of sexual quiescence, i.e., anestrus.

During canine pregnancy, the length of the luteal lifespan determines the duration of gestation. It ends rapidly at about 60 days after ovulation, when the so far slow luteal regression is interrupted by a precipitous P4 decline shortly before parturition, referred to as the prepartum luteolysis. This, in dogs, as in most other mammals, is a prerequisite of parturition. Importantly, in contrast to pseudo-preg-

nancy, the concomitant surge of PGF2α in maternal plasma indicates its key role during luteolysis and/or parturition [34].

Although the mean P4 concentrations tend to be numerically higher at pregnancy, mostly due to strong individual variations, they only rarely differ statistically between pregnant and pseudo-pregnant dogs [35]. Sometimes, however, differences can be seen, especially after days 25–30 of gestation, that is, following implantation (which in the dog takes place around day 17–18 of pregnancy) and placentation. It has been hypothesized that the elevated prolactin (PRL) concentrations measured during the same phase of pregnancy might be responsible for this increase [2, 29]. In this context, it is noteworthy that, because of high individual variations in the strongly elevated PRL levels observed in overtly pseudo-pregnant bitches (*lactatio falsa*), similar to P4, PRL cannot be used as a reliable endocrine marker for pregnancy determination in dogs.

In addition, individual E2 levels fluctuate strongly during most of the diestrus period (Fig. 8.3). Following the preovulatory peak, E2 tapers progressively downward over 9–12 days to basal values of 8–9 pg/ml when cytological diestrus is definitely established [27]. This "shift" from estrus to diestrus is characterized by a change in the vaginal cytology picture from 80–100 % of superficial cells to 80–100 % of parabasal and intermediate cells observed at diestrus [28]. Coinciding with luteal formation and increasing P4, from approximately day 10 E2 again increases significantly and stays elevated in both pregnant and nonpregnant dogs, with average levels ranging between 15 and 40 pg/ml depending on the breed, but never reaching the preovulatory levels [2, 23, 27] (Fig. 8.3).

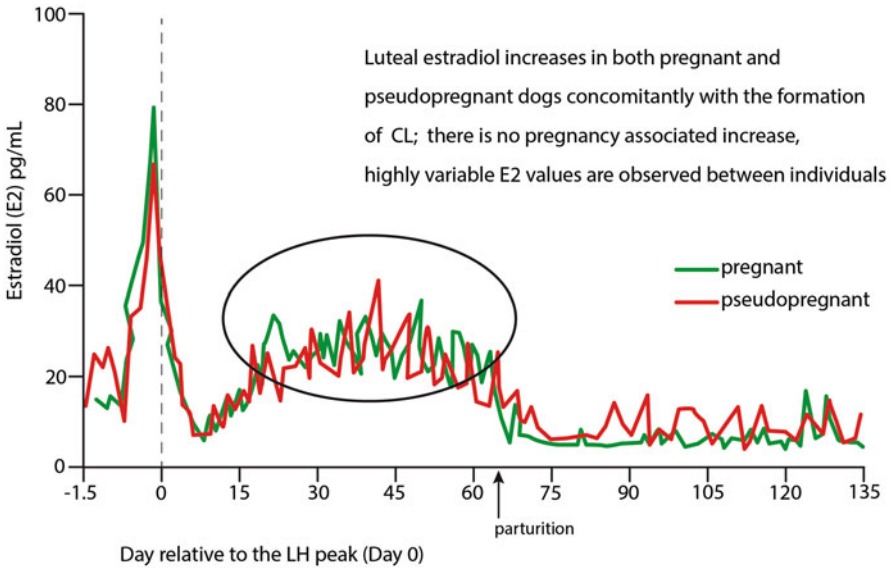

Fig. 8.3 Diagrammatic representation of estradiol (E2) profiles in pregnant and pseudo-pregnant bitches. (Modified after [27])

As mentioned before, there is no pregnancy-associated increase in E2; its profiles, at least in part, parallel those of P4. Beginning on day 60 of the extended luteal lifespan, E2 starts to decline under both conditions. Importantly, and in contrast to other domestic animal species, in pregnant dogs a prepartum drop in E2 is observed during prepartum luteolysis [18]. No hint of placental aromatase activity (CYP19arom) was found [18], and neither could aromatase expression be identified in canine placenta [19]. Its abundant expression was, however, confirmed in the canine CL [11, 19]. This finding, together with the prepartum E2 decline, further indicates its luteal origin. Both P4 and E2 seem to exert paracrine and autocrine effects on canine luteal structures as expression of their respective receptors (PGR, ERα/ESR1, ERβ/ESR2) was found throughout the luteal phase in both steroidogenic and nonsteroidogenic cells [10, 11]. Furthermore, the luteotropic effects of P4 on canine CL arise from the diminishing effects of anti-gestagens on its functionality. Thus, treatment with a PGR blocker unequivocally results in a preterm luteolysis (or abortion) [36, 37]. It is noteworthy that, in addition to the lack of a prepartum increase in estrogens seen in other species, in the dog the parturition-associated increase of cortisol in maternal plasma is not mandatory for normal parturition and can be observed only irregularly [18, 38]. This increase, when present, was attributed by Hoffmann and coworkers to maternal stress [18]. On the other hand, however, some effects of locally produced cortisol cannot be excluded, and it is plausible that the circulating levels observed in maternal blood do not fully reflect its concentrations at the feto-placental level. Accordingly, the placental expression of glucocorticoid receptor is elevated in the dog during normal prepartum luteolysis, but not in response to anti-gestagen treatment when applied to mid-pregnant dogs [39]. This finding suggests that cortisol may be involved in the local withdrawal of P4 at the time of physiological parturition, thereby resembling endocrine mechanisms found in humans [40]. In this context, it needs to be emphasized that, in addition to the divergent profiles of P4 in pregnant and pseudo-pregnant dogs, also the E2 and cortisol secretion patterns indicate the presence of different, species-specific endocrine regulatory mechanisms associated with the cessation of CL function and initiation of parturition.

8.5 Luteal Development: Morphological Aspects and Functional Implications

As in other species, the canine CL originates in ruptured ovarian follicles. The histological analysis of ovarian structures on the day of ovulation (determined by P4>5 ng/ml) reveals the presence of both freshly ovulated and preovulatory follicles, characterized by the aforementioned strong folding of theca interna layers [17], separated from the follicular cavity by the basement membrane [30]. Concomitantly, the shape of luteinizing theca cells changes from elongated to rounded. Following ovulation, in addition to further luteinization of follicular wall structures, the luteinizing granulosa cells can be clearly observed, still at least partly separated from the theca cells by remnants of the basement membrane [30].

As presented in Fig. 8.2, the abruptly increasing luteal P4 secretion is supported by strong proliferative and vasculogenic activities, as indicated by enhanced expression of the Ki67 proliferation marker and increased staining for the endothelial cell marker, endoglin [11, 41]. The vasculogenic and angiogenic activities are reflected in increased expression of vascular endothelial growth factor-A (VEGFA) and its two receptors (VEGFR1/Flk1 and VEGFR2//KDR/Flk1) in steroidogenic and non-steroidogenic cellular components, as reported for the nonpregnant canine CL [42, 43]. This increase seems, at least to some extent, to be driven by hypoxia, which may be concluded from the clearly detectable presence of the hypoxia-inducible factor-1α (HIF1α) [43]. Similarly, during pregnancy expression of the VEGF system is upregulated in steroidogenic and vascular components of the CL and increases with luteal formation, being strongest at the postimplantation stage of pregnancy (days 18–25 of embryonic life) [44]. This stage is also the time when the P4 demand increases to support the establishment of canine gestation through P4-dependent uterine secretory activity. The increased metabolic needs of the CL are reflected in the increased expression of the facilitative glucose transporter GLUT1 (SLC2A1), responsible for glucose uptake [43]. The vascular activity facilitates increased blood flow, as indicated by elevated expression of endothelin receptor B (ETB) in early canine CL of both pregnant and pseudo-pregnant dogs. Of the two endothelin (ET) receptors (ETB and ETA), ETA is responsible for vasoconstriction, whereas occupation of ETB receptors results in nitric oxide-mediated vasodilation [45]. Interestingly, within the canine CL ETB is localized both in lutein cells and vascular endothelium. Along with ETB, increased expression of one of its ligands, ET2, was noted in forming canine CL and was localized predominantly in endothelial cells, thereby implying a functional interplay between these two compartments [46]. The enhanced provision of ETs is signaled by the concomitantly increased presence of their activating enzyme, endothelin-converting enzyme 1 (ECE1), at both locations [46].

Functionally, the dynamically rising P4 output depends on the increased expression of steroidogenic acute regulatory protein (STAR) and 3-β-hydroxysteroid-dehydrogenase (3βHSD, HSDB2) [31, 36, 47, 48], which are key factors regulating steroidogenesis. Their expression throughout the luteal phase closely matches the peripheral P4 concentrations during pregnancy and in nonpregnant cycles. Functional aspects concerning the canine STAR promoter are not yet fully established. Its proximal fragment, homologous with the murine counterpart bearing transcriptional activity comparable to the full-size promoter, has been cloned and characterized [31]. It reveals several putative binding sites for transcription factors, such as C/EBP, SF1, GATA, SREBP, CRE-1, and CRE-3 (transcriptionally active half-sites of CREB found in the murine counterpart): all these are known as positive regulators of STAR expression. Additionally, binding sites for one of the strongest inhibitors of STAR expression, DAX1, were identified [31]. As expected, the cloned fragment of canine STAR promoter proved to be responsive to one of the most potent canine luteotropic factors, namely PGE2 [31].

Morphologically, only one type of steroidogenic cell can be found in mature canine CL, so unlike in other species no distinction between small and large lutein cells is possible. The process of their differentiation from both types of progenitor cells remains to be elucidated. In early CL, on day 5 after ovulation, steroidogenic

cells are irregularly shaped and are 5–10 μm in size. Their cytoplasm includes many small lipid droplets, indicating high rates of metabolic and steroidogenic activity [8, 49]. Although still in the hemorrhagic state, characterized by extravasated erythrocytes, the capillary bed is already well developed. As the lutein cells continue to grow, their size increases, reaching 20 μm at day 15; they become mature at around day 25 of the luteal lifespan with diameters approximately 30–40 μm. At that time, the luteal tissue appears dense, steroidogenic cells are polyhedral, and the vascular bed is fully established, providing virtually every lutein cell with a direct vascular supply, as indicated by the high density of endoglin staining [8, 41]. The number of small lipid droplets decreases; however, their activity remains high as indicated by the increased numbers of mitochondria and smooth endoplasmic reticulum. Proliferation and vascularization rates slow down in mature canine CL. Once the highest steroidogenic activity is over, the slowly ongoing luteal regression is characterized by signs of luteal degeneration. This is first reflected in structural changes of the endoplasmic reticulum, which by day 30 starts to exhibit "whorl-like" structures, and from day 45 on includes large lipid droplets as a further sign of degeneration [8, 10, 49]. The ER also loses its proximity to the nucleus and moves toward the periphery of lutein cells. The number of mitochondria decreases, and the cytoplasm of the lutein cells becomes filled with large vacuoles, another sign of cellular degeneration. The intercellular distances between lutein cells increase along with elevated numbers of matrix and connective tissue components [8]. The density of the vascular bed and the expression of vasculogenic and vasoactive factors decrease together with diminishing steroidogenic activity, as indicated by reduced STAR and 3βHSD expression. Around day 60–65, luteal degeneration is already strongly advanced with large, irregularly shaped lutein cells and an increased incidence of pyknotic nuclei [8]. At the subcellular level, the number of mitochondria is strongly diminished, and degenerative vacuoles fill virtually the entire cytoplasm. Already at this stage the matrix and connective tissue components may indicate the slowly ongoing transition toward corpus albicans formation.

Interestingly, in nonpregnant bitches, all the aforedescribed changes take place in the absence of strong apoptotic events, which can be observed only sporadically [10, 49], indicating that the slow luteal regression is a passive, preprogrammed, degenerative process; this is opposite to the situation in pregnant bitches, where the prepartum luteolysis and accompanying PGF2α increase are associated with massive apoptotic activity within the CL, which can be observed microscopically and is evidenced by the strong expression of active caspase-3 [8]. Interestingly, at least at the mRNA level, the vascular epithelial growth factor (VEGF) system and, therefore, vasculogenic activity remain unaffected during normal and (within the first 24 h of the anti-gestagen treatment) induced luteolysis, compared with its expression at mid-gestation [8]. At the protein level, however, the expression of VEGFA and its VEGFR1 receptor decreases, indicating possible divergence between the mechanisms regulating their mRNA expression and the turnover rates of the respective proteins [44] during cessation of canine luteal function. Concomitantly, the functionality of blood vessels reacts strongly to the luteolytic insult in both situations (i.e., during normal and induced luteolysis), reflected in increased endothelial expression of the vasoconstrictive ETA [46], which remains unaffected during late

luteal regression in pseudo-pregnant bitches. It has therefore been concluded that also where vascular activity is concerned, the extended luteal regression remains primarily a passive process [46].

Among other regulatory components that affect canine CL structurally and functionally are immune system-derived factors. Although under-investigated, CD4- and CD8-positive cells, as well as MHC II-positive cells, cumulatively representing predominantly lymphocytes and macrophages, are present in the CL throughout the luteal phase. Although cells bearing all three differentiation markers can be identified in early CL, renewed infiltration of CD8- and MHC II-positive immune cells could be found in regressing CL (at days 45 and 65 for CD8, and 65 and 75 for MHC II) [10, 41]. By applying qualitative PCR, the expression of IL-8, IL-10, IL-12, TNF-α, and TGF-β, but not of IL-1β, IL-2, and IL-4, could be confirmed [50]. Clearly, further investigations are needed to determine the role of immune system components in canine luteal function.

8.6 Hypophyseal Hormones

Both PRL and LH are luteotropic within the canine CL. There is, however, controversy concerning the exact timing and the extent to which both factors are required for luteal maintenance in the dog [51–55].

Thus, during the first 2–4 weeks of its development, the canine CL appears to be at least in part refractory to hypophyseal influence as early hypophysectomy (on day 4 after ovulation) resulted in only temporary suppression of P4 secretion, which was attributed to postoperative stress. This was followed by a 6 to 10-day recovery phase, with the luteal lifespan, however, being shortened compared with controls [55]. In the same study, when dogs were hypophysectomized on day 18 after ovulation, the suppression of CL function was permanent. Based on these observations, it has been concluded that in the dog luteal function is autonomous during a certain period, at least regarding hypophyseal support, or in a broader sense, gonadotropic support. Taking into account the length of the recovery phase observed after early hypophysectomies, it has been postulated that the refractory phase ends on about day 24–28 after ovulation [55]. A contradiction exists, however, with results in a study by Concannon [52], in which hypophysectomies performed between days 10 and 50 of the luteal phase always resulted in premature cessation of CL function within 3–17 days, supporting the conclusion that canine CL is chronically dependent upon gonadotropic support. Further studies are needed to clarify these discrepancies.

Nevertheless, during the second half of diestrus, hypophyseal hormones are needed for maintaining canine CL function, with PRL being the predominant luteotropic factor. The latter is absolutely required from day 25 of the luteal lifespan onward, as clearly presented in functional studies utilizing the dopamine agonist, bromocriptine, for suppression of PRL secretion [53]. Interestingly PRL, but not LH, was able to reverse the negative effects of bromocriptine on P4 production [56]; this further supports the afore-presented, postulated time-dependent luteal sensitivity to

hypophyseal support. Suppression of LH function at comparable time points or later during the luteal lifespan (i.e., on days 25, 30, 31 of the luteal phase [53], or day 42 after the onset of estrus [51], or between days 30–34 and at day 40 after the LH surge [54]), resulted either in temporarily decreased P4 release, or did not affect circulating P4 levels, indicating the subordinate role of LH as a luteotropic agent compared with PRL. Its role, however, in regulating canine CL function is illustrated by the stimulatory effects evoked by LH treatment on PRL secretion and its direct positive effects on P4 production observed in some studies [52, 54]. PRL, on the other hand, did not stimulate LH secretion and neither did it directly stimulate P4 levels, indicating its role in maintenance or support of CL function and slowing down of luteal regression, rather than active stimulation of P4 secretion [56]. This finding is supported by the observation that the progressive luteal regression that takes place during the second half of diestrus cannot be prevented despite the increasing bioavailability of PRL and LH [57–61]. Especially in pregnant bitches, the levels of PRL increase continuously and significantly during the second half of gestation toward parturition, displaying maximal values of about 50 ng/ml close to term [57]. In pseudo-pregnant bitches, it remains at basal levels during most of the luteal lifespan, increasing only about two- to threefold from initial values of 2–4 ng/ml to their maximal levels of approximately 9 ng/ml at the time point corresponding to parturition [57, 58]. Interestingly, PRL can be produced by the CL, but its expression is low and does not seem to contribute significantly to the overall circulating levels (our data, unpublished). Apparently, some local autocrine or paracrine effects cannot be excluded, especially because the PRL receptor (PRLR) is continuously expressed in CL [62]. The expression of PRLR is time dependent, at both mRNA and protein levels, being abundantly present in early CL and decreasing significantly during regression toward the end of the luteal life in nonpregnant and pregnant dogs, respectively. It could be speculated that the decreased expression of PRLR during the course of luteal regression might be, at least in part, responsible for the diminishing sensitivity of the CL toward hypophyseal support, contributing thereby to its degeneration. On the other hand, however, the degenerative processes might be responsible for falling PRLR expression. Analogous to what has been described for other species, such as pigs or monkeys [63, 64], it could be hypothesized that the enhanced PRL secretion observed during pregnancy derives from an increasing secretion of relaxin from placental syncytiotrophoblast [25], simultaneously or slightly earlier (days 25–30 after the preovulatory LH surge), which stimulates, in turn, hypophyseal PRL production.

8.7 Intraluteal and Extraluteal Prostaglandins

As in other species, in the dog, prostaglandins (PGs) appear to be major players regulating canine CL function. The locally produced, that is, intra-CL, PGs are especially involved in its formation and establishing P4 production, but not in the cessation of luteal function. Consequently, the early luteal phase is associated with strongly increased cyclooxygenase 2 (COX2/PTGS2) and PGE2 synthase (PGES, PTGES) [31, 36, 65], accompanying progressively rising P4 levels. Their

expression decreases significantly in regressing CL and remains low until the end of the luteal lifespan in pregnant and nonpregnant dogs. A similar expression pattern, indicating the involvement of PGs in CL formation, was identified for the PG transporter (PGT) [31]. The expression of HPGD (15-prostaglandin dehydrogenase), the enzyme responsible for degradation of PGs, seemed to be negatively correlated with PTGES and PGT expression, possibly increasing the bioavailability of PGs in the canine CL [8]. At the cellular level, PGE2 stimulates STAR expression, and phosphorylation (i.e., activation) as evidenced by its increased protein expression and steroidogenic output from cultured canine lutein cells isolated during early diestrus [31], proving the luteotropic capacity of PGE2 in canine CL.

The PGE2-mediated regulation of STAR is cAMP/PKA dependent, and two of the PGE2 receptors (EP2/PTGER2 and EP4/PTGER4) known to act via this pathway are clearly detectable in the canine CL throughout the luteal phase [31, 66]. Interestingly, the expression of 3βHSD remains unaffected by PGE2 treatment [31]. A functional in vivo proof, and compelling evidence for the luteotropic function of PGs in canine CL, have been provided by applying the selective COX2 blocker firocoxib in nonpregnant dogs up to day 30 after ovulation [30, 67]. This treatment resulted in inhibition of the steroidogenic machinery, reflected in lowered STAR and 3βHSD expression and reduced P4 concentrations. Additionally, the expression of PTGES and PRLR was significantly suppressed. The latter, together with stimulatory effects of PGE2 on PRLR expression presented in the same study in in vitro cultured lutein cells, indicates possible indirect effects of PGE2 on local PRL availability, by regulating the expression of its receptor [30, 67]. Additional indirect effects of PGE2 in the dog CL arise from the observation that it increased ETB expression in early lutein cells in vitro, possibly contributing thereby to the increased blood flow in the forming CL [46].

It is noteworthy that, although only low or no expression of PGF2α-synthase (PGFS/AKR1C3) can be detected in canine CL throughout the luteal phase under both pregnancy and pseudo-pregnancy, the PGF2α receptor (FP, PTGFR) can be clearly detected throughout the luteal lifespan [36, 68, 69]. PGFS/AKR1C3 is the only canine-specific PGF2α-synthase identified so far and is responsible for the direct conversion of PGH2 to PGF2α [68].

The sole target of PGF2α appear to be on lutein cells, where the FP receptor is localized [69]. Its constitutive expression points toward a basic capability of the canine CL to respond to PGF2α during its entire lifespan. Indeed, PGF2α is luteolytic in the dog even as early as day 5 of the luteal lifespan [70, 71], although this requires high dosages or repeated treatments accompanied by strong side effects.

8.7.1 Prepartum Luteolysis

Although the nonpregnant canine CL apparently lacks an internal PGF2α source, allowing it to persist for a long time, the prepartum increase in circulating PGF2α seems to originate in the pregnant uterus, where the increased COX2 expression is predominantly localized in fetal trophoblast cells [37] (Fig. 8.4).

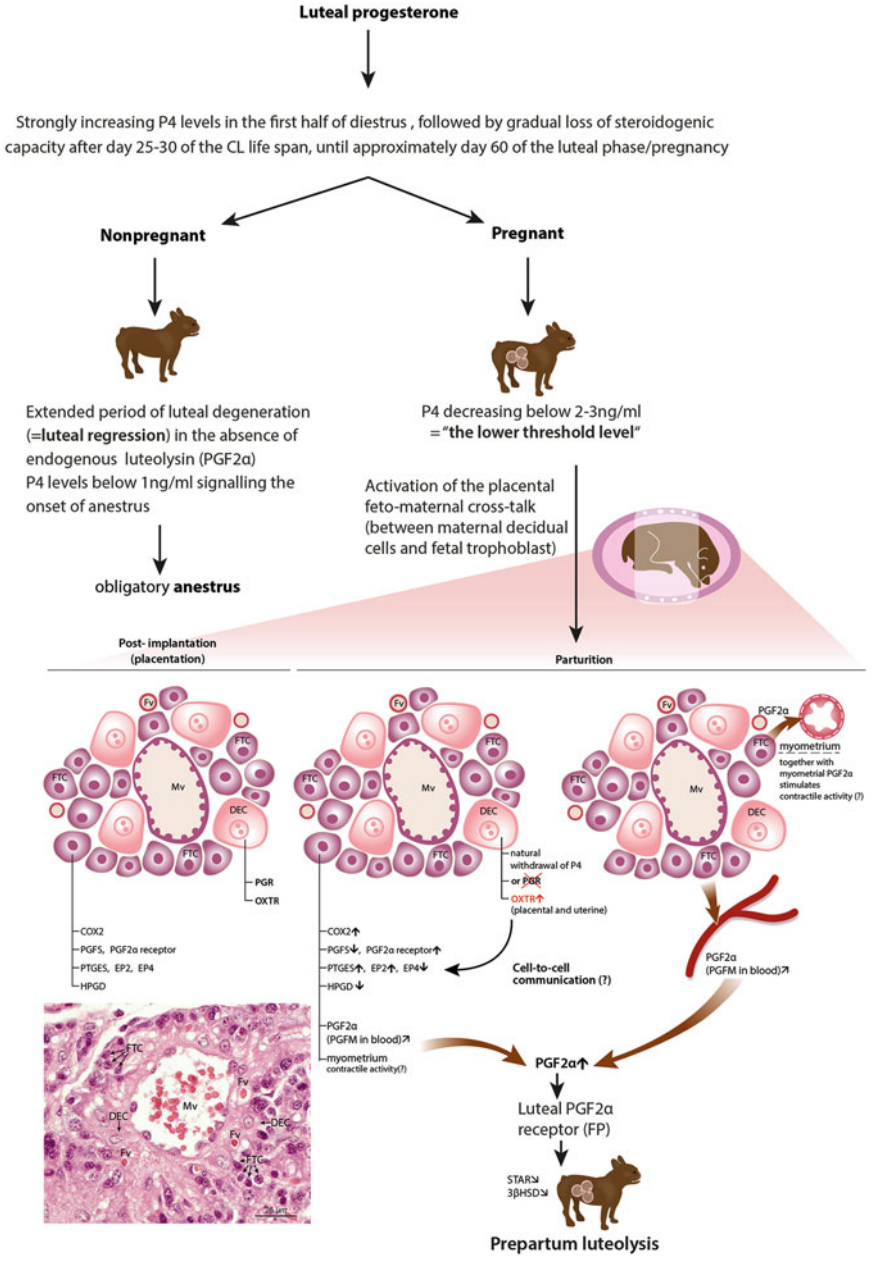

Fig. 8.4 Schematic illustration of the differential mechanisms regulating luteal function in pregnant and pseudo-pregnant dogs. A proposed model of the placental endocrine cascade involved in the prepartum output of the luteolytic PGF2α is presented. A fragment of the canine placenta endotheliochorialis is represented schematically and depicted in the micrograph. Shown are maternal decidual cells (DEC, the only cells of the canine placenta expressing progesterone receptor,

The uterine expression of COX2 is targeted mostly to the myometrium, indicating its contractile functions [72]. PGFS/AKR1C3 does not seem to be responsible for the utero-placental synthesis of PGF2α at the time of prepartum luteolysis, as its expression is downregulated at that time. Instead, the concomitantly increased expression of microsomal PTGES implies the presence of alternative pathways involved in prepartum PGF2α release in the dog, for example, those utilizing PGE2 as a substrate for PGF2α synthesis. Indeed, during prepartum luteolysis the respective biochemical capabilities of canine uterine and placental homogenates have been confirmed [37, 68, 73].

As demonstrated by applying an anti-gestagen (aglepristone) to mid-pregnant dogs, P4 signaling seems to play a major role in the underlying feto-maternal communication leading to the prepartum PGF2α output. While the P4 receptor (PGR) is localized solely in the maternal stroma-derived decidual cells, interfering with its function evokes changes in the uterine and placental PG system similar to those observed during normal prepartum luteolysis, resulting in enhanced PGF2α synthesis and, unequivocally, leading to pre-term luteolysis [37] (Fig. 8.4). The role of the placental oxytocin receptor (OXTR) as a possible signaling molecule mediating the prepartum PGF2α production arises from its colocalization with PGR in the maternal placenta and increased expression during both normal and induced parturition [74].

8.8 Perspectives

Having discussed the most important endocrine mechanisms governing luteal function, it becomes obvious that many of the regulatory aspects, especially those related to the cessation of CL function in pregnant and pseudo-pregnant bitches, remain to be further elucidated. In line with this, global transcriptomic studies involving next-generation sequencing (RNA-Seq) have been initiated aiming at identifying novel potential regulatory pathways and new candidate genes involved in the underlying cellular processes (our data, unpublished). Genes differentially expressed in CL collected from pseudo-pregnant bitches during late luteal regression (day 65 after ovulation) were compared with those expressed in CL derived from normal prepartum

Fig. 8.4 (continued) PGR, and oxytocin receptor, OXTR), and fetal trophoblast cells (FTC). Intercellular communication during the onset of parturition is presented, including cross-communication between these two cell types, resulting in strong induction of fetal placental prostaglandin (PG) synthesis and coinciding with the high prepartum PGF2α output. The expression of several regulatory factors is indicated. Blocking PGR function in the placenta materna (decidual cells) leads to similar cellular effects (at least with respect to utero-placental PG synthesis) as during normal parturition. A detailed explanation is provided in the text. *Mv* maternal blood vessel, *Fv* fetal vessels, *COX2* cyclooxygenase 2 (PTGS2), *PGFS* PGF2α-synthase (AKR1C3), *FP* PGF2α-receptor, (*PTGFR*) *PTGES* PGE2-synthase, *EP2* and *EP4* respective PGE2 receptors (PTGER2 and PTGER4), *HPGD* 15-prostaglandin dehydrogenase (deactivator of PGs). (Modified after Kowalewski [7, 8])

luteolysis. Most of the functional terms identified during late luteal regression were related to the cellular and extracellular matrix remodeling processes. On the other hand, prepartum luteolysis was dominated by expression of genes related to immune and inflammatory responses, indicating an ongoing acute process, contrasting thereby with the passive formation of the corpus albicans, and further pointing towards the luteolytic nature of circulating PGF2α. This was also indicated by the higher expression of genes related to steroid receptor activity in samples derived from late luteal regression, which were acutely suppressed during prepartum luteolysis. When compared with samples obtained from dogs in which luteolysis was induced at mid-gestation using the anti-gestagen aglepristone, the inflammatory events prevailed in those samples derived from normal prepartum luteolysis. Among the most important overrepresented functional terms resulting from the anti-gestagen-mediated withdrawal of P4 function were events related to the inhibition of transcriptional activity, negative regulation of gene expression, and negative regulation of cell proliferation. In both luteolytic groups, genes related to lipogenesis and steroid synthesis were affected. It thus seems that, even though similar at the functional level, that is, resulting in diminished steroidogenic output due to apoptotic events preventing STAR production and function, luteolysis evoked by PGR blockage is more strongly related to the deprivation of luteotropic P4 effects than to the PGF2α-related inflammatory reaction observed in natural parturition.

8.9 Feline Luteal Function: Species-Specific Peculiarities and Comparative Aspects

The most important endocrine events characterizing the reproductive cycle of the domestic cat are presented in Fig. 8.5.

8.9.1 Periovulatory Events

Domestic cats are typically seasonally polyestrous, especially when kept in temperate zones. Variations, however, can occur between latitudes, depending on the length of photoperiods to which the females are exposed, resulting in year-round cycles observed under equatorial or near-equatorial photoperiods. Thus, long-day photoperiods are stimulatory for estrus, and melatonin-suppressing effects on estrogen synthesis, post-coital LH release and, thereby, cyclicity were described [75]. In temperate climate zones, besides the late autumn and winter anestrus, and in the absence of mating or pseudo-pregnancy, cats are polyestrous. The breeding season usually starts in January to February and continues until September [28]. An average interestrous interval, with low (below 20 pg/ml) or basal E2 concentrations, usually lasts 8 days [28] but can be as long as 2–4 weeks [76]. During estrus, average E2 concentrations range approximately between 20 and 80 pg/ml [77]. The LH release starts

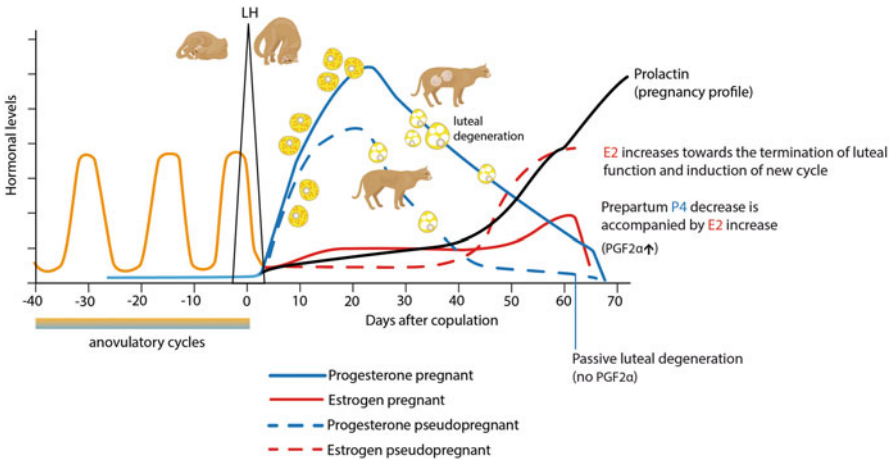

Fig. 8.5 Diagrammatic representation of the reproductive cycle in the domestic cat. Dynamic hormonal changes characteristic of pregnancy and pseudo-pregnancy are depicted. The degenerative processes associated with luteal regression are indicated: type I vacuolation (small vacuoles positive for lipid staining) is associated with increased steroidogenic output and is observed during formation and maintenance of the CL; type II vacuolation displaying large degenerative vacuoles negative for lipid staining can be observed during luteal regression in both pregnant and pseudo-pregnant queens [13]

within minutes following coitus, peaks at 2–4 h, and returns to baseline within 16 h or less [78, 79]. Ovulation begins about 24 h from the initial increase of LH and continues until approximately 32 h from the initial copulation; only one coitus can be enough to induce prolonged LH release and can result in ovulation [78]. However, more frequently, multiple copulations are needed to achieve the higher LH levels required for induction of the ovulation process, with about 50 % of queens ovulating after a single copulation [79]. The number of matings does not influence the number of ovulated follicles and thereby the subsequent number of CL.

Although traditionally considered as induced ovulators, spontaneous ovulations can be observed in queens even at frequencies of 35 % to approximately 60 % when they are kept in proximity to each other, and in pheromonal but not physical contact with males [80, 81].

8.9.2 Postovulatory Endocrine Patterns During Pregnancy and Pseudo-Pregnancy, and Sources of Circulating Hormones

Following ovulation, P4 starts to increase within 1–2 days [82] reflecting the formation of the functional CL. In cats that ovulated but did not conceive, CL of pseudo-pregnancy are formed, while in pregnant queens *corpora lutea graviditatis* develop.

The duration of the initial increase in P4 production is similar in pregnant and pseudo-pregnant cats until approximately days 10–12 of gestation, when implantation takes place. Afterward, in pregnant animals P4 concentrations increase dynamically, reaching peak values of approximately 30–40 ng/ml at day 21. Thereafter, a gradual decrease begins, with circulating P4 concentrations falling to approximately 13 ng/ml at day 50, and decreasing further toward parturition (days 63–65). Baseline P4 concentrations are not prerequisite for the onset of parturition [77]. These baseline levels, that is, <1 ng/ml, are observed immediately after parturition [83]. A similar initial P4 secretion pattern is observed during pseudo-pregnancy with peak levels, however, lower than during pregnancy, reaching concentrations of about 20–30 ng/ml on day 21 [82, 83]. This is followed by a gradual decline of luteal activity with P4 dropping to <1 ng/ml by days 36–46 post coitum [82, 84]. Thus, the luteal phase in pseudo-pregnant cats lasts about half of its length in pregnant queens [5]. Following pseudo-pregnancy, ovarian activity recommences within 7–10 days [84].

The basic steroidogenic capacity of CL seems to reflect the circulating P4 profiles [16]. The expression of STAR increases toward mid-gestation (3–4 weeks), but not toward the mid-luteal phase during pseudo-pregnancy (days 10–15 of the pseudo-pregnant luteal lifespan); 3βHSD is highest in mid-pregnancy and mid-pseudo-pregnancy, with higher relative amounts of the respective mRNA observed in pregnant animals [16].

Factors at least partly originating from the pregnant uterus and placenta were suggested to be responsible for the differences in the CL lifespan and its P4 output during pregnancy versus pseudo-pregnancy [12]. Thus, contrasting with its canine counterpart, the feline placenta is capable of producing both P4 and E2 [16, 85]. Expression of the respective steroidogenic factors and enzymes (STAR, 3βHSD, and aromatase) has been confirmed [16, 85]. Interestingly, STAR and 3βHSD are localized only in the maternal part of the placenta, namely in decidual cells [16]. The placental P4 and E2 levels and secretion patterns do not, however, mirror their circulating levels [16, 85]. Especially for P4, an inverse relationship between the placental and circulating levels was obvious in the study by Braun and coworkers [85]. It seems, therefore, that in cats as in dogs, the peripheral P4 during pregnancy is predominantly of luteal origin; this is supported by the fact that ovariectomies result in a strong decrease in plasma P4 [86, 87]. However, locally, that is, intraplacentally produced P4 seems to have a supplemental role in supporting pregnancy with mostly local effects. It appears to be sufficient to protect pregnancy in some queens, but not in all, depending on the stage of gestation. Thus, 100 % of cats aborted when ovariectomies were performed on day 35 of gestation, 80 % aborted following ovariectomy on day 40, 40 % aborted after surgery on day 45, and 60 % of queens aborted when ovaries were removed on day 50 of gestation [86].

