

# Novel Signaling Pathways That Control Ovarian Follicular Development, Ovulation, and Luteinization

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## ABSTRACT

The interactions of peptide and steroid hormone signaling cascades in the ovary are critical for follicular growth, ovulation, and luteinization. Although the pituitary gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH) play key regulatory roles, their actions are also dependent on other peptide signaling pathways, including those stimulated by insulin-like growth factor-1 (IGF-1), transforming growth factor-beta (TGF- $\beta$ ) family members (e.g., inhibin, activin, growth differentiation factor-9, bone morphogenic proteins), fibroblast growth factor, and Wnts (via Frizzled receptors). Each of these factors is expressed and acts in a cell-specific manner at defined stages of follicular growth. IGF-1, estrogen, and FSH comprise one major regulatory system. The Wnt/Frizzled pathways define other aspects relating to ovarian embryogenesis and possibly ovulation and luteinization. Likewise, the steroid receptors as well as orphan nuclear receptors and their ligands impact ovarian cell function. The importance of these multiple signaling cascades has been documented by targeted deletion of specific genes. For example, mice null for the LH-induced genes progesterone receptor (PR) and cyclo-oxygenase-2 (COX-2) fail to ovulate. Whereas PR appears to regulate the induction of novel proteases, COX-2 appears to regulate cumulus expansion. This review summarizes some new aspects of peptide and steroid hormone signaling in the rodent ovary.

## I. Overview

Ovarian follicular growth is controlled by the production of intraovarian growth regulatory factors such as insulin-like growth factor-1 (IGF-1) (Adashi *et al.*, 1985; Baker *et al.*, 1996; Zhou *et al.*, 1997; Burks *et al.*, 2000; Guidice, 2000), steroids (Richards, 1994), members of the transforming growth factor-beta (TGF- $\beta$ ) family (Elvin *et al.*, 1999; Lewis *et al.*, 2000; Chapman and Woodruff, 2001; Otsuka *et al.*, 2001), and the Wnt/Frizzled family (Vainio *et al.*, 1999; Hsieh *et al.*, in press). These factors act by autocrine, paracrine, and intracrine mechanisms. In addition, follicular growth is controlled by endocrine factors such as the pituitary gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Richards, 1994). Recent mutant mouse models as

well as studies identifying new components of IGF-1, FSH, and estradiol (E) signal transduction pathways provide novel insights into how these hormones might interact to control follicular growth and are the focus of this review.

## II. Interactions of the IGF-1, Estradiol, and FSH Signaling Cascades

The functional links between the FSH and IGF-1 signal pathways are supported by the observations that IGF-1, IGF-1 receptor (Igf-1r), and FSH receptor co-localize to granulosa cells of small growing follicles and preovulatory follicles (Zhou *et al.*, 1995,1997). In mice null for the FSH receptor or the FSH $\beta$  subunit, follicular growth is impaired beyond the preantral stage and is associated with altered expression of specific genes (Kumar *et al.*, 1997; Burns *et al.*, 2001) (Figure 1). Whereas IGF-1 expression is not altered by hypophysectomy or lack of FSH $\beta$  subunit (Zhou *et al.*, 1997), levels of Igf-1r are reduced and can be restored by treating rats with pregnant mare serum gonadotropin (PMSG), which stimulates follicular growth (Zhou *et al.*, 1997). Mice null for either IGF-1 (*Igf-1*) or insulin-receptor substrate-2 (*Irs-2*) exhibit severe growth retardation, including follicular growth in the ovary (Baker *et al.*, 1996; Zhou *et al.*, 1997; Burks *et al.*, 2000). In contrast, although mice null for growth hormone exhibit reduced growth rates and have low serum levels of IGF-1, ovarian function is normal, likely a consequence of GH-independent production of IGF-1 by granulosa cells within the ovary (Zhou *et al.*, 1997; Zaczek *et al.*, 2001). Finally, IGF-1 enhances FSH action in granulosa cells by mechanisms that are not entirely clear (Orly, 2000) but must involve specific interactions of these two signaling pathways (Richards, 2001a; Richards *et al.*, in press (b)) (Figure 1).

IGF-1 impacts multiple signaling cascades, one of which includes the phosphoinositide-3 kinase (PI3-K) cascade (LeRoith *et al.*, 1995; Vanhaesebroeck and Alessi, 2000). PI3-K generates phosphoinositides that activate phosphoinositide-dependent kinases (PDK)1 and -2. PDK1 and -2, in turn, phosphorylate and activate protein kinase B (PKB), which then phosphorylates and inactivates targets such as glycogen synthase kinase (GSK)-3 $\beta$ , BAD, and caspase 9 as well as members of the Forkhead (FKHR) winged-helix transcription factor family (Vanhaesebroeck and Alessi, 2000). The IGF-1 signaling cascade has been highly conserved, as revealed by functional and structural homologies of genes present in mammals (IGF-1R, PI3-K, phosphate and tensin homologue deleted on chromosome 10 (PTEN), PDK1, PKB, FKHR) and *C. elegans* (Daf-2, Age-1, Daf 18, PDK1, Akt, Daf 16) (Guarente and Kenyon, 2000) (Figure 1). In *C. elegans*, this pathway impacts cell survival, energy homeostasis, and longevity and is intimately linked to the neural and reproductive systems. Lack of the IGF-1 receptor (Daf-2) in *C. elegans* leads to prolonged cell survival that is dependent on Daf16 and Daf12, homologues of FKHR and