As in dogs, the feto-placental unit is the main source of circulating relaxin in the cat, although it is also locally produced in the feline CL [88, 89]. It is not detectable during the estrous cycle or pseudo-pregnancy [89]. Relaxin becomes detectable at about day 20–25 of gestation, then increases rapidly, reaches a plateau between days 30–35, staying elevated until 10–15 days before parturition when it starts to decrease gradually toward term, and is undetectable 24 h after delivery [89]. Analogous to

the dog, the mRNA and protein have been found solely in fetal trophoblast cells as the cellular source of relaxin [90].

There is a pregnancy-specific increase in PRL: it is elevated during the last one third of pregnancy, beginning to rise from baseline values of around 7 ng/ml during the 6th week of gestation, and displaying strongly elevated levels from the 7th week, with values of 31 ng/ml on average, and reaching maximal values of around 43 ng/ml for the last 3 days of gestation [91]. During pseudo-pregnancy, PRL fluctuates on a daily basis, but remains generally at its basal levels of around 7 ng/ml [91]. PRL is needed not only for initiation of mammary gland growth and lactogenesis, but also acts as a luteotropic factor important for the maintenance of feline pregnancy; interfering with its secretion, for example, by applying bromocriptine during its pregnancy-related elevated secretion, leads to abortion [92, 93]. In contrast to PRL, LH fluctuates throughout the luteal phase, however, no pregnancy- or pseudo-pregnancy-related increase is observed; instead, it remains low [77, 94].

As already indicated, in the domestic cat, similar to the dog, E2 seems to be also primarily of luteal origin. It is high around the time of mating and decreases afterward in both pregnant and pseudo-pregnant queens. The level of E2 remains low during the first 35–40 days of pseudo-pregnancy; average values of 13–24 pg/ml can be detected. Thereafter, toward the termination of luteal function, it becomes more variable [83, 94]. A similar secretion pattern, with somewhat higher values, is observed during pregnancy; in the second half of gestation E2 concentrations start to vary, commencing with decreasing P4 concentrations, and increase toward parturition [77, 83].

PGF2α is luteolytic as early as days 21–25 of the luteal phase in pseudo-pregnant cats, leading to significant depression of circulating P4 [82]. When applied at days 11–15 of pseudo-pregnancy, PGF2α resulted in only temporary suppression of P4 secretion [82]. In pregnant cats, 100 % aborted when treated with PGF2α from day 33 of gestation [93]. As in dogs, parturition is associated with a prepartum luteolytic mechanism. A significant increase in fecal and serum PGF2α metabolite (PGFM) is observed during the last trimester of pregnancy [95, 96], beginning at about day 41 and reaching a peak about 3 days before parturition [96]. Similarly, elevated PGF2α concentrations can be detected in the feline placenta, mirroring the serum profile and indicating its luteolytic function [95]. This placental signal is missing in pseudo-pregnant queens, with fecal PGFM remaining at basal levels [96]. Moreover, it needs to be emphasized that in cats, as in dogs, ovarian cyclicity is maintained following hysterectomy, precluding the existence of a uterine luteolysin in the absence of pregnancy [97].

8.9.3 Morphological and Functional Implications

As in other species, in cats the residual cells of ovulated ovarian follicles give rise to CL formation. Similar to other species, but in contrast to the dog, the feline CL is composed of large and small lutein cells, both populations possessing steroidogenic activity.

Morphologically, the developmental stages of CL resemble those described for the dog. In this context, an interesting feature of cat CL is the presence of two types of vacuole that, analogous to canine CL, have been identified in CL of both pregnant and pseudo-pregnant queens [13]. The first type (type 1), characterized by small lipid droplets, stains positively with Sudan II and thus reveals the lipid nature of their content, and was associated with the high steroidogenic capacity of the cells. The second type of vacuole (type 2) are larger and scattered throughout the cytoplasm, remaining negative to lipid staining, and are associated with the process of cell degeneration [13]. In pregnant queens, this type of vacuolation replaced the first type by day 38 of gestation, concomitant with greatly decreased P4 production. Strong signs of luteal degeneration were observed by day 48 of pregnancy, with deformed lutein cells containing small, condensed nuclei and increased numbers of non-steroidogenic cells. A similar shift in morphological features of the CL was found in pseudo-pregnant cats during luteal regression.

8.10 Conclusions

Despite obvious differences between dogs and cats concerning their reproductive patterns and the underlying endocrine regulatory mechanisms, at least when it comes to the process of luteal regression, there is a similarity between the two species. In both species, the CL has an inherent lifespan that is not modulated by any luteolysin of uterine origin, dissimilar to most other domesticated animals. Because of this, in both dogs and cats, the luteal phase is greatly prolonged, resulting in physiological pseudo-pregnancy. In contrast, in pregnant animals of both species, there is an active prepartum luteolysis that causes gestation to end and allows parturition to ensue. As this mechanism is absent in nonpregnant females, the length of the luteal lifespan during canine and female pseudo-pregnancy seems to be regulated by aging processes, causing the CL to degenerate and structurally remodel toward *corpus albicans* formation. The inherent luteal lifespan of the CL in nonpregnant cats seems to be, however, much shorter than in the dog. The reason for the persistence of P4 and maintenance of pregnancy over the time span of pseudo-pregnancy may lie in factors of placental origin, including placental steroidogenesis. Finally, also *per analogiam* with the canine species, intraluteally produced PGs appear to be more involved in formation of the feline CL, with increased activity of PGE2, than in the luteolytic action of PGF2α during its termination [98].

Finally, the control of CL function in dogs and cats appears to represent a more primitive mechanism than in other domesticated animals, in which luteotropic and/or luteolytic agents have evolved to play a role in its longevity or demise, respectively.

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Chapter 9
Luteolysis in Ruminants: Past Concepts, New Insights, and Persisting Challenges

Rina Meidan, Eliezer Girsh, Roni Mamluk, Nitzan Levy, and Svetlana Farberov

Abstract It is well established that in ruminants, and in other species with estrous cycles, luteal regression is stimulated by the episodic release of prostaglandin F2α (PGF2α) from the uterus, which reaches the corpus luteum (CL) through a countercurrent system between the uterine vein and the ovarian artery. Because of their luteolytic properties, PGF2α and its analogues are routinely administered to induce CL regression and synchronization of estrus, and as such, it is the basis of protocols for synchronizing ovulation. Luteal regression is defined as the loss of steroidogenic function (functional luteolysis) and the subsequent involution of the CL (structural luteolysis). During luteolysis, the CL undergoes dramatic changes in its steroidogenic capacity, vascularization, immune cell activation, ECM composition, and cell viability. Functional genomics and many other studies during the past 20 years elucidated the mechanism underlying PGF2α actions, substantially revising old concepts. PGF2α acts directly on luteal steroidogenic and endothelial cells, which express PGF2α receptors (PTGFR), or indirectly on immune cells lacking PTGFR, which can be activated by other cells within the CL. Accumulating evidence now indicates that the diverse processes initiated by uterine or exogenous PGF2α, ranging from reduction of steroid production to apoptotic cell death, are mediated by locally produced factors. Data summarized here show that PGF2α

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stimulates luteal steroidogenic and endothelial cells to produce factors such as endothelin-1, angiopoietins, nitric oxide, fibroblast growth factor 2, thrombospondins, transforming growth factor-B1, and plasminogen activator inhibitor-B1, which act sequentially to inhibit progesterone production, angiogenic support, cell survival, and ECM remodeling to accomplish CL regression.

Keywords Prostaglandin F2α • Luteal steroidogenic cells • Luteal endothelial cells • Progesterone • Vascularization • Extracellular matrix

9.1 Origin and Identification of Luteolytic Substances: An Historical Overview

Articles published in the late 1960s indicate that, in many species, the nongravid uterus is involved in the cyclical regression of corpora lutea (CL) [1]. For example, hysterectomy prolongs the lifespan of sheep and guinea pig CL [2]. In addition, grafting endometrial tissue into hysterectomized animals terminates luteal function [1]. It was later discovered that the proximity of the ovary to the endometrium is important so that transplantation of the ovary and uterus, together to the neck, results in regular luteal regression, whereas the CL was maintained when the ovary alone was transplanted to the neck [3]. Furthermore, the CL fails to regress when the ovary is simply surgically separated from its local uterine horn in the abdomen or when the uterus was absent [4]. It was therefore clear that the luteolytic effect of the uterus is a local phenomenon. Lukaszewska and Hansel [5] reported that a low molecular weight substance, extracted from the uterus, has a luteolytic effect. Then the stage was set for Pharriss and Wyngarden's proposition [6] that because prostaglandin F2α (PGF2α) is an abundant uterine prostaglandin and has a pronounced venoconstricting effect, it is potentially a substance that might control luteal regression. Several later reports showed that administering PGF2α shortens the CL lifespan and curtails progesterone secretion. Pharriss and Wyngarden [6] reported that PGF2α administered subcutaneously to rats at 1 mg/kg/day induces a dramatic shortening of pseudo-pregnancy. McCracken and colleagues reported that infusing PGF2α could induce complete luteal regression in sheep with ovarian transplants having vascular anastomosis to the vessels of the neck [7, 8]. There is substantial variability in the frequency and amplitude of PGF2α pulses, as deduced from its main plasma metabolite, 15-keto-13,14-dihydro-PGF2 (PGFM), associated with ruminant luteolysis, but typically, there are 48 discrete pulses that occur at 6- to 14-h intervals [9].

Recently, this issue has been reexamined. To mimic the physiological concentration and the pulsatile release of PGF2α that occurs during natural luteolysis, the Wiltbank laboratory [10] employed multiple low-dose pulses of PGF2α. They found that although the initial PGF2α pulse had a distinct effect on luteal mRNA concentrations, the second and ensuing pulses of PGF2α established distinct patterns of gene expression that result in luteolysis [10]. This study therefore shows that

repeated exposure to PGF2α is necessary for continuance of luteolytic pathways within the CL, consistent with the concept of auto-amplification [10, 11]. Such amplification also distinguishes the responses to PGF2α at mid-cycle from the CL at an early luteal stage [11]. The need for several PGF2α pulses (five in this case) at a precise frequency to induce luteal regression in sheep was also reported [12].

A recent study found that transport of PGF2α pulses from the uterus to the ovary at the time of luteolysis in ruminants is regulated by prostaglandin transporter (PGT)-mediated mechanisms. The pharmacological inhibition of PGT protein prevents PGF2α pulses from reaching the endometrium and maintains a functional CL [13].

The CL is also a site of PGF2α synthesis; therefore, the function of luteal PGF2α and its contribution to luteolysis were investigated by several laboratories. The CL contains the rate-limiting enzyme for prostaglandin synthesis: prostaglandin-endoperoxide synthase-2 (PTGS2; or cyclooxygenase-2, COX-2) as well as the specific PGF2α synthases (PTGFS-AKR1B1 and PTGFS-AKR1C3) that convert PGH2 into PGF2α [14]. However, the data concerning their specific upregulation toward luteolysis in sheep and cows have been inconsistent [15–17]; moreover, even if luteal PGF2α biosynthesis is elevated at the time of luteolysis, there is no proof that it participates along with uterine PGF2α in the demise of the CL.

In summary, it is now well established, in ruminants and in other species with estrous cycles, that luteal regression is stimulated by the episodic release of PGF2α from the uterus, which reaches the CL through a countercurrent system between the uterine vein and the ovarian artery [8, 12].

9.2 Luteolysis: Definition

Luteolysis or luteal regression is defined as the loss of function and the subsequent involution of the CL [18, 19]. It is inhibited in the presence of embryonic signals and occurs only in cyclic animals [20, 21]. Cessation of CL function is necessary because the functional CL, producing progesterone, suppresses the final stages of ovulatory follicle development, which sustains a new fertilizable oocyte. The CL must also be physically eliminated to maintain the ovary at its proper size. Endogenous PGF2α or its exogenous administration initiates a cascade of events leading to the irreversible demise of the CL. During this process the CL undergoes dramatic changes in its steroidogenic capacity, vascularization, ECM remodeling, and cell viability, resulting in a tissue composed mainly of connective tissue—the corpus albicans [22–24]. Although luteolysis is a continuous process, a distinction can be made between functional and structural luteolysis, which differ in their temporal and mechanistic features. Functional regression is a phase in which progesterone production declines rapidly (within several hours) [18, 22, 23]. During the second, longer phase of structural regression, the CL decreases in size, ECM composition changes, and cellular integrity is lost; it is during this phase that the various luteal cell types undergo apoptosis (see Sect. 9.4).

9.2.1 Early Luteal Phase Refractoriness Toward PGF2α

The bovine CL is resistant (or refractory) to the luteolytic actions of PGF2α before day 5 of the estrous cycle [25–28]. The refractory period exists even though the early bovine CL contains the receptors for PGF2α and can respond to its injection, by changing hormone secretion [oxytocin and basic fibroblast growth factor 2 (bFGF or FGF2)] as well as by changing the gene expression patterns [11, 27–29]. This phenomenon is also seen in other species, for example, before day 8 of the 20-day luteal phase in marmoset monkeys [30] and before day 4 in pregnant or pseudo-pregnant rats [31].

To obtain a better understanding of early CL resistance to PGF2α-induced luteolysis, Mondal et al. compared the transcriptome of PGF2α-responsive versus PGF2α-refractory CL (day 11 and day 4, respectively) before and at two time points post PGF2α: 4 and 24 h. [11]. At 4 and 24 h post PGF2α, 221 (day 4), 661 (day 11), 248 (day 4), and 1421 (day 11) PGF2α-regulated genes were identified [11]. There were specific functional gene categories and pathways (immune related, angiogenesis, apoptosis, and many others) that were regulated only by PGF2α on day 11 CL, particularly at 24 h post injection [11]. This work revealed, however, that a considerable proportion of transcripts (25 %), regulated at 4 h after PGF2α in the day 11 cow CL, were similarly regulated in day 4 CL that failed to regress. The significant, but transient gene expression response on day 4 suggests that although the initial response occurred (at 4 h), it was subsequently blocked or failed to amplify (at 24 h) [11]. It was further discovered that the young CL activates survival mechanisms (such as FGF2) (see below in Sect. 9.5) that enables it to evade luteal regression.

9.3 Mechanism Underlying PGF2α Actions: Functional Luteolysis

9.3.1 Direct Effects on Progesterone-Producing Cells

The tight correlation between PGF2α pulses and the reduction of luteal progesterone output, its quick decline in response to bolus luteolytic PGF2α doses, and the presence of PGF2α receptors (FP, PTGFR) in steroidogenic luteal cells all support the concept that PGF2α has direct anti-steroidogenic actions on luteal cells. Indeed, starting from the 1980s, numerous studies have investigated a possible link between PGF2α and progesterone synthesis. Several propositions were made, suggesting the involvement of PGF2α in LH receptor actions [32], Ca^{2+}/protein kinase C (PKC) second-messenger systems [33, 34], and lipoprotein-stimulated progesterone production [35]. However, despite the massive research effort (see previous reviews [19, 36, 37]), no agreed mechanisms by which PGF2α exerts a direct anti-steroidogenic effect could be determined. At the same time, there were reports demonstrating that PGF2α paradoxically increases progesterone production [38–41]. It was then proposed that these inconsistent results [24, 36, 38–42] were caused by the variable degrees of

homogeneity in the luteal cell preparations used, which typically contain other non-steroidogenic cell types. For instance, contradictory results were obtained with large ovine luteal cells enriched by elutriation or luteinized bovine granulosa cells, a pure cell population that provides a valid model for large luteal cells [43, 44]. In the first cell type, activation of the PKC effector pathway inhibited progesterone secretion, whereas in the second cell type, PGF2α and PKC effectors elevated agonist-stimulated cAMP and progesterone synthesis [40]. Although these differences could be related to differences in species, Girsh et al. reported that inhibition of progesterone production by PGF2α could be observed in two in vitro models: bovine luteal slices from mid-cycle (maintaining luteal architecture and cell types) and co-cultures of luteal steroidogenic and endothelial cells. These studies imply that the presence of endothelial cells is critical for PGF2α-dependent inhibition of progesterone. Luteal bovine endothelial cells express PTGFR, reported first by Mamluk and colleagues. More specifically, they showed that the three functionally important cell types of the CL (small and large steroidogenic and endothelial cells) express PTGFR. Thereafter, it was confirmed in other studies utilizing CL-derived bovine and porcine endothelial cells [45–47], demonstrating not only their presence but functions such as induction of NOS activity and apoptosis. Functional PTGFR has also been localized to endothelial cells in other tissues such as the eye [48] and endometrial adenocarcinomas [49].

The mediatory role of endothelial cells in the response of uterine (or exogenous) PGF2α may explain its contradicting actions regarding progesterone synthesis: stimulatory when acting directly on steroidogenic cells (paracrine/autocrine mode of action) (Fig. 9.1), and inhibitory when acting as an endocrine hormone reaching the gland via its blood vessels (Fig. 9.1).

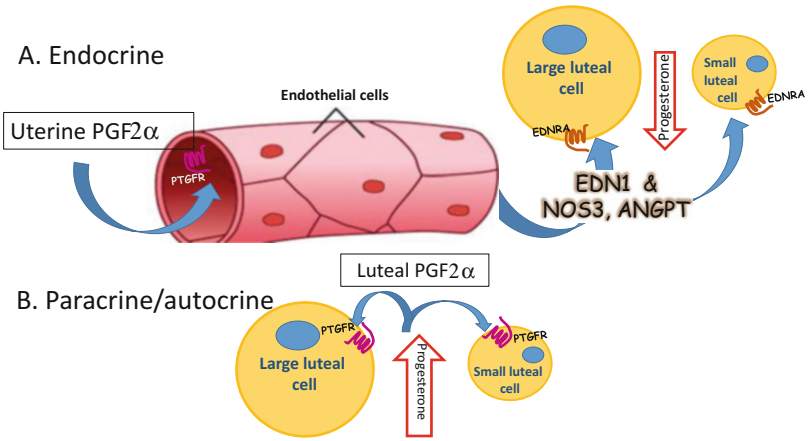

Fig. 9.1 Illustrative summary of the contradictory actions of PGF2α on progesterone production. (**a**) Endocrine mode of action: uterine or exogenously administered PGF2α, reaching the corpus luteum via blood vessels, induces vasoactive compounds such as endothelin-1 (EDN1), angiopoietin (ANGPT), and eNOS (NOS3). EDN1 through its EDNRA receptor present on luteal steroidogenic cells acts to decrease progesterone synthesis. (**b**) Paracrine/autocrine mode of action: locally produced PGF2α, or when cultured in vitro, via its receptor PTGFR expressed on small and large steroidogenic cells acts directly on steroidogenic cells and stimulates progesterone synthesis

9.3.2 Indirect Effects on Progesterone Production: Luteal Endothelial Cells

Adequate blood supply is required for the optimal formation, development, and function of the CL. Indeed, the development of the CL is accompanied by extensive angiogenesis, resulting in a network of capillaries that serve as the delivery route for biological effectors from within and outside the ovary [23, 50, 51]. Endothelial cells lining these capillaries are estimated to comprise half the cells within the CL [52, 53]. Because of its strategic location, the endothelium layer can integrate a myriad of physical and biochemical signals within an organ. Endothelial cells sense changes in blood flow, blood pressure, and oxygen tension [52, 54], to which they respond by the appropriate upregulation of vasoactive compounds such as endothelin-1 (EDN1), angiopoietin (ANGPT), and eNOS (NOS3).

9.3.3 Endothelin-1

The endothelium is the primary source of EDN1 [55]; therefore, being a highly vascular tissue [56] the CL can produce EDN1. Nevertheless, EDN1 synthesis by the CL is not merely a reflection of endothelial cell density, but rather it is hormonally regulated. The highest levels of EDN1 mRNA were found in the bovine CL on day 18 of the cycle, as compared with days 5 and 10 of the cycle [57, 58]. High EDN1 levels in the regressed CL suggest that PGF2α could induce EDN1 expression. Indeed, since the initial observation [57], many reports have confirmed that PGF2α, either in vitro or in vivo, quickly augments luteal expression of EDN1 mRNA and peptide content, as has been shown in various species and experimental systems [59, 60]. The in vivo administration of a luteolytic dose of PGF2α to ewes and heifers during mid-cycle markedly elevates the expression of EDN1 [58, 61]. Moreover, the ability of PGF2α to exert its luteolytic actions correlated with its ability to stimulate EDN1 synthesis [28]. Challenging the CL before day 5 of the cycle with a luteolytic dose of PGF2α failed to induce EDN1 or its receptors (endothelin receptor type A, EDNRA), in contrast to CL collected on days 7–14 of the cycle [28]. Utilizing in vivo MDS implanted in the CL, which functions like an artificial capillary, Ohtani et al. [58] documented real-time, intraluteal changes in EDN1 and progesterone concentrations. Following luteolytic PGF2α administration, there was a rapid (after 4 h) increase in EDN1. Markedly elevated EDN1 levels were accompanied by a simultaneous inhibition in progesterone produced by the CL [58, 62]. In vitro studies confirmed that EDN1 has a direct inhibitory effect on steroidogenic luteal cells [24, 61, 62] (Fig. 9.2). Physiological concentrations of EDN1 inhibit both basal and LH-stimulated progesterone secretion in a dose-dependent manner. The highly selective EDNRA antagonist, BQ-610, completely blocks the inhibitory effects of EDN1 on both basal and LH-stimulated progesterone production [61, 62]. Therefore, it appears that PGF2α stimulates luteal EDN1, which acts via EDNRA to decrease luteal progesterone synthesis during spontaneous and PGF2α-induced regression of the CL (Fig. 9.2).

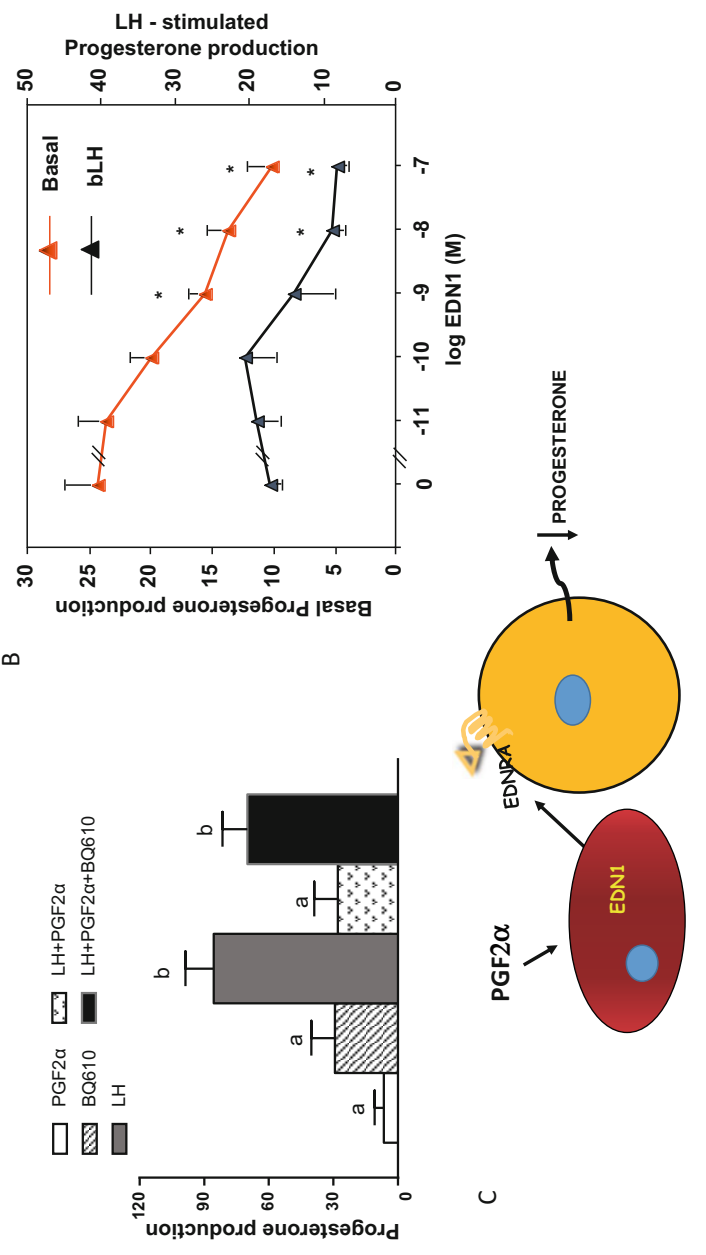

Fig. 9.2 Endothelin-1 (EDN1) directly inhibited progesterone production and mediated the anti-steroidogenic action of PGF2α in luteal slices. (**a**) PGF2α significantly inhibited luteinizing hormone (LH)-stimulated progesterone secretion in luteal slices; the addition of BQ610 (a selective EDNRA receptor antagonist) reversed this inhibitory effect. (**b**) Varying physiological concentrations of EDN1 inhibited both basal (*red line*) and LH-stimulated (*black line*) progesterone secretion from dispersed luteal cells in a dose-dependent manner. (**c**) Graphic summary of the data presented in (**a**) and (**b**): PGF2α stimulates luteal EDN1, which acts via EDNRA to decrease luteal progesterone synthesis

Why does PGF2α fail to induce EDN1 in the young CL? CL refractoriness toward PGF2α may be related to its endothelial cell function, as it coincides with the angiogenic process at this stage. Nevertheless, the exact underlying mechanism, whether it is the paucity of endothelial cells per se or their inability to respond to PGF2α, or both, remains unknown.

With an apparent role for EDN1 in the process of luteal regression, efforts have been made to manipulate ovarian function in vivo using this peptide or its receptor antagonists. For instance, intraluteal EDN1 injections reduce progesterone secretion and facilitate the luteolytic process during the mid-luteal phase in the cow [63], and when the EDNRA antagonist LU 135252 was injected into the corpora lutea of naturally cycling animals, structural luteolysis as well as progesterone synthesis was markedly delayed [64]. In a different study, treatment with an antagonist for EDNRA (BQ-610) alone or in combination with EDNRB (endothelin receptor type B) antagonist (BQ-788), using Alzet mini-osmotic pumps implanted in the ewe's ovary, markedly reduced luteal progesterone content. In contrast, with BQ-788 treatment alone, no effect on progesterone content was observed [61, 63–65]. Although EDN1 alone caused luteal regression in pseudo-pregnant rabbits, in most of the studies EDN1 infusion alone only reduced progesterone production and potentiated sub-luteolytic doses of PGF2α injection. However, these studies could not reproduce the full luteolytic effects of PGF2α or completely inhibit PGF2α-induced luteolysis with EDN receptor antagonists. It remains to be determined whether it is the short half-life of a small peptide such as EDN1 that caused these experiments to be only partially successful or whether there are other reasons. However, it is clear that the chemical nature of EDN1 and its systemic effects preclude its in vivo use for estrous cycle manipulations. Perhaps the development of site-directed delivery of long-acting EDN1 or small molecules that activate EDNRA will make these approaches possible in the future.

9.3.4 Angiopoietins (ANGPTs)

Similarly to EDN1, ANGPTs are secreted by endothelial cells and act as a vasoconstrictor [66]. Angiopoietins have important and varied functions in regulating the integrity of the microvascular network. ANGPT1 expression is thought to be crucial in the interaction between endothelial cells and the surrounding mural cells driving vessel maturation and stabilization. ANGPT1 is the major agonist for the tyrosine kinase receptor, TEK (TIE2), which is found primarily on endothelial cells. ANGPT2, on the other hand, is a TEK antagonist; thus, it disrupts vascularization. The ANGPT system members, the two ligands, angiotensin-converting enzyme (ACE), and the receptors, are all localized in the CL [67]. ANGPT2 was transiently increased after PGF2α injection in cows and sheep. Furthermore, ANGPT2 reduced progesterone released from perfused bovine CL. Although these data tend to support the role of ANGPT2 in luteolysis, its importance remains unclear because of the considerable species differences [51, 68–70], and also because ANGPT2 is an antagonist. Therefore, it is difficult to predict the resulting TIE2/TEK activity. Most studies have

tried to deduce the activity by calculating the ratio between ANGPT2/ANGPT1; however, whether it provides an accurate measure remains unclear. In addition, the effects of ANGPT2 with respect to angiogenesis are dependent on the presence or absence of another key angiogenic factor, vascular endothelial growth factor-A (VEGFA), which further complicates drawing conclusions regarding these findings.

9.4 Effects of PGF2α on the Endothelial–Immune Interface

There is ample evidence demonstrating the important role of immune cells and the related signaling molecules (cytokines) during luteolysis. Endothelial cells have important immunological functions: they actively participate in both innate and adaptive immunity [71]. Activated endothelial cells induce the production of pro-inflammatory cytokines and chemokines, which amplify the immune response by attracting and mediating the extravasations of immune cells. Besides immune cell trafficking, endothelial cells also induce cytokine production in immune cells [71]. Recognition of endothelial antigens by T cells expedites their infiltration into tissues [72]. Recently, gene expression data from transcriptome analyses have provided comprehensive evidence for the infiltration and activation of many immune cell types in the CL following PGF2α administration [11, 73]: these include macrophages (CD14), T lymphocytes (CD1B, CD2, CD3E, CD3G, CD8, CD 48, CD69), dendritic cells (CD83), and natural killer cells (CD2, CD244) [11]. Most of these genes either were not induced at all on day 4 or the genes were stimulated to a lesser extent than on day 11 (CD14, CD69, CD3E). In contrast to the mature gland, immune-related genes were not found any longer at 24 h post PGF2α in the early, day 4 CL [11]. The presence of immune cells in the mature CL [11, 74, 75] may account for the more robust response to PGF2α and the persistent enrichment of PGF2α-regulated immune-related genes on day 11 but not on day 4 CL. Although immune cells lack PTGFR mRNA [76] and the capacity to respond to PGF2α directly, they can be activated by other cells within the CL, including steroidogenic and endothelial cells [76–79].

Notably, PGF2α induced the upregulation of numerous endothelial adhesion molecules such as CCL2 in the responsive CL [11, 80], SELE, SELP, ICAM, and several integrins (ITGA7, ITGAL, ITGB2, ITGB5).

These molecules facilitate leukocyte recruitment and endothelial transmigration [81, 82]. CCL2 was specifically studied in relationship to luteolysis. The monocyte chemotactic protein-1 (MCP-1/CCL2) is a member of the C–C chemokine family, and it is a potent chemotactic factor for monocytes and CD4+ T cells. Its expression was increased near luteolysis in all species that were examined [75, 80, 83–86]. The cellular source of CCL2 in the CL was reported in the endothelial cells of women [85] and cows [76, 80, 86], as well as in luteal steroidogenic cells [83]. Additionally, it was shown that CCL2 can be tethered on endothelial cells by glycosaminoglycan (GAG) side chains of proteoglycans. CCL2 is induced rapidly, within 1–4 h, by PGF2α [80]; if indeed it occurs in endothelial cells, it would strengthen the argument again in favor of the direct action of PGF2α on endothelial cells.

9.5 Actions of PGF2α on Blood Vessels

Luteal regression brings about profound and dynamic changes in blood vessels and endothelial cell morphology and function. In naturally occurring and PGF2α-induced luteolysis, there is an acute increase in luteal blood flow (most likely induced by nitric oxide, NO) before any change in the progesterone concentration becomes apparent. Previous studies indeed showed that PGF2α stimulated eNOS [87].

However, this increase is transient, and soon afterward the blood flow decreases from 8 h post PGF2α onward [88]. Vessel vasoconstriction, mediated by PGF2α itself and EDN1, limits oxygen and the nutrient supply to the gland. However, these changes, although dramatic, do not conclude the participation of the vascular tree in the process of luteal regression.

Accumulating data highlight another aspect related to luteal vasculature: PGF2α modifies the expression of key factors in angiogenesis regulation [29, 70, 89, 90]. Notably, these changes were highly dependent on the luteal stage [29] (Fig. 9.3). The set of factors modulated include pro-angiogenic FGF2 and VEGFA as well as the anti-angiogenic compounds pentraxin 3 (PTX3) and thrombospondins (THBS1, -2), their cell adhesion receptor (CD36), and transforming growth factor-B1 (TGFB1). These studies suggest a potential functional relationship between angiogenesis and

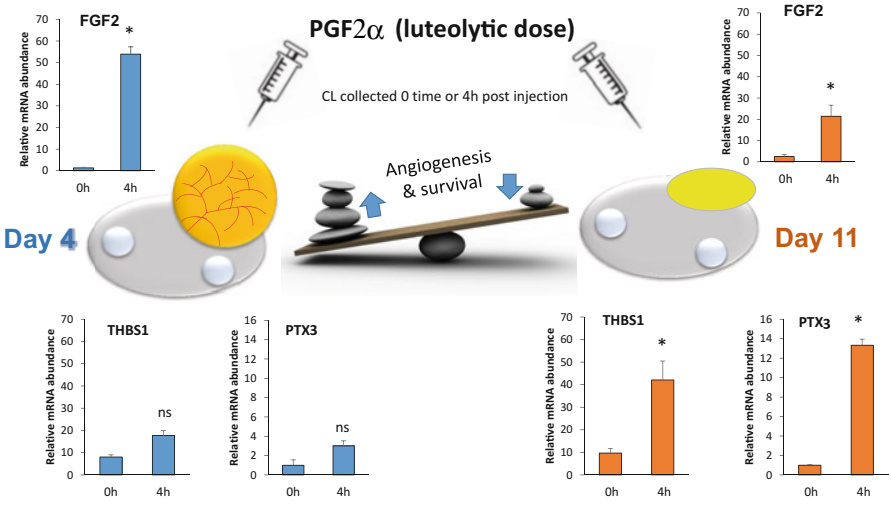

Fig. 9.3 PGF2α regulated angiogenesis-modulating factors in the corpus luteum in a stage-dependent manner. Effect of PGF2α administration on the expression of pro-angiogenic fibroblast growth factor 2 (FGF2) and anti-angiogenic thrombospondin-1 (THBS1) and pentraxin 3 (PTX3) in PGF2α-refractory (day 4) and PGF2α-responsive (day 11) bovine corpus luteum collected before (0 h) and 4 h after PGF2α administration. FGF2 was dramatically increased following PGF2α on day 4 CL. In contrast, the anti-angiogenic factors PTX3 and THBS1 were only induced in the day 11 corpus luteum. Consequently, stage-specific regulation of FGF2 or its modulators by PGF2α may help tilt the balance between pro- and anti-angiogenic processes, thus controlling the ability of the CL to resist or advance toward luteolysis

the luteolytic response to PGF2α. Interestingly, these genes were expressed in both dispersed luteal endothelial and steroidogenic cells; however, THBS1 and FGF2 were more abundant in luteal endothelial cells [29].

FGF2 activity in the extracellular milieu is controlled by its interaction with various extracellular matrix proteins and binding factors. PTX3 and THBS are two prominent examples of such factors [91]. PTX3 binds FGF2 with high affinity and specificity [92–94]. This interaction prevents the binding of FGF2 to its cognate tyrosine kinase receptors, leading to inhibition of the angiogenic activity of the growth factor [29, 91, 95]. THBS1, another potent angiogenesis inhibitor, belongs to a family of extracellular matrix proteins [96]. It binds to various components of the extracellular matrix, such as fibronectin and proteoglycans. In this way, THBS1 is stored in the extracellular matrix, where it folds and changes its conformation [97]. Among thrombospondin family members, THBS2 has a domain structure equivalent to THBS1 and shares most THBS1 functions [96]. THBS1 is a multimodular protein that exerts its anti-angiogenic activity through multiple mechanisms involving different active sequences in different domains [98]. The core of the THBS1 contains type I and type III repeats. Type I repeats and their peptide mimetics have been successfully used to block angiogenesis and tumor growth in preclinical models [99], and in ovarian follicles [100]. Type III repeats of THBS1 (mimicked by small molecule 27) bind FGF2 and inhibit angiogenesis by isolation of FGF2 [101]. Using luteal endothelial cells, it was found that THBS1 and small molecule 27 inhibit FGF2-induced proliferation and migration in vitro (Fig. 9.4) [29]. The THBS1 gene is hormonally regulated; it is the direct target of PGF2α and FGF2 in luteal cells, as demonstrated by in vitro studies [29, 102]. Furthermore, in granulosa cells, THBS1 expression was inhibited by luteinizing signals (LH and insulin). Functionally, THBS1 inhibits FGF2 expression and its activities (i.e., its ability to promote cell proliferation, migration, and survival) [102] (Fig. 9.4). Moreover, recent evidence also showed the ability of THBS1 to activate latent TGFB1 in luteal endothelial cells, which results in its TGFB1-dependent actions [103]. TGFB1 was shown to disrupt the angiogenic potential of microvascular endothelial cells of bovine CL [104]. When treated with TGFB1, the luteal capillary network becomes unstable, with complete regression, demonstrating that TGFB1 induces the disassembly of luteal capillary-like structures [104]. These results suggest that THBS1 is expressed in a physiologically relevant manner and that it functions in the luteolytic process. The two main luteal pro-angiogenic factors, VEGFA and FGF2, were inversely affected by PGF2α. VEGFA mRNA was reduced after PGF2α injection [29, 70, 90]. Importantly, this occurred regardless of the luteal stage [29, 70]. Neuvians et al. [90] also reported that, along with VEGFA, its type 2 receptor decreased during luteolysis, further diminishing VEGFA function.

Expression of FGF2 in response to PGF2α exhibited a completely different pattern; somewhat surprisingly, FGF2 was elevated in the regressing CL. These findings were reported for both sheep and cows [10, 29, 70, 90]. However, this FGF2 elevation should be seen in the correct context, as shown in studies from Meidan's laboratory [29, 102, 103]. First, in the immature day 4 gland, PGF2α induced a robust increase in FGF2, much higher (fivefold) than the day 11 gland; in comparing protein

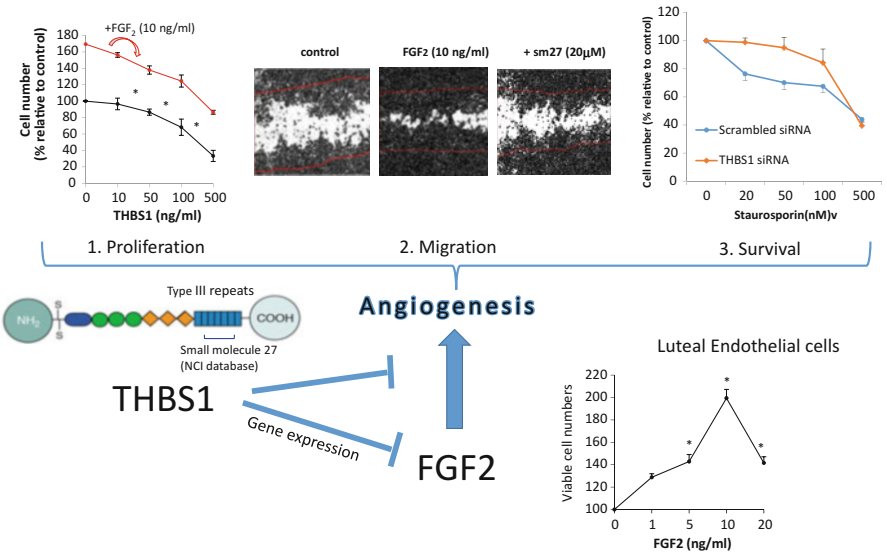

Fig. 9.4 Thrombospondin-1 (THBS1) is a potent anti-angiogenic luteal factor that antagonizes FGF2 actions. THBS1, a multimodular protein, exerts its anti-angiogenic activity through multiple mechanisms in different domains: THBS1 inhibits proliferation. (*1*) Basal and FGF2-induced proliferation of cultured luteal endothelial cells in a dose-dependent manner. (*2*) Migration: addition of small molecule 27 (mimicking FGF2-binding domain in type III repeats of THBS1) reduced luteal endothelial cell migration as compared to FGF2 alone. (*3*) Survival: THBS1-silenced luteal endothelial cells showed better survival rates; the number of viable cells after treatment with staurosporine was significantly higher in THBS1-silenced cells compared to control (scrambled siRNA). THBS1 also suppressed FGF2 mRNA expression in luteal endothelial cells

levels, the increase on day 4 was even more pronounced [29]. Second, inhibitors of angiogenesis, namely, PTX3, THBS1 and -2, and TGFβ1, were elevated only in PGF2α-responsive glands [29, 103, 105], which is further expected to inhibit FGF2 actions. Reduced angiogenic support in response to PGF2α, responding to lower levels of FGF2 and VEGFA, along with increased expression of anti-angiogenic factors (THBS1, -2, PTX3, and TGFβ1) in day 11 PGF2α-responsive CL, is expected to destabilize luteal vasculature and reduce its hormonal output, which is characteristic of luteal regression [10, 23, 29]. Additionally, these events, along with the vasoconstrictive and anti-steroidogenic actions of EDN1 [60, 63], are expected to further reduce progesterone secretion and promote the regression of mature CL.

Conversely, the robust elevation of FGF2 by PGF2α at an early stage (particularly when no FGF2 inhibitors exist) may act as a survival signal for both luteal endothelial cells [29, 106] and steroidogenic cells [107, 108]. Support of blood vessel growth and its stabilization are expected to enhance the supply of nutrients and hormones to the gland, thus promoting its survival and contributing to its ability to become resistant to luteolysis. It still remains to be understood why FGF2 is so abruptly elevated in the early CL; the interplay between FGF2 and THBS1 may be one explanation, but this issue requires further research.

In summary, stage-specific regulation of FGF2 or its modulators by PGF2α may help tilt the balance between pro- and anti-angiogenic processes, thus controlling the ability of the CL to resist or advance toward luteolysis (Fig. 9.3).

9.6 Mechanism Underlying PGF2α Actions: Structural Luteolysis

9.6.1 ECM Remodeling

Structural regression requires extensive tissue remodeling and is essential for the ovary to maintain its proper size. There is continuous crosstalk between cells depositing and degrading ECM components and the differentiated phenotype of the ECM supporting cells. Disruption of the ECM leads to dedifferentiation and ultimately involution and apoptosis of the CL as in many other tissues.

Investigations in several species revealed the stimulation of gene expression and the activity of matrix-degrading proteins during luteolysis. Matrix metalloproteases (MMPs), their inhibitors [109–113], and the plasminogen activator system (PAs) were elevated in luteolysis [109, 114, 115]. Most MMPs and PA members were increased within hours, but some, such as tPA [109] as well as MMP activity [111], were elevated as soon as 30 min post PGF2α administration. These early changes in the tissue inhibitors of metalloproteinases (TIMPs) and MMPs led to the proposition that alterations in the structure of the ECM by PGF2α may be involved in decreasing progesterone during functional luteolysis. However, there is no direct proof for this contention, and ECM remodeling, initiated early during the luteolytic process, is more likely to have a part in the structural demise of the gland. Nonetheless, these studies did not expose a mechanism for PGF2α-induced elevation of ECM-degrading enzymes and their inhibitors. A recent study described the molecular mechanism resulting in the stimulation of one of these proteins, plasminogen activator inhibitor type 1 (PAI-1). Endothelial PAI-1 is a member of the serine protease inhibitor family and is encoded by the SERPINE1 gene. The mechanism underlying the induction of SERPINE1 involves THBS1 and TGFB1, as detailed next. TGFB1 signaling engages phosphorylated SMAD2 and SMAD3, which form a heteromeric complex with SMAD4, which consequently transactivates SERPINE1 promoter [116–118]. Similar to both THBS1 and TGFB1, SERPINE1 expression showed marked luteal stage-specific expression with extensive responses to PGF2α on day 11 [103]. Similarly, THBS1 and SERPINE1 were elevated in sheep CL in response to luteolytic signals [119]. Moreover, the stimulation of these genes was suppressed during early pregnancy, requiring the maintenance of the CL [119]. Farberov and Meidan [103] recently reported that these three genes (THBS1, TGFB1, SERPINE1) are highly expressed in luteal endothelial cells. SERPINE1, being a known downstream target of TGFB1, was indeed elevated in luteal endothelial cells by active TGFB1. However, we also observed that THBS1 elevates SERPINE1, an effect that was abolished by the TGFB1 receptor-1

kinase inhibitor (SB431542). Additional findings further implied that THBS1 activates TGFB1 in luteal endothelial cells: THBS1 increases the effects of latent TGFB1 on phosphorylated SMAD2 (phospho-SMAD2) and SERPINE1. THBS1 silencing significantly decreases SERPINE1 and reduces the phospho-SMAD2 levels. Last, THBS1 actions on SERPINE1 were inhibited by LSKL peptide (TGFB1 activation inhibitor); LSKL also counteracted latent TGFB1-induced phospho-SMAD2 [103]. Taken together, these studies propose the following molecular cascade of events: THBS1 and TGFB1 are directly induced by PGF2α in luteal cells [29]; THBS1 then activates latent TGFB1, which binds its receptors, phosphorylates SMAD2, and upregulates SERPINE1 [103]. Furthermore, TGFB1 enhances its own mRNA expression and that of THBS1, which leads to further induction of SERPINE1 [103]. This feed-forward loop provides a means to sustain and amplify the expression of SERPINE1 (PAI-1) during luteolysis (Fig. 9.5).

How can PAI-1 promote structural luteolysis? There are at least two mechanisms (Fig. 9.5). In the first, PAI-1 inhibits urokinase plasminogen activator (uPA), which converts plasminogen to plasmin, a strong proteolytic enzyme [120–122]. Thus, SERPINE1 is a potent profibrotic factor; excessive PAI-1 expression contributes to the accumulation of collagen and other ECM proteins and thus preserves scarring [123–125]. The scarring, resulting from the profibrotic actions of SERPINE1, may

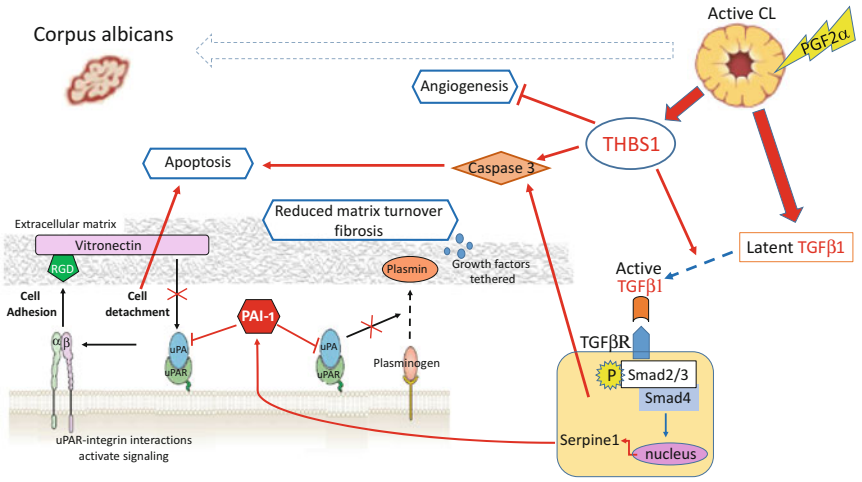

Fig. 9.5 Illustrative summary on the control of expression and roles of thrombospondin-1 (THBS1), transforming growth factor-B 1(TGFB1), and SERPINE1 in structural luteolysis of the bovine corpus luteum. THBS1 and latent TGFB1 are directly induced by PGF2α in luteal cells. THBS1 inhibits angiogenesis and induces caspase 3 and apoptosis in luteal cells. THBS1 also activates latent TGFB1, which binds its receptors, phosphorylates SMAD2 and SMAD3, and upregulates SERPINE1 [encoding plasminogen activator inhibitor-1 (PAI-1)]. PAI-1 blocks the conversion of plasminogen to plasmin by inhibiting urokinase plasminogen activator (uPA), thereby contributing to the accumulation of collagen and other ECM proteins and thus promoting scar formation and the corpus albicans. Additionally, by inhibiting the interaction between the uPA–uPA receptor complex and vitronectin, PAI-1 disrupts cell adhesion, leading to apoptosis

promote the transition of active CL to corpus albicans. In the second mechanism, PAI-1 inhibits the interaction between the uPA–uPA receptor complex and vitronectin, thus decreasing cell attachment and the interaction between αvβ3 integrin and vitronectin, which are important for cell adhesion [126]. Therefore, PAI-1 can reverse integrin-mediated adhesion and can initiate cell detachment leading to apoptosis (Fig. 9.5).

9.6.2 Apoptosis

Several lines of evidence have shown that programmed cell death (apoptosis) occurs during structural luteolysis. In cattle, DNA ladder formation was observed in naturally regressing CL and also 24 and 48 h after PGF2α administration [127]. PGF2α regulates numerous proteins associated with cell survival and apoptosis in cows [105, 128, 129], sheep [119], pigs [130], rodents [131, 132], and primates [133, 134]. Apoptosis in the CL is initiated by both extrinsic (death ligand) and intrinsic (mitochondrial) pathways. Fas ligand, Fas, tumor necrosis factor-α (TNFA), TNFARI, interferon-gamma, and most factors of the extrinsic pathway were markedly upregulated shortly after the PGF2α treatment (starting from 30 min up to 12 h) [128, 135–137]. The intrinsic pathway of CL apoptosis might be attributed to an increase in oxidative stress [138] and the beginning of p53 accumulation in the nucleus of the compromised cells [139]. The factors of the intrinsic pathway, p53, Bax, and Bcl-XL, were mostly later upregulated at 24–48 h after PGF2α [140]. Whether the extrinsic or intrinsic pathway is more important for structural luteolysis remains to be clarified. Nevertheless, there is a consensus regarding the role of caspases in this process. Caspases are crucial mediators of apoptosis. Among them, caspase-3 is a frequently activated death protease, catalyzing the specific cleavage of key cellular proteins in the apoptotic cell by both extrinsic and intrinsic pathways [141]. Caspase-3 is required for typical hallmarks of apoptosis such as chromatin condensation and DNA fragmentation. One could therefore predict that caspase-3 will be activated during luteolysis, as was indeed found in all species examined thus far: rat [142], equine cyclic corpora lutea [143], cattle [134, 144], and ewes [145, 146].

Clearly, a fine balance between numerous pro- and anti-apoptotic or survival factors determines the fate of the CL exposed to PGF2α. Importantly, however, when incubated in vitro with luteal cells of various species, PGF2α is not at all apoptotic; therefore, other factors most likely mediate cell death provoked by PGF2α in vivo. TGFB1 and THBS1 are good candidates for such a role. First, THBS1 and TGFB1 expression exhibited marked PGF2α-dependent and luteal stage-specific expression with extensive stimulation observed on day 11 bovine CL [11, 29, 102]. THBS1 and TGFB1 are also directly stimulated in vitro by PGF2α and its analogues using various luteal cell models [11, 29, 102]. Second, both compounds promote apoptosis in vitro, but THBS1 exhibits significantly stronger pro-apoptotic effects on luteal endothelial cells than does TGFB1 (80 % maximum inhibition of cell numbers versus

30 %, respectively) [103]. These apoptotic effects were accompanied by proportional caspase-3 activation [103]. THBS1 is a known pro-apoptotic factor in numerous tissues; for instance, the systemic treatment of mice with THBS1 increased the number of apoptotic endothelial cells in areas of tumor neovascularization, and endothelial cells (of diverse origin) treated in vitro with THBS1 become apoptotic [98, 100]. In luteinized granulosa cells as well in luteal endothelial cells, THBS1 reduced viable cell numbers and induced their apoptotic cell death, that is, elevated caspase-3 activation and the appearance of apoptotic 4′,6-diamidino-2-phenylindole (DAPI)-stained nuclei [102]. Furthermore, manipulation of endogenous THBS1 by small inhibitory RNA in luteal endothelial cells was directly related to their ability to withstand apoptotic cues [102]. The notion of THBS1 as a negative regulator of the survival and viability of luteal cell types is also illustrated by its antagonistic relationship with FGF2 (previously discussed). Taken together, the findings reported here highlight the role of THBS1, TGFB1, and SERPINE1 in structural luteolysis, and their gene products are expected to promote vascular instability, as well as apoptosis and matrix remodeling of the regressing CL (Fig. 9.5).

9.7 Concluding Remarks

In mammalian species, including cattle, the CL holds the key to reproductive success. Progesterone secreted by the CL controls ovarian cyclicity and is absolutely required during pregnancy. Because of its unique role, CL formation, maintenance, and regression are tightly regulated. In the early 1970s McCracken first reported that PGF2α pulses from the uterus are obligatory for luteolysis in sheep. Since then, PGF2α and its analogues have been widely used in laboratory and farm animals, and numerous research studies have been devoted to advancing our understanding of the underlying mechanisms associated with luteolysis. Important novel findings regarding the luteolytic cascade and the development of luteolytic capacity were reviewed here. Accumulating evidence now indicates that the diverse processes initiated by uterine or exogenous PGF2α, ranging from reduction of steroid production to apoptotic cell death, are mediated by factors produced locally by one or more luteal cell types. The complexity of cell interactions needed to achieve luteolysis may explain why it is so difficult to establish an in vitro model that can mimic the events that occur in PGF2α-responsive or PGF2α-resistant CL.

Despite the knowledge gained, much remains to be discovered. The lack of a means to block luteolysis in the PGF2α-responsive CL or promote luteolysis in the PGF2α-resistant gland continues to impede improvements in reproductive treatment and technologies, and this remains a scientific challenge.

Beyond reproduction, because luteolysis represents a unique case of physiological tissue involution that occurs periodically in the adult female, it may hold the answers needed to devise effective tools for involution of pathological tissues such as tumors and some non-tumor tissues.

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Chapter 10
Corpus Luteum Rescue in Nonhuman Primates and Women

Richard L. Stouffer and Jon D. Hennebold

Abstract The primate corpus luteum undergoes a process at the end of a nonfertile menstrual cycle termed luteolysis, which involves considerable structural and functional changes that lead to a loss in the ability to produce the steroid hormone progesterone. Because progesterone is critical for events involved in embryo implantation and sustaining pregnancy, the survival and continued function of the corpus luteum are required throughout the first weeks of pregnancy, after which the placenta becomes responsible for the maintenance of gestation. Extension of the functional lifespan of the primate corpus luteum is achieved through the secretion of chorionic gonadotropin (CG) from the conceptus. CG signals through the luteinizing hormone–chorionic gonadotropin receptor (LHCGR) located on luteal cells to override the cellular and molecular events that are responsible for the demise of the corpus luteum during nonfertile cycles. Thus, in this chapter, the source of various CG forms and the regulation of their production, as well as the mechanisms through which LHCGR signaling regulates cellular activities in the primate corpus luteum during early pregnancy are reviewed. Also, current and possible uses of hCG forms for diagnosis and treatment of infertility and pregnancy disorders are considered.

Keywords Primate • Pregnancy • Maternal recognition of pregnancy • Corpus luteum • Luteolysis • Luteal rescue • Chorionic gonadotropin • Luteinizing hormone–chorionic gonadotropin receptor • Progesterone • Relaxin

10.1 Introduction

A vital process in the ovary of mammalian species is the differentiation of the corpus luteum from the wall of the ovulatory follicle and its production of hormones, notably progesterone (P4), that are essential for the initiation and maintenance of intrauterine pregnancy. However, species have evolved diverse mechanisms for controlling the functional lifespan of the corpus luteum in adult females during both

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their ovarian cycle and pregnancy [1]. In primates, including women, the functional lifespan of the corpus luteum during the ovarian (menstrual) cycle is sufficient for oocyte–sperm interaction and fertilization, movement of the early (pre-blastocyst stage) embryo through the oviduct, preparation of the uterus for implantation, plus blastocyst attachment and the early trophoblastic invasion of the endometrium. However, the corpus luteum is a transient gland. The regression of the corpus luteum near the end of the menstrual cycle in primates (approximately 2 weeks after initial differentiation from the luteinizing follicle) typically occurs before the developing placenta acquires the ability to produce progesterone, which keeps the uterus in a quiescent, supportive state for gestation. Thus, the functional lifespan of the corpus luteum must be extended in a fertile cycle for a limited time, until its essential activities (e.g., progesterone production) are usurped by the placenta (i.e., the luteal–placental shift). A critical event for fertility in primates occurs when the early conceptus signals to the mother (i.e., maternal recognition of pregnancy) that intrauterine pregnancy is beginning and extends luteal function until the luteal–placental shift.

Considering the importance of these processes, it is unfortunate that our understanding of the early events in maternal recognition of pregnancy in primates remains so limited (for earlier review, see Stouffer and Hearn [2]). A major factor is the critical differences between nonprimates and primates in the mechanisms employed to extend the luteal lifespan in fertile cycles. In the absence of nonprimate models, investigators have relied on nonhuman primates, but studies on early pregnancy are limited by cost and logistical difficulties. Finally, studies focusing on the regulation of pregnancy initiation in women, and their oocytes and developing embryos, are limited in scope for ethical reasons. Nevertheless, some progress has occurred during the past 15 years in characterizing the structure–function of the embryonic signal for maternal recognition of pregnancy, that is, chorionic gonadotropin (CG), plus CG-receptor signaling and actions promoting the "rescue of the primate corpus luteum" in early pregnancy. This chapter builds on recent reviews [3, 4] while emphasizing the authors' experience using a nonhuman primate model, the rhesus macaque, for ovarian research.

10.2 Corpus Luteum of the Menstrual Cycle

During the fertile menstrual cycle, the embryonic signal responsible for extending the functional lifespan of the primate corpus luteum is preceded by or acts within a milieu of endocrine and local factors that promote or suppress the structure–function of the corpus luteum [5].

10.2.1 Luteotropic Factors

It is generally accepted that the corpus luteum in many primates (e.g., Old World monkeys to great apes to humans) is dependent upon luteinizing hormone (LH) secreted by the anterior pituitary for its development, maintenance, and steroidogenic

function during the menstrual cycle [5]. We recently used gene microarrays to elucidate the transcriptome in the ovulatory luteinizing follicle [6] and in the corpus luteum [7] of rhesus macaques at specific stages of the luteal phase, as well as the dynamics of mRNAs following LH depletion or replacement [8]. These databases, which are publicly available, identify cellular pathways and processes that are promoted (e.g., components in steroid biosynthesis, such as steroidogenic acute regulatory protein, or STAR) or suppressed (e.g., immune factors or response such as interleukin-1 receptor antagonist, or IL1RN) by LH signaling. However, further studies are needed to discern initial (e.g., early-response genes) versus later (e.g., genes maintaining luteal structure) processes regulated by LH, as well as the proteome and protein activity related to the dynamics of the transcriptome.

Global characterization of LH-regulated gene products identified local ligand–receptor systems that mediate, at least in part, the trophic actions of LH. One factor receiving considerable attention during the past two decades is the steroid hormone progesterone [9]. Evidence indicates that locally produced progesterone is not only critical for ovulation, as it is in many mammalian species, but also promotes the development and maintenance of luteal structure–function in primates. The authors employed steroid ablation–progesterone replacement protocols to identify the transcriptome of LH-regulated, steroid/progestin-dependent versus steroid-independent gene products in the rhesus macaque corpus luteum [8]. Similarly, siRNA techniques using adenoviral vectors for transduction successfully "knocked down" the nuclear progesterone receptor (PGR) mRNA/protein in the macaque preovulatory follicle and reaffirmed the function of critical P-nuclear PGR signaling in follicle rupture and luteal development [10]. However, further studies are needed to evaluate the role of nuclear as well as nonnuclear (e.g., progesterone receptor membrane component 1, or PGRMC1 [11]) progesterone receptors and their signaling in the corpus luteum of the cycle. Similarly, LH promotes the synthesis or expression of other ligand–receptor systems that could serve trophic functions [e.g., the prostaglandin-E (PGE)–PGE receptor (PTGER) pathway] [12], while suppressing others [e.g., the corticotrophin-releasing hormone/urocortin (CRH/UCN)-receptor (R)-binding protein (BP) system] [13], and the PGF2α-PTGFR system [12] that could have antigonadotropic roles. The expression and actions of various local factors could be interrelated as recent evidence suggests LH-stimulated progesterone suppresses the numbers of immune cells [14, 15] and cytokines [15, 16] in the primate corpus luteum.

10.2.2 Luteolytic Processes

The corpus luteum in primates ceases function and structurally regresses, that is, undergoes functional and structural luteolysis, either (a) at the end of the non-fecund menstrual cycle, or, if pregnancy occurs, (b) after the luteal–placental shift. The processes of functional and structural regression appear temporally distinct, as circulating progesterone levels decline to baseline by 3 days before onset of menstruation, whereas appreciable luteal mass remains into the next follicular phase [14]. A major deficit in our understanding of the regulation of the primate corpus luteum is

the mystery surrounding the signal(s) or event(s) that initiate luteolysis [5]. The control of luteal regression in primates (Old World monkeys to humans) is remarkably different from that in many nonprimate mammals. A uterine luteolytic factor (PGF2α) is not released if timely implantation is absent, because hysterectomy does not alter the functional lifespan of the corpus luteum of the menstrual cycle [17]. This realization led to Knobil's proposal [18] that a "self-destruct" mechanism exists within the primate ovary that controls luteal lifespan. Two factors produced by the primate corpus luteum, estrogen and PGF2α, received initial attention. There are caveats to both factors, but recent evidence that (a) one estrogen receptor isoform ESR2 (also known as ERβ) is abundantly expressed by luteal tissue and downregulated by progesterone [19], and (b) the balance between synthesis/signaling of luteotropic (PGE2) and luteolytic (PGF2α) prostaglandins shifts as the corpus luteum ages [12], supports further evaluation of these factors. However, a number of other factors/processes that can be pro-luteolytic also emerge as the corpus luteum progresses through its lifespan during the menstrual cycle (Fig. 10.1) [5].

Recent studies suggest a role for immune cells in regulating the luteal lifespan, but again there may be species differences between primates [14, 15] and other species. The numbers of several types of immune cells (except lymphocytes) increase markedly in the macaque corpus luteum late in the menstrual cycle, but only after progesterone levels decline for 3 days [14], suggesting a major part in structural luteolysis. This information does not rule out earlier roles in controlling luteal development or functional regression, as proposed in domestic animals [20].

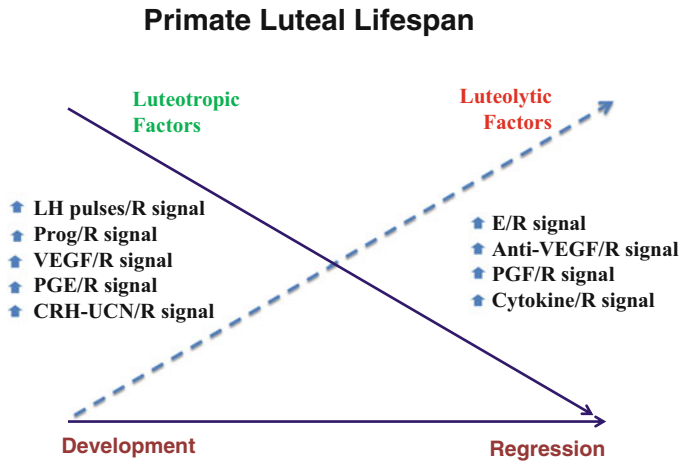

Fig. 10.1 Schematic of proposed changes in the balance between luteotropic and luteolytic signals in the primate corpus luteum during its lifespan in the non-fecund menstrual cycle. *VEGF* vascular endothelial growth factor, *R* receptor, *Prog* progesterone, *PGE* prostaglandin E, *CRH-UCN* corticotropin-releasing hormone/urocortin, *E* estrogen, *PGF* prostaglandin F. (From Stouffer et al. [5]. Figure reprinted from Reproductive Biology, Vol. 13, Issue 4. Stouffer RL, Bishop CV, Bogan RL, Xu F, Hennebold JD. Endocrine and local control of the primate corpus luteum, p. 256. 2013, with permission from Elsevier)

But if so, it may involve natural killer (NK) cells, the most abundant immune cell type in the functional corpus luteum in macaques [14], which are also found in the human corpus luteum [21]. The factors that promote immune cell migration and activity in primate luteal tissue remain poorly understood. One factor may be the cytokine C-C motif ligand 2 (CCL2; also known as monocyte chemoattractant 1 or MCP1), which is elevated in the regressing monkey [7] and human [15] corpus luteum. Evidence suggests that luteotropic factors (progesterone, PGE) suppress, whereas luteolytic agents (PGF) promote, CCL2 expression [15].

The authors updated a scenario proposed by Hamberger and colleagues [22] several years ago to summarize the shift from pro-luteotropic to pro-luteolytic factors that may control the lifespan of the primate corpus luteum (Fig. 10.1). Other factors may also be involved, as Duncan and colleagues [23] recently proposed that bone morphogenetic proteins (BMP2, -4, -6) are mediators of luteolysis in women. It is clear, however, that the loss of circulating LH support per se does not control luteolysis. There is a reduction in frequency of LH pulses secreted by the pituitary from early luteal phase to mid-late luteal phase just before the onset of luteal regression. Moreover, by mid-late luteal phase, each pulse of progesterone secretion is entrained to an LH pulse, such that there are intervals replete with and depleted of LH and progesterone [24]. However, sustaining LH levels by either generating endogenous LH pulses [25] or administering LH three times per day [26] did not prolong the luteal lifespan in monkeys. Alternatively, there is a decline in luteal tissue and cell responsiveness to LH as the corpus luteum ages. For example, dispersed cells from the macaque corpus luteum at mid-late luteal phase are less responsive to LH; the dose–response curve for cAMP and progesterone production is shifted, compared to cells from the early luteal phase [27]. The decreased sensitivity appears caused by LH-receptor desensitization, not downregulation, as receptor content does not decline until after progesterone levels decrease [28]. Thus, LH-receptor desensitization may be an early event associated with functional regression, whereas receptor downregulation may be a later event during structural involution. Once again one wonders what process or signal controls the onset of LH-receptor desensitization, and is this a critical signal for luteolysis?

10.3 Corpus Luteum of Early Pregnancy

10.3.1 Rescue by Chorionic Gonadotropin (CG)

Dissimilar from other species, the secretion of an LH-like hormone, chorionic gonadotropin (CG), by the implanting blastocyst and developing placenta "rescues" the corpus luteum in many primates from its impending demise and extends its functional lifespan in early pregnancy (for review of earlier work, see [2]). The steroidogenic function of the corpus luteum during early gestation is similar to that during the menstrual cycle, albeit leading to somewhat higher levels of circulating progesterone and estrogens (Fig. 10.2). Its peptidergic function, as indicated by relaxin and inhibin A production, is also markedly enhanced. It is generally regarded that progesterone is the only luteal

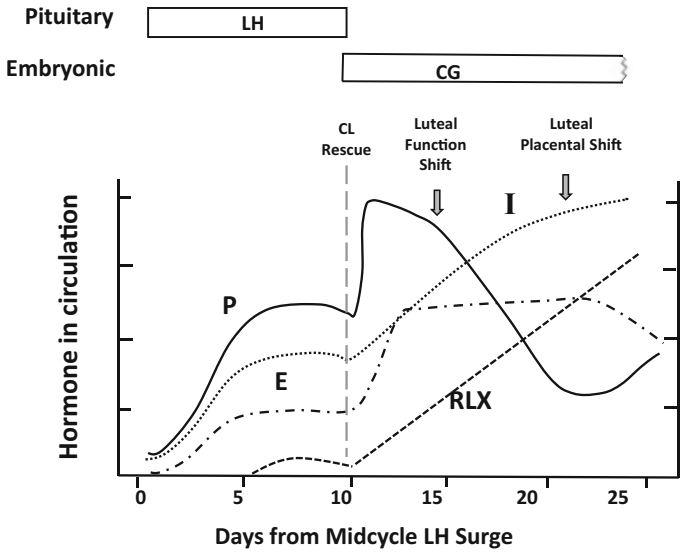

Fig. 10.2 Schematic of the changes in circulating levels of progesterone (P4), estrogen (E), relaxin (RLX), and inhibin (I) during the luteal phase of the fertile menstrual cycle in rhesus monkeys (CG). (Adapted from Stouffer and Hearn [2]. Figure reprinted from The Endocrinology of Pregnancy, Vol. 9 of the series. Stouffer RL, Hearn JP. Endocrinology of the Transition from Menstrual Cyclicity to Establishment of Pregnancy in Primates, p. 44. 1998, with permission from Springer)

product essential for the initiation and maintenance of early pregnancy. However, it now appears that relaxin of luteal origin acts on several tissues, including the embryo, uterus, and cardiovascular system, and may optimize maternal–fetal function and maternal adaptations to pregnancy [29]. Further studies are needed to assess the regulation and roles of luteal hormones during early pregnancy in primates.

10.3.2 CG Structure and Production

Chorionic gonadotropin (CG) is a heterodimeric glycoprotein composed of two subunits, termed α and β, that associate noncovalently. It is a member of the glycoprotein hormone family, which includes pituitary-derived follicle-stimulating hormone (FSH), luteinizing hormone (LH), and thyroid-stimulating hormone (TSH). Jiang and colleagues [4] summarize the discovery of these hormones and how their clinical application, especially for CG, spurred their purification, synthesis, and structure–activity analyses. Notably, CG shares a common 92-amino-acid α-subunit with all three pituitary glycoprotein hormones. Their specific bioactivity, linked to receptor binding and signaling, relates to differences among their β-subunits. Within the cluster of seven genes encoding β-subunit-like products on human chromosome 19, one encodes the β-subunit of LH (LHβ), four encode CG β-subunits (CGβ), and two are pseudogenes.

The amino acid sequences of human (h) LHβ and CGβ are very similar (82% homology). The major difference is that CGβ includes the entire 145-amino-acid (aa) protein, whereas the 24-aa leader sequence of LHβ is removed to yield a 121-aa product. It is now clear that circulating CG (as well as LH) exists as a mix of isoforms generated by (a) natural sequence variation, (b) posttranslational modification of the gene products, and (c) metabolism to yield truncated or "nicked" molecules. Not surprisingly, data are accruing that the bioactivity differs between isoforms.

As summarized by Choi and Smitz [3], there are at least four physiologically relevant isoforms of hCG: (a) "regular" hCG, (b) hyperglycosylated or H-hCG, (c) free hCGβ, and (d) pituitary hCG. The hCG molecule is highly glycosylated, a characteristic that also distinguishes it from LH. The α-subunit of both hormones contains two N-linked glycosylated sites, whereas CGβ also contains two N-linked and four O-linked sites. The increased sialic acid content of hCG influences its receptor-binding activity, increases its biological activity, and prolongs its half-life in the circulation compared to LH. The H-hCG isoform has the same amino acid composition as regular hCG, but even larger N- and O-linked oligosaccharides, thus increasing the molecular weight from 36,000–37,000 kDa to 40,000–41,000 kDa. Also, the sources of the various isoforms appear to differ. Fournier and colleagues [30] recently reviewed the literature and proposed a model wherein regular hCG is produced by the forming syncytiotrophoblast, whereas H-hCG is synthesized primarily in the extravillous cytotrophoblast. They also summarize evidence that these isoforms have different biological functions: (a) regular hCG appears to be the primary form for maternal recognition of pregnancy, extending luteal function, as well as other events in the reproductive tract to promote pregnancy initiation and early gestation, whereas (b) H-hCG is synthesized to affect local placental development including trophoblast invasion and increased vascularization. The typically low levels of sulfated hCG, of pituitary origin, increase in women during the perimenopausal interval and then plateau. The role(s), if any, for pituitary hCG are unknown, but its production over time mimics that of hLH.