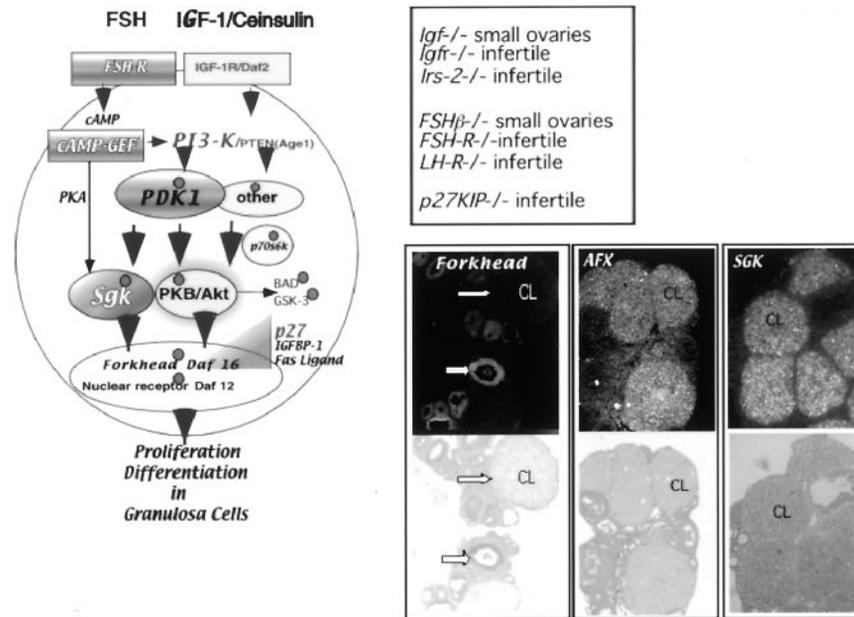


FIG. 1. Follicle-stimulating hormone (FSH) and insulin-like growth factor-1 (IGF-1) signaling pathways operate in granulosa cells. FSH/LH and IGF-1 impact the proliferation and differentiation of granulosa cells, as indicated by specific knockout mouse models and the effects of these hormones in hypophysectomized rats, as reviewed in the text. Note that the IGF-1 pathway is highly conserved from *C. elegans* to mammals. Common downstream targets of FSH and IGF-1 in granulosa cells include components of the PI3-kinase cascade that lead to the phosphorylation of protein kinase B (PKB) and serum and glucocorticoid-induced kinase (Sgk) as well as the transcription factor, Forkhead (FKHR), which is selectively expressed at high levels in granulosa cells of growing follicles. FSH but not IGF-1 induces Sgk, which reaches its highest levels of expression in luteal cells. These terminally differentiated cells preferentially express AFX (Foxo-4) and FKHL1 (Foxo-3) – but not FKHR – as well as a putative target of AFX, p27KIP. [Adapted with permission from Richards JS, Sharma SC, Falender AE, Lo YH 2002 Expression of FKHR, FKHL1 and AFX genes in the rodent ovary: evidence for regulation by IGF-1, estrogen and the gonadotropins. *Mol Endocrinol*, in press; Gonzalez-Robayna JJ, Falender AE, Ochsner S, Firestone GL, Richards JJ 2000 FSH stimulates phosphorylation and activation of protein kinase B (PKB/Akt) and serum and glucocorticoid-induced kinase (Sgk): evidence for A-kinase independent signaling in granulosa cells. *Mol Endocrinol* 14:1283–1300. Copyright The Endocrine Society.]

a nuclear hormone receptor, respectively (Guarente and Kenyon, 2000; Lin *et al.*, 2001) (Figure 1).

The mammalian homologue of the nuclear receptor Daf12 is not yet known. However, three members of the Forkhead family have been identified in the mouse: FKHR (Foxo-1), FKHL1 (Foxo-3), and AFX (Foxo-4) (Kaestner *et al.*, 2000; Brunet *et al.*, 2001). A current model of IGF-1 action indicates that

phosphorylation of Forkhead by PKB (or related kinases) restricts nuclear localization of these factors (Nakae *et al.*, 2000; Brownawell *et al.*, 2001). This impedes transcriptional activation of specific Forkhead target genes, such as Fas ligand (FasL), an inducer of apoptosis (Brunet *et al.*, 1999), p27KIP, an inhibitor of cell cycle progression (Medema *et al.*, 2000) and insulin-like growth factor binding protein-1 (IGFBP-1), a presumed inhibitor of IGF-1 (Kops *et al.*, 1999; Guidice, 2000). Recently, we have shown that FKHR, FKHRL1, and AFX are expressed in the rodent (mouse and rat) ovary in a cell-specific manner at defined stages of follicular growth and luteinization (Richards *et al.*, in press (b)). Expression of FKHR mRNA is restricted to granulosa cells of growing follicles and is not detected in luteal cells. In contrast, FKHRL1 and AFX are highest in corpora lutea, where the PKB-related kinase, serum and glucocorticoid-induced kinase (Sgk), is also expressed in abundance (Figure 1). In addition, we have shown that FKHR expression is regulated by E, IGF-1, and the gonadotropins (Richards *et al.*, in press (b)). E markedly increases FKHR mRNA and protein in granulosa cells of preantral follicles of the hypophysectomized (H) rat. Thus, expression of FKHR mRNA, protein, and phosphorylation are not strictly associated with follicles that are undergoing apoptosis or appear destined for atresia. Rather, FKHR is highest in granulosa cells of follicles in the hypophysectomized and estradiol-treated (HE) rats that exhibit increased proliferation (Rao *et al.*, 1978), increased expression of cyclin D2 (Robker and Richards, 1998a,b) and increased staining for proliferator cell nuclear antigen (PCNA) (Robker and Richards, 1998a,b). FKHR is also elevated in preovulatory follicles of PMSG-treated mice, adult mice, and pregnant mice at day 22 of gestation (Figure 1). The high levels of FKHR in granulosa cells suggest a key role in promoting follicle growth. Moreover, E not only induces FKHR mRNA but also upregulates other notable components of the IGF-1 signaling system, including IGF-1R $\beta$  subunit and the glucose transporter, Glut-1 (Richards *et al.*, in press (b)). The coordinated up-regulation of FKHR with IGF-1R $\beta$  and Glut-1 indicates further that E enhances granulosa cell function in the H rat model by regulating three different targets that control cellular energy flow, glucose metabolism, and cell survival. Because IGF-1 helps maintain high expression of estrogen receptor beta (ER $\beta$ ) mRNA, at least in cultured granulosa cells, E and IGF-1 comprise an autocrine regulatory system in granulosa cells that promotes cell survival and proliferation (Richards *et al.*, in press (b)) (Figure 2).