Little is known regarding the factors or mechanisms controlling CG gene expression and protein production beginning as early as the two-cell embryo [31, 32] within the chronology of pregnancy. Studies of the promoter regions of the CGα and β genes suggest that transcription factors, such as AP2 and SP1, recognize specific response elements to stimulate gene activity. Also, a number of steroid hormones (e.g., progesterone, estradiol), growth factors, and cytokines (e.g., EGF, IL-6, TGFβ1), and perhaps oxygen-sensitive pathways regulate CG production in model systems, such as cultures of villous trophoblast. Also, a local placental [33]–embryonic [34] loop involving GnRH was proposed as a regulator of CG, analogous to hypothalamic GnRH regulation of pituitary LH/CG production. How or whether any of these factors have key roles in the onset of GG production in the implanting blastocyst and developing syncytiotrophoblast is unknown. However, the mechanisms that sustain, if not initiate, CG production in the placenta must vary somewhat among species, as judged by the differences in patterns and levels of CG circulating during gestation in a variety of primate species (Fig. 10.3). Peak CG levels are highest in women, 10-fold less in great apes (e.g., chimpanzees), and at least 100-fold lower in baboons and Old World and New World monkeys. Although CG is first detected around the

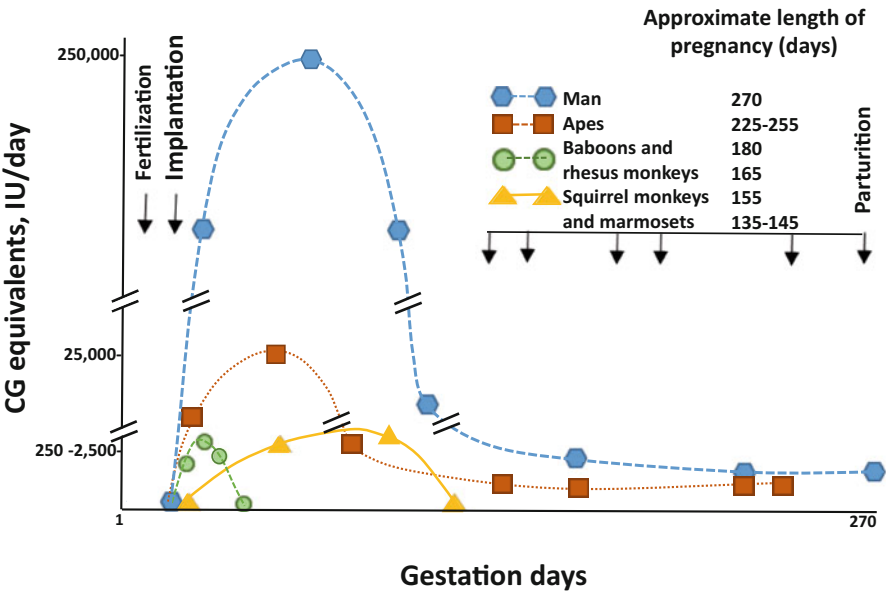

Fig. 10.3 Patterns and quantities of chorionic gonadotropin excreted during gestation by a variety of primate species. The time scale in the *upper-right corner* denotes the approximate duration of pregnancy and parturition (*arrows*) for these species. (Adapted from Stouffer and Hearn [2]. Figure reprinted from The Endocrinology of Pregnancy, Vol. 9 of the series. Stouffer RL, Hearn JP. Endocrinology of the Transition from Menstrual Cyclicity to Establishment of Pregnancy in Primates, p. 39. 1998, with permission from Springer)

time of implantation in all primates, the duration of CG production varies from throughout pregnancy in women and apes to only the first trimester in macaques. Based on emerging evidence for CG action in the human placenta, one can hypothesize that additional "extra-gonadal" roles for CG evolved in higher primates but are limited to maternal recognition of pregnancy in others.

10.3.3 LHCG-Receptor Binding and Signaling

As reviewed by Choi and Smitz [3], both LH and CG bind to and activate a common receptor, the LHCGR, which is a 675-aa G-protein receptor and a member of the rhodopsin subfamily of glycoprotein hormone receptors that includes FSHR and TSHR. It consists of seven domains spanning the cell membrane and an unusually large extracellular domain containing leucine-rich repeats and glycosylation sites. Jiang, Dias, and He [4] recently reviewed the efforts of structural biologists to understand the interactions of gonadotropin hormones with their receptors at an atomic level. After the crystal structure of hCG was defined [35, 36], an initial view of the hCG–LHCGR complex was predicted [37] in which several aspects of ligand binding

and orientation appear confirmed by subsequent studies. Recent research focused on gonadotropin interaction with its receptor ectodomain, so a remaining task is to elucidate the crystal structure of the native LHCGR. Nevertheless, current views support a two-step model: (1) ligand binding to the R high-affinity hormone-binding subdomain induces a conformational change in the hormone to form a "binding pocket," and (2) subsequent interactions produce a "pull and lift" force that frees tethered extracellular loops, thereby releasing an hypothesized inhibitory influence on the ectodomain leading to the activated conformation of the seven-transmembrane domain [4]. Although this model relates to the monomeric receptor, gonadotropin receptors (including LHCGRs) [38] can exist as dimers or larger number oligomers, which may explain biological phenomena such as negative cooperativity [4].

The expression of LHCGRs in theca cells and granulosa cells of the differentiating antral follicle, as well as luteal cells of the corpus luteum, is well established in primates [3]. LHCGR expression is not constitutive but dynamic, and primarily regulated in the maturing follicle by FSH or LH through both transcriptional and posttranscriptional processes. Similarly, the degradation of mRNA and protein products may be regulated, as well as the production of LHCGR variants by alternate splicing of mRNA [3]. Further studies are needed to understand the regulation of LHCGR expression, including the possible role of splice variants (see following) [39] in the primate corpus luteum. Similarly, mounting evidence supports the "extragonadal" expression of LHCGR [3]. These data are particularly relevant in primates, where CG is an endogenous molecule and is increasingly implicated in "extra-gonadal" functions in the female reproductive tract, placenta, and fetus to promote pregnancy and fetal development [40].

Evidence indicates that LHCGR is capable of binding H-hCG, as well as LH and regular hCG, but not the free β-subunit of LH or CG. However, H-hCG may also activate TGFβ-RII in the cytotrophoblast to promote angiogenesis [41], while being a poor stimulator of progesterone production by human luteinized granulosa cells [42]. Thus, different hCG forms may have different receptor affinity, leading to potential differences in signaling activity (see below) and biological function.

Until recently, it was generally considered that, because of their structural similarity, the postbinding effects and actions of hLH and hCG were very similar if not functionally equivalent. It remains clear that the major effect of LH-/CG-receptor binding is the activation of the G protein G_s, which in turn activates adenylate cyclase and thereby increases the production of cyclic AMP [3]. The cAMP/cAMP-dependent protein kinase A pathway is a major, indeed critical, cellular mechanism in LH-/CG-stimulated ovulation and steroid hormone (progesterone) synthesis. However, LH-/CG-receptor binding also activates phospholipase C/inositol phosphate signaling independent of the cAMP/PKA pathway. Given the higher levels of LH or CG required to activate PLC [43], it was hypothesized that this pathway is primarily active during the LH surge in the menstrual cycle or rising CG levels in early pregnancy. Recently, other cellular pathways involving ERK1/2 and AKT were identified in LHCGR signaling that may have a part in "nonsteroidogenic" processes, such as cell proliferation, differentiation, and survival in the follicle [44]. A possible role in the corpus luteum, that includes distinguishing LH versus CG

signaling pathways as the mechanism of CG rescue of the corpus luteum in early pregnancy, was recently proposed [44]. Grzesik et al. [45] concluded the differences between LH and CG signaling were determined by interaction of the L2-beta loop of the gonadotropin with the hinge region of the LHCG receptor.

10.3.4 Mechanisms of CG Rescue of the Corpus Luteum

10.3.4.1 Luteal Structure and Remodeling

Luteal rescue serves to maintain the overall cellular integrity and morphology of the primate corpus luteum, similar to that observed during the mid-luteal phase of the menstrual cycle. CG maintains the weight of the primate corpus luteum [46–48], whereas at the cellular level, CG also preserves luteal cell size and morphology [47, 48]. At the subcellular level, electron microscopic evaluation of luteal cells within rhesus monkey corpora lutea obtained at day 13 of pregnancy revealed they are similar in appearance to those at the mid-luteal phase (day 10 post-LH surge); that is, the cells possessed large, round nuclei and abundant lipid droplets ≥1 μm in size [49]. Although the general morphology and cellular organization appear to be similar between corpora lutea at mid-luteal phase and those obtained following CG rescue, there are differences in junctional complexes that are important for forming and maintaining cell-to-cell contacts. In the human corpus luteum at mid-luteal phase, the tight junction proteins claudin 1 (CLDN1) and occludin (OCLN) exhibited significant levels of immunostaining that were evenly distributed among granulosa-lutein cells [50]. After CG rescue, both CLDN1 and OCLN1 staining was significantly reduced. Similarly, expression of endothelial adhesion molecules claudin 5 (CLDN5) and vascular endothelium-cadherin (CDH5) were significantly reduced in the vasculature of corpora lutea obtained following CG rescue relative to the high level of expression observed in corpora lutea collected midway through the luteal phase of the menstrual cycle. From these findings, it was suggested the loss in tight junctions might facilitate the release of key endocrine factors by the corpora lutea (i.e., progesterone, relaxin) during early pregnancy [50].

During luteolysis, a number of mechanisms, including apoptosis and autophagy, have been proposed to account for the loss of the cellular constituents comprising the primate corpus luteum [51, 52]. At the ultrastructural level, both apoptotic and non-apoptotic death of luteal cells was observed in corpora lutea obtained from women undergoing induced and natural luteal regression. CG rescue of the corpus luteum prevented apoptosis, but not autophagy [52, 53]. Although the importance of apoptosis in primate luteolysis has been challenged [54], several studies reported its existence during the regression of the corpus luteum at the end of the menstrual cycle [55]. Apoptosis is controlled by several intracellular proteins, including those that serve as either (a) antiapoptotic factors such as B-cell lymphoma-2 (BCL2), myeloid cell leukemia-1 (MCL1), and Bcl-xL, or (b) proapoptotic factors such as Bcl-2-associated X protein (BAX), Bcl-2 interacting killer (BIK), and Bcl-2-associated death protein

(BAD). Cell death by apoptosis is determined by the ratio of pro- and antiapoptotic factors within a given cell. In women, *BCL2* mRNA expression was at its lowest point in the regressing corpus luteum and its highest level of expression was in the corpus luteum of early pregnancy [56]. In contrast, *BAX* mRNA levels were at their highest in the regressing corpus luteum and their lowest in the corpus luteum of early pregnancy. Moreover, BCL2 and BAX protein levels paralleled mRNA levels in the corpus luteum at these stages [56]. These findings differed from an earlier report noting that the expression of BCL2 did not change in the human corpus luteum of the menstrual cycle or after luteal rescue following CG administration [57]. Another study reported that the human corpus luteum also expresses the antiapoptotic protein MCL1 during early pregnancy, although the analysis was performed on a single isolated corpus luteum [58]. Thus, whether luteolysis is mediated solely via apoptosis and, if so, the precise mechanism by which CG prevents it from occurring, remain to be determined. Moreover, the role of autophagy in maintaining primate luteal structure–function is virtually unknown. Only a single paper has reported on this issue in the primate corpus luteum, suggesting that the protein beclin-1 (BECN1), which is required for the formation of the autophagosome, is expressed in granulosa-lutein cells during early pregnancy and, therefore, may be involved in promoting cell survival rather than cell death [59].

In addition to preserving luteal cells, CG may alter the dynamics of other cell types in the corpus luteum of early pregnancy. For example, CG exposure simulating early pregnancy prevented the rise in CCL-2 expression and immune cells in the human corpus luteum [15]. Also, luteal rescue results in either maintenance or growth of the luteal vasculature, perhaps depending on the species, as well as the markers used to assess angiogenesis. In women treated with CG to simulate early pregnancy, the effect on endothelial cell proliferation was minimal in the rescued corpus luteum [60]. However, in another study, CG treatment of women led to increased luteal endothelial cell area and proliferation as determined by CD34 and Ki-67 immunostaining, respectively [61]. Moreover, human corpora lutea isolated during early pregnancy (6–8 weeks) possessed greater numbers of both blood vessels and the supportive pericytes relative to corpora lutea obtained at mid-luteal phase of the menstrual cycle [62]. In this study, the increased angiogenesis associated with CG rescue was accompanied by increased mRNA levels for vascular endothelial growth factor A (*VEGFA*). In the corpus luteum of early pregnancy in women, increased expression of angiopoietin (ANGPT)-1 and decreased expression of ANGPT2 were noted relative to their expression in those at other stages of the luteal phase in a non-fecund cycle [62]. ANGPT1 serves to stabilize pericytes and endothelial cells of blood vessels, whereas ANGPT2 is a natural antagonist of ANGPT1 that serves to destabilize formed blood vessels [63, 64]. To provide a quantitative assessment of luteal vascularity during early pregnancy, three-dimensional power Doppler ultrasonography was employed weekly in a longitudinal study of women from 5 to 11 weeks of pregnancy, with pregnancy being defined based on last menstrual period and fetal crown–rump length [65]. From these studies, it was determined the vascular volume in the ovary containing the corpus luteum was at a maximum at week 5 and declined continuously thereafter. Thus, it appears

that increased capacity, if not development, of the vasculature in women occurs early in pregnancy, in the first weeks that CG is produced by the conceptus. In contrast to the corpus luteum of women during early pregnancy, the marmoset and rhesus monkey corpus luteum display minimal or no change in endothelial and pericyte areas or in endothelial cell proliferation relative to the mature corpus luteum obtained in the absence of pregnancy [66, 67], indicating that there may be primate species-specific differences in the degree of vessel development and maturation in the corpus luteum during early pregnancy. Although some studies have investigated VEGFA and ANGPT1/2 expression and action in the corpus luteum of pregnancy in primates, a number of recently discovered factors and pathways involved in angiogenesis and vessel stabilization (e.g., apelin, vasohibin, the ROBO/SLIT pathway, prohibitin-1, prokineticin-1, and the DELTA/JAGGED/NOTCH pathway) [1, 5] await investigation with regard to their role in maintaining the integrity of the vasculature following luteal rescue by CG.

One cellular process that appears regulated by CG during corpus luteum rescue in early pregnancy is tissue or extracellular matrix (ECM) remodeling. During natural luteolysis or following gonadotropin withdrawal, a wave of ECM and cellular remodeling occurs, which is associated with a concomitant increase in luteal protease expression and activity [68–70]. In rhesus monkeys, matrix metalloproteinase-9 (MMP9, gelatinase B) and MMP2 (gelatinase A) mRNA and protein expression are low during luteal formation and the period of peak steroid synthesis, but increase significantly after cessation of progesterone production at luteolysis. In women, MMP2 and MMP9 expression also increased in the corpus luteum during luteal regression [68]. However, administration of exogenous CG maintained luteal function and was associated with a significant reduction in MMP2, but not MMP9, expression and activity. Moreover, connective tissue growth factor (CTGF), which is associated with tissue and ECM remodeling in wound healing [71], reaches maximal expression in the human corpus luteum at the time of regression [72]. When women received CG to simulate early pregnancy, *CTGF* mRNA levels decline significantly in luteal fibroblasts and endothelial cells. It was subsequently demonstrated that CG did not downregulate CTGF expression in luteal fibroblasts directly, but required an unidentified paracrine factor produced by granulosa-lutein cells.

10.3.4.2 Luteal Function

In addition to maintaining the structure of the corpus luteum during early pregnancy, CG serves to sustain the level of progesterone production required for supporting pregnancy. The ability of CG to extend the period of luteal function clearly requires continued cholesterol uptake and translocation to the mitochondria where the first step of steroidogenesis occurs, primarily through action of steroidogenic acute regulatory protein (STAR). Once translocated across the mitochondrial membranes, cholesterol is then converted to pregnenolone by the cytochrome P450 cholesterol side-chain cleavage enzyme (CYP11A1; also known as $P450_{scc}$). Pregnenolone is subsequently converted to progesterone via 3β-hydroxysteroid dehydrogenase

(HSD3B) [73]. In terms of supporting steroidogenesis within the corpus luteum, administration of CG to women during the late luteal phase maintains expression of STAR, CYP11A1, and HSD3B mRNA and protein at levels that are comparable to those in the mid-luteal phase corpus luteum [74]. In rhesus monkeys undergoing a simulated early pregnancy protocol, CG induced the expression of several key steroidogenic enzymes in the corpus luteum relative to those removed from animals not receiving supplemental CG [75, 76]. The mRNA level for HSD3B2 is rapidly upregulated by CG, but it was transient and returned to pretreatment levels after 3 to 5 days of CG exposure. In contrast, mRNAs encoding STAR, CYP11A1, and CYP19A1 (also known as aromatase, critical for estradiol production) exhibited delayed (>3 days of CG treatment) and sustained expression. The expression pattern for these steroidogenic enzymes correlated with the observed level of circulating progesterone and estradiol, with progesterone levels peaking 2 days after initiation of CG treatment and estradiol levels reaching a maximum at 8 days of CG treatment [75].

The primate corpus luteum is not only a site of steroid synthesis; as noted earlier, it also serves as a target of steroid action through the expression of several nuclear hormone receptors including the genomic progesterone receptor (PGR), a plasma membrane-associated progesterone receptor (progesterone membrane component 1, PGRMC1), both estradiol receptors ESR1 (ERα) and ESR2 (ERβ), as well as the androgen receptor (AR) [9, 19, 77–80]. A critical function for steroid action in the primate corpus luteum was demonstrated in rhesus monkeys receiving exogenous CG at the mid-late luteal phase with or without simultaneous treatment of a trilostane, a compound that blocks the synthesis of steroid hormones by inhibiting HSD3B activity [48]. Although CG was capable of maintaining luteal weight and preventing the appearance of luteal cell histology consistent with luteolysis, CG plus trilostane resulted in a significant decrease in luteal weight and luteal cell size [48]. Inhibition of steroid synthesis through the administration of trilostane to rhesus monkeys during either the early or mid-luteal phase leads to luteolysis despite the presence of LH levels sufficient to support luteal function. Administering a synthetic progestin (R5020) along with trilostane maintains luteal weight and prevents the luteal cell loss that occurs following treatment with trilostane alone [70], further supporting the hypothesis that progesterone is a luteotropic agent [9, 81]. It is currently unclear to what extent progesterone action is involved in CG rescue of luteal function and whether any progesterone-dependent effects are mediated through the genomic progesterone receptor (PGR) or the membrane-associated form (PGRMC1), because both are expressed in the primate corpus luteum during simulated early pregnancy [48, 75, 82].

It is possible that the effects of steroid ablation during luteal rescue leading to the demise of the corpus luteum are dependent on the actions of steroids other than progesterone. Duncan and coworkers reported that the estrogen receptor isoform ESR2 was expressed in the late-stage corpus luteum obtained from women and that its levels were unchanged in response to CG rescue [80]. More recently, studies were published that support a role for glucocorticoids (e.g., cortisol) and the glucocorticoid receptor (nuclear receptor subfamily 3, group C, member 1; NR3C1) in maintaining luteal function in response to CG. In women receiving exogenous CG beginning at the mid-luteal phase and continuing for 5–8 days, it was noted that

there is a significant increase in luteal expression of the enzyme 11β-hydroxysteroid dehydrogenase-1 (HSD11B1; converts inactive cortisone to biologically active cortisol) relative to stage-matched corpora lutea obtained from women not receiving CG [83]. NR3C1 mRNA and protein were detectable in mid- and late-stage corpora lutea as well as those from women receiving exogenous CG. Immunohistochemistry revealed significant expression of HSD11B1 in the granulosa-lutein cells, whereas NR3C1 immunostaining was localized to numerous cell types, including granulosa-lutein cells, fibroblasts, and endothelial cells, as well as in resident macrophages. Thus, cortisol generated within the corpus luteum via HSD11B1 during early pregnancy may act through NR3C1 to prevent luteolysis in response to CG.

The afore-described pathways and events critical for the extension of luteal lifespan and function controlled by CG and CG-inducible steroids were investigated on a case-by-case basis. With the development of genomic approaches (i.e., DNA microarray technology) that allow for the simultaneous assessment of most, if not all, transcripts in a given rhesus macaque cell type or tissue, it became possible to systematically determine changes in gene expression in the primate corpus luteum in response to CG and steroid hormones. The changes in the levels of mRNAs in corpora lutea obtained from monkeys undergoing simulated early pregnancy were assessed using the Affymetrix GeneChip Rhesus Macaque Genome Array [75]. CG treatment occurred for 1, 3, 6, and 9 days beginning on day 9 after the mid-cycle surge of LH. Following only 1 day of CG treatment, the levels of 419 mRNAs were significantly different (≥2-fold change) compared to control corpora lutea, with 292 mRNAs being upregulated and 127 being downregulated. When comparing subsequent days of treatment, continued CG administration resulted in a limited effect on gene expression, with fewer than 100 mRNAs changing significantly between 3 and 6 days or 6 and 9 days of CG treatment. However, when comparing mRNA levels between stage-matched corpora lutea obtained from animals not receiving CG and those receiving CG, the effects on gene expression were significant. For example, when comparing corpora lutea obtained from days 14 to 16 of the luteal phase of animals not receiving CG (period of declining progesterone synthesis) to those obtained at the same stage of the luteal phase that had received CG for 6 days, there were 2078 mRNAs with increased levels and 452 that decreased. As expected, a number of proteins encoded by differentially expressed mRNAs were related to steroid production (e.g., STAR, CYP11lA1, HSD3B2). CG administration also led to a transient re-expression of the prostaglandin PGE synthesis–receptor signaling system, which is associated with corpus luteum development during the early to mid-luteal phase of the menstrual cycle [12], but suppressed the expression of mRNAs encoding proteins associated with immune function.

To further clarify the direct versus indirect effects (i.e., steroid-mediated processes) of CG that prevent luteal demise and extend the structure–function of the primate corpus luteum in early pregnancy, a DNA microarray study was performed using RNA isolated from the corpus luteum of rhesus macaques receiving CG, CG plus the steroid synthesis inhibitor trilostane, or CG and trilostane plus the synthetic progestin R5020 [84]. Corpora lutea were collected at 1 or 6 days after the initiation of CG treatment, which commenced on day 9 of the luteal phase. The results of this

study revealed that the majority of CG-regulated luteal mRNAs are regulated independently of local steroid actions wherein trilostane significantly affected the expression of 50 mRNAs after 1 day and 87 mRNAs after 6 days of CG treatment relative to corpora lutea from animals receiving CG alone. Moreover, the number of genes in the corpus luteum affected by progesterone replacement in CG plus trilostane-treated animals (i.e., CG + trilostane + R5020) relative to those receiving CG plus trilostane alone (i.e., CG + trilostane) was relatively small and included 46 mRNAs after day 1 of CG treatment and 129 mRNAs after 6 days of CG treatment. Although the steroid-regulated genes in the macaque corpus luteum during CG rescue may be few, they are likely essential for sustaining luteal function during early pregnancy based on the fact that trilostane treatment initiates premature structural regression of the corpus luteum during simulated early pregnancy [48]. Also, these data demonstrate that regulation of gene expression in the rescued corpus luteum in early pregnancy differs from the corpus luteum of the menstrual cycle in primates, because in the latter the number of genes whose expression is affected by steroid depletion is considerably greater (>300) [8]. Thus, the availability of high-throughput genomic methods has allowed for a greater understanding and comparison of how different genes are regulated (i.e., gonadotropin- versus steroid dependent) in the primate corpus luteum throughout the menstrual cycle and during pregnancy (e.g., STAR) (Fig. 10.4). Such approaches will also provide the means for investigating the role that individual intraluteal factors (i.e., androgens, glucocorticoids, PGs) play in mediating the effects of CG that are necessary in primates for luteal survival and function during early pregnancy.

10.4 Clinical Applications

Urinary, and more recently recombinant, preparations of hCG are widely used, as a bolus injection, to mimic the endogenous LH surge in controlled ovarian stimulation cycles, for the purpose of collecting meiotically mature oocytes for assisted reproductive technologies (ARTs) in infertility patients [85]. The hCG bolus also promotes luteinization of the antral follicles in COS cycles and, because of the long half-life of hCG, will sustain elevated progesterone levels in the circulation for several days. However, "downregulation" of pituitary LH secretion by either GnRH analogues administered during the follicular phase or elevated steroid levels into the luteal phase causes circulating P levels to decline as CG disappears. To sustain P and its actions, P is often provided as a luteal-phase supplement, which is preferred [86], in part because hCG treatment can cause morbidity from ovarian hyperstimulation syndrome (OHSS) [87] and mask the ability of clinical "early pregnancy test" kits to detect endogenous hCG excreted in the urine.

However, the use of exogenous CG to promote pregnancy initiation at the time or following implantation is controversial. Although rare, two variants of the hCG β-subunit are associated with increased risk of recurrent miscarriage [88]. However, a recent Cochrane review of existing clinical trials [89] indicates that the evidence

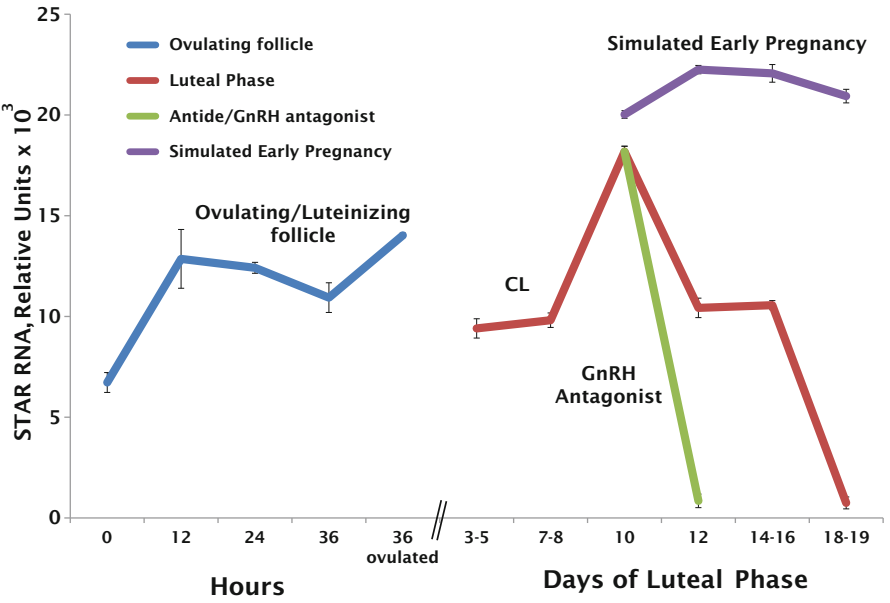

Fig. 10.4 Example of the dynamic expression of a specific gene product (STAR mRNA, relative units) during the lifespan of the macaque corpus luteum, plus the effects of LH depletion during the menstrual cycle and hCG administration during simulated early pregnancy. Similar data analyses can be generated for any number of genes of interest from the publicly available NCBI GEO databases (e.g., GSE2276, GSE10367, GSE25335). (From Stouffer et al. [5]. Figure reprinted from Reproductive Biology, Vol. 13, Issue 4. Stouffer RL, Bishop CV, Bogan RL, Xu F, Hennebold JD. Endocrine and local control of the primate corpus luteum, p. 267. 2013, with permission from Elsevier)

supporting hCG supplementation to prevent recurrent miscarriage remains equivocal. Because increasing evidence suggests that hCG from the embryo has a critical local role in the uterus to promote pregnancy initiation, as well as extending luteal function, clinical researchers hypothesized that exogenous hCG would facilitate fertility, especially in ART patients in which "lower-quality" embryos are transferred into the uterus. Initial reports suggested that intrauterine hCG administration during transfer of early, cleavage-stage embryos offered some benefit, but recent studies observed no improvement when hCG was administered 2 days before or on the day of blastocyst transfer [90], regardless of embryo quality. Nevertheless, it is now apparent that a number of factors (e.g., BMI, smoking) influence fertility, and that the circulation and maternal–fetal interface includes several different hCG molecules. Further studies with better characterized embryos, including their CG production, and select CG moieties are needed to address this issue. For example, Evans and colleagues [91] propose that H-hCG could prove useful in treating pregnancy disorders.

Similarly, a number of studies are addressing the premise that alterations in a particular type of hCG molecule can help identify dysfunctional pregnancies [92]. There are reports that a low ratio of H-hCG to total hCG (<0.5) is associated with pregnancy loss [93], whereas low levels of hCG β-subunit occur during ectopic

pregnancies [92]. However, there is remarkable variation in CG levels between individuals with normal pregnancies, as well as in the ability of clinical assays to reliably detect the various forms of hCG [94]. Our increasing understanding of the types and roles of the various hCG moieties should aid in the development of better assays for diagnosis of early pregnancy and associated disorders, as well as novel treatments for sub- or infertility.

10.5 Final Perspective

Although progress during the past 15 years has increased our knowledge of the types of CG moieties and CG- versus LH-receptor signaling in cells (but not necessarily its specific target cells), it remains unclear how the appearance of CG around the onset of implantation rescues the primate corpus luteum from impending luteal regression and extends its functional lifespan until the later luteal–placental shift in progesterone production. There is evidence that LH- and CG-receptor signaling and activation of intracellular pathways can differ [44], but do these differences actually occur in luteal cells and are they critical for corpus luteum rescue in early pregnancy? Alternatively, is the qualitative and quantitative change in gonadotropin exposure, from low pulses of LH secretion three or four times per day to continuous increasing levels of CG, sufficient for corpus luteum rescue? Notably, Zeleznik [95] reported that either LH or CG could rescue the macaque corpus luteum when given in exponentially increasing doses. Although research on the corpus luteum seems to be losing popularity, perhaps because of recognition that progesterone replacement can replace this endocrine gland in clinical scenarios, there are important unresolved issues, especially in primates, regarding the processes controlling the function and lifespan of the corpus luteum during the menstrual cycle and its rescue in early pregnancy.

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Chapter 11
Corpus Luteum and Early Pregnancy in Ruminants

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Abstract This review examines the function of the corpus luteum (CL) with emphasis on pregnancy in ruminant models and the possible impact of pregnancy in conferring luteal resistance to prostaglandin F2α (PGF2α). Critical processes involved with formation of the CL impact the capacity to secrete progesterone. Similarly, complete luteolysis is critically important in the event that pregnancy does not occur so that a new ovulation and opportunity for pregnancy is established. It is well known that serum progesterone must reach a critical nadir if ovulation and fertilization are to occur. Following fertilization, the function of the CL in providing adequate progesterone is critical in setting up an endometrial environment so that pregnancy is maintained. Benefits of supplemental progesterone during early pregnancy are inconsistent in ruminants. However, recent studies indicate that supplemental progesterone following artificial insemination (AI) may depend on the

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presence of the CL and the amount of progesterone released from the CL. The primary signal for maternal recognition of pregnancy, interferon-tau (IFNT), is secreted from the ruminant conceptus (embryo proper and extraembryonic membranes). IFNT disrupts release of PGF2α from the endometrium and is antiluteolytic through inhibiting uterine expression of the estradiol receptor (ESR1) or the oxytocin receptor (OXTR). Endocrine action of IFNT on peripheral blood mononuclear cells and on the CL may also contribute to immunomodulatory function and longer-term sustainability and function of the CL as pregnancy progresses.

Keywords Interferon • Corpus luteum • Pregnancy • Ruminants • Interferon-stimulated genes • Peripheral blood mononuclear cells • Leukocytes

11.1 Exposure to Progesterone Changes Gene Expression in the Uterus

Continuous exposure of the uterus to progesterone for 8–10 days during the estrous cycle causes downregulation of the progesterone receptor (PR) in the endometrium, allowing estradiol to bind to the estrogen receptor (ESR1), and resulting in the synthesis and insertion of the oxytocin receptor (OXTR) in the endometrium [1, 2]. Oxytocin (OXT) binds to its endometrial receptor, activating the synthesis and pulsatile release of PGF2α into the uterine ovarian vein [3, 4]. PGF2α crosses over into the ovarian artery from the ovarian vein via a countercurrent exchange mechanism [5, 6]. This unique mechanism allows PGF2α to be delivered directly to the CL without first passing through the systemic circulation. The local effects of PGF2α result in the demise of the CL, leading to a new estrous cycle if the mother/dam is not pregnant.

In ruminants, the utero-ovarian plexus provides an intimate association of the uterine vein and ovarian artery, which allows delivery of small molecules such as PGF2α from the uterus to the ovary to cause luteolysis [7–9]. Wiltbank and Casida [10] reported that hysterectomy, which disrupts the utero-ovarian plexus, delayed estrus in ewes for more than 100 days. The original CL at the time of hysterectomy was maintained until the time of necropsy, and the ovary had minimal follicular activity. If total hysterectomy was performed on days 13.5–15, then the CL had a lifespan of approximately 148 days, which was similar to the length of gestation [7, 11]. Other studies demonstrated that lifespan of the CL varies when one uterine horn is removed and is dependent on proximity of the removed horn to the CL [12]. Removal of the uterine horn that is ipsilateral to the CL-containing ovary extends luteal lifespan. However, if the uterine horn contralateral to the CL is removed, then the CL regresses in 15–17 days, which is similar to the normal cycle. These data are interpreted to mean that luteolysis requires an intimate connection between the CL and the nonpregnant ipsilateral uterine horn.

11.2 Downregulation of Progesterone Receptor Leads to Upregulation of Prostaglandin F2α (PGF2α) Pulses and Luteolysis in Sheep

Progesterone regulates expression of many receptors involved in coordinating the estrous cycle and secretion of prostaglandins. Progesterone prevents expression of the ESR1 and the oxytocin receptor (OXTR) in the endometrium, which generally precedes PGF2α release. Withdrawal of progesterone or interruption of PR activity results in an increase in the expression of these receptors [2]. Continuous exposure to progesterone causes a downregulation of the endometrial PR by day 11 of the estrous cycle [13]. Downregulation of endometrial PR is followed by an increase in ESR1 and OXTR expression and PGF2α release on days 13, 14, and 14–16, respectively [2, 13–16]. The onset of OXT and PGF2α pulses is concomitant with an increase in endometrial OXTR, suggesting that PGF2α synthesis and release can be driven by OXT signaling [16, 17]; this allows increased pulse amplitude and frequency of PGF2α secretion, which initiates luteal regression.

11.2.1 Nature of the Luteolysin and Luteolysis in Ruminants

PGF2α has been implicated as the initiator of luteolysis in many species including the ovine [6, 9, 18]. PGF2α-induced luteolysis appears to have three mechanisms of action: (1) auto-induction of PGF2α synthesis by the CL; (2) reduction of steroidogenesis; and (3) reduction of blood flow to the CL. In 1970, McCracken indicated that PGF2α was the luteolytic agent and would cause luteolysis when delivered to the CL [9]. Functional regression of the CL is strongly associated with a decrease in progesterone production [19]. Structural changes occur in the CL after the initial drop in progesterone concentrations. Binding of uterine-derived PGF2α to the CL induces several downstream effects in both large luteal cells (LLC) and small luteal cells (SLC). In LLCs, PGF2α interfaces with its receptor to induce a suicidal loop of production of PGF2α from the CL by upregulating the PTGS2 (prostaglandin synthase-2) pathway [20, 21]. PGF2α action also entails increased intake of calcium in LLC, which induces apoptosis, activates protein kinase C (PKC) and associated cellular responses, inhibits progesterone synthesis, and induces OXT release [20, 22]. In 1986, Moor demonstrated that uterine PGF2α release in ewes increases before OXT and oxytocin-associated neurophysin, indicating that PGF2α initiates release of OXT [4]. The OXT produced from LLC binds to the OXTR on the SLC, causing the release of calcium and activation of the PKC pathway, both of which lead to cell death via apoptosis [20, 23]. For a detailed description of gene expression during luteolysis in ruminants, see [24, 25].

11.3 Rescue of the CL During Pregnancy in Ruminants

Moor and Rowson [26], as well as Mapletoft and coworkers [27], used ovine embryo transfer experiments coupled with ligation of uterine horns to clarify the role of the conceptus in protecting the CL during establishment of early pregnancy. It was inferred from these studies that no systemic, conceptus-derived mediator rescued the CL, because ligation of the gravid horn protected the CL ipsilateral to the conceptus while the contralateral CL regressed. However, a systemic role of the conceptus in resistance for the CL to PGF2α and longer-term survival of the CL during early pregnancy cannot be excluded. For example, several investigators have described the CL of pregnancy to be more resistant to lytic effects of PGF2α [28–31]. Exactly why and how this resistance to PGF2α occurs in the CL during pregnancy is unknown.

There is no direct luteotropic counterpart to human chorionic gonadotropin in ruminants [32]. Rather, conceptus-derived IFNT regulates the antiluteolytic alteration of PGF2α release from the endometrium during pregnancy [33, 34]. IFNT may also have direct actions on the CL in context of conferring resistance to PGF2α [35, 36]. The latter possibility seems likely because the release of PGF2α from the endometrium is not ablated by pregnancy and the CL is capable of local production of PGF2α [37]. Actually, PGF2α is found in greater concentrations in uterine venous drainage from day 13 pregnant compared with estrous cycling ewes [38]. Also, the basal production of PGF2α is higher in pregnant compared to nonpregnant ewes, possibly because of the continued expression of PTGS2 in the uterine luminal and glandular epithelium and the production of prostaglandins by the conceptus [39].

11.4 Contribution of Conceptus to Lifespan of the CL

The conceptus must be present from day 12 through day 17 in the ewe for a successful pregnancy to be recognized and maintained [12, 40–42]. In support of this concept, infusion of IFNT into the uterine vein for only 3 days starting on day 12 of the estrous cycle was not sufficient to cause a delay in return to estrus (Antoniazzi and Hansen, unpublished data). IFNT was initially named protein X after its discovery on day 13 of pregnancy as the major conceptus secretory protein [33] and renamed as trophoblast protein-1 before being classified as an IFN and named IFNT [34, 43–45]. IFNT is the maternal recognition signal in ruminants that indicates the presence of a viable embryo(s), resulting in the differential expression of endometrial proteins [46–50], prevention of luteolysis, and continued production of progesterone [32, 36, 39]. IFNT can be detected in media from cultured ovine conceptuses by day 10 and increases in secretion through day 16 of pregnancy [51]. IFNT accumulates in uterine flushings to detectable concentrations between days 13 and 14 of pregnancy [52]. IFNT silences *ESR1* transcription and consequently inhibits the production and insertion of OXTR into the endometrium, thus disrupting the pulsatile release of PGF2α [53]. The paracrine actions of IFNT alter PGF2α pulsatility in the ewe [54] and may actually reduce PGF2α concentrations in the cow [55].

Maternal recognition of pregnancy is clearly a paracrine mechanism, but it could also be sustained through endocrine induction of luteal resistance to prostaglandin through interferon-stimulated genes (ISGs) [25, 35, 36, 52, 56–58]. Even though the mechanism for maternal recognition of pregnancy varies among mammals, the upregulation of interferon-stimulated gene 15 (ISG15) in the endometrium appears to be a universal response to the presence of an embryo, as has been seen in humans and baboons [59, 60], cows [46, 61], sheep [62], swine [63] and mice [64–66]. IFNT stimulates production of ISGs in the glandular epithelium [48, 61, 62]. Several of these ISGs have been identified, such as ISG15 [46, 62, 67], myxovirus (influenza virus) resistance (MX1) [68], and 2′,5′-oligoadenylate synthetase (OAS) [48, 69, 70].

IFNT elicits its actions through the type 1 interferon receptor, which shares two subunits, IFNAR1 and IFNAR2. These subunits are expressed in the luminal epithelium, subglandular epithelium, and stroma of the ovine uterus during the estrous cycle and pregnancy in day 14–15 ewes [71]. In ovine endometrial cells, IFNT causes tyrosine phosphorylation and nuclear translocation of signal transduction and activator of transcription (STAT)-1, -2, -3, -5, and -6 as well as increased transcription of STAT1 and STAT2 [72, 73]. Interferon-stimulated gene factor 3 (ISGF3) and STAT1 form homodimers and bind to IFNT-stimulated response elements (ISRE) and gamma-activated sequences (GAS) to drive the expression of ISGs. In response to pregnancy (IFNT), mRNAs encoding these signal transducers and stimulated ISGs increase in concentration primarily in endometrial stroma and glandular epithelium in the ewe [74]. However, the ISGs are not strongly inducible in luminal epithelium and subluminal glandular epithelial cells because of expression of interferon regulatory factor 2 (IRF2) in these cells, which strongly inhibits IFN stimulatory response elements in ISGs. The consequences of a lack of induction of ISGs in ovine luminal epithelium have not been completely resolved in the context of regulation of release of PGF2α and antiluteolytic mechanisms of IFNT.

Several ISGs in addition to STATs and IRFs have been identified in the ruminant uterus. ISG15 can be found in its free 15-kDa form and conjugated to target proteins in the uteri of pregnant cows on days 17–45 [61]. ISG15 protein was localized to the glandular epithelium with light staining in the luminal epithelium and stroma during the timeframe of peak IFNT expression around day 18 of pregnancy [61]. IRF2 is a repressor of ISGs and is present in the luminal epithelium and subglandular epithelium, thus restricting the ability of IFNT to increase ISGs in the luminal epithelium, but not in the stroma and glandular epithelium regions of the ovine uterus [74].

ISGs such as STATs and IRFs and RNA helicases are upregulated in the endometrium and peripheral blood mononuclear cells (PBMC) [35] as well as in the CL [25, 52, 57, 58] of pregnant compared to nonpregnant ewes. IFNT and possibly ISGs enter the blood stream and condition T cells and macrophages, potentially activating a first line of defense against viruses to prevent early embryonic mortality or persistent viral infection of the embryo [35].

11.5 Progesterone and Early Conceptus Survival

The zona-enclosed embryo is thought not to be dependent on oviductal or uterine secretions. However, following hatching, the developing and elongating conceptus starts to release signals and the endometrium responds to these signals to provide a more complex and nourishing histotroph when compared to the progesterone-primed uterus of the estrous cycle. The mechanisms through which progesterone prepares the uterus for pregnancy and is associated with fertility have been extensively examined. The uterine epithelia secrete or selectively transport molecules into the uterine lumen that are collectively known as a histotroph. The uterine histotroph nourishes the free-floating conceptus and contains amino acids and glucose, cytokines, enzymes, growth factors, lymphokines, transport proteins for vitamins and minerals, and extracellular matrix molecules. Progesterone activates genes contributing to production of the histotroph, which supports the early pregnancy. However, crucial conceptus-derived signals such as IFNT work in concert with progesterone to fully engage a nurturing environment during elongation of the conceptus, formation of the placenta, and attachment/implantation to the luminal epithelium of the endometrium.

The benefits of exogenous supplementation of progesterone to pregnancy rates have been considered for decades (reviewed in [75–77]. Briefly, the actions of progesterone depend on the amount that is circulating, which also is regulated by the amount synthesized and metabolized. It is very clear that serum progesterone concentrations need to be low at the time of AI. Even very low (>0.9 ng/ml) serum concentrations of progesterone at the time of AI will significantly reduce fertility. Carvalho and coworkers [78] recently described an advantage to pregnancy rate by adding a second PGF2α treatment to ensure luteolysis just before induced ovulation using the Ovsynch gonadotropin-releasing hormone (GnRH) protocol (day −10, GnRH; day −3, PGF2α; day −0.7, GnRH; day 0, timed AI) in dairy cows. Following AI, supplementation of high serum progesterone concentrations has varied effects on fertility. When used with the Ovsynch protocol, supplementation of dairy cows with progesterone using CIDR (controlled internal drug release) implants improved pregnancy rates in the cases in which no CL was present at the time of PGF2α injection [79]. There was no benefit of supplementation with progesterone when a functional CL was present at the time of PGF2α. However, if the CL was absent or producing subluteal-phase concentrations of serum progesterone at the first GnRH injection of the Ovsynch protocol [79, 80], then there was a benefit to supplementing with progesterone to improve fertility in dairy cows. The positive impact of serum progesterone supplementation during the estrous cycle depends on when during the luteal phase this is applied and may be most effective when concentrations of progesterone are impaired at the time of initiation of Ovsynch treatments. During pregnancy, there may be a greater advantage to supplementing serum progesterone during the late luteal phase when the conceptus is elongating and producing IFNT. For example, increasing serum progesterone concentrations during this period using human chorionic gonadotropin (hCG) [81] has a tendency to increase IFNT production from cultured day 18 bovine conceptuses. This finding was consistent with an earlier study describing a positive effect of hCG treatment on production of IFNT from

cultured ovine embryos [82] but no effect of supplemental serum progesterone on the production of IFNT. Similarly, even though there was a positive effect of hCG treatment on day 5 of the estrous cycle in context of increased serum progesterone, treatment at that time before embryo transfer on day 7 had no impact on IFNT production by conceptuses flushed on day 14 and cultured for 24 h [83]. Although progesterone is critical for the establishment of pregnancy, the evidence for a benefit of supplementation of serum progesterone during early pregnancy is conflicting, and current interpretation suggests a moderate positive response in context of supporting development of more advanced conceptuses, which are typically correlated with increased production of IFNT.

In addition to progesterone, other intrauterine factors such as prostaglandins and cortisol influence conceptus responses to the endometrium. For example, PTGS2, the rate-limiting enzyme in endometrial prostaglandin synthesis, is upregulated in the bovine endometrium in response to pregnancy and IFNT [84, 85]. Thus, IFNT may stimulate greater endometrial PGE2 compared to PGF2α production, possibly providing local support for the CL during early pregnancy. A role for prostaglandins in elongation of the conceptus has been suggested in context of a local paracrine role during early pregnancy [86]. A direct role of PGE2 on the CL has not yet been described during early pregnancy in ruminants.

11.6 Development of Resistance of the CL to PGF2α During Pregnancy

In 1988, Zarco demonstrated that PGF2α is secreted in nonpregnant ewes in a pulsatile manner, whereas in pregnant ewes, PGF2α is released in a more constant pattern that steadily increases [87, 88], indicating that the pulsatile release of PGF2α, which is diminished in the pregnant ewe, may be required for luteolysis. Prostaglandin pulses associated with luteolysis of cyclic ewes occur every 7–8 h [89]. McCracken reported that uterine arterial infusion of OXT into ewes on day 16 of pregnancy did not elicit the same production of PGF2α that occurred during the estrous cycle in ewes at 16 days post estrus [90], and that this may be caused by the lower concentration of endometrial OXTR in pregnant versus cyclic ewes. The enzyme responsible for metabolizing PGF2α, prostaglandin dehydrogenase (PGDH), is elevated in the CL of pregnant ewes when compared to day 13 of the cyclic ewes, thus indicating higher metabolism of PGF2α [31, 37]. Furthermore, the dose of exogenous PGF2α necessary to cause luteal regression in pregnant ewes is elevated, especially surrounding the time of maternal recognition of pregnancy, compared to nonpregnant ewes [91].

The CL of pregnant ruminants is resistant to the luteolytic effects of PGF2α [31]. However, the biochemical mechanisms involved in luteal resistance to PGF2α are not well described. The lytic effects of intrafollicular injection of 200 μg PGF2α were tested in ewes on day 12 of pregnancy or the estrous cycle. In nonmated ewes and ewes without embryos present, serum progesterone declined, and 79–89 % of

ewes returned to estrus within 2.4–2.9 days after PGF2α treatment. In contrast, 63 % of pregnant ewes with normal-sized embryos did not return to estrus after PGF2α treatment and also evidenced a decline in serum progesterone that then apparently rebounded after 24 h. It was concluded from these studies that an antiluteolytic factor from the conceptus might overcome the lytic action of PGF2α [28]. When examining the response to dose of PGF2α treatment, Silvia and Niswender [91] reported that CL from nonpregnant ewes responded (luteolysis) to lower doses of PGF2α on day 12 post estrus compared to CL from pregnant ewes.

It was concluded that the CL of pregnancy was more resistant to the luteolytic effect of PGF2α. When ewes were treated with PGF2α on days 13 or 16, serum progesterone concentrations declined regardless of pregnancy status. However, serum progesterone rebounded to levels before treatment with PGF2α in only day 13 and day 16 pregnant ewes [91]. This rebound in serum progesterone concentrations did not occur when the same experiment was repeated on days 16 and 26 of pregnancy. This result was interpreted to mean that the resistance of the CL to luteolytic effects of PGF2α may develop between day 10 and 13 of pregnancy and is lost between day 16 and 26 of pregnancy.

One obvious target for disruption of PGF2α-induced luteolysis is through its receptor. However, the numbers of receptors for PGF2α do not appear to change in response to pregnancy status on days 10 and 13 [92]. By day 15, there were actually increased concentrations of PGF2α receptors on the CL in response to pregnancy when compared to the estrous cycle. An increase in PGF2α-receptor mRNA concentrations also was observed in CL by day 16 of pregnancy when compared to the estrous cycle [93]; which was consistent with former PGF2α receptor-binding studies. Numbers of PGE2 receptors did not change in the CL in this study. These findings were interpreted as evidence that the CL of pregnant ewes is not protected from the actions of PGF2α at the time of maternal recognition of pregnancy by a reduction in numbers or affinity of PGF2α receptors but through another mechanism.

Alterations in expression of PGF2α-degrading and PGF2α-synthesizing enzymes have also been evaluated in the endometrium and CL. The manner in which PGF2α is secreted is dependent upon an animal's physiological status. Pregnant and nonpregnant ewes have very different patterns of PGF2α release [89, 94]. Although peak production of PGF2α occurs at day 14–15, regardless of pregnancy status, ewes secrete PGF2α in a pulsatile fashion during the estrous cycle, whereas pregnant ewes have a more constant, slowly increasing pattern in the release of PGF2α based on the presence of PGF2α metabolites [87, 88]. More PGF2α is found exiting the uterus through the uterine vein in day 13 pregnant versus nonpregnant ewes [38]. The change in peaks of PGF2α during pregnancy may be associated with increases in metabolism by PGDH within the uterus and CL. Also, the presence of PGF2α in the uterine vein during pregnancy may necessitate a self-preservation mechanism for the CL to make PGDH. For example, PGDH mRNA is elevated in the CL on day 4 of the estrous cycle and day 13 of pregnancy when compared to day 13 of the estrous cycle in ewes [37]. Enzyme assays to determine PGDH activity revealed that production of PGFM was greater on day 4 in CL during the estrous cycle and on day 13 in CL from pregnant ewes compared to CL on day 13 of the

estrous cycle. PTGS2 mRNA concentrations in the CL did not differ between pregnant and nonpregnant ewes on day 12 or 13 post estrus [37, 95]. Silva and colleagues [37] reported a significant increase in PGDH mRNA and enzyme activity in day 13 pregnant ewes compared to day 13 non-pregnant ewes, and Costine et al. [95] did not detect these differences on day 12. Similarly, Romero et al. [25, 95] saw no difference in PGDH mRNA concentrations using microarray approaches in CL on days 12 and 14 of pregnancy and the estrous cycle. Aside from a change in synthesis or degradation of PGF2α or numbers or sensitization of available receptors on luteal cells, there are other potential mechanisms by which the CL may become resistant to PGF2α during maternal recognition of pregnancy. Redundant or complementary mechanisms may be in place to maintain the CL during pregnancy. PGE1 [96] or PGE2 [97], in addition to other conceptus-derived factors such as IFNT [25, 35, 52, 56, 57], may act on the CL to confer increased resistance of the CL to PGF2α as pregnancy progresses.

11.7 Endocrine Release of IFNT into the Uterine Vein

When ovine [98] and bovine [99, 100] ISG15 mRNA concentrations were first described to be upregulated in PBMC in response to early pregnancy, it was hypothesized that IFNT or an IFNT-induced cytokine was released into the uterine vein and had endocrine action during early pregnancy. Data to further support the concept for an endocrine role of pregnancy was provided in cattle through examining genes expressed in PBMC compared to endometrium on day 18 of pregnancy in dairy cows [35]. In this study, several hundred endometrial (674 genes upregulated and 721 downregulated ~1.5 fold; $P<0.05$) and PBMC (375 genes upregulated and 784 downregulated ~1.2 fold; $P<0.05$) genes were differentially expressed based on pregnancy status on day 18 of pregnancy. Many of the genes upregulated in response to pregnancy in PBMC were the same as those upregulated in endometrium and were ISGs. Pregnancy also induced 55 genes in CL on day 12 and 734 genes on day 14. ISGs (i.e., ISG15 and MX1) represented many of the genes induced by pregnancy that also were induced when culturing luteal cells with IFNT, but not PGE2 [25].

Other groups also have described a pregnancy-associated increase in ISGs in peripheral tissues. For example, Green et al. [101] reported a significant increase in ISG mRNA concentrations in leukocytes in response to pregnancy on day 18 in dairy heifers, although this was not observed in lactating dairy cows. In contrast, on day 19 of pregnancy, lactating dairy cows supplemented with progesterone had no change in leukocyte ISG mRNA concentrations; however, pregnancy caused an upregulation in concentrations of ISG mRNA [102]. A more extensive analysis of ISGs in leukocytes was completed using beef cows, which demonstrated an increase in mRNA concentrations between days 15 and 22 with a peak concentration by day 20 of pregnancy [103]. Use of leukocyte ISG mRNA concentrations to predict pregnancy status was more accurate when coupled with ultrasound examination for the presence of a CL on day 20 of pregnancy. Also, upregulation of ISGs in the liver by

day 18 of pregnancy was recently demonstrated in cattle [104], which is consistent with previous reports of upregulation of liver ISGs in response to pregnancy in sheep [52, 57]. Based on data from our laboratory in addition to these newer studies, one might make the inference that the detection of ISGs in blood is a reasonable indicator of pregnancy status in ruminants. However, there is a very large false-positive rate because of strong induction of innate immune responses, including upregulation of ISGs in response to infections and proinflammatory stressors in dairy cows (see [35, 105]).

To further delineate the mechanism by which ISGs were upregulated in extrauterine tissues, antiviral activity was evaluated in uterine vein blood from day 15 pregnant sheep. Significant amounts of IFNT (~200 μg/24 h) were released into the uterine vein on day 15 of pregnancy [58]. These results were actually similar to a previous paper that also described significant antiviral activity in uterine vein blood of pregnant sheep [106]. Preadsorption of uterine vein blood from day 15 pregnant ewes with a highly specific monoclonal antibody against recombinant ovine (ro) IFNT (provided by Dr. Fuller Bazer, Texas A&M University) significantly reduces antiviral activity [57]. By using a very specific and sensitive radioimmunoassay, IFNT has been detected in uterine flushings and uterine vein blood from pregnant sheep but was not detected in uterine flushings or uterine vein blood from nonpregnant sheep [52]. Similarly, by using mass spectroscopy, we have resolved IFNT in uterine vein serum from day 16 pregnant sheep based on detection of eight peptides ranging in size from 1147.75 to 2490.72 Da with a probability of amino acid match from 91 to 97 % (four peptides with >95 % match). Based on these studies, we suspect that IFNT is responsible for the bulk of the antiviral activity and is released in amounts large enough to elicit a peripheral endocrine response.

11.8 Infusion of IFNT into the Uterine Vein Provides Luteal Resistance to PGF2α

ISG mRNAs increased in the CL following intrauterine infusions or subcutaneous treatment with roIFNT [107]. In this study, intrauterine infusion of IFNT caused a delay of return to estrus whereas subcutaneous delivery did not, suggesting a primary paracrine role for IFNT on the endometrium. Endocrine actions induced by IFNT were further studied [57] using mini-osmotic pump infusion of roIFNT for 24 h or 7 days into the uterine vein. Utilizing the average weight of 60 kg and blood volume of 58 ml/kg for ewes, the blood volume was estimated to be 3.48 l. Oliveira and colleagues previously estimated that the release of IFNT into the uterine vein on day 15 is approximately 200 μg/day [58]. Based on these data, osmotic pumps were loaded to deliver 200 μg/day, which results in a release of 8.3 μg/h into the uterine vein. Employing the estimated blood volume as calculated here, systemic levels of IFNT in circulation would stabilize around 2.4 ng/ml/h. The calculated systemic level of IFNT in circulation is biologically relevant in the context of a dissociation constant (K_d) for the receptor of 3.7×10^{-10} M [108] and estimated 50 % occupancy of the receptor at 6.3 ng IFNT/ml, although, based on the concept of spare receptors,

only 1 % of IFNT receptors need to be occupied to elicit a biological response, which would reflect physiological levels of IFNT in the blood as low as 63 pg IFNT/ml. To achieve endocrine delivery of 200 μg/day of recombinant interferon tau (roIFNT), osmotic pumps were surgically installed into the abdominal cavity to infuse either BSA or roIFNT via a catheter into the uterine vein on day 10 of the estrous cycle in sheep.

Seven days of infusion of roINFT into the uterine vein from day 10 to day 17 of the estrous cycle resulted in 80 % (four of five ewes) of ewes having extended estrous cycles lasting through day 32, whereas all ewes infused with BSA returned to estrus by day 19 [57]. The single ewe that did not respond to this delivery of IFNT through delay of return to estrus had low serum progesterone concentrations at the time of pump installation that continued to decline over time, meaning that this ewe presented a "short cycle" and had already started the luteolytic process at the start of delivery of IFNT into the uterine vein. This experiment was the first demonstrating that endocrine delivery of low concentrations of IFNT systemically can induce a significant (long-term) delay in returning to a normal estrous cycle. Subsequent studies demonstrated that IFNT action through the induction of ISG15 consistently occurred in CL ipsilateral and contralateral to the side of infusion of IFNT into the uterine vein [56]. This result was interpreted to mean that IFNT probably did not cross over from the uterine vein to ovarian artery to act on the CL. Rather, the action of IFNT on the CL is thought to be a systemic rather than a local utero-ovarian plexus transport. Also, serum progesterone concentrations did not differ in roIFNT- versus BSA-infused ewes, which was consistent with reports by others showing that IFNT does not have a luteotropic role in the context of increasing steroidogenesis and production of progesterone [54, 109, 110].

To further study the mechanisms associated with endocrine action of IFNT, a series of experiments with delivery of IFNT into the uterine vein and jugular vein, as well as subcutaneous delivery of IFNT, were completed in the presence or absence of an exogenous challenge with PGF2α [56, 57]. It was reasoned that if IFNT had a direct action on the CL, then this would be reflected in blocking the effect of exogenous challenge with PGF2α in causing a decline in serum progesterone. Delivery of 20–200 μg/day roIFNT starting on day 10 of the estrous cycle into the uterine vein, jugular vein, or through a subcutaneous route in the neck effectively blocked the decline in serum progesterone caused by PGF2α injection on day 10.5 or 11 (depending on the study) in the BSA-infused controls [56, 57].

To examine mechanisms of IFNT action on the CL, CLs were collected at various times following infusion with IFNT and treatment with PGF2α. Following a 24-h infusion of IFNT, there was an induction of ISG15 mRNA (as well as other ISGs) in endometrium and ipsilateral and contralateral CL relative to the side of the osmotic pump as well as the liver. These ISGs may contribue to development of resistance of the CL to luteolysis,

Because daily temporal responses to pregnancy (IFNT) had not been described in sheep in context of IFNT concentrations and then regulation of genes in endometrium and CL, we recently completed a study to see if ISGs induced by pregnancy [52] were similar to those induced by mini-osmotic pump infusion of roIFNT [56, 57]. By using an IFNT radioimmunoassay, IFNT was detected in uterine flushings

by day 14 and in uterine vein serum by days 15–16 of pregnancy. ISG mRNA concentrations were detected in endometrium by day 13 of pregnancy, which was one day prior to upregulation of ESR1 and OXTR mRNA concentrations on day 14 of the estrous cycle. ISG mRNA concentrations in the CL and liver also were detected by day 14 and in peripheral blood mononuclear cells by day 15 in pregnant ewes. Concentrations of mRNAs for ISGs such as STAT1, STAT2, IRF7, IRF9 [52], and melanoma differentiation factor 5 (MDA5; DDX58), retinoic acid-inducible gene (RIGI/IFIHI), and ISG15 were greater in the CL on day 14 of pregnancy compared to the estrous cycle (Fig. 11.1). It was concluded that ISGs induced by pregnancy were similar to those induced by miniosmotic pump infusion of roIFNT.

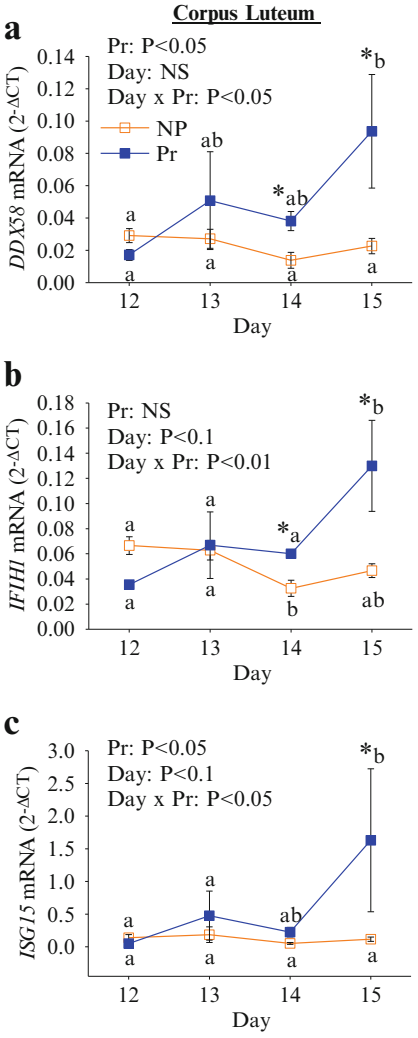

Fig. 11.1 Upregulation of ISGs mRNA concentration in corpora lutea as pregnancy progresses in sheep. Different superscripts represent differences ($P < 0.05$) across days of pregnancy. *Values differ because of pregnancy status on any given day. *Pr* pregnant, *NP* nonpregnant (not exposed to semen). Data reprinted with permission from [52]

To further examine the response of the CL to pregnancy, microarray studies were completed that demonstrated that pregnancy on day 14 was associated with differential expression of 734 genes, many of which were ISGs induced when culturing luteal cells with IFNT but not PGE2 [25]. In the CL of ewes, interleukin 6, luteinizing hormone/chorionic gonadotropin hormone receptor, pentraxin-related protein 3, and vascular endothelial growth factor gene expression was stabilized during early pregnancy, but diminished as the estrous cycle progressed and in response to culture of luteal cells with luteolytic hormones [25]. Culture of ovine LLC, SLC, and mixed luteal cells (MLC) with recombinant ovine (ov) IFNT [56] and bovine LLC and SLC with bovine (bo) IFNT (Fig. 11.2) resulted in increases in ISG15 mRNA (concentration-dependent) and protein (time-dependent) concentrations.

Based on these results, in addition to paracrine action on the endometrium to disrupt pulsatile release of PGF2α, pregnancy may also circumvent luteolytic responses through activation or stabilization of gene expression associated with interferon, chemokine, cell adhesion, cytoskeletal, and angiogenic pathways in the CL (Fig. 11.3).

Yang and coworkers [113] described upregulation of ISG15 in the bovine CL by day 16 of pregnancy, which continued to be upregulated through day 60 of pregnancy, which is consistent with other reports in cattle [114] and the induction of ISG15 in CL by pregnancy in the sheep [25, 52, 58]. However, attempts to induce ISG15 using bovine CL from day 15 of the estrous cycle through culture with rboIFNT did not succeed; whereas culture of ovarian stroma, endometrium and mammary cells all had greater ISG15 protein concentrations when cultured with rboIFNT. The lack of a bovine luteal cell response to culture with IFNT is in con-

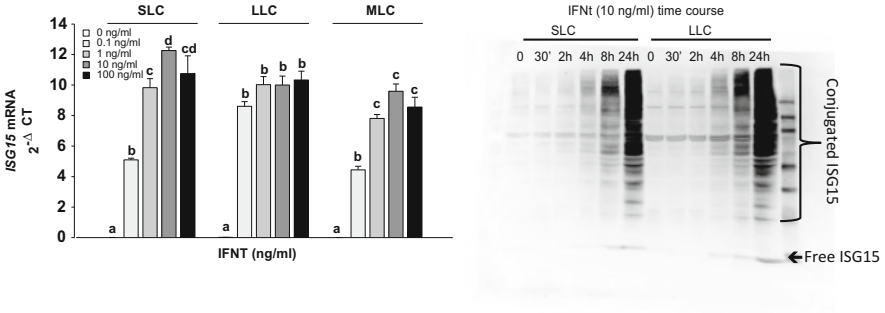

Fig. 11.2 ISG15 mRNA concentrations in ovine small (SLC), large (LLC), and mixed (MLC) luteal cells cultured with varying concentrations of rovIFNT (*left panel*) and ISG15 protein induced in bovine SLC and LLC following culture with 10 ng/ml rboIFNT over time (*right panel*). The left panel represents quantitative real-time PCR. (Reprinted with permission from [56]). The right panel represents data from cultured bovine luteal cells prepared by J.S. Davis (University of Nebraska Medical Center). Bovine luteal cells were purified by centrifugal elutriation [111]. After overnight attachment in media containing 10 % fetal bovine serum, the cells were pre-equilibrated in serum-free media for 2 h and treated with IFNT (10 ng/ml) for up to 24 h. Western blot analysis was performed using 5F10 mouse monoclonal antibody against rbISG15 [61]. ISG15 becomes covalently bound to targeted proteins, which are represented by conjugated ISG15 [112]. Means with different superscripts differ ($P < 0.05$)

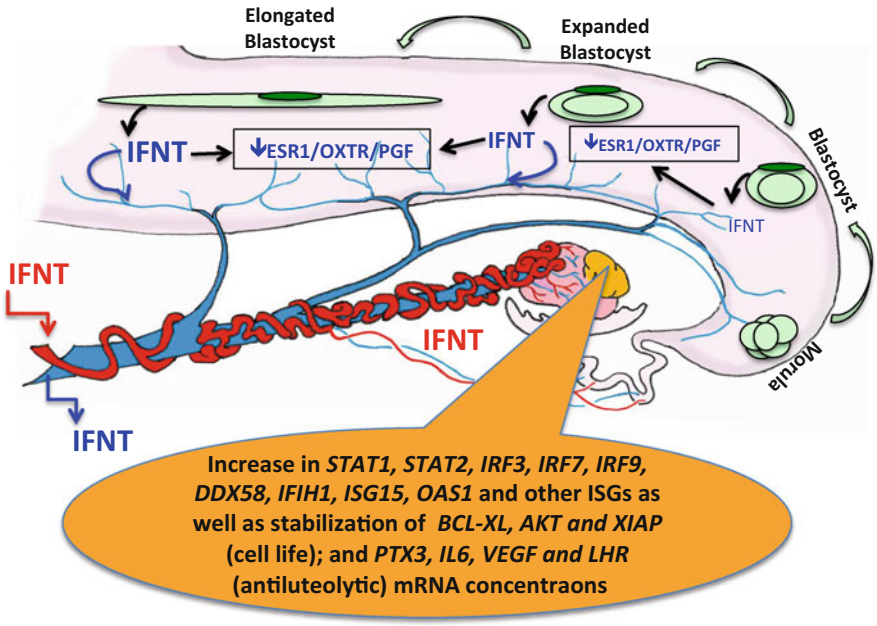

Fig. 11.3 Model of paracrine action of pregnancy (IFNT) on endometrium and endocrine action on the corpus luteum in ruminants

trast to studies using day 10–12 ovine CL, where culture with IFNT strongly upregulated ISGs. For this reason, we used the same rboIFNT (provided by Dr. R.M. Roberts, University of Missouri) and antibody against boISG15 (5F10 [61]) and describe herein that 24 h culture of bovine luteal cells with rboIFNT does indeed cause an upregulation in ISG15 free and conjugated proteins in both SLC and LLC. The only difference in this study compared to the Yang study was use of a ten-fold-lower concentration of rboIFNT and a different source of bovine corpora lutea.

11.9 Conclusions

IFNT is released from the conceptus trophectoderm cells in significant amounts about the time that the blastocyst starts to expand. IFNT binds type 1 IFN receptors and stimulates STATs and IRFs that upregulate transcription of specific set of genes (i.e., the ISGs) or downregulate genes encoding stimulators of synthesis and release of PGF2α through binding to IFN stimulatory response elements. IFNT inhibits the upregulation of endometrial ESR1, and this is thought to occur through inhibitory action of induced and phosphorylated transcription factors directly on the promoter of this gene in sheep. In cattle, the action of IFNT might reside on the

OXTR gene [115]. The bovine *OXTR* gene promoter region was cloned and found to contain an ISRE, ESR1 response element half-sites, and SP1 sites, and could only be transactivated by estrogen if cells were cotransfected with ESR1 and steroid receptor coactivator 1 [116]. Curiously, IRF2 overexpression, which is typically inhibitory in other systems, increased activity of the bovine *OXTR* promoter, but a direct effect of IFNT on promoter activity was not reported.

Regardless, in both sheep and cattle, the synthesis and pulsatile release of PGF2α is disrupted through paracrine action of IFNT on the endometrium, and this is considered to be an early maternal response to pregnancy and primary antiluteolytic mechanism that protects the CL in ruminants. As the conceptus develops and elongates, IFNT concentrations accumulate in the uterine lumen to the point where IFNT passes through the basement membrane of the endometrium and enters the endometrial venous drainage. Also, there may be variation in the expression of junctional complex proteins that allow the endometrial cells to become more or less leaky to movement of IFNT into the uterine venous blood [117]. IFNT responses, such as induction of ISG15 mRNA in the endometrium (paracrine action), can be detected as early as day 13 of pregnancy. This endometrial response to IFNT is followed 1–2 days later in extrauterine tissues (endocrine action) on days 14–15. The direct action of IFNT on the CL may confer resistance of the CL to the luteolytic pulses of PGF2α (see Antoniazzi et al. [34]). In addition, IFNT may control antiapoptotic mechanisms and cell survival genes to ensure luteal cell differentiation that prolongs luteal lifespan. Other studies remain to be done to determine where the lytic pathway downstream from binding of PGF2α to its receptor may be blocked during early pregnancy to confer resistance to luteolysis. Luteal resistance during early pregnancy may occur in response to ISGs as proximal to the PGF2α receptor as disruption of G-protein interaction to induce phospholipase C and consequent activation of protein kinase C; or as distal as possibly stabilizing cell survival gene expression and proteins to allow for recovery of the CL to any insults by PGF2α so that production of progesterone continues during early pregnancy. Whether IFNT acts alone or in concert with other CSP to directly protect the CL remains to be determined. Ultimately, longer-term luteal survival and resistance to lytic effects of PGF2α may be driven by pregnancy-induced ISGs, cell survival genes, and antiluteolytic mechanisms in the ruminant CL.