In contrast to E and basal levels of FSH, ovulatory levels of LH cause dramatic decreases in the expression of FKHR that, in preovulatory follicles, is irreversible as luteinization proceeds (Richards *et al.*, in press (b)). Whether or not this is critical for alterations in granulosa cell function is unknown. However, it is intriguing to note that granulosa cells become resistant to apoptotic insult when they are stimulated with FSH/LH to undergo luteinization (Porter *et al.*, 2000). At this stage of differentiation, factors that impact proliferation (E, IGF-1,

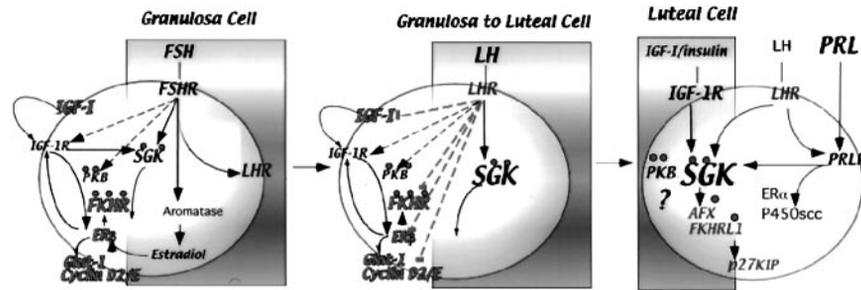


FIG. 2. Schematic of changes in the IGF-1 and FSH pathways in granulosa/luteal cells. Note that IGF-1, FKHR, ER $\beta$ , Glut-1, and cyclinD2/E are coexpressed in proliferating granulosa cells. Estradiol (E) upregulates IGF-1R $\beta$ , FKHR, and Glut-1, key components of the IGF-1 signaling pathway. IGF-1 upregulates ER $\beta$  and maintains FKHR. FSH at basal concentrations can maintain FKHR directly or indirectly via E. FSH and IGF-1 both stimulate the phosphorylation and activation of PKB and Sgk and their target, FKHR, whereas FSH alone induces Sgk (Alliston *et al.*, 1997,2000; Burns *et al.*, 2001). LH acts to downregulate expression of IGF-1, FKHR, ER $\beta$ , and cyclin D2. As granulosa cells luteinize, they gain increased levels of Sgk, AFX (Foxo-4), and FKHL1 (Foxo-3), which may impact the expression of p27KIP. [Reprinted with permission from Richards JS, Sharma SC, Falender AE, Lo YH 2002b Expression of FKHR, FKHL1 and AFX genes in the rodent ovary: evidence for regulation by IGF-1, estrogen and the gonadotropins. *Mol Endocrinol*, in press. Copyright The Endocrine Society.]

cyclin D2) and apoptosis (FKHR, FasL) are lost, whereas factors that are expressed in luteal cells and presumed to impact luteinization (FKHL1, AFX, Sgk, IGFBP-1, p27KIP, p21CIP, Jun D, Fra2) are acquired (Figure 2) Robker and Richards, 1998a; (Guidice, 2000; Sharma and Richards, 2000; Richards *et al.*, in press (b)). Therefore, it is possible that FKHR, FKHL1, and AFX have different functions that are dependent on the cell type, stage of cell differentiation, or specific associated proteins. In this regard, one study reports that AFX upregulates p27KIP (Medema *et al.*, 2000). In another study, Tanaka *et al.* (2001) show that expression of FKHL1 does not activate p21CIP or p27KIP promoter activity or regulate FasL expression. Therefore, the precise relation of Forkhead proteins to the regulation of these genes remains uncertain. Recently, FKHR has been shown to interact with and selectively modify the functional activity of other transcription factors, specifically members of the nuclear steroid receptor superfamily (Schoor *et al.*, 2001; Zhao *et al.*, 2001). FKHL1 and AFX may interact with the same or different transcription factors. Thus, the function of FKHR proteins likely depends not only their specific transcriptional activities but also on the hormonal milieu, the cell context, and the levels of proapoptotic and antiapoptotic factors (Hsueh *et al.*, 1994; Pru and Tilly, 2001) (Figure 2).

The gonadotropins, as well as IGF-1, impact the phosphorylation of Forkhead proteins and thus can control their functional activity (Richards, 2001a). Specifically, we have shown previously that FSH as well as IGF-1 can impact the

PI3-K pathway (Gonzalez-Robayna *et al.*, 1999,2000). FSH, like IGF-1, stimulates phosphorylation of PKB Ser-473 that is blocked by the PI3-kinase inhibitor LY294002 but not by the protein kinase A (PKA) inhibitor, H89. Rather H89 enhances FSH and IGF-1 phosphorylation of PKB (Gonzalez-Robayna *et al.*, 2000). FSH also induces the expression of the PKB-related kinase Sgk (Alliston *et al.*, 1997,2000; Gonzalez-Robayna *et al.*, 1999,2000; Burns *et al.*, 2001). Importantly, Sgk reaches its highest levels of expression in corpora lutea in association with the increased levels of FKHL1 and AFX (Figure 1). Based on these functions of FSH, it is not surprising that FSH stimulates the phosphorylation of FKHR at Thr-24 and Ser-256 in granulosa cells in a kinetic manner that mimics the action of IGF-1 in these same cells (Richards *et al.*, in press (b)). Although the precise mechanism(s) by which FSH stimulates PKA-independent activation of PI3-K remains to be clearly documented, the cAMP-regulated guanine nucleotide exchange factors (cAMP-GEFs) (de Rooij *et al.*, 1998,2000; Kawasaki *et al.*, 1998) provide a potential new link between FSH stimulation of adenylyl cyclase and activation of PI3-K via ras-related small guanine nucleotide triphosphatases (GTPases). Whether PKB, Sgk, or other kinases specifically mediate the phosphorylation of FKHR in granulosa cells *in vivo* also needs to be verified (Figures 1 and 2).

### III. Regulated Expression of Wnts and Frizzleds in the Ovary

Other factors that have been shown to impact ovarian cell function and follicular organization are members of the Wnt and Frizzled family of signaling molecules (Figure 3). Wnts are secreted, extracellular signaling molecules that act locally to control diverse developmental processes such as cell fate specification, cell proliferation, and cell differentiation (Cadigan and Nusse, 1997; Miller *et al.*, 1999). Wnts transduce their signals by binding to G protein-coupled receptors of the Frizzled family to activate distinct signaling cascades (Slusarski *et al.*, 1997; Lin and Perrimon, 1999; Liu *et al.*, 2001). In the canonical pathway Wnts/Frizzleds act to hyperphosphorylate dishevelled (Dvl) (Mao *et al.*, 2001), a cytoplasmic scaffolding protein and glycogen synthase kinase 3-beta (GSK-3 $\beta$ ) leading to the release of  $\beta$ -catenin. Soluble  $\beta$ -catenin heterodimerizes with members of the T-cell factor/Lymphoid enhancer factor (Tcf/Lef) family of transcription factors to regulate expression of selected target genes such as c-myc (He *et al.*, 1998). Wnt/Frizzled activation of the  $\beta$ -catenin pathway is further modulated by co-receptors (proteoglycans and/or arrow/LRP-5/LRP-6) (Lin and Perrimon, 1999; Tsuda *et al.*, 1999; Alexander *et al.*, 2000; Pinson *et al.*, 2000; Tamai *et al.*, 2000; Wehrli *et al.*, 2000; Mao *et al.*, 2001) and by antagonists such as secreted frizzled-related proteins (sFRPs) (Rattner *et al.*, 1997). Some Wnts activate Frizzled receptors that signal via intracellular calcium, protein kinase C (PKC), and/or calmodulin-dependent kinases (CAMK) (Kuhl *et al.*, 2000)

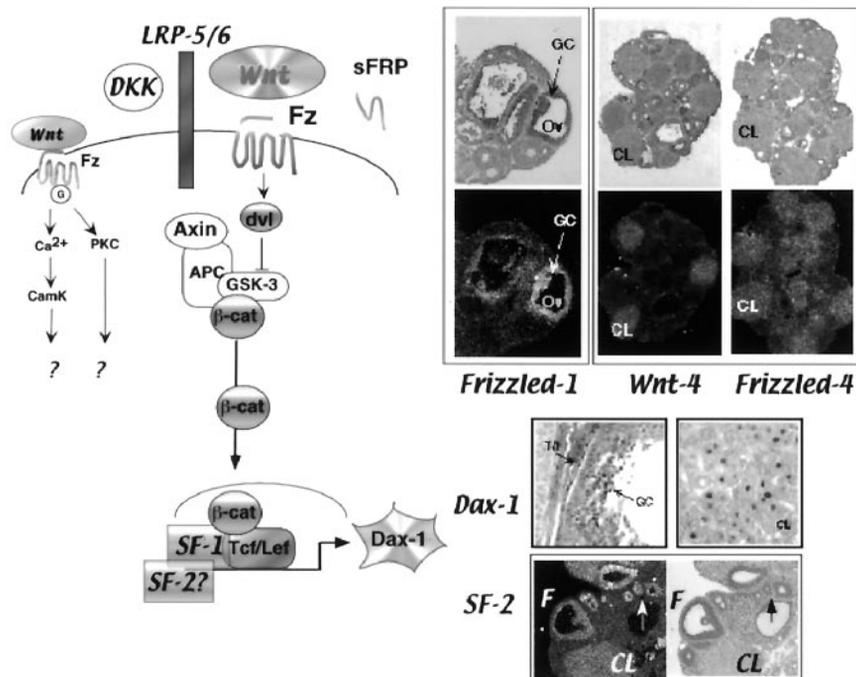


FIG. 3. Wnts and Frizzled receptors are expressed at specific stages of follicular growth and luteinization. Wnts are secreted ligands that activate Frizzled (Fz) receptors. The activation of these G protein-coupled receptors (GPCR) is associated with Wnt activation of the low-density lipoprotein (LDL)-related receptors (LRP5/6). Each of these can be inhibited by other secreted proteins – namely, soluble Frizzled-related proteins (sFRPs) and Dickkopf (Dkk), respectively. Wnts activate the Axin and dishevelled (Dvl), glycogen synthase kinase (GSK)-3,  $\beta$ -catenin pathway (via LRP5/6 and Frizzled receptors, respectively), leading to transcriptional activation of T-cell factor/lymphoid enhancer factor (Tcf/Lef)-regulated genes such as c-myc and Dax-1. Wnts also can activate Frizzled receptors that stimulate calcium-mediated pathways. In the ovary, Frizzled-1 is induced by the LH surge in granulosa cells of ovulating follicles. Which Wnt might activate this receptor is not yet known. Wnt 4 is expressed in small primary follicles as well as in corpora lutea and may activate Frizzled-4 that is also expressed in luteal cells. Although Dax-1 expression is increased by overexpression of Wnt-4, its pattern of expression in the adult ovary is not strictly related to Wnt-4. Note that steroidogenic factor (SF)-1 (NR5A1) and possibly SF-2 (NR5A2) act coordinately with Wnt-4 and Tcf/Lef to enhance transcription of DAX. SF-2 is highly expressed in granulosa cells of proliferating follicles as well as corpora lutea but is noticeably absent in theca-interstitial cells of the mouse. These data confirm those published for the mare (Boerboom *et al.*, 2000). [Adapted with permission from Hsieh M, Johnson MA, Greenberg NM, Richards JS 2002 Regulated expression of Wnts and Frizzleds at specific stages of follicular development in the rodent ovary. *Endocrinology*, in press. Copyright The Endocrine Society.]