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Chapter 12
Corpus Luteum Regression and Early Pregnancy Maintenance in Pigs

Adam J. Ziecik, Emilia Przygrodzka, and Monika M. Kaczmarek

Abstract Development of the porcine corpus luteum (CL) requires the initial preovulatory LH surge and support of many biologically active agents including tonic secretion of LH, ovarian steroids, growth factors, and prostaglandins. A lack of embryo presence in the uterus leads to CL regression, characterized by disrupted progesterone production (functional luteolysis) and further degeneration of luteal and endothelial cells (structural luteolysis) triggered by prostaglandin F2α (PGF2α). The porcine CL expresses abundant levels of PGF2α receptors in the early and mid-luteal phase of the estrous cycle but remains insensitive to a single treatment of exogenous PGF2α until about day 12 of the estrous cycle. The nature of porcine CL resistance to PGF2α remains unknown, and the mechanism of luteolytic sensitivity acquisition involves infiltration of immune cells into the CL. Former theories of luteolysis inhibition and maternal recognition of pregnancy in the pig have proposed that possible mechanism for prevention of luteal regression is connected with a limited PGF2α supply to CL, evoked by its sequestering in the uterus. Later studies besides the increased synthesis of prostaglandin E2 (PGE2) by the conceptus and endometrium revealed simultaneously decreased expression of PGF2α synthesis enzymes. This chapter summarizes available knowledge on the porcine CL maintenance and regression and present our recent studies leading to a novel 'two signal-switch' hypothesis, based on the interplay of both PGF2α and PGE2 postreceptor signaling pathways. Several practical aspects of how to prolong and enhance CL function and improve pregnancy maintenance are also discussed.

Keywords Corpus luteum • Luteolytic sensitivity • Luteolysis inhibition • Pregnancy establishment • Embryo signals • Prostaglandins • Two-switches hypothesis • Pig

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12.1 Pro-Luteal Environment in the Reproductive Tract in Advance of Maternal Recognition of Pregnancy

In sexual reproduction, specific organs have been developed to allow the introduction and passage of egg and sperm to reach each other before fertilization. These organs support the physiological demands of gametes and later developing embryo(s); however, nurturing factors may not only originate from local reserves. The maternal reproductive tract hosts a critical crosstalk with the embryo that starts at the very early stages of pregnancy. Although the response of the reproductive tract toward embryos at the very early stages of pregnancy is poorly understood, several investigators suggested the presence of early communication among gametes, embryos, and the female reproductive tract before the main maternal recognition of pregnancy signal occurs in pigs. Some early gametes or embryos and mother communication pathways can also be involved in luteal function.

Before embryo signals are systemically recognized by the mother and luteal function is maintained through pregnancy, mating or insemination affects several local processes in the porcine reproductive tract. Studies performed in many species, including pigs [1], suggests that embryo–maternal communication exists at the very early stages of pregnancy, long before the well-known embryonic signals can be detected. In pigs, as in other mammals, deposition of semen into the female reproductive tract triggers a cascade of cellular and molecular events that in many respects resembles a classic inflammatory response [2, 3]. Within hours after mating, neutrophils are recruited into the uterine lumen [4–6]. In endometrial stroma, however, an accumulation of macrophages and dendritic cells, granulocytes, and lymphocytes occurs [2, 7]. It was shown that inseminate constituents, such as seminal plasma, modulate the endometrial influx of polymorphonuclear leukocytes after insemination [8, 9]. Leukocyte recruitment is elicited after seminal factors signal uterine epithelial cells to induce expression of a number of proinflammatory factors, including granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-6 [10]. Furthermore, prostaglandin synthesis and angiogenesis pathways were also affected in a transient as well as a more prolonged manner in the porcine oviduct and endometrium [11–13]. On the other hand, the effects of intrauterine seminal fluid on the ovary were manifested as clear changes in the development and steroidogenic competence of the corpus luteum (CL) [14].

The effects of uterine exposure to seminal plasma on prostaglandins synthesis and secretion persist over the course of the prereceptive period and are of considerable interest for achievement of the pro-luteal embryotrophic milieu in the reproductive tract. Altered expression of prostaglandin endoperoxide synthase 2 (PTGS2, or cyclooxygenase-2, COX-2), and PGF2α synthase (PTGFS) in the endometrium on days 5 or 10 after seminal plasma exposure was accompanied by an increased PGE2 level on day 10, this being crucial for modulation of the PGE2:PGF2α ratio shortly before the maternal recognition of pregnancy [12]. Thus, it seems likely that seminal plasma constituents can sensitize the endometrium for forthcoming pregnancy by amplifying the uterine synthesis of crucial antiluteolytic/luteoprotective PGE2 and supporting key events occurring during early pregnancy, such as embryo development and maternal recognition of pregnancy. Whether the increased number

of viable embryos and improved embryo growth observed by O'Leary and coworkers [10] 9 days after seminal plasma exposure might be linked with increased PGE2 levels and the PGE2:PGF2α ratio in the endometrium needs further investigation.

Interestingly, the effect of intrauterine seminal plasma exposure on CL development and ovarian steroidogenesis was also observed [14]. It was shown that plasma progesterone levels are higher and peaked earlier in gilts treated with seminal plasma. Concomitantly, this was associated with an increase in average weight of CL, without a concurrent increase in ovulation rate, suggesting that the number and output of steroidogenic luteal cells are greater in animals exposed to seminal components. Some authors proposed that the effect of seminal plasma presence in the uterine horns persisting over the course of early pregnancy might be partly the consequence of elevated local progesterone synthesis, which could act to differentially regulate several progesterone-responsive uterine parameters [15], including the observed altered cytokine, angiogenic factors, and prostaglandin synthesis pathway gene expression [10–13].

Moreover, significant elevation in the abundance of activated macrophages in the thecal and perifollicular stromal tissue 34 h after seminal plasma treatment suggests that these cells and their secretory products influence the architecture and functionality of the vascular stroma and theca tissues of the ovary with direct or indirect effects on granulosa cells, showing accelerated progesterone synthesis when cultured in the presence of human chorionic gonadotropin (hCG) [14]. Recently, the CL of macrophage-depleted mice have been shown to produce substantially less progesterone, have disrupted blood vasculature, and exhibit changes in the local expression of genes encoding angiogenic regulators [16]. The reduced progesterone production was fully responsible for the infertility defect in mice because pregnancy was restored and supported to term through exogenous progesterone administration. On the other hand, our findings showed clearly that seminal plasma can alter vascular endothelial growth factor A (VEGFA) ligand–receptor system expression and vascular density in the porcine endometrium and oviduct [12, 13]. These findings indicate the substantial function of semen in controlling macrophage luteal populations and its paramount function at this time to provide trophic support for formation of the vascular network pivotal to CL development, progesterone synthesis, and the establishment of viable pregnancy.

Recently, downregulation of a set of immune-related genes expressed in the presence of a 6-day-old blastocyst were observed in the porcine endometrium [1]. Additionally, changes observed in the uterine horn while the embryo was still in the oviduct imply that there is a local effect of the embryo on the oviduct that is extended to the uterine horn. These changes have been suggested to help prepare the uterus for the acceptance of the embryo, a semi-allograft in the maternal organism.

Taking into account the aforementioned facts, it seems likely that uterine response to the presence of semen and embryos could directly and indirectly influence the milieu of the reproductive tract. If we take under consideration the potential involvement of lymphatic pathways and countercurrent transfer of 'programming information' from uterine lymphatics into ovarian arterial blood [17], a hypothesis involving the indirect effects of semen and embryos on the female reproductive tract seems more likely to be accurate. Using this route, cytokines, PGs, and other biologically active molecules (of uterine, seminal plasma, or embryonic origin) may

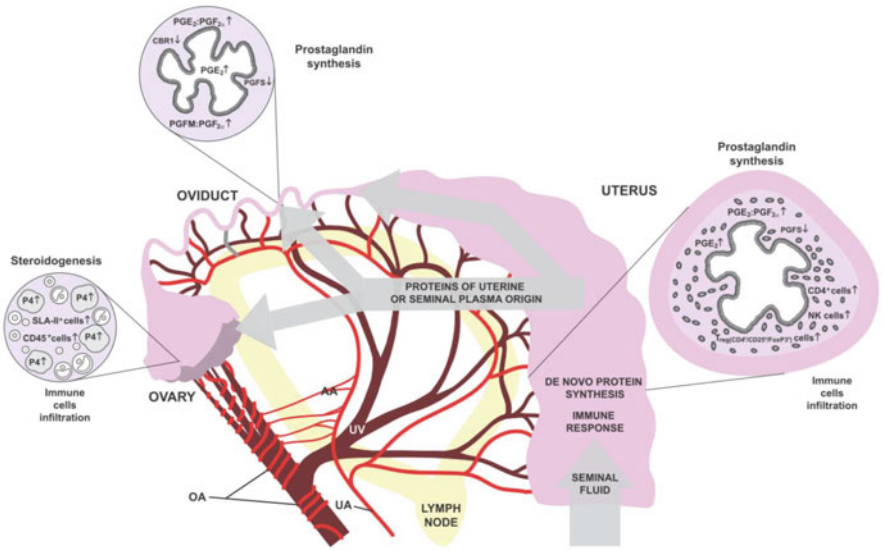

Fig. 12.1 The proposed potential mechanism of seminal plasma-mediated increase in early embryo survival and quality: involvement of steroidogenesis and prostaglandin pathways as well as immune cell infiltration. Seminal plasma initiates the immune cell infiltration (e.g., T and NK cells) and de novo protein synthesis in the endometrium. For instance, the prostaglandin synthesis pathway is affected, as PGE2 levels are higher and consequently the PGE2 to PGF2α ratio is increased in the endometrium. The biologically active molecules of uterine or seminal plasma origin can also reach the ovarian and oviduct tissues directly or via the arteries, having entered the uterine (UA) and ovarian arteries (OA) from uterine lymphatics or veins (UV) by means of countercurrent exchange. These sequences of events change prostaglandin synthesis in the oviduct as well as immune cell infiltration and progesterone synthesis in the ovary. *P4* progesterone, T_{reg} T-regulatory cells, T_h T-helper cells, *NK* natural killer cells, *AA* arterio-arterial anastomoses connecting uterine and ovarian arteries, *SLA-II* swine leukocyte antigen class II. (Modified from Ziecik et al. [20])

reach the oviduct and ovarian tissues via the arteries, having entered the utero-ovarian artery from the uterine lymphatics or veins by means of countercurrent exchange [18, 19], and in consequence affect several pathways in the female reproductive tract, including progesterone and PG synthesis, as well as angiogenesis, leading to successful pregnancy outcomes (Fig. 12.1).

12.2 Porcine CL Development, Regression, and Maintenance

12.2.1 Development

According to the old paradigm, formation of porcine CL requires only an initial surge of the preovulatory LH triggered by the pituitary, and then its further existence is independent on LH until day 12 of the estrous cycle. Such an opinion was

drawn from the experiment when pigs hypophysectomized on the first day of estrus developed apparently normal CL up to day 12 of the estrous cycle, which then regressed by day 16 [21]. Conclusion on the maintenance of LH dependence of CL was justified by the fact that the majority of mature gilts and postpartum sows exhibit the maximal LH levels at the first observance of estrus [22]. The question whether the porcine CL is fully 'autonomous,' as was believed earlier [23], still remains open. Evidence that (1) the passive immunization of the gilt with anti-pLH serum on day 8 of the estrous cycle dramatically decreased progesterone level in the blood [24] and (2) LH in a time- and dose-dependent manner increased secretion of progesterone by cultured luteal slices collected at mid-luteal phase [25] indicates that this pituitary gonadotropin may still have an important if not decisive function in the maintenance of porcine CL function. The supportive role of many biologically active agents including ovarian steroids [26–28], growth factors (e.g., IGF-I) [29], and prostaglandins [25, 30, 31] in luteal function maintenance was also well documented.

12.2.2 Regression

It is believed that regression of porcine CL occurring on days 15–16 of the estrous cycle results from an increase in pulsatile endometrial secretion of PGF2α [32]. However, the highest pulses of PGF2α occur after a decline of progesterone level in the blood plasma, that is, when the functional luteolysis of CL is completed [33]. So far, oxytocin [34], TNFα [31, 35, 36], and LH [37, 38] are considered as the potential modulators of endometrial prostaglandin production. A strong relationship between oxytocin, oxytocin receptors, and PGF2α release in vitro was reported for the cultured explants of porcine endometrium collected on days 15–16 of the estrous cycle [39, 40]. However, the agreement between peaks of oxytocin and inactive metabolite of PGF2α (PGFM) peaks in the blood of gilts reached only about 30 %, whereas blocking oxytocin receptors neither prevented luteolysis nor changed the duration of the estrous cycle [41]. Furthermore, a much higher agreement was found between peaks of LH and PGFM (75.5 %); thus, the "luteolytic" role of LH can be limited only to the period of the late luteal phase of the porcine estrous cycle [33].

It is believed that in pigs, as in many species, luteolysis is triggered mainly by PGF2α. Although porcine CL express abundant levels of PGF2α receptors also in the early luteal phase [42, 43], a luteal tissue remains refractory to a single treatment with exogenous PGF2α for the first 12–13 days of the estrous cycle. Furthermore, experiments employing the in vivo microdialysis system [31] and in vitro incubation of luteal slices of porcine CL [25] indicate an increased progesterone secretion after PGF2α treatment during the mid-luteal phase of the estrous cycle, that is, before acquisition of luteolytic sensitivity (LS). An acquisition of LS to PGF2α is still not a fully determined phenomenon in pigs. Moreover, it does not depend on a number of PGF2α-binding sites in luteal cells as suggested earlier [42, 43].It is a very complex process, but it seems likely that PGF2α induces different molecular pathways in porcine CL with and without acquired LS [44–48]. For example,

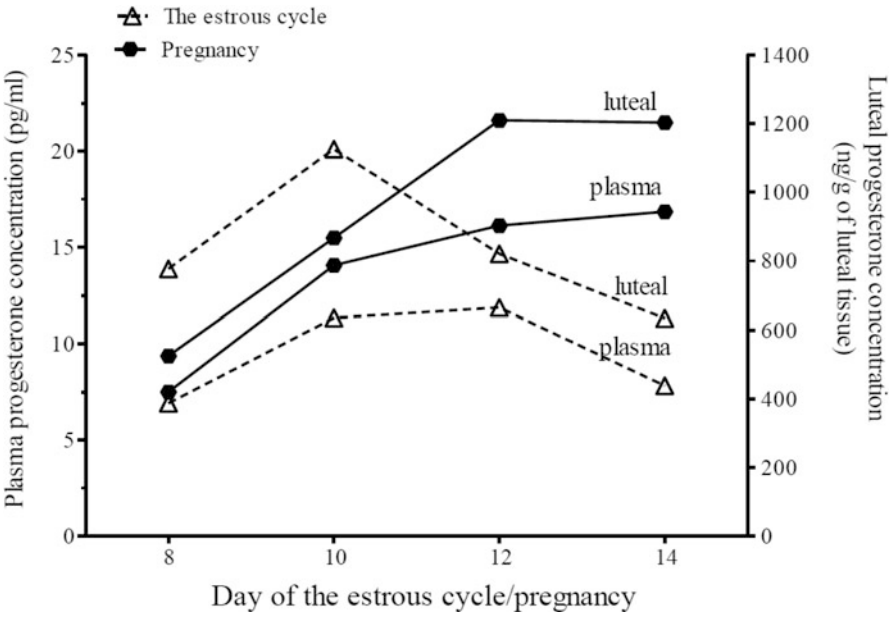

Fig. 12.2 Blood plasma and luteal concentration of progesterone on days 8–14 of the estrous cycle and pregnancy in pigs. (Adapted from Przygrodzka et al. [52, 53])

PGF2α affects the signaling pathway and its own synthesis [44, 49], as well as estradiol-17β [45], progesterone [46], endothelin-1 (EDN1) [50], chemokine CCL2 and its receptor (CCR2) [51] levels, but only in porcine CL being already sensitive to the luteolytic action of PGF2α.

Wuttke and colleagues [31] suggested that PGF2α-induced estradiol-17β secretion is stimulatory to progesterone production in young and middle-aged CL. The macrophage-delivered TNFα stimulates progesterone secretion in the early and middle-aged CL. Lack of estradiol-17β supply causes functional luteolysis triggered by PGF2α and TNFα [31]. It is interesting that the significant decrease of luteal progesterone content begins after day 12 of the estrous cycle contrary to the parallel day of pregnancy (Fig. 12.2). Factors inducing apoptosis (Bax and Bcl-2; TNF family) are also involved in the process of LS acquisition in pigs [48, 54].

It has become more generally accepted that elevated macrophage infiltration into porcine CL throughout the estrous cycle [55, 56], similarly to other species, coincides with the development of LS during the estrous cycle [29]. Macrophages are the major source of TNFα in the porcine corpus luteum [56]. A decreased luteal concentration of progesterone on day 14 of the estrous cycle is proceeded by an expression of *TNFA* and *IFNG* mRNA on day 12, suggesting that those cytokines are required for LS acquisition in pigs [52]. Recently, Przygrodzka and colleagues [53] identified TNFα receptor-1 signaling, apoptosis signaling, and production of nitric oxide (NO) and reactive oxygen species (ROS) among the canonical pathways activated in CL collected as early as on day 12 of the estrous cycle.

12.2.3 Maintenance

Establishment of pregnancy requires the maintenance of a functional CL beyond its normal cyclic lifespan to sustain production of progesterone. Progesterone stimulates secretory activity of the endometrium that is crucial for embryonic development and implantation. The first described embryonic signal in the pig is estrogen (mainly estradiol-17β) secreted by the conceptus on days 11 and 12 of pregnancy [57], that is, 2 days before CL begins to regress in nonpregnant gilts/sows.

The maternal recognition of pregnancy coincides with a rapid transformation of the conceptus from the spherical to tubular and then filamentous forms between days 10 and 12 after fertilization, when the first estradiol-17β peak secretion of conceptus origin is noted. The second peak of estradiol-17β secretion by conceptus appears in the maternal circulation on days 15–30 of pregnancy [28], when the lifespan of the CL is already extended. It is not clear whether the second elevation of embryo-originated estrogens in the maternal blood is related to CL maintenance during this period of pregnancy. In the pig, embryo implantation (days 14–18) and placentation take place during days 14–30 of pregnancy. It is believed that this second peak of estrogen conceptus secretion is rather needed for early embryo development. Moreover, estrogen was shown to affect the porcine CL in two ways, acting as a luteotropic or antiluteolytic agent. The luteotropic action of estradiol-17β depends on its direct action on CL by enhancing production of progesterone, as previously found in in vivo [26] and in vitro studies [25].

The indirect effects of estradiol-17β on porcine CL function range from an increase of luteal LH receptor concentration [58], and a decrease in PGF2α release from the uterus into the peripheral circulation [59], to the control of prostaglandin synthesis in the endometrium [60] and conceptus [61]. The period of estrogen secretion is also correlated with an increase of estrogen receptor expression in the luminal and glandular epithelium of the endometrium [62] and the conceptus itself [63].

12.2.4 Theories of Maternal Recognition of Pregnancy

The demonstration of aromatase activity in the preimplantation pig blastocyst [57] was confirmed and extended later by others [42], leading to the nomination of estradiol-17β as the embryonic signal necessary for the maternal recognition of pregnancy in the pig [64]. Observation of higher PGF2α concentrations in the uteroovarian vein between days 12 and 18 of the estrous cycle than in pregnant animals suggested that PGF2α is directed primary toward the uterine vessel drainage (endocrine direction) and to CL in nonpregnant animals [65]. After reaching the CL, PGF2α initiates a cascade of events leading to luteolysis. A low PGF2α accumulation in the uterine lumen (exocrine direction) during the estrous cycle [23] seemed to confirm the foregoing supposition.

According to the original Bazer and Thatcher [59] concept of maternal recognition of pregnancy, estrogens produced by the pig blastocyst as early as on day 11 (tubular and filamentous blastocysts) of pregnancy alter the direction of PGF2α secretion in pregnant pigs toward the uterine lumen, preventing PGF2α entrance to the uterine venous drainage and weakening its luteolytic effect on the CL. The authors suggested that the luteostatic effect of estrogens originating from the blastocyst (or exogenous estrogen) is mediated on the uterine endometrium level.

Another explanation for the abundance of PGF2α in the uterus is its retrograde transfer from the venous blood and uterine lymph into the uterine lumen, as well as accumulation of PGF2α by the uterine veins and arterial walls [18, 66]. The high PGF2α level found in the uterine lumen during early pregnancy would be a consequence of PGF2α uptake from the arterial blood, supporting the uterus, and its removal into the uterine lumen. Similarly, as suggested by Bazer and Thatcher [59], transfer of PGF2α to the uterine lumen may strongly reduce the peak of its concentration in the peripheral blood during pulsatile release of PGF2α from the uterus. Nevertheless, Hunter and Poyser [15] suggested that the exocrine redirection of the uterine PGF2α secretion may not provide a full explanation for maintenance of the CL in pregnant pigs and pointed out that this route of delivery for the luteolytic agent may not always be effective.

The aforementioned theories of maternal recognition of pregnancy of both 'endocrine versus exocrine' and 'retrograde' transfer of PGF2α in the porcine reproductive tract were proposed in the last decades of the twentieth century, before the "omics era" had dawned, and were focused on the sequestering of PGF2α in the uterus (early pregnancy) or its redirection toward the ovary (late luteal phase of the estrous cycle). Nevertheless, many researchers were encouraged to undertake new studies in the next decades using state-of-the-art methods.

12.3 The Roles of PGF2α and PGE2 and Their Receptors in Porcine CL Function

A part of the potential mechanism by which the conceptus prevents luteolysis is changing prostaglandin synthesis in favor of the luteo-protective PGE2. The porcine conceptus and endometrium synthesize large amounts of PGE2 before implantation [61, 67]. Additionally, the porcine myometrium secretes more PGE2 than PGF2α during early pregnancy [68]. The PGE2:PGF2α ratio is increased in the uterine lumen and vein [30, 69] as well as in the trophoblastic tissue on days 10–13 of gestation [61].

Evidence for a luteotropic/antiluteolytic effect of PGE2 in the pig was demonstrated by Akinlosotu and coworkers [70]. Moreover, a direct effect of exogenous PGE2 delivered in implants to luteal tissue in protecting porcine CL from the luteolytic dose of PGF2α was shown by Ford and Christenson [27]. However, the direct intrauterine application of PGE2 was incapable of extending luteal function in nonpregnant gilts [71] and simultaneously caused an elevation of PGF2α concentration in the uteroovarian venous blood [72], probably overcoming the luteotropic effect of PGE2.

On the other hand, an infusion of PGE2 into the ovarian artery elevated the concentration of progesterone in the ovarian venous blood on days 13 and 14 of pregnancy [73]. During early pregnancy, the expression of microsomal PGE2 synthase (*mPGES1*) is intermediate in the porcine endometrium on days 10–11, low on days 14–17, and increases after day 22 [67]. Its mRNA and protein levels were significantly elevated (28 fold versus days 14–15) on days 10–13 in spherical/tubular and filamentous conceptuses [61]. *mPGES1* leads to the higher PGE2:PGF2α ratio in spherical/tubular day 10–13 conceptuses at the time of maternal recognition of pregnancy. Also, the PGFM:PGF2α ratio, which is an index of 15-hydroxyprostaglandin dehydrogenase activity, was very low in spherical/tubular and filamentous conceptuses and markedly enhanced after day 14 of pregnancy [74]. These results suggest an increased metabolism of PGF2α during implantation to prevent the luteolytic effect of native PGF2α. The pattern of *mPGES1* expression in conceptuses maximizes, in such a way, the biological effect of luteotropic PGE2 and overlaps with the occurrence of the biphasic profile of estrogen synthesis and secretion by blastocysts [75].

In contrast, the low expression of carbonyl reductase, PG 9-ketoreductase (CBR1), an important enzyme catalyzing nicotinamide adenine dinucleotide phosphate (NADPH)-dependent reversible conversion of PGE2 into PGF2α in conceptus on days 10–13 of pregnancy, also indicates a significant contribution of the preimplantation conceptus to the synthesis of PGE2 during the maternal recognition of pregnancy in the pig [61]. Simultaneously, moderate changes in levels of PGF2α and PGE2 synthases occur in the porcine CL [76] and endometrium [67]. In addition to the possible endocrine role of conceptus-delivered PGs in porcine CL function, it can be important in altering gene expression in the endometrium before pregnancy recognition [77].

Another explanation for a mechanism preventing luteolysis can be distribution of PGF2α and PGE2 transporters, that is, ABCC4 (ATP-binding cassette subfamily C member 4 out of the cell, at the cell surface) and SLCO2A1 (solute carrier organic anion transporter family member 2A1, into the cell), respectively, which were abundant in the porcine endometrium on day 12 of pregnancy when conceptuses elongate and initiate implantation in pigs [78]. However, estradiol-17β did not increase *ABCC4* and *SLCO2A1* mRNA levels in cultured explants of porcine endometrium [78]. On the other hand, a high content of both PGs transporters is in agreement with the elevated concentrations of PGF2α and PGE2 in the uterus of pregnant gilts [79].

Earlier pioneering studies by Gadsby and coworkers [42, 43] could suggest that the level of PGF2α receptors (PTGFR) is fundamental in sensitizing the porcine CL to the luteolytic action of PGF2α, because a decreased expression of this receptor was found in luteal cells of pregnant and pseudo-pregnant pigs. In contrast, Przygrodzka and coworkers [53] did not observe a significant decrease of PTGFR at either mRNA or protein levels (Fig. 12.3) in the CL of early pregnant gilts in comparison to CL of cyclic gilts. The present results support a similar distribution of PTGFR mRNA in cyclic and early pregnant sheep [80]. It is worth noting that Gadbsy and colleagues [42, 43] used a different technique, investigating the capacity of [^{3}H]PGF2α-binding sites in isolated porcine luteal cells. It is unclear whether a high number of binding sites reflects their ability to activate postreceptor signaling

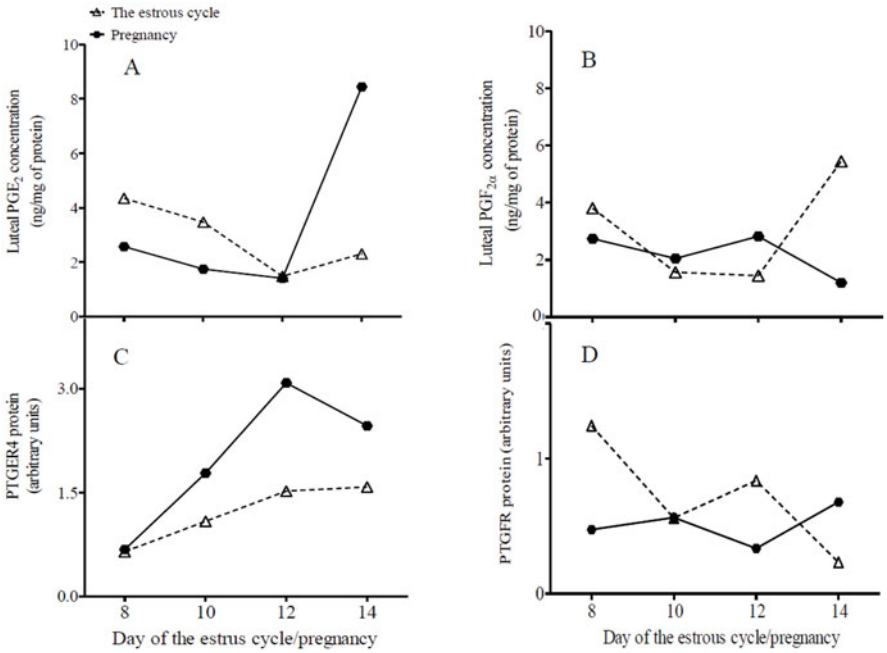

Fig. 12.3 Concentrations of PGE2 (**a**) and PGF2α (**b**) and the content of PTGER4 (**c**) and PTGFR protein (**d**) in corpus luteum on days 8–14 of the estrous cycle and pregnancy in pigs. (Adapted from Przygrodzka et al. [52])

mechanisms because recent studies suggested new, alternatively spliced PTGFR, leading to stimulation or inhibition of CL function [81].

On the other hand, expression of PTGS2 mRNA and protein [53], as well as the content of PGF2α, was elevated in the porcine CL at the time of luteolysis (Fig. 12.3). It brings evidence that confirms the concept of possible increase of PGF2α synthesis in CL with acquired LS [44].

Surprisingly, PTGFR mRNA [53] and protein levels (Fig. 12.3) were more abundant in the CL of pregnant gilts than the cyclic counterparts. Because the expression of PTGFR is prominent in endothelial cells of porcine CL [82], PGF2α may be involved also in luteal function maintenance after overcoming luteolysis during early pregnancy through its participation in angiogenesis [83, 84]. Interestingly, treatment with PGF2α elevated the synthesis of progesterone and content of cAMP-response element-binding protein (CREB) in cultured luteal slices from pregnant pigs [53].

Simultaneously, a decreased level of PTGFR (Fig. 12.3) in CL collected on day 14 of the estrous cycle could be an effect of negative feedback between increasing concentrations of intraluteal PGF2α and PTGFR expression, as previously described in porcine luteal tissue [44]. The earlier report of Zorilla and coworkers [49] suggested that activation of different post-PTGFR signaling pathways, for example, an

increase of the specific protein kinase C (PKC) ε expression, is more important for acquisition of luteolytic sensitivity in porcine CL than just a precise level of PTGFR.

Przygrodzka and coworkers [53] showed a fourfold higher concentration of PGE2 in the porcine CL on day 14 of pregnancy than on the parallel day of the estrous cycle (Fig. 12.3). Similarly, PGE2 content was higher only in CL ipsilateral to the gravid horn of unilateral pregnant gilts [85]. Because PGE2 content did not correspond to the mPGES1 expression in the luteal tissue, analogical as in ovine CL [86], the synthesis of PGE2 in the conceptus and endometrium, rather than in CL, seems to contribute to the process of luteal function rescue during the maternal recognition of pregnancy in the pig. A local transfer of PGE2 from the uterus to the ovary [73] may be involved in this mechanism. Moreover, a significantly increased content of one isoform of PGE2 receptors (PTGER4) was found in porcine CL collected on days 12 and 14 of pregnancy (Fig. 12.3). The presence of a second isoform of PGE2 receptor (PTGER2) was also documented earlier [36], and both receptors were shown to participate in cAMP production in cultured luteal slices [53]. It is worth emphasizing that secretion of luteal progesterone is stimulated by PGE2 through a cAMP-mediated pathway in many species [85]. As PGF2α increased the content of cAMP response element-binding protein (CREB) in CL of early pregnant pigs [53], it is possible that 'luteolytic' PGF2α can enhance accumulation of cAMP, already stimulated by PGE2, via both Ca^{2+} and PKC activation [87] as well as increase availability of CREB for its further activation by luteotropic hormones. Our recent in vitro studies [25] showed that PGF2α enhanced progesterone secretion by precision-cut luteal slices obtained from the mid-luteal phase CL, but diminished progesterone secretion by luteal slices obtained at the late luteal phase. The observed effects were consistent with results of in vivo experiments employing the microdialysis system in pigs [31].

The aforementioned studies clearly indicate a stronger role of conceptus- and uterus-delivered PGE2 in the rescue of porcine CL. Figure 12.4 presents the 'two signal-switch' hypothesis on the role of post PGF2α and PGE2 signaling pathways in CL regression and its overcoming during maternal recognition of pregnancy in the pig. It seems likely that the PTGFR level is less responsible for events leading to regression of CL than a sudden shift in the post-PTGFR signaling pathways occurring in CL after acquisition of luteolytic sensitivity, most probably under the influence of cytokines and endothelin-1. At the time when cells within the CL are 'sensitive' to PGF2α ("the LS switch"), its postreceptor signaling pathway leads to activation of the PKC pathway via diacyloglicerol (DAG) and inositol (1,4,5)-triphosphate (IP3) formation as well as elevated concentration of Ca^{2+}-activating protein-serine threonine kinase (RAF1) and initiating signaling cascades RAF1/MAPK1/ERK1/2. Activated proteins ERK1/2 can be translocated to the nucleus, where they phosphorylate transcription factors such as ETS domain-containing protein (ELK-1) and affect transcription of early response genes, that is, cellular oncogene *FOS* and *JUN*. In contrast, the aforementioned post-PTGFR pathway, leading finally to functional and structural destruction of CL, is blocked during the period of pregnancy establishment or CL rescue. By means of an embryonic signal ("the RESCUE switch"), PGE2 through PTGER2 and PTGER4 may activate protein kinase A, leading to inhibition

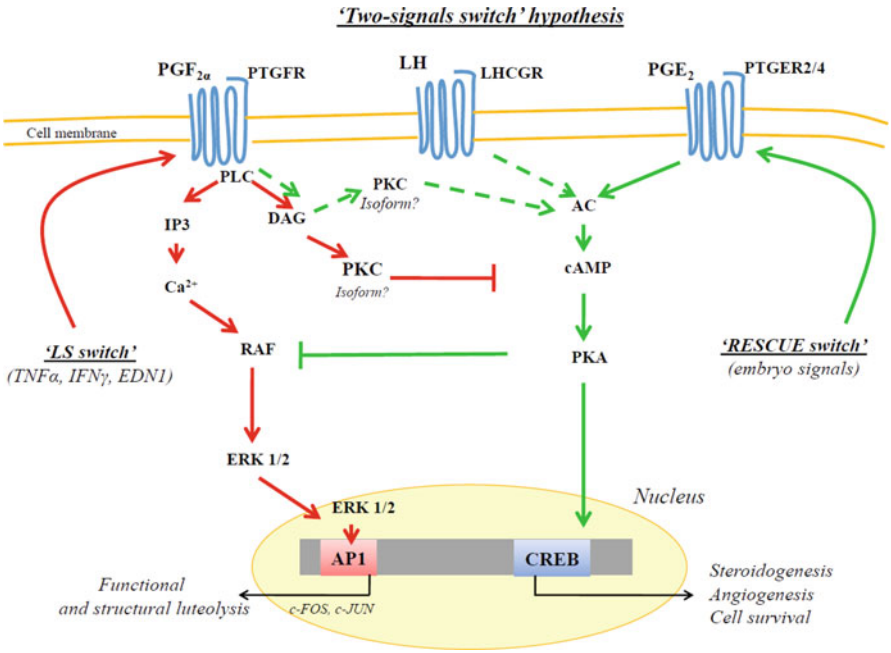

Fig. 12.4 'Two signal-switch' hypothesis of PGF2α and PGE2 involvement in regression or rescue of porcine CL. Before acquisition of luteolytic sensitivity (LS), PGF2α acts on luteal cells through its specific transmembrane G protein-coupled receptor (PTGFR) amplifying LH-stimulated cAMP accumulation (*dotted green arrows*) and supports porcine CL function until day 12 of the estrous cycle, i.e., until acquisition of LS. Turning the 'LS switch' on by mediators of luteal regression (*red pathway*) changes the post-PTGFR sequence of events leading to inhibition of cAMP accumulation and to luteolysis. However, during pregnancy the embryo(s) signals estradiol-17β and PGE2, produced mainly by conceptuses and the endometrium, to turn the 'RESCUE switch' on (*green solid arrows*) and induce the post-PTGER2/4 pathway leading to protein kinase A (PKA) activation. The *letter green pathway* leads to inhibition RAF, blockage, or turning off the 'LS switch' and activation of CREB for maintenance of steroidogenesis, angiogenesis, and cell survival. *PLC* phospholipase C. (Adapted from Przygrodzka et al. [52, 53])

of main downstream elements of the post-MAPK signaling pathway, most likely via direct blockage of MAPK activator–RAF [88, 89] and turning of "the LS switch." In consequence. instead of apoptotic genes induction, the expression of CREB is enhanced to support steroidogenesis, angiogenesis, and cell survival.

Additionally, our previous studies suggest that the luteoprotective action of PGE2 may involve a stimulation of VEGF expression in luteal cells on day 10–12 of pregnancy [90]. Interestingly, downregulation of a strong endogenous antagonist of VEGF soluble receptor (sFLT1) in the CL on day 12 of pregnancy may increase the amount of bioavailable VEGF in the porcine CL [91]. As a result, prolonged progesterone production is enhanced by increasing luteal capillary permeability and delivery of cholesterol to the luteal cells as well as facilitated PG transport from the circulation.

12.4 Genes Involved in Rescue of Corpus Luteum

Beside modulation of post-PGF2α and -PGE2 receptor signaling pathways, as well as an increase of intraluteal concentration of progesterone, the presence of live embryos in the uterus can enhance the expression of crucial genes involved in steroidogenesis [i.e., scavenger receptor class B, member 1 (*SCARB1*), steroidogenic acute regulatory protein (*STAR*), hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1 (*HSD3B1*), and luteinizing hormone/choriogonadotropin receptor (*LHCGR*)] in porcine CL collected on day 14 of pregnancy [53]. Found in the same study, the elevated levels of nuclear receptor subfamily 5, group A, member 1 (*NR5A1* = *SF-1*), an activator of steroidogenic genes transcription, suggest that its presence can be important to sustain progesterone production as suggested for bovine CL [92]. Similarly, the high abundance of progesterone receptor membrane component 1 (*PGRMC1*) in porcine CL collected on day 14 of pregnancy [53] could be essential to enhance steroidogenesis [93] and can be the gateway of antiapoptotic action mediated by progesterone in luteal cells [74].

Recently, Przygrodzka and coworkers [52, 53] examined the expression of 50 genes associated with synthesis and action of steroids, PGs, angiogenesis, and apoptosis in the porcine CL collected at the mid- and late luteal phases of the estrous cycle and parallel days of early pregnancy. Venn diagrams revealed that *EDN1*, cytochrome P450 19A1 (*CYP19A1*), estrogen receptor 2 (*ESR2*), *PTGS2*, *JUN*, and *FOS* were downregulated on day 14 of pregnancy, whereas among upregulated genes kinase insert domain receptor (*KDR*), angiopoietin 2 (*ANGPT2*), pentraxin 3 (*PTX3*), *HSD3B1*, low density lipoprotein receptor (*LDLR*), *STAR*, estrogen receptor 1 (*ESR1*), *LHCGR*, progesterone receptor (*PGR*), *PGRMC1*, progesterone receptor membrane component 2 (*PGRMC2*), *NR5A1*, nuclear factor of kappa light polypeptide gene enhancer in B cells 1 (*NFKB1*), prostaglandin F synthase (*PTGFS*), and hydroxyprostaglandin dehydrogenase 15-(NAD) *(HPGD*) were identified. For example, Fig. 12.5 presents the relatively constant expression of 13 genes connected to steroidogenesis, angiogenesis, and PG metabolism on day 12 of pregnancy and the estrous cycle but their up- or downregulation 2 days later.

Moreover, in silico analysis revealed that T-cell migration, activation of leukocytes, and infiltration of lymphocytes were already inhibited in CL obtained on day 12 of pregnancy. Also, the production of NO and ROS in macrophages was among decreased ingenuity pathways analysis of biological functions and pathways in CL collected on days 12 and 14 of pregnancy.

Although the involvement of immune cells in the regulation of regression and rescue of porcine CL remains practically unknown [55], the data presented here suggest the potential role of immune system cells in the control of luteal lifespan in the pig. It seems likely that crosstalk between immune cells products, that is, cytokines and factors involved in post-PG receptor-signaling pathways are crucial for CL lifespan during the estrous cycle and pregnancy in pigs and other mammals.

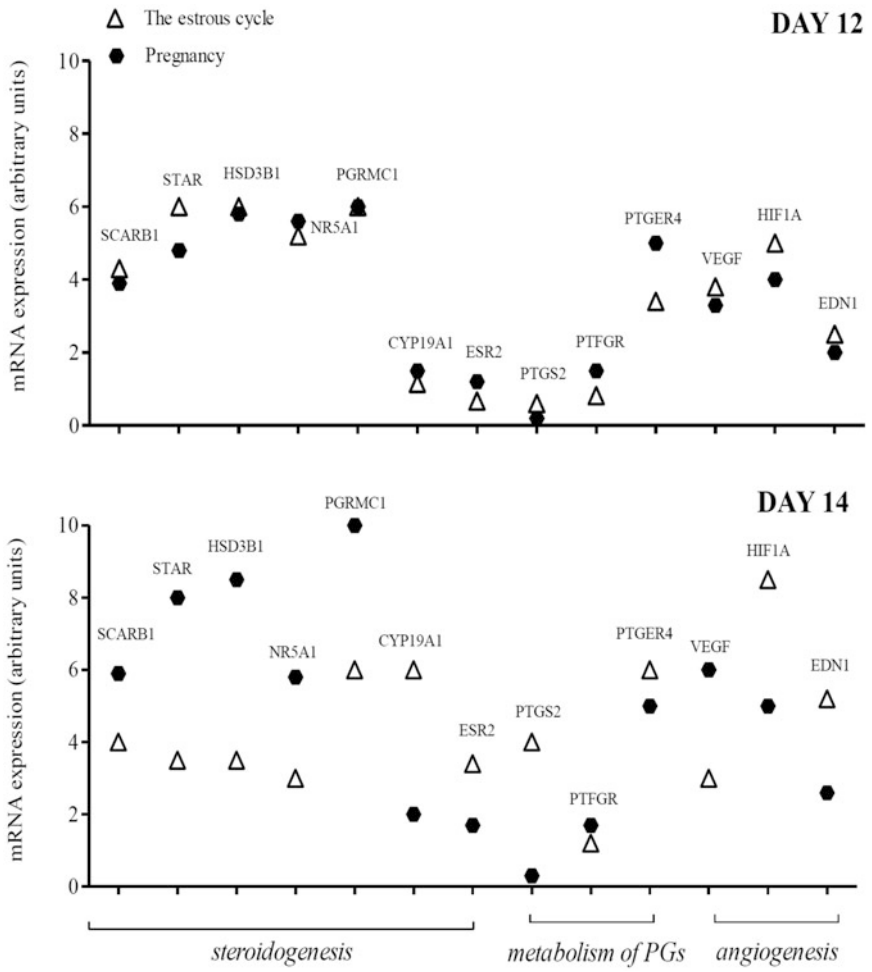

Fig. 12.5 Schematic presentation of mRNA expression of 13 genes potentially involved in the function of porcine corpus luteum on day 12 (*upper panel*) and day 14 (*lower panel*) of the estrous cycle and pregnancy

12.5 The Effect of hCG Administration on Luteal Function Maintenance During the Estrous Cycle and Pregnancy

Because the majority of embryonic losses (20–30 %) in pigs occurs between days 12 and 30 of gestation [94] and the level of progesterone is positively correlated with embryonic survival during the first week of pregnancy [95], many attempts

with progesterone supplementation were performed to support pregnancy. Intriguingly, some studies showed improved embryonic rates [95], whereas others indicated its negative influence on fertilization [96] and embryo survival rates [97]. A single injection of hCG on day 12 of the estrous cycle prolonged the lifespan of CL; consequently, extended progesterone production and delayed luteolysis in the pig were observed [98, 99]. Except increased progesterone concentration on days 15–17 of the estrous cycle, elevated amounts of estradiol-17β in the blood plasma were indicated on days 14 and 15 of the estrous cycle. Because estradiol-17β down-regulates endometrial PTGFS and CBR1 protein concentration [76], both estradiol-17β alone and the increased ratio of PGE2:PGFM could be responsible for prolonged luteal function in hCG-treated cyclic gilts.

A sufficient supply of progesterone and continuous maintenance of CL are necessary for the establishment of pregnancy in the pig [20]. A minimum of 4 ng/ml progesterone in the blood plasma has been found to be crucial to maintain pregnancy in pigs [100]. Moreover, the concentration of progesterone is positively correlated with embryonic survival during the first month of gestation [95]. However, a single administration of hCG did not affect progesterone content in the systemic circulation of pregnant gilts [98, 101]. Similarly, injection of hCG during the first 8 days of pregnancy has not affected the concentration of progesterone in the blood plasma [102]; this may be caused by an increased metabolism of progesterone and its active transport into the uterus. In contrast, administration of hCG on day 12 of pregnancy led to elevated amounts of progesterone caused by an increased number of additional CL in ruminants [103, 104]. In pregnant gilts, injections of 500 or 1000 IU hCG did not affect the number of CL [101], but elevated amounts of estradiol-17β on days 14 and 15 of pregnancy were observed [98, 101]. A similar effect was revealed in pregnant sheep [103].

On the other hand, a single intramuscular injection of 750 IU hCG increased embryonic viability on day 30 of pregnancy in the pig [98, 101]. Moreover, in the luteal tissue of pregnant gilts given 750 IU hCG, augmented expression of STAR and LH/hCG receptors was found, with simultaneously increased angiogenesis, a reduced percentage of CL cells in the stage of early and late apoptosis, and elevated percentage of viable cells [98].

The majority of studies performed so far on the effect of various hormones on embryo survival in pigs were concentrated exclusively on the period up to day 30 of pregnancy [98, 101, 105–107]; thus, it was still not clear whether this effect can be maintained until the end of pregnancy. Recently, studies have clearly showed that hCG does not have a negative effect on the pregnancy rate, but administration of hCG on day 12 or 20 of pregnancy results in an elevated litter size and significant increase in the number of total piglets born, respectively [108]. Additionally, the number of piglets weaned tended to be increased in sows treated with hCG on day 20 of pregnancy. This study revealed that hCG administration during early pregnancy does not have a negative effect on pregnancy performance in gilts and sows and can be even beneficial for pregnancy outcome.

12.6 Other Attempts to Prolong CL Function in Pigs

Early studies showed that the luteal function in pigs is continued until day 60 of pregnancy after removal of all fetuses on day 30 of gestation [107], which may indicate that intrauterine stimulus is not needed to maintain the porcine CL between days 30 and 60 of gestation. Similar observations were made in pigs during pseudo-pregnancy caused by injections of pharmacological doses of estrogens between days 11 and 15 of the estrous cycle [109, 110]. Estrogen treatment on days 12–15 of the estrous cycle sufficiently suppresses the luteolytic effects of the uterus and allows continuation of the luteal function for a period similar to that observed after hysterectomy [111]. However, we have to bear in mind that estrogen-induced pseudo-pregnancy does not fully mimic the endocrine events associated with early pregnancy [112].

The possibility of using a vaginal route delivery of biologically active factors applying low doses of PGE2 and estradiol-17β to affect luteal function in cyclic gilts was also studied. Prolonged luteal function and extended synthesis of progesterone were observed in two of five gilts simultaneously receiving PGE2 and estradiol-17β on days 11–16 of the estrous cycle [113]. Intravaginal application of PGE2 and 17β-estradiol in pregnant primiparous sows revealed their possible supporting effects on luteal function when administered in the second crucial period adjusted to a natural increase of embryonic estrogens in the blood, that is, on days 16–25 of gestation [28], the time when the establishment of pregnancy occurred and embryo implantation ends in pigs. Although there were no significant differences in the number of total piglets born, a clear tendency to increased numbers of live-born and weaned piglets was noticed [108]. Summarizing, the intravaginal application of estradiol-17β and PGE2 on days 17–23 of pregnancy seems to be a promising approach to improve embryo survival, but a practical treatment protocol should be elaborated in the future.

12.7 Concluding Remarks

The pro-luteal environment in the reproductive tract in advance of maternal recognition of pregnancy caused by gametes, embryos, and seminal plasma is beneficial but not sufficient for prolonged CL lifespan. In pigs, pregnancy recognition is the result of conceptus secretion of estrogens on day 11 and 12, which affects PG synthesis and transport in favor of luteoprotective PGE2.

The development of advanced ‘omics’ tools in the past 25 years has revolutionized research methods also in the reproductive biology of pigs. The former basic theories of the maternal recognition of pregnancy in pigs are valuable but seem to be insufficient to fully understand the process of CL rescue, dependent on cooperation of many pleiotropic factors at systemic, local, and intracellular molecular levels.

Further studies are needed to explain how conceptuses and endometrial factors regulate differential PGs synthesis, the way of their release on days 11–14 of estrous cycle and pregnancy, or differential response of CL to PGF2α and PGE2 in those

periods. Perhaps the first time presentation of the 'two signal-switch' hypothesis of PGs involvement in CL rescue will be a small contribution to understanding the complexity of CL control and function and give impetus to further large-scale investigation. One of the most important challenges in understanding the mechanism of CL function in the pig and other species is to establish a hierarchy and timing of molecular relationships between numerous mediators of luteal regression (cytokines, chemokines, endothelin-1) and rescue (embryo signals, angiogenic factors, gonadotropins), as well as post-PG receptor intracellular pathway elements.

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Chapter 13
The Corpus Luteum and Women's Health

W. Colin Duncan

Abstract The corpus luteum of women is remarkable. The dominant follicle shows high levels of cellular proliferation, and its transition into the corpus luteum involves intense angiogenesis, higher than that seen in the most aggressive solid tumors. The corpus luteum, however, does not undergo malignant change. Its formation and resolution involve acute inflammatory responses and yet these processes are completed with no evidence of scarring. It is the most active endocrine gland in the body with a huge capacity for steroid synthesis, making up to 40 mg progesterone each day, and there are no disorders of clinical function. It has an essential role in human reproduction and the establishment of pregnancy, and yet we still do not fully understand how it works at a molecular level. The corpus luteum is fundamental to women's health but research into the human corpus luteum is decreasing. However, although increased molecular understanding of the corpus luteum in women may facilitate the development of novel contraceptive paradigms and strategies to reduce the incidence and impact of ovarian hyperstimulation syndrome in assisted conception, it is likely that understanding the corpus luteum will provide generic insights into processes that affect the health of men and women throughout and beyond their reproductive years.

Keywords Corpus luteum • Human • Luteinizing hormone • Luteinized granulosa cells • Angiogenesis • Vascular endothelial growth factor • Human chorionic gonadotropin • Ovarian hyperstimulation syndrome • Inadequate • Progesterone • Luteinized unruptured follicle

13.1 The Human Corpus Luteum

The corpus luteum in women, as in other mammalian species, produces large amounts of progesterone to promote the establishment of pregnancy. To do this it has large, metabolically active, steroidogenic cells surrounded by an extensive capillary network, which is required to bring trophic molecules, nutrients, and building

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blocks and to disseminate secreted products. In women, the corpus luteum is induced by, and maintained by, luteinizing hormone (LH) from the pituitary gland. It is formed from the dominant follicle and loses its functional and structural integrity after 14 days.

During the luteal phase of the ovarian cycle, the human corpus luteum undergoes marked tissue and vascular remodeling [1]. Normal luteolysis occurs in the absence of a uterus and in the presence of maintained LH concentrations. In women, formation of the corpus luteum sets in place an inherent process that results in inevitable luteolysis after 14 days in a non-conception cycle.

In a conception cycle, maternal recognition of pregnancy involves the early conceptus sending a trophic signal to the corpus luteum to rescue it from luteolysis [1, 2]. That signal is human chorionic gonadotropin (hCG), which functions as a long-acting LH-like molecule to maintain the structural and functional integrity of the corpus luteum. In early pregnancy removal of the corpus luteum will induce miscarriage, and ongoing luteal function is required to maintain pregnancy [3]. At around 8 weeks of gestation, the luteo-placental shift occurs, as the placenta is producing enough progesterone to support pregnancy, and luteectomy has no effect on the continuation of the pregnancy.

13.2 Clinical Correlates of the Ovarian Cycle in Women

The ovarian cycle in women can readily be assessed using serial ovarian ultrasound scans and serum hormone assessment across the menstrual cycle. At the time of menstruation there are numerous antral follicles in the ovaries measuring 4–8 mm in maximum diameter (Fig. 13.1a). These follicles have developed from primordial follicles through gonadotropin-independent and gonadotropin-sensitive phases of growth [4]. It is difficult to determine how long this process takes in vivo but evidence from tissue transplantation studies suggests that it is longer than 3 months [5]. These antral follicles are gonadotropin dependent and will not continue to grow without gonadotropin stimulation.

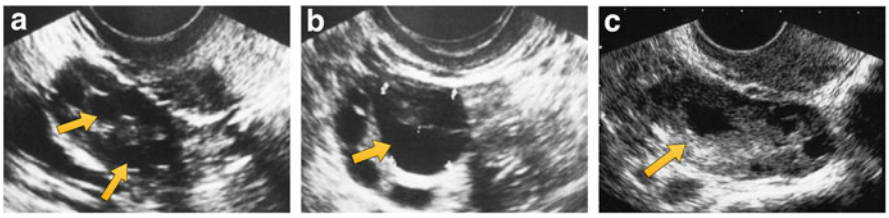

Fig. 13.1 Ultrasound scan images of the human ovary. (**a**) The right ovary in the early follicular stage with several 4- to 8-mm follicles (*arrows*). (**b**) The right ovary in the preovulatory phase with a dominant follicle (*arrow*) measuring 17 mm. (**c**) The right ovary in the luteal phase with the corpus luteum clearly visible (*arrow*)

The corpus luteum secretes steroid (progesterone, estradiol) and peptide (inhibin A) hormones that have negative feedback on the pituitary to suppress pituitary gonadotropin secretion during the luteal phase, which means that, in contrast to most mammalian species, follicular growth is inhibited during the luteal phase of the ovarian cycle. These hormones decline during luteolysis, and it is this decline in luteal steroid hormones that destabilizes the endometrium and induce menstruation [6]. However the fall in luteal hormones increases pituitary gonadotropin secretion, to stimulate the growth of the small antral follicles present in the ovaries at menstruation, and the follicular phase of the ovarian cycle begins [4].

The rate of follicle growth can vary from cycle to cycle and woman to woman. Indeed, differences in the length of a menstrual cycle are a consequence of variations in the length of the early to mid-follicular phases. However, when the lead follicle reaches 12 mm in diameter, generally about day 9 or 10 of the menstrual cycle, it then grows at an average of 2 mm in diameter each day. This growth is associated with rapidly increasing estradiol concentrations that exert negative feedback on the pituitary to reduce follicle-stimulating hormone (FSH) secretion (Fig. 13.2a, b).

Follicles more than 12 mm in diameter will already be developing LH receptors on granulosa cells, and LH will maintain follicle growth and function in the presence of declining FSH concentrations. This mechanism is responsible for follicular selection and unifollicular ovulation, but it means that follicles of 12 mm may ovulate, and follicles reaching 14 mm or more will ovulate, in response to an LH surge.

Increasing estradiol secretion from the dominant follicle promotes a switch to positive feedback at the hypothalamus and pituitary that results in a gonadotropin surge (Fig. 13.2a, b). The dominant follicle will generally measure between 17 and 23 mm in diameter (Fig. 13.1b) at the time of the LH surge, which generally occurs on day 14 of a normal menstrual cycle. The LH surge induces disparate processes involved in ovulation, which can be split into three components [8].

One component of ovulation is reactivation of oocyte maturation wherein the oocyte, which has been maintained in the diplotene stage of prophase, progresses to the metaphase of the second meiotic division. Another component is luteinization of the granulosa cells where they develop the enzymatic machinery to synthesize progesterone (Fig. 13.3). Until the LH surge the steroidogenic role of granulosa cells is focused on the aromatization of androgen products from theca cells (Fig. 13.3). The third and final component of the ovulatory response, induced by the LH surge, is follicular rupture, which is an acute inflammatory process involving the breakdown of the apical follicle wall [9].

The corpus luteum is formed from the remaining cells of the ruptured follicle once the cumulus oocyte complex has been released. Development of the corpus luteum involves hemorrhage into the follicle, thickening of the steroidogenic cell layers, and marked neovascularization. The mature corpus luteum varies in size and appearance but it usually measures between 10 and 35 mm in diameter [10] and can clearly be visualized on an ultrasound scan in vivo (Fig. 13.1c). However, there are not yet any definitive ultrasound indices that suggest luteal health or cycle outcome [11]. At ovulation the follicle collapses, and the corpus luteum is usually smaller

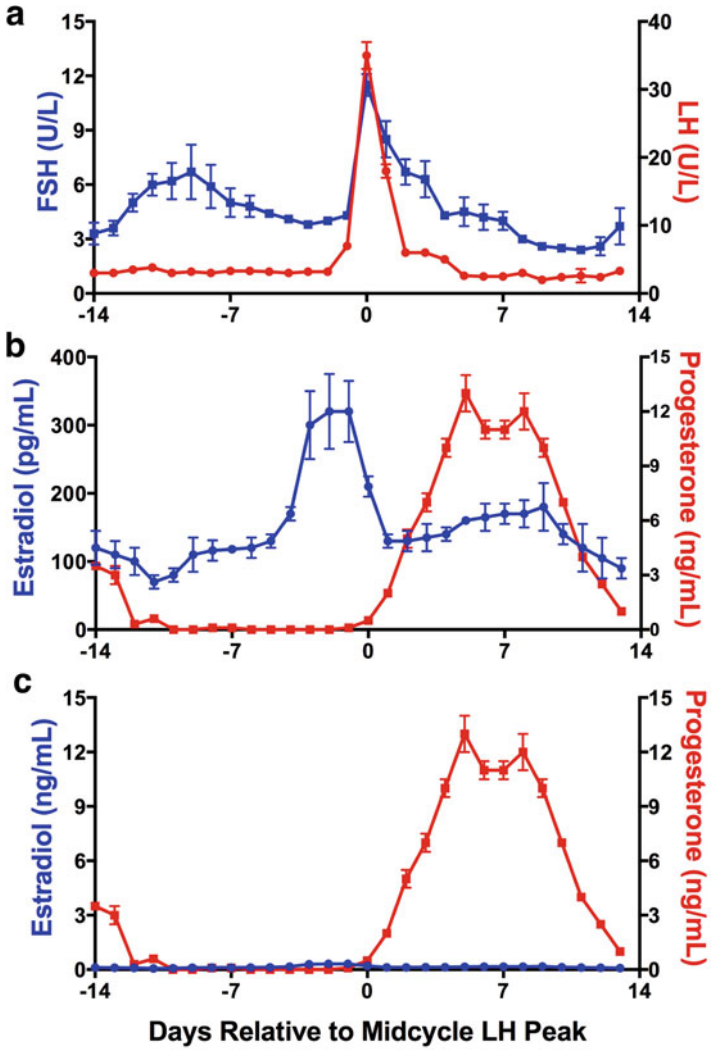

Fig. 13.2 Hormone profile of the menstrual cycle of women. (**a**) Concentrations of FSH and LH. (**b**) Concentrations of estradiol and progesterone. (**c**) Concentrations of estradiol and progesterone plotted on the same scale, highlighting the dominance of progesterone. (Data adapted from [7])

than the dominant follicle. The mean follicle volume at ovulation, measured by three-dimensional ultrasound, was 6.94 cm^3 whereas the mean mid-luteal corpus luteum volume was 4.87 cm^3 [12].

The corpus luteum has a marked blood flow, as demonstrated by color Doppler analysis, that is sometimes referred to as a 'ring of fire' around the corpus luteum [10]. The resistance index of blood vessels, measured by power Doppler, decreases from ovulation to the mid-luteal phase [13], with vascular flow 3.1 times higher at 7 days

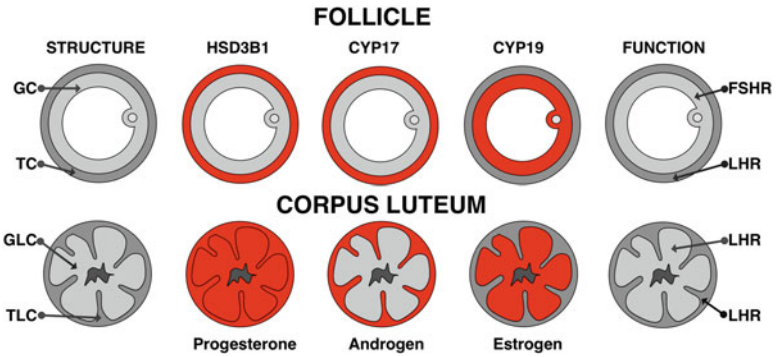

Fig. 13.3 Steroidogenic cells in the human follicle and corpus luteum. In the follicle the theca cells (TC) have the enzymatic machinery to make progesterone (HSD3B1) and convert it to androgen (CYP17) under LH receptor (LHR) regulation. The granulosa cells (GC) convert androgen into estrogen (CYP19) under FSH receptor (FSHR) regulation. In the corpus luteum the theca–lutein cells (TLC) have the same steroidogenic capacity as the TC but the granulosa–lutein cells (GLC) can make progesterone as well as convert androgen into estrogen under LHR regulation

after ovulation than around the dominant follicle before ovulation [12]. This high blood flow to the corpus luteum is important in luteal function and is maintained in the presence of severe peripheral vascular disease [11]. Serum progesterone concentrations are negatively correlated to the resistance index of luteal vessels [13].

During the late luteal phase, serum progesterone falls (Fig. 13.2b, c) and luteal vascular resistance index increases [13]. One week after peak luteal progesterone production, progesterone secretion has ceased (Fig. 13.2b, c), and the corpus luteum has become a small avascular remnant that is difficult to clearly identify on an ultrasound scan (Fig. 13.1a). In women functional and structural luteolysis are difficult to distinguish, and the loss of functional and structural integrity occur in parallel. The loss of luteal steroid hormones promotes endometrial destabilization and induces menstruation [6]. At the time of menstruation there is a cohort of antral follicles of 4–8 mm, ready to respond to the rise in pituitary gonadotropins, and the ovarian cycle commences again (Figs. 13.1 and 13.2).

In a conception cycle the structure and function of the corpus luteum is maintained by an endocrine signal secreted from the trophoblast cells of the implanting conceptus. hCG rescues the corpus luteum from luteolysis but to achieve this it is required in exponentially increasing concentrations [14, 15]. In clinical practice hCG dynamics are used to investigate the early stages of human pregnancy as it normally doubles every 48 h in the first 6 weeks of pregnancy [16]. In early pregnancy the resistance index of the luteal vessels remains at the mid-luteal level until 8 weeks of pregnancy, when the levels are equivalent to the late luteal phase [13], and the vascular flow to the corpus luteum is greatest at 5 weeks of gestation [17].

There seems to be a window where hCG is able to rescue the corpus luteum. Exogenous hCG could stimulate progesterone secretion in the early luteal corpus luteum and the late luteal corpus luteum, but the effects were much less marked than

that seen in the mid-luteal corpus luteum, when normal luteal progesterone secretion is at its peak [18]. We were able to mimic maternal recognition of pregnancy using daily doubling concentrations of hCG from LH+7 that resulted in similar hCG concentrations to normal early pregnancy [1, 19]. Although the corpus luteum can be rescued by LH infusion, the short half-life of LH (21 min) compared to hCG (12 h) [20] means that adequate bioactive LH concentrations cannot be achieved to support prolonged luteal function [14].

At 8 weeks of gestation, the luteo-placental shift occurs, where the placenta becomes an increasing source of progesterone and takes over the role of pregnancy maintenance, and at this stage luteectomy will not cause miscarriage [3]. Circulating progesterone concentrations will decline before this shift and reach a nadir at 7 weeks of gestation [17], despite increasing hCG concentrations, as this peaks around 8 weeks of gestation [17]. At this time the volume of the corpus luteum is decreasing and rising concentrations of the placental protein PAPP-A can be detected in the serum, suggesting increasing placental function [17].

The corpus luteum loses its functional integrity between 8 and 10 weeks of gestation, and placental steroids and inhibin prevent ovarian follicular activity during pregnancy. Although the corpus luteum is nonfunctional for the remainder of the pregnancy, and it undergoes marked involution, it can sometimes still be identified as a small remnant on the ovary at the time of birth. This observation, and the fact that there seem to be more small remnants of past corpora lutea in the ovaries of women with oligo-ovulatory polycystic ovary syndrome (PCOS) [21], suggest that follicular growth may be involved in the final remodeling and removal of luteal remnants from the ovary.

13.3 Molecular Correlates of the Ovarian Cycle in Women

How the human corpus luteum is regulated at a fundamental molecular level is not yet fully understood. One reason for this is that access to human luteal tissue during the luteal phase of a normal menstrual cycle is difficult and during normal early pregnancy it is impossible. In our studies of the human corpus luteum we enucleated the whole corpus luteum from the ovary of normally cycling women who were undergoing laparotomy for hysterectomy for benign gynecological conditions, typically fibroids and heavy menstrual bleeding [1]. We were able to assess the stage of the luteal phase by using serial preoperative urine samples to detect the date of the LH surge. In some women we treated the women with exponentially increasing concentrations of hCG from LH+7 for 5–8 days before surgery to simulate early pregnancy [15].

We were able to track human luteal structure and molecular function across the luteal phase and examine the effects of hCG during luteal rescue. These studies were very challenging but, with those of other groups working on the corpus luteum of women (e.g., [2, 22, 23]), increased our understanding of human luteal physiology. However, because of advances in medical treatment, the number of women

having a hysterectomy who are cycling normally, and not on hormonal treatment, is now vanishingly small, and thus we, and others, can only rarely access fresh human luteal tissue for research purposes.

Another reason is that there is marked diversity in the molecular regulation of the mammalian corpus luteum, and common experimental species, such as rodents and ruminants, fundamentally differ from women in their molecular physiology [24, 25]; thus, insights from animal models, although important, have major caveats. Although there are parallels with human luteal function, there are also key differences. One example is that progesterone concentrations increase on administration of LH or hCG in the luteal phase in women [18], but the concentrations decrease in the rat because the LH receptors are downregulated [26]. In women LH receptors are not downregulated in the presence of excess ligand [22, 27], which is logical because maternal recognition of pregnancy and the maintenance of luteal function depends on large amounts of ligand.

Thus, insights into the molecular physiology of the human corpus luteum come from limited sources such as clinical studies using imaging and hormone assessment to investigate luteal structure and function in vivo and the vanishing collections of carefully timed fresh or archived human corpora lutea. However, many important observational and interventional studies have used nonhuman primates as highly relevant experimental models of human luteal function [28, 29]. These nonhuman primate studies are truly important, although at present it is difficult to source funding for research into normally functioning tissues.

An important source of experimental insights into human steroidogenic luteal cells comes from luteinized granulosa cells (LGCs) that are collected during follicular aspiration during assisted conception treatment [30, 31]. LGCs secrete progesterone in response to LH or hCG and survive for around 14 days in culture. Manipulation of LH or hCG concentrations in vitro can mimic the luteal phase and replicate luteal steroidogenic cell function at different stages of the luteal phase [15, 32]. In addition, these cells can be used in co-culture to study paracrine molecular interactions between luteal steroidogenic cells and other cells, such as endothelial cells [33] and fibroblasts [30, 34].

Some key observations have been highlighted by research into the human corpus luteum. The first is that the corpus luteum is absolutely dependent on LH for its structural and functional integrity. Removal of LH using gonadotropin hormone-releasing hormone (GnRH) antagonist will induce luteolysis and replacement of LH will prevent luteolysis [35]. Maternal recognition of pregnancy is a luteotropic mechanism involving hCG, which also binds to and signals through the LH receptor. Thus, the LH receptor, and its endocrine stimulation, are of fundamental importance in human luteal function [15].

Although LH concentrations and pulse frequency are reduced in the luteal phase, luteolysis occurs in the presence of maintained LH concentrations [36], confirming that luteolysis is inherent to the corpus luteum itself and not a response to an altered endocrine environment. Expression of LH receptors is maintained across the luteal phase, luteolysis is initiated, and progesterone concentrations begin to fall, in the presence of LH receptors [27] and all the elements of the progesterone production

cellular machinery [37]. It seems that luteal steroidogenic cells are programmed to become incrementally less sensitive to LH and increasingly cannot maintain normal luteal function and progesterone production [15]. Indeed, it requires logarithmically increasing concentrations of hCG to maintain stable, and then declining, luteal steroidogenic cell function during early pregnancy [17]. We do not fully understand the molecular basis for the loss of LH-receptor function as the corpus luteum ages.

The LH receptor is localized to the steroidogenic cells of the corpus luteum [27]. In women, the theca–lutein cells and the granulosa–lutein cells remain separated (Fig. 13.3), and both cell types express the LH receptor, but other cells in the corpus luteum do not express LH receptors [1, 15]. Both the theca–lutein and the granulosa–lutein cells produce progesterone whereas the theca–lutein cells secrete androgens and the granulosa–lutein cells convert androgens into estrogens (Fig. 13.3). The luteal alterations associated with functional LH withdrawal during luteolysis, as well as hCG exposure during pregnancy, involve changes to non-steroidogenic cells, such as endothelial cells, which proliferate and then regress [28], macrophages that accumulate during luteolysis [38], and fibroblasts which secrete growth factors and enzymes involved in connective tissue remodeling [30, 34]. This understanding highlights the role for paracrine molecules from the steroidogenic cells, regulated by the LH receptor, in the regulation of disparate cell function in the human corpus luteum.

It is clear that multiple important paracrine molecules are differentially regulated in the corpus luteum during luteolysis and luteal rescue [2, 15, 29]. Luteolytic roles are known for some paracrine factors such as connective tissue growth factor (CTGF) [34], the Slit/Robo system, activin [30], bone morphogenetic proteins [39], galectins [40], matrix metalloproteinases [41], and endothelin, with luteotropic roles for other factors such as cortisol, prostaglandin E [15], progesterone, insulin-like growth factor [2], and vascular endothelial growth factor [28]. There are less defined roles for other molecules such as NO, various cytokines [2], and prostaglandin $F_{2\alpha}$, in the absence of prostaglandin E withdrawal. It is likely, however, that alterations in these important effector molecules are consequences of differential LH action and do not represent the initial step on the pathway to luteolysis.

One fundamentally important paracrine molecule that deserves further discussion is VEGF [42]. The granulosa cells of the dominant follicle are avascular, and the blood vessels are localized to the theca cell layer of the follicle and separated from the granulosa cells by a basement membrane (Fig. 13.4). At ovulation the basement membrane breaks down and the endothelial cells invade the granulosa cell layer. The luteinizing steroidogenic cells become terminally differentiated and do not further divide (Fig. 13.4). The marked increase in the size of the granulosa cell later is secondary to cellular hypertrophy and folding. However, there is marked cellular proliferation in the developing corpus luteum, and dual labeling confirms that the dividing cells are endothelial cells (Fig. 13.4) [43].