(Figure 3). In this regard, it is of interest that granulosa cells express CAMK IV and mice null for CAMK IV have impaired fertility with abnormal follicular and luteal development (Wu *et al.*, 2000).

The Wnt/Frizzled cellular signaling pathways impact the development of reproductive organs. For example, Wnt-4 is essential for side branching in the mammary gland, a process that likely involves regulation of bone morphogenetic protein (BMP) or fibroblast growth factor (FGF) signaling molecules (Coleman-Krnacik and Rosen, 1994; Weber-Hall *et al.*, 1994; Phippard *et al.*, 1996; Brisken *et al.*, 2000; Wakefield *et al.*, 2001). In mammary tissue, Wnt-4 is co-localized with progesterone receptor (PR) and is a target of PR action (Brisken *et al.*, 2000). In the kidney, Wnt-4 is a mesenchymal signal essential for epithelial cell differentiation (Kispert *et al.*, 1998). Furthermore, in this tissue, Wnt-4 can be replaced by Wnt-1, Wnt-3a, Wnt-7a, or Wnt-7b, indicating redundant or overlapping pathways with specific Frizzled receptors (Kispert *et al.*, 1998). In pituitary gland development, Wnt-5a and BMP-4 play critical roles in cell fate, whereas Wnt-4 is important for expansion of ventral pituitary cell phenotypes. Wnt-7a-deficient mice are infertile due to abnormal development of the oviduct and uterus (Parr and McMahon, 1998). Mutations of Wnt-2 and Frizzled-5 were shown to impact placental angiogenesis (Monkley *et al.*, 1996; Ishikawa *et al.*, 2001).

Wnt-4 is also essential for the embryonic development of the ovary. Female mice null for Wnt-4 have sex-reversed ovaries that, at birth, are depleted of oocytes and contain supporting cells expressing genes characteristic of testis development such as Mullerian inhibiting substance (MIS) (Vainio *et al.*, 1999). Since mice null for Wnt-4 die at birth, we have analyzed by reverse transcription-polymerase chain reaction (RT-PCR) and *in situ* hybridization the expression of Wnt-4 in the adult ovary. Our results show that Wnt-4 is expressed in granulosa cells of small primary follicles containing one or two layers of cells and in granulosa cells of preovulatory follicles (Figure 3) (Hsieh *et al.*, in press). Wnt-4 expression is increased in granulosa cells by the LH surge and reaches its highest level in corpora lutea (Figure 3). Unlike the mammary gland, Wnt-4 is not a target of PR in the ovary (Hsieh *et al.*, in press), perhaps because PRA rather than PRB plays a primary role in the follicle, whereas PRB plays a greater role in mammary tissue (Mulac-Jericevic *et al.*, 2000; Conneely *et al.*, 2001). Wnt-4 may control different aspects of granulosa cell and luteal cell function, depending on which Frizzled receptors are present. Although it is not yet clear which Frizzled receptor is present in primary follicles, our results show that Frizzled-1 is induced transiently by the LH surge (Hsieh *et al.*, in press). It is localized to granulosa cells of ovulating follicles between 4–12 hours after exposure to the LH surge, just prior to ovulation. Thus, Frizzled-1 may control the expression of genes that impact the ovulation process. In contrast, Frizzled-4 is preferentially expressed at elevated levels in corpora lutea (Hsieh *et al.*, in press). Thus,

Frizzled-4 may be a receptor for Wnt-4 in this tissue (Figure 3). Mice null for Frizzled-4 also exhibit reproductive (ovarian?) defects but the exact nature of these are not yet known (J. Nathans, personal communication).

What are the functions of Wnt-4 in the adult ovary? In the embryonic gonad, the expression pattern of Wnt-4 is similar to that of DAX-1 (Swain *et al.*, 1996; Vainio *et al.*, 1999). More recently, overexpression of Wnt-4 in gonadal cells upregulated the expression of DAX-1 (Jordan *et al.*, 2001), indicating that Wnt-4 may regulate DAX-1 in the ovary as well. This observation is supported by the ability of  $\beta$ -catenin to enhance SF-1-stimulated transactivation of the DAX-1 promoter via Tcf/Lef promoter elements (Morohashi *et al.*, 2001). As a co-repressor of the orphan nuclear receptor SF-1, ovarian DAX-1 may antagonize the transcriptional activation of genes that are regulated by SF-1 such as aromatase (CYP 19) (Fitzpatrick and Richards, 1994; Carlone and Richards, 1997), P450scc (CYP21) (Clemens *et al.*, 1994; Richards, 1994), 17 $\alpha$ -hydroxylase (CYP17) (Zang and Mellon, 1996), FSH receptor (Heckert, 2000; Levallet *et al.*, 2001), MIS (Shen *et al.*, 1994; Watanabe *et al.*, 2000), and inhibin- $\alpha$  (Ito *et al.*, 2000). Since the expression of these genes is low in small growing follicles of immature mice and rats (Richards, 1994,2001b), it is tempting to speculate that a Wnt-4/Frizzled pathway is acting in these follicles to control Dax-1 and hence the activity of SF-1.

However, the role of Wnt-4 in the ovary appears to be complex. DAX-1 remains expressed (albeit at a lower level) in mice null for Wnt-4 (Vainio *et al.*, 1999; Jordan *et al.*, 2001), likely due to the potent control of DAX-1 expression by SF-1. Furthermore, only some but not all SF-1 regulated genes are elevated in the Wnt-4 null mice (Vainio *et al.*, 1999). Mice null for DAX-1 appear to have normal ovarian function (Yu *et al.*, 1998). Recent studies have identified and also shown that the equine ovary expresses not only SF-1 (NR-5A1) but also SF-2 (NR-5A2) (Boerboom *et al.*, 2000). Whereas SF-1 is high in theca cells, SF-2 is highest in granulosa cells and corpora lutea. We have recently confirmed these data in the rat and mouse by RT-PCR and *in situ* hybridization analyses (Figure 3). Thus, although the expression of Wnt-4 in small follicles may suppress steroidogenesis at this stage of development, a critical role for DAX-1 and SF-1 or SF-2 is not entirely clear. Even more striking, Wnt-4 as well as Frizzled-4 are elevated in luteal cells that are highly steroidogenic, contain nuclear Dax-1 protein, and express SF-2 as well as SF-1 (Hsieh *et al.*, in press) (Figure 3). Therefore, it is possible the Wnt-4/Frizzled pathway(s) operating in small follicles is (are) different than the Wnt-4/Frizzled-4 pathway that appears, but has not yet been proven, to be dominant in luteal cells. In addition, the specific downstream effectors of Wnt/Frizzled signaling may change as follicles terminally differentiate to luteal cells, thereby controlling distinct patterns of gene expression. Recent studies have shown that luteal cells exhibit elevated expression of specific kinases, including Sgk (Gonzalez-Robayna *et al.*, 1999; Alliston

*et al.*, 2000) and a MAP kinase pathway (Maizels *et al.*, 2001). Whether these kinases are targets (or mediators?) of Wnt/Frizzled signaling is also not known. In summary, the localization and regulation of Wnt-4, Frizzled-4, Frizzled-1 and others (Hsieh *et al.*, in press) in the adult rodent ovary, combined with the evidence for critical roles for Wnt-4 (Vainio *et al.*, 1999) and FGF-9 (Colvin *et al.*, 2001) in ovary and testis development, respectively, indicate that Wnt/Frizzled signaling is important for the growth and development of ovarian follicles. The identification of these ovarian-derived regulatory molecules provides a new intraovarian regulatory network that needs to be more clearly defined.

#### IV. Genes Involved in Ovulation

To prepare for ovulation, the ovary undergoes a series of closely regulated events. Small follicles must mature to the preovulatory stage, during which time the oocyte, granulosa cells, and theca cells acquire specific functional characteristics. The oocyte becomes competent to undergo meiosis, granulosa cells acquire the ability to produce E and respond to LH via the LH receptor, and theca cells begin to synthesize increasing amounts of androgens that serve as substrates for the aromatase enzyme in the granulosa cells (for a review, see Eppig, 1991; Richards, 1994). Remarkably, many events are spatially restricted to specific microenvironments within the follicle or surrounding interstitial compartments to allow successful expulsion of the cumulus-oocyte complex from the ruptured follicle (Hess *et al.*, 1999; Hizaki *et al.*, 1999; Sato *et al.*, 2001; Zhou *et al.*, 2001).

##### A. GENES CONTROLLING CUMULUS EXPANSION

The cumulus cells surrounding the oocyte and the matrix that the cumulus cells produce prior to ovulation comprise a special functional unit. The pioneering studies of many investigators have shown that the matrix upon which the cumulus cells move is formed by at least three major components (Figure 4). These include hyaluronic acid (HA) (Hess *et al.*, 1999; Salustri *et al.*, 1999) and at least two HA-binding proteins, namely, tumor-necrosis factor-stimulated gene (TSG)-6 (Fulop *et al.*, 1997; Yoshioka *et al.*, 2000) and the serum-derived inter- $\alpha$ -inhibitor (I $\alpha$ I), also known as inter- $\alpha$ -trypsin inhibitor (ITI) or serum-derived hyaluronic acid binding protein (SHAP) (Hess *et al.*, 1999; Sato *et al.*, 2001; Zhou *et al.*, 2001). HA is a high molecular weight (several million daltons), linear, unbranched glycosaminoglycan. In the ovary, HA is produced by the cumulus cells and granulosa cells adjacent to the antrum (Ochsner *et al.*, 2001). Expansion occurs only when I $\alpha$ I enters the follicle or when serum is added to cumulus-oocyte complexes (COC) to stabilize the matrix by covalent

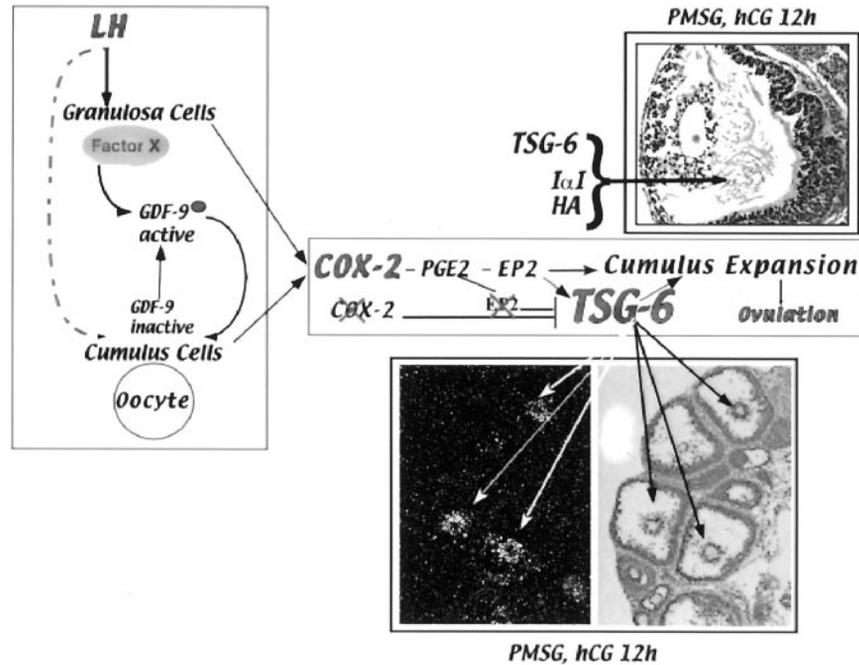


FIG. 4. LH induction of COX-2 is essential for cumulus expansion and ovulation. COX-2 is obligatory for the synthesis of prostaglandins, namely, PGE2 that binds the EP2 receptor. The induction of COX-2 by LH may be direct or indirect via activation of oocyte-derived factor, growth differentiation factor (GDF)-9, as discussed in the text. Mice null for COX-2 and EP2 fail to ovulate and exhibit impaired cumulus cell expansion, a process that requires the synthesis and cross-linking of key matrix components. These are hyaluronic acid (HA) and the HA binding proteins, inter- $\alpha$ -inhibitor (I $\alpha$ I) and tumor suppressor gene (TSG)-6. I $\alpha$ I is provided by serum and enters the follicles upon dissolution of the basement membrane in response to LH. The heavy chain of I $\alpha$ I is then covalently linked to HA by a converting enzyme present on granulosa cells. TSG-6 is induced by LH in cumulus cells of ovulating follicles. The induction of TSG-6 is dependent on the induction of COX-2 and the expression of the EP2 receptor. Of note, TSG-6 expression is absent in cumulus cells of COX-2 and EP2 knockout mice (Ochsner *et al.*, 2001).