In the dominant follicle, the granulosa cells are in contact with each other, whereas in the corpus luteum each granulosa cell is in direct contact with an endothelial cell and there are as many endothelial cells as steroidogenic cells. Thus, the corpus luteum is highly vascular; indeed it has been estimated that the corpus luteum

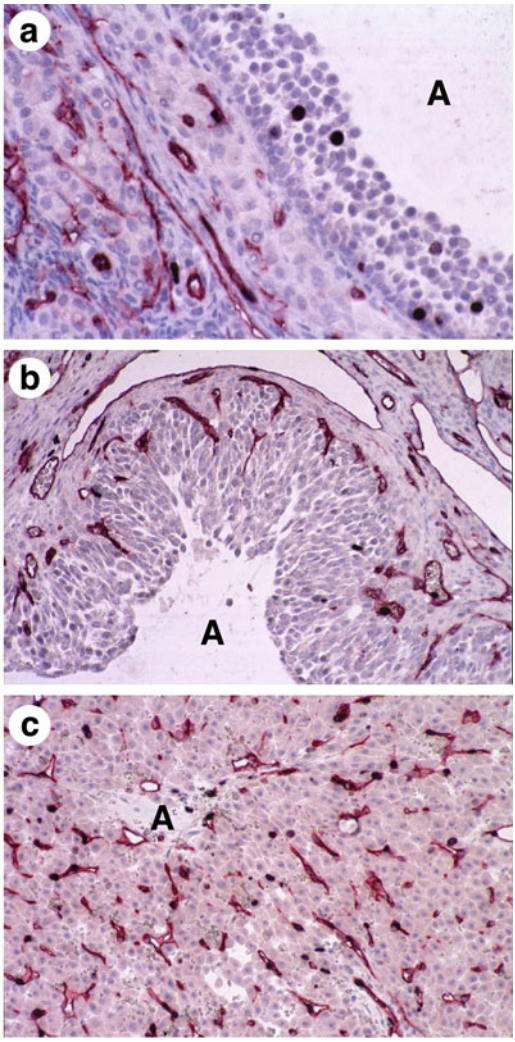

Fig. 13.4 Micrographs of the developing nonhuman primate corpus luteum. Proliferating cells are stained black and endothelial cells are stained red. (**a**) Wall of the dominant follicle showing small avascular proliferating granulosa cells next to the antrum (A). (**b**) Just after ovulation the luteinized granulosa cells undergo hypertrophy and stop dividing. The basement membrane below the granulosa cells has broken down, and endothelial cells enter the luteinized granulosa cell later next to the collapsing antrum (A). (**c**) Several days later the corpus luteum has formed with little evidence of the antrum (A); it has become vascularized and all proliferating cells are endothelial cells. (Photographs courtesy of Prof H.M. Fraser; adapted from [43])

has as much as eight times the blood supply per unit mass than the kidney [11, 15]. As well as having a marked Doppler signal showing increased flow to larger blood vessels in the periphery of the corpus luteum [10], contrast ultrasound shows intense microvascular perfusion throughout the corpus luteum [44]. This intense angiogenesis is a key and defining feature of the corpus luteum.

The primary angiogenic molecule in the human corpus luteum is VEGF. VEGF is secreted from luteal steroidogenic cells and acts on receptors on the endothelial cells [28]. Luteal VEGF secretion initially is under both hormonal and hypoxic control, enhanced by the increased metabolic activity of the steroidogenic cells [45, 46]. VEGF is responsible for the luteal angiogenesis response and the establishment of the microvascular network in the early to mid-luteal phase [42]. When VEGF is

inhibited, using therapeutic molecules that bind to VEGF and prevent it interacting with its receptors, during luteal development a poorly functioning corpus luteum with a markedly reduced vascular network is noted [28].

As well as stimulating the microvascular network, VEGF has a role in maintaining endothelial cell survival and luteal cell integrity [47]. VEGF action is required in the post-angiogenic phase of luteal function. Inhibition of VEGF in the mid-luteal phase results in increased cell death of the endothelial cells, followed by the cell death of steroidogenic cells [47]. LH-dependent VEGF expression is therefore involved in maintaining the structural and functional integrity of the corpus luteum in the normal luteal phase. In simulated early pregnancy, hCG promotes additional VEGF synthesis, and there is further luteal VEGF secretion and a second wave of angiogenesis [48].

VEGF is not only a growth factor that regulates angiogenesis: it also regulates vascular permeability [49]. In the corpus luteum, and particularly in the corpus luteum of pregnancy, there is downregulation of the endothelial cell tight junctions, such as claudin 5 [50]. The paracrine role of VEGF in this process has been demonstrated using cultures of luteinized granulosa cells co-cultured with an endothelial cell monolayer insert [33]. When hCG was added to the endothelial cells, there was no effect on endothelial cell tight junction expression or permeability, but when it was added to the luteinized granulosa cells it reduced the endothelial cell tight junction expression and increased permeability, and the effect was blocked by using molecules to bind VEGF [33]. In addition, blocking VEGF in vivo upregulated endothelial cell tight junctional proteins in the corpus luteum [51]. VEGF has a key role in the regulation of the microvasculature throughout the lifespan of the corpus luteum.

13.4 Clinical Relevance of the Corpus Luteum in Women

The clinical and molecular correlates of the ovarian cycle already described here highlight ten key observations that are of particular clinical importance in women's health and reproduction.

13.4.1 Follicular Growth Is Suppressed in the Luteal Phase

Ovulation, with luteinization and follicular rupture, is a consequence of the LH surge that results from positive feedback by high levels of estradiol. When FSH injections are used to maintain elevated FSH concentrations in the presence of increased circulating estradiol, multifollicular development is encouraged, and this technique is used during ovarian stimulation in assisted conception to optimize the collection of oocytes. As multifollicular development is associated with increased estradiol concentrations, there is a high incidence of a premature LH surge before the follicles have fully matured.

The advent of treatment to prevent premature LH surges was a key factor in the development of ovarian stimulation regimes used in assisted conception. Although GnRH antagonists, administered during gonadotropin stimulation [20], are increasingly used, the standard treatment is the use of GnRH agonists [52]. GnRH agonists will cause an initial surge of gonadotropins but then desensitize the pituitary to GnRH and switch off gonadotropin secretion and thus the LH surge. The problem was the timing of the GnRH injections to switch off the pituitary before stimulation was instigated. If the treatment is started during the follicular phase, the surge of gonadotropins is associated with the development of persistent follicular cysts as follicle growth was transiently stimulated [53].

Starting GnRH agonists in the luteal phase seems to improve the outcome of the stimulation cycle [54]. In addition, treatment resulted in predictable menses at the time of gonadotropin suppression and facilitated planning for ovarian stimulation. Throughout the world, the long protocol GnRH agonist treatment cycles, in which treatment is started in the luteal phase, remain popular and facilitate standardized treatment protocols [55]. This insight was translated from detailed preclinical studies on the effect of GnRH on the nonhuman primate ovarian cycle [56].

13.4.2 Follicular Rupture Is an Inflammatory Response

The LH surge has direct effects on granulosa cells to promote the establishment of molecular pathways for the synthesis of progesterone during luteinization. LH stimulates adenylyl cyclase to synthesize cyclic adenosine monophosphate (cAMP), and the direct introduction of cAMP into granulosa cells causes them to luteinize [57]. It is likely that this is associated with the breakdown of intercellular communications between granulosa cells because removal of granulosa cells from the follicular environment causes them to spontaneously luteinize [57].

As the oocyte does not express LH receptors, the oocyte maturation associated with ovulation is not direct but is mediated through the neighboring granulosa cells. The LH surge causes breakdown of cellular communications between the oocyte and granulosa cells, and because granulosa cells are involved in the maintenance of oocyte arrest, maturation is stimulated [58]. The effect of the LH surge on follicular rupture is not nearly as direct as the effect on granulosa cell steroidogenesis and oocyte maturation. Follicular rupture is the final part of the ovulation process, occurring 36 h after the start of the LH surge. This knowledge is widely used in assisted conception where oocyte maturation and luteinization are required but follicular rupture is not. Thus, in an IVF cycle, oocyte retrieval is carried out 35 h after the artificial LH surge (by hCG injection) before follicular rupture occurs [59].

Follicular rupture is a complex process involving an inflammatory reaction, and although numerous inflammatory mediators are involved, local prostaglandin synthesis has a key role [60], which is important as the use of potent antiinflammatory agents in the periovulatory phase has no significant impact on oocyte maturation or luteinization but can prevent follicular rupture [61]. As a result, the oocyte is trapped within the

developing corpus luteum, with subfertility associated with anovulation in the presence of normal progesterone concentrations [62]. Known as luteinized unruptured follicle syndrome, this requires ultrasound scan monitoring of follicular rupture with temporary withdrawal of antiinflammatory drugs in the periovulatory phase.

13.4.3 Development of the Corpus Luteum Involves Intense Angiogenesis

Ovarian hyperstimulation syndrome (OHSS) is a life-threatening complication of ovarian stimulation in assisted conception. The risks to life are primarily caused by extravasation of fluid from the vascular space, which increases the viscosity of blood and predisposes to thrombosis of the cerebral vasculature or thromboembolism [63]. The development of ascites causes a tense fluid-filled abdomen, which can impair renal blood flow and function. The development of pleural effusions (Fig. 13.5a) can cause breathlessness and impaired oxygen concentration. The primary pathophysiology involved in OHSS is increased vascular permeability [64].

Moderate to severe OHSS occurs in 3–8 % of all IVF cycles as a consequence of multifollicular development [64]. Before ovarian stimulation starts, some women can be identified as being at risk of OHSS, as it is more likely to occur in young women or women with a high antral follicle count (>24 antral follicles), such as those with PCOS or high circulating anti-Müllerian hormone (AMH) concentrations (>3.5 ng/ml, >25 pmol/l) [65]. During ovarian stimulation, women with increased circulating estrogen concentrations (>5000 pg/ml, >18,000 pmol/l) or an increased number of follicles (>14 follicles with diameter >11 mm) are at risk of OHSS [64, 65].

OHSS does not occur until the process of ovulation is stimulated with hCG because OHSS is linked to the vascularization of the developing corpora lutea. As VEGF is the key molecule involved in the establishment of the luteal microvasculature [28], its concentrations are markedly increased when multiple corpora lutea are developing at the same time. Indeed, circulating VEGF is increased after the hCG injection in

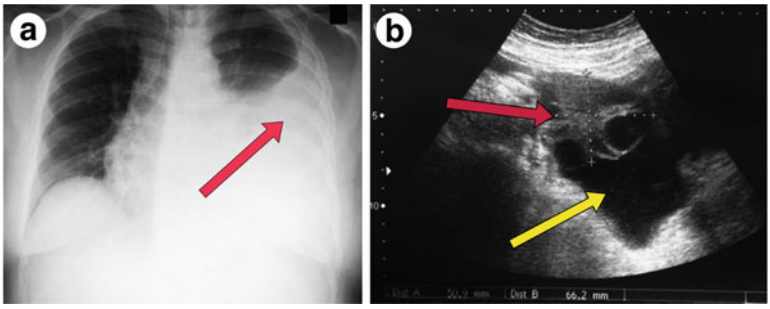

Fig. 13.5 Extravasation of fluid in severe OHSS. (**a**) Chest X-ray shows large left-sided pleural effusion (*arrow*) in a breathless woman with OHSS. (**b**) Abdominal ultrasound scan showing enlarged ovary (*upper arrow*) surrounded by intraperitoneal fluid (*lower arrow*) in a woman with ascites secondary to OHSS

women during IVF, and the magnitude of serum VEGF can predict OHSS [65]: it is the vasoactive properties of VEGF that increase capillary permeability and cause extravasation of fluid into the third space and hypercoagulability [64].

The diagnosis of OHSS and its severity takes into consideration the blood hematocrit, hypoproteinemia, the size of the ovaries, the presence of ascites (Fig. 13.5b), and urine output [64, 65]. There are two types of OHSS, early and late forms. The early form occurs within 9 days of the hCG injection and is caused by the initial stimulation of vasoactive molecules involved in luteal neovascularization. We know that during early pregnancy hCG stimulates further luteal angiogenesis, and VEGF secretion [48], and late-onset OHSS occurs after 10 days from initial hCG injection in the presence of increasing hCG from an early pregnancy. Management is symptomatic, and supportive, with monitoring, thromboprophylaxis, analgesia, and hydration, with the possibility of paracentesis or pleural drainage as required [65], until it spontaneously resolves as the corpora lutea become increasingly less responsive [66].

When a risk of OHSS is identified, the stimulation regimen can be individualized and made milder in an attempt to limit excessive follicular development [64]. As LH action is enhanced by insulin and insulin-like growth factors [67], a strategy to target these co-factors can reduce hCG-stimulated VEGF concentrations, and thus the risk of OHSS. Metformin treatment during ovarian stimulation reduces these co-factors, particularly in women with PCOS who are insulin resistant, with higher circulating insulin concentrations, and reportedly reduces the incidence of OHSS by 63 % [68]. As dopamine has direct effects on endothelial cell permeability, probably by altering VEGF receptor phosphorylation, dopamine agonists such as cabergoline have also been suggested as a way to reduce OHSS [66, 69].

As hCG is a long-acting molecule, another strategy is to reduce the duration of luteal stimulation around ovulation by trying to mimic the shorter-acting LH surge. Exogenous LH is too short acting to produce an adequate stimulus to set in place the pathways to ovulation [64]. As the GnRH agonist has a much higher affinity for the receptor than a GnRH antagonist, in a cycle using GnRH antagonists a shorter-acting endogenous LH surge can be simulated by an injection of GnRH [20], or more recently kisspeptin [70]. Other strategies involve cycle cancellation and avoidance of the hCG trigger, coasting by withholding gonadotropin injections for several days, to allow atresia of some follicles before hCG administration, or freezing all embryos to avoid any chance of late-onset OHSS [64].

13.4.4 The Corpus Luteum Secretes Large Amounts of Progesterone

The corpus luteum is formed from the dominant follicle after ovulation, and the measurement of serum progesterone is used clinically for the diagnosis of ovulation. One in six couples will engage with clinical infertility services at some point during their reproductive life. Routine assessment of subfertility involves confirming regular ovulation, and this is achieved by the measurement of serum progesterone concentrations. Progesterone concentrations in excess of 30 mnol/l (9.4 ng/ml)

are used to determine the presence of a mid-luteal corpus luteum and thus confirm ovulation [71].

As progesterone concentrations peak during the mid-luteal phase (Fig. 13.2b, c), in a standard 28-day menstrual cycle progesterone assessment is measured at day 21. The variation in the length of a menstrual cycle relates to the length of the follicular phase, and ovulation generally occurs 2 weeks before menstruation, so progesterone is measured 1 week before the expected menstruation: in a regular 35-day menstrual cycle this would be on day 28 and in a 23-day cycle on day 16. When a cycle is irregular, and the date of menstruation cannot be predicted, serial progesterone measurements, generally weekly, are required to confirm that ovulation occurs.

In the luteal phase of the menstrual cycle there is a 0.5 °C rise in basal body temperature and, although now much less frequently used clinically than in the past, some women will record changes in their body temperature to confirm ovulation. However, temperature is variable, and difficult to chart consistently; a rise in progesterone occurs after the time of peak fertility and there is no correlation between the magnitude of the temperature rise and progesterone concentrations [72]. It is likely that it is not progesterone itself, but a switch from estrogen to progesterone dominance, that causes the rise in basal body temperature.

There are some additional systemic effects of progesterone outside its role in uterine function and basal body temperature regulation. Progesterone is able to relax smooth muscle, and women are more likely to be constipated in the luteal phase of the cycle and during pregnancy. This effect, as well as the fact that it will displace cortisol from cortisol-binding globulin, means that asthma may improve after ovulation and deteriorate in the late-luteal phase and during menstruation [73]. In addition, progesterone is involved in lobular differentiation in breast tissue, and it also has a proliferative role, meaning that breast tenderness in common after ovulation [74].

There are progesterone receptors in numerous areas of the brain, and there is no doubt that changes in sex hormones can affect mood in some women. The physical, behavioral, and psychological changes that occur in the second half of the luteal phase are known as premenstrual syndrome. These symptoms are experienced by most women and include bloating, mood swings, and an increase in irritability, and in around 5 % of women these symptoms are bad enough to interfere with their ability to live a normal life [75]. Although a healthy lifestyle, exercise, and psychological therapies such as cognitive behavioral therapy and antidepressants can help, there is a role for ovarian suppression in severe cases or when contraception is required.

13.4.5 Progesterone Prepares the Endometrium for Implantation

As luteal progesterone is involved in the endometrial transition from a proliferative to a secretory phenotype, with increasing decidualization, it has a major role in facilitating implantation [6]. This role suggests that if the corpus luteum is not

producing enough progesterone, implantation will be reduced and the woman will be subfertile. Although this concept is known as the inadequate corpus luteum or luteal-phase defect, it remains contentious whether inadequate luteal function in women can be a cause of subfertility [76]. Certainly the corpus luteum secretes more progesterone than is required for fertility, and there is no good evidence that progesterone replacement in a natural cycle would improve the chances of conception.

Most clinicians would agree that there is no intrinsic pathology of the corpus luteum that is associated with reduced fertility in women [76]. However, low progesterone concentrations in the luteal phase, or a short luteal phase, can occasionally be detected. The concept of the inadequate corpus luteum, or reduced luteal progesterone secretion, is however flawed, and a more accurate interpretation would be the concept of the inadequate follicle or the inadequate LH surge. The classic time when a woman has inadequate luteal function is during the resumption of ovarian function while breastfeeding, and this results from partial suppression of gonadotropins [77].

Women who are breastfeeding, or who have pathological elevations of prolactin, have suppressed LH concentration and as such are less able to generate an adequate LH surge. Similarly, women with recovering hypogonadotropic hypogonadism, secondary to low body fat as seen in anorexia, over-exercise, or chronic illness, are also less likely to be able to generate an adequate LH surge [78]. In contrast, women with PCOS tend to have higher baseline LH concentrations and pulsatility, with a reduced area under the curve for the LH surge; the same is true in the perimenopausal state where basal LH concentrations are elevated [67, 78]. In addition, women taking 5-day courses of anti-estrogen fertility drugs, such as clomifene, tamoxifen, or letrozole, in the early follicular phase for ovulation induction, may have reduced capacity for estrogen-regulated positive feedback to generate the LH surge, particularly if follicular growth is rapid [67].

In all these cases there is a potential for follicular growth without ovulation and the generation of simple follicular cysts. However, in some cases there is enough LH to start the luteinization process, and a poorly functioning corpus luteum may be formed. If the cause of the reduced luteal follicular function is an inadequate LH surge, the injection of hCG in the presence of a dominant follicle can normalize the luteal phase.

In other cases follicular growth and follicular estradiol secretion are suboptimal. Although this may be a result of ovarian aging, poor follicular reserve, and a consequence of naturally declining fertility, with little scope for intervention, in some cases the gonadotropin stimulation of the follicle is inadequate [78]. The conditions described here that are associated with a reduced LH surge can also be associated with reduced follicular growth in the late follicular phase and estradiol synthesis. In these circumstances, restoration of gonadotropin stimulation using either anti-estrogen ovulation induction agents, FSH injections, FSH and LH injections, or pulsatile GnRH can help drive normal follicular growth [78]. These strategies are often combined with a timed hCG injection to reproduce the LH surge.

13.4.6 Hemorrhage Occurs Within the Developing Corpus Luteum

After ovulation, rupture of the dominant follicle is associated with shrinking of the follicular area during the folliculo–luteal transition [12]. However, in one in four or five cycles the corpus luteum does not shrink and may expand to become cystic. It is not clear whether this represents luteinized unruptured follicles in the absence of antiinflammatory drugs or whether follicular rupture has occurred but there has been increased hemorrhage within the corpus luteum giving rise to an enlarged hemorrhagic cystic corpus luteum.

If women with unexplained subfertility were more prone to cystic corpora lutea, this might be a cause of subfertility. However, up to 40 % of normal fertile women develop a cystic corpus luteum during ultrasonic assessment of the menstrual cycle [79], which is similar to the incidence in unexplained subfertility [80]. In addition, in the first trimester of pregnancy simple or hemorrhagic luteal cysts were seen in 29 % of patients, and indeed their presence was associated with ongoing pregnancy [81]. As a tubal ectopic pregnancy is on the same side as the corpus luteum in 80 % of cases [16], the corpus luteum in early pregnancy can help locate the site of an ectopic pregnancy.

It has been claimed that the blood trapped within a cystic corpus luteum can predispose to development of an endometrioma of the ovary [82]. However, it can be difficult to distinguish a luteal hemorrhagic cyst from a developing endometrioma, and a plausible molecular pathophysiology linking the two is lacking. Overall, although luteal cysts may be associated with unilateral lower abdominal discomfort in the luteal phase of the cycle or in early pregnancy, at present it seems most likely that they are a natural occurrence and a variance of the normal corpus luteum.

13.4.7 The Corpus Luteum Is Highly Vascular

As the corpus luteum is very vascular, any rupture of the corpus luteum causes marked intraabdominal hemorrhage, which is an acute gynecological emergency [83]. Rupture of the corpus luteum is rare but it is a differential diagnosis of ruptured tubal ectopic pregnancy [16]. It is a life-threatening condition that seems to occur more commonly in women with coagulation defects, with hemoglobulinopathies, or who are taking anticoagulants [84]. It can occur during a normal menstrual cycle but it is more common in early pregnancy [83]. As luteal formation is associated with increased VEGF and vascular permeability [64], the luteal phase is normally associated with a small amount of free fluid in the pelvis. However, fluid surrounding the ovary suggests an abnormal amount of fluid, which may be blood, and major hemorrhage is suggested by fluid around the spleen and referred shoulder pain from diaphragmatic irritation [16]. Blood in the pelvis usually contains focal collections of more dense material such as early blood clots. Luteal rupture with intraabdominal hemorrhage requires surgery, even in the presence of a coexisting normal intrauterine pregnancy.

13.4.8 The Corpus Luteum Maintains Early Pregnancy

Around 15–20 % of early pregnancies will miscarry in the first trimester and most of these are sporadic, with chromosomal or morphological abnormalities [85]. Because about 1 % of couples suffer from recurrent miscarriage, defined as three or more miscarriages, which is higher than chance alone, there must be some underlying causes that predispose couples to miscarriage. Although there may be genetic, structural, or immunological causes, for most couples with recurrent miscarriage no defined cause is identified [85]. In theory, as luteal progesterone is absolutely required to support early pregnancy before the luteal–placental shift, and removal of progesterone induces a miscarriage, it is possible that inadequate progesterone action in early pregnancy is a cause of early miscarriage.

Certainly serum progesterone concentrations are lower in pregnancies that miscarry than in viable pregnancies. However, although serum progesterone concentrations have been reported to be lower in women with miscarriage [86], most studies have shown no reduction in luteal function progesterone in women suffering from recurrent miscarriage [85]. The hCG dynamics of a pregnancy that will miscarry may be abnormal, and the low progesterone concentrations are generally thought to be a consequence rather than a cause of miscarriage [16].

However, it has been reported that decidualization may be abnormal in women with recurrent miscarriage [86], and there may be an endometrial resistance to progesterone. Women with threatened miscarriage, and those with recurrent miscarriage, are often eager for treatment, and the use of progesterone supplementation to prevent miscarriage in early pregnancy is very common. In the UK a large placebo-controlled randomized study is addressing this issue, PRISM (for threatened miscarriage), but the results are not yet reported. The PROMISE study however has reported and early progesterone supplementation was no different to placebo in the prevention of recurrent miscarriage [87].

It is clear, however, from the current literature that although several studies have used progesterone and related steroids, orally, intramuscularly, and vaginally, in the attempt to prevent miscarriage, they are underpowered and there are no convincing data so far to suggest their routine administration in threatened miscarriage [86, 88, 89]; thus, further data are required in regard to prophylaxis in recurrent miscarriage [89]. The fetal effects of exogenous progesterone in early pregnancy are not yet clear, and as there have been reports of an association of progesterone treatment in early pregnancy with hypospadias, ongoing safety monitoring is required [90].

13.4.9 The Luteo-Placental Shift Occurs Around 8 Weeks of Gestation

When an embryo is replaced in an artificial menstrual cycle, a corpus luteum is absent and luteal function has to be replaced. Luteal function is required to promote secretory and decidual changes in the endometrium to facilitate implantation and

pregnancy maintenance for the first 8 to 10 weeks of gestation. Although in addition to progesterone the corpus luteum secretes several different peptide and steroid hormones including relaxin, inhibin A, prokineticin, VEGF, androgen, and estrogen [15], it is progesterone that is the key luteal product in the establishment and maintenance of pregnancy [3]. Although progesterone alone can promote implantation and maintain pregnancy, there is some evidence that the addition of estrogen may increase the effectiveness [91].

The mechanism of action of the additional estradiol is not clear. Estradiol has a part in uterine and endometrial vascularization [6] and it may be that this is involved. However, estradiol has been shown to upregulate the progesterone receptor, and its role may be to further promote progesterone action [6]. In clinical practice it is now routine in egg donation recipients, and in embryo replacement in an artificial cycle, that both progesterone and estrogen are replaced for the first 10–12 weeks of gestation.

13.4.10 The Corpus Luteum Also Secretes Androgen

Androgen is not replaced in artificial cycles, but the theca–lutein cells of the corpus luteum make and secrete androgens [1]. Although the corpus luteum shares some features with solid tumors, including marked neoangiogenesis, a remarkable feature of the corpus luteum is that malignancies are relatively unknown. For such a rapidly changing tissue, the fact that it does not become neoplastic is remarkable. There is, however, one extraordinary tumor of the rescued corpus luteum of pregnancy known as a luteoma of pregnancy [92]. Luteomas occur rarely in pregnancy, are entirely benign, and regress normally after delivery [92].

It is likely that luteomas are driven by hCG during pregnancy and consist mainly of luteinized theca cells, which means that they produce large amounts of androgens [93]. They often affect both ovaries and seem to be more common in women with PCOS. They vary in diameter but can be quite large, with a mean diameter of 6.5 cm in one study [92]. Although sometimes only diagnosed at delivery, they may be diagnosed during pregnancy because of maternal virilization [93], which can be quite marked but regresses well after delivery. The placenta expresses large amounts of aromatase and thus the fetus is relatively protected from the maternal androgen; normal female offspring have been reported in the presence of significantly increased maternal androgen concentrations [93]. However, this system can be swamped, and luteomas of pregnancy can cause virilization of a female fetus and intersex disorders [92]. As these tumors are rare and spontaneously regress, their management is unclear, and surgical management is discouraged.

13.5 Insights Involving Assisted Conception and the Corpus Luteum

13.5.1 What Human Luteal Physiology Tells Us About Assisted Conception

We know that the corpus luteum is absolutely dependent on LH from the pituitary gland. However, modern ovarian stimulation regimes involve the use of GnRH agonists and GnRH antagonists to prevent a premature LH surge by blocking pituitary LH secretion. Whether the LH surge, for luteinization and oocyte maturation, is stimulated using a GnRH agonist bolus, during a GnRH antagonist cycle, or with a longer acting injection of hCG, there is a gap after the LH/hCG stimulation to induce the ovulatory responses and the hCG stimulation of early pregnancy where the corpus luteum is deprived of trophic hormone (Fig. 13.6). Knowledge of luteal physiology would suggest that progesterone production in the early to mid-luteal phase may therefore be relatively deficient.

Indeed, meta-analysis of assisted conception cycles shows that luteal support after oocyte retrieval improves pregnancy rates [94]. As the pathophysiology involves reduced progesterone as a consequence of reduced trophic hormone, it is unsurprising that either hCG injections [OR 1.75 (1.09–2.81)] or progesterone supplementation [OR 2.95 (1.09–2.81)] improves pregnancy rates [94]. However, as VEGF is stimulated by LH/hCG, OHSS is less common after supplementation with progesterone when compared to hCG [OR 0.45 (0.26–0.79)] [94]. Currently it is standard practice to use progesterone for post-oocyte collection luteal support. Although this can be by injection, most clinics use micronized natural progesterone gels or pessaries given vaginally [94].

As progesterone will be stimulated initially by the artificial LH surge, it makes sense that progesterone support should start after rather than before this surge. Indeed, a systematic review confirms that the pregnancy rate was better when progesterone was started on the day of, rather than the day before, oocyte retrieval [95]. As hCG will stimulate progesterone for at least 3 days after administration it would be expected that there would be no difference in starting on day 2 or 3 after the oocyte collection rather than starting on the day of oocyte collection, and this indeed was the case [95]. However, starting after this time resulted in a 16% decrease in pregnancy rate, as would be expected [95]. As the use of a GnRH agonist trigger is increasing, most clinics routinely start progesterone on the day of, or the day after, oocyte retrieval regardless of regimen.

Luteal physiology would suggest that if the corpora lutea cannot be fully rescued by hCG in early pregnancy then luteal support should occur until after the luteoplacental shift, as in egg donation cycles. However there is no change in pregnancy rate or outcome if luteal support is discontinued after 2 weeks [96]. This finding suggests that once hCG is in the circulation progesterone support is not required as

the corpora lutea will be producing enough progesterone [95] (Fig. 13.6). However, in one survey progesterone support was continued until 10–12 weeks of gestation in 67 % of cycles, when the fetal heartbeat was detected in 22 % of cycles and discontinued at pregnancy test in only 12 % of cycles [96]. In another survey 40 % of units continued progesterone support until 12 weeks [97].

If there were only one or two follicles, as steroidogenic cells are aspirated with the oocyte, one would expect deficient luteal function and perhaps luteal support

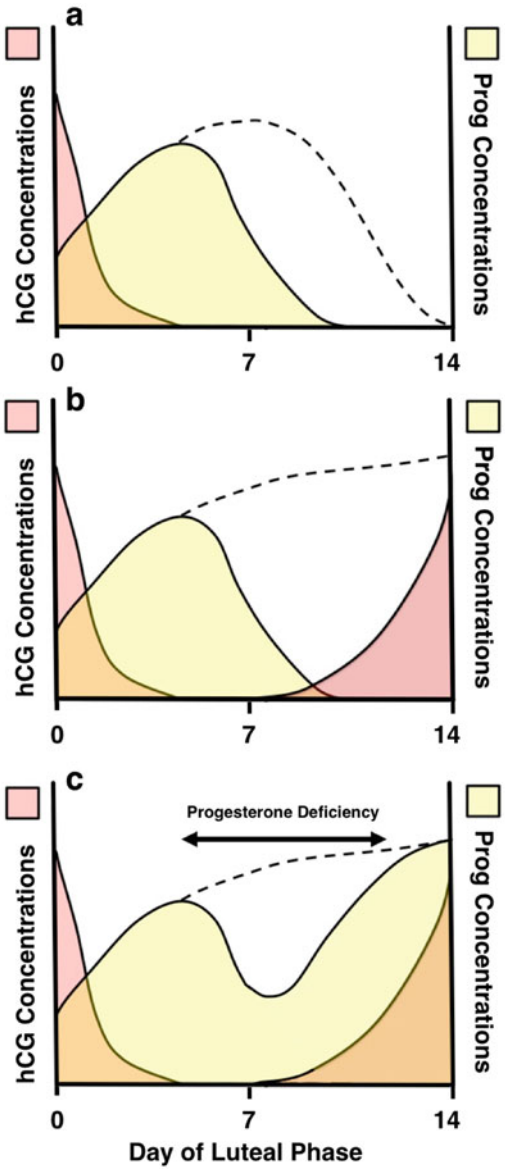

Fig. 13.6 Insights into the requirement for luteal support during assisted conception. (**a**) In a downregulated cycle, exogenous hCG induces progesterone production, but in the absence of LH progesterone output falls earlier than during a natural cycle (*dotted line*). (**b**) In a conception cycle, endogenous hCG rises exponentially from LH+7 to maintain progesterone output (*dotted line*). (**c**) Endogenous hCG will rescue the corpus luteum in a downregulated cycle in assisted conception to maintain progesterone, but there is a time of relative progesterone deficiency in the early to mid-luteal phases

should continue until after 8 weeks gestation. However, there do not seen to be any clinical studies addressing this. Overall knowledge of luteal physiology suggests there is a 5-day window in the early to mid-luteal phase where luteal support is required (Fig. 13.6). At present, however, such short-term support is not part of clinical practice.

13.5.2 What Assisted Conception Tells Us About Human Luteal Physiology

We know that without trophic support the corpus luteum will undergo luteolysis and that an injection of GnRH antagonist in the luteal phase will induce rapid luteolysis. However, as pregnancy can occur without luteal support, there is still some residual luteal function in the early to mid-luteal phase despite the absence of LH or hCG. This finding suggests that the corpus luteum has a window in the early to mid-luteal phase where it is resistant to luteolysis as a consequence of LH withdrawal. In addition, it can thereafter be fully rescued by hCG during early pregnancy.

13.6 Importance of Ongoing Research into the Human Corpus Luteum

Despite all the advances in cellular and molecular biology, we still do not fully understand how the human corpus luteum is regulated at a fundamental level. At a translational level, this has implications for the understanding of human fertility and assisted conception as well as the developments of novel contraceptives targeting the luteal phase. However, research into the human corpus luteum is declining, in contrast to other research into fertility (Fig. 13.7). Although this might be because of the challenges of obtaining tissue and the lack of suitable nonprimate models, it is likely that the alignment of research funding toward diseases and disease processes is involved.

Research into the human corpus luteum suffers as it does not have recognized functional disorders and there are very few disease processes that affect it. As it is fundamental for fertility, mutations affecting its function are not passed on to future generations. However, understanding how angiogenesis is regulated, how remodeling occurs, why malignancy does not happen, and why inflammation does not scar, in such a dynamic tissue will give important generic insights that go well beyond the ovary and reproduction. Further basic research into the human corpus luteum is important, and as a paradigm for important physiological regulation of pathological processes, it is unparalleled.

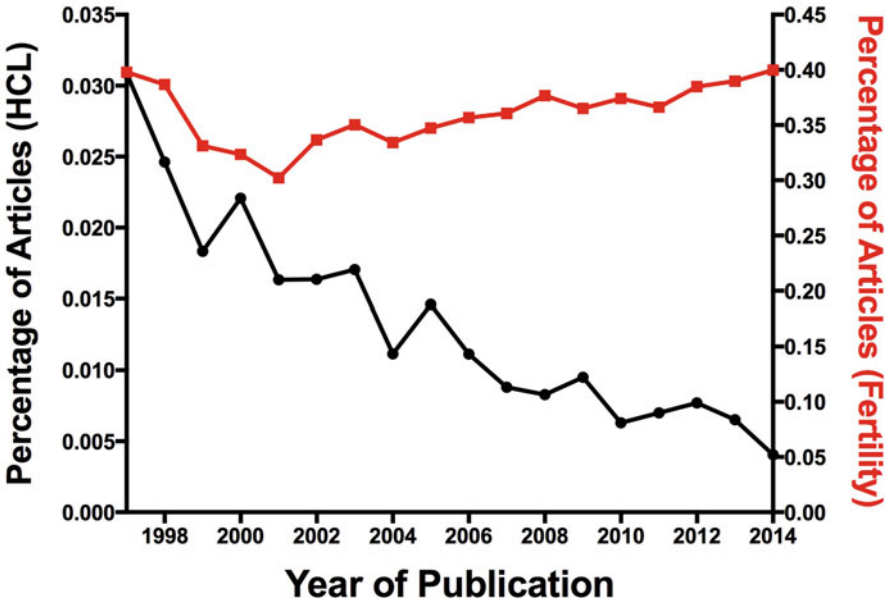

Fig. 13.7 Results of PubMed searches show the drop in percentage of publications on the 'human corpus luteum' (HCL) in recent years although publications on 'fertility' are maintained

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