coupling to the heavy chain (HC) of I $\alpha$ I (Hess *et al.*, 1999; Salustri *et al.*, 1999). The cumulus-derived matrix also contains other factors such as the proteoglycans brevican and versican (MacArthur *et al.*, 2000).

Cumulus expansion is induced as a consequence of the LH surge and is dependent on the induction of specific genes (Figure 4). These include cyclooxygenase-2 (COX-2) the rate-limiting enzyme in the synthesis of prostaglandins such as prostaglandin E2 (PGE2) (Sirois *et al.*, 1992; Joyce *et al.*, 2001; Ochsner *et al.*, 2001); HA synthase-2 (HAS-2), which catalyzes the production of HA (Weigel *et al.*, 1997); and TSG-6, which is an HA binding protein (Lee *et al.*,

1992; Yoshioka *et al.*, 2000). Ovulation is impaired in mice null for COX-2 (Dinchuk *et al.*, 1995; Morham *et al.*, 1995), EP2, the PGE2 receptor (Hizaki *et al.*, 1999; Tilley *et al.*, 1999) as well as I $\alpha$ I (Sato *et al.*, 2001; Zhou *et al.*, 2001). In each mutant mouse, the COCs within preovulatory follicles fail to undergo cumulus expansion in response to LH (Davis *et al.*, 1999). Ovulation and cumulus expansion can be restored in the COX-2 mice by exogenous administration of PGE2 or IL-1 $\beta$  (Davis *et al.*, 2001), indicating that prostaglandins and other signaling pathways are obligatory for both events. These observations provide clear evidence that one critical site for PGE2 action in the ovulating follicle is the COC. One target of PGE2 action may be TSG-6, since expression of TSG-6 is selectively reduced in the cumulus cells (but not granulosa cells) of COX-2 and EP2 null mice (Ochsner *et al.*, 2001). Note that TSG-6 remains selectively expressed in cumulus cells but not granulosa cells of ovulating follicles 12 hours after exposure to the LH-like molecule, human chorionic gonadotropin (hCG). These results indicate that PGE2 regulates expression of TSG-6 within the cumulus microenvironment and that TSG-6 may play some critical role in expansion of the matrix. Both TSG-6 and HA are expressed several hours prior to any visible physical expansion of the matrix (i.e., dispersion of the cumulus cells away from the oocyte). This suggests that the presence of these molecules is not sufficient for matrix formation or the movement of cumulus cells away from the oocyte (Figure 4).

The other critical component is I $\alpha$ I. Ovulation can be restored in the I $\alpha$ I-deficient (bikunin null) mice by adding serum (Sato *et al.*, 2001; Zhou *et al.*, 2001). I $\alpha$ I is normally excluded from follicular fluid because of its size and the avascular nature of the granulosa cell layer. It enters upon dissolution of the basal lamina during ovulation (Hess *et al.*, 1999). I $\alpha$ I is composed of several subunits: the light chain (LC) known as bikunin, which is covalently associated with the heavy chains (HC) via a chondroitin-sulfate moiety. In the presence of HA, I $\alpha$ I undergoes a substitution reaction in which the HC (SHAP) is covalently bound to HA releasing the bikunin (Sato *et al.*, 2001). The high degree of covalent linkage between the heavy chains and HA in the COC is unprecedented (Chen *et al.*, 1996) and suggests that the enzyme activity controlling this process is elevated within the follicle. Indeed, studies by Larsen and colleagues have shown high activity of the HC-HA conversion process in mural granulosa cells (Chen *et al.*, 1996). Although the enzymatic activity that catalyzes the covalent linkage to HA is essential, the biochemical identity of this converting enzyme is not yet known. Nor is it known if the enzyme is hormonally regulated in the mural granulosa cells. Such a condition would also be an important factor in controlling matrix formation.

Collectively, these observations indicate that HA and I $\alpha$ I, as well as COX-2/PGE2/EP2-induced gene products (TSG-6 and others?) are critical for

COC formation or cumulus cell differentiation; lack of any one of these factors precludes expansion.

The molecular mechanisms by which LH induces expression of HAS-2, COX-2, and TSG-6 genes may be either direct via LH receptors present on cumulus cells (although this possibility is controversial) or indirect via the activation of other signaling events in the follicle (Figure 4). The latter recently has gained credence, since the previously unknown soluble oocyte-derived factor that is essential for cumulus expansion (Eppig, 1991; Salustri *et al.*, 1999) has been provisionally identified as growth differentiation factor-9, GDF-9 (Elvin *et al.*, 1999). In cultures of rodent granulosa cells, GDF-9 can induce the expression of both HA and COX-2 (Elvin *et al.*, 1999). However, *in vivo* GDF-9 is expressed in oocytes beginning at the small primary follicle stage and continues in the oocyte after ovulation. If GDF-9 is the stimulatory factor, this raises the dilemma of why the expression of HA and COX-2 is restricted to ovulating follicles (i.e., those stimulated by the LH surge). One possible scenario that would link the obligatory requirement of LH action with that of the soluble oocyte-derived factor (i.e., GDF-9) is that this factor may need to be modified before it becomes activated. GDF-9, like other members of the TGF- $\beta$  family, is synthesized as a pro-peptide and therefore it is likely present at the surface of the oocyte-cumulus cell junctions, possibly attached to proteoglycans (Park *et al.*, 2000) as a latent factor. Induction by LH of a specific protease (factor X) (Figure 4) may be necessary to activate and/or release GDF-9, thereby allowing it to interact with cellular receptors and induce HAS-2 and COX-2 in cumulus cells. Full resolution of the pathways by which LH induces COX-2 in granulosa cells versus cumulus cells will necessitate stage-specific knockouts of GDF-9. Knockouts of TSG-6 also are needed to convincingly show a role for this protein in cumulus expansion *in vivo*.

## B. GENES EXPRESSED IN THECA CELLS

The specific ovulatory role of the theca cells is not well defined. Whereas matrix metalloproteinase (MMP)2 is expressed exclusively in the theca cells of preantral, preovulatory, and ovulating follicles (Lui *et al.*, 1998), the other MMPs and their inhibitors (TIMPs) exhibit more complex expression patterns for which a function is not yet clear. Based on the regulated expression of several aldo-keto reductase enzymes in the theca cells, it is possible that they act as a protective shield to ensure that toxic levels (Richards *et al.*, in press (a)) of compounds do not reach the granulosa cells or the oocyte at an inopportune time. These changes also may serve to protect the theca cells themselves and the ovary in general from exposure to toxic compounds.

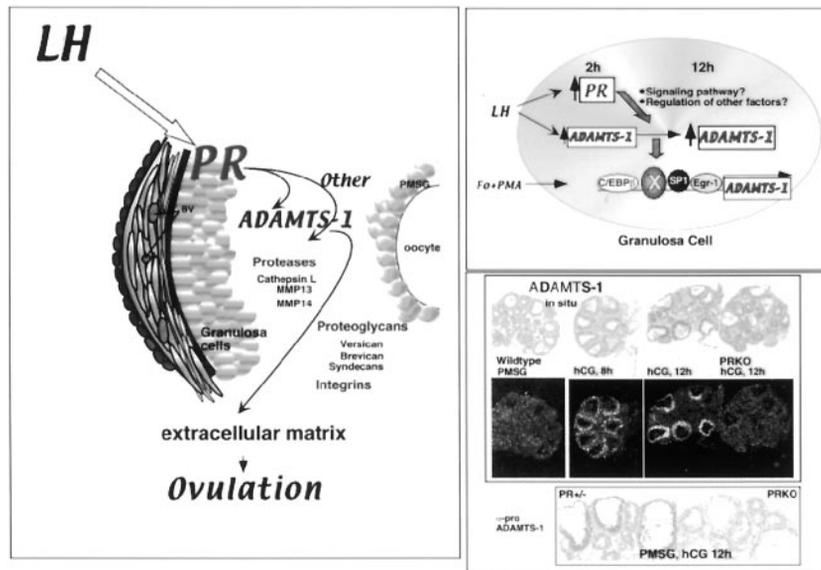


FIG. 5. LH induction of progesterone receptor (PR) and ADAMTS-1 is essential for ovulation. Expression of ADAMTS-1 is low in pregnant mare serum gonadotropin (PMSG)-treated mice but increases markedly by 8–12 hours after human chorionic gonadotropin (hCG), as shown by *in situ* hybridization and immunohistochemistry using an antibody to the prodomain of the protein ( $\alpha$ -pro ADAMTS-1). Expression of ADAMTS-1 is impaired in the progesterone receptor knockout (PRKO) mice (Robker *et al.*, 2000). The molecular mechanisms by which PR induces ADAMTS-1 appear to involve an indirect pathway, since consensus PR response elements (PRREs) are not found in the proximal ADAMTS-1 promoter. The functional role(s) of ADAMTS-1 remains to be identified, since this protease has multifunctional domains. In other tissues, it is a potent antiangiogenic factor, can degrade aggrecan and brevican, and may impact the integrin system in a manner similar to thrombospondin. Thus, PR and ADAMTS-1 have the potential to regulate many steps in the ovulation process.

### C. LUTEINIZING HORMONE-REGULATED GENES IN GRANULOSA CELLS

The LH receptor is essential for ovulation and luteinization (Lei *et al.*, 2001). The LH-induced transcription factors in granulosa cells include early growth regulatory factor-1 (Egr-1) (Espey *et al.*, 2000a), CAAT enhancer binding protein beta (C/EBP $\beta$ ) (Sirois and Richards, 1993), and progesterone receptor (PR) (Park and Mayo, 1991; Natraj and Richards, 1993) (Figures 2 and 5). Like COX-2, each of these components of the ovulatory process is induced rapidly but is expressed only transiently, with peak levels of message and protein observed approximately 4 hours after the LH surge. Each of these mediators appears to be involved in the functional activity of granulosa cells of ovulating follicles, as revealed by knockout studies (for a review, see Richards *et al.*, 1998,2000,in

press (a)). Other transcription factors such as the activator protein-1 (AP1) family members (e.g., Fra2 and JunD) are induced rapidly by the LH surge but then remain elevated in the nondividing, terminally differentiated granulosa cells during the postovulatory luteal phase (Sharma and Richards, 2000).

### 1. Progesterone Receptor (PR)

PR is a member of the nuclear receptor superfamily and regulates the numerous functions in reproductive tissues, including the uterus, mammary gland, and ovary. In the ovary, LH rapidly and selectively induces PR in mural granulosa cells of preovulatory follicles (Park and Mayo, 1991; Natraj and Richards, 1993). The molecular mechanisms by which LH acts to induce PR are not yet entirely clear but, in contrast to other tissues, strong evidence for a role of ER is lacking. E alone does not induce PR in intact cells *in vivo* or *in vitro* (Natraj and Richards, 1993). Nor does E induce activity of PR-promoter luciferase constructs transfected into granulosa cells (Clemens *et al.*, 1998; S.C. Sharma and J.S. Richards, in preparation). In addition, PR mRNA and protein can be induced by hCG in granulosa cells of PMSG-treated ER $\beta$ KO mice (J.S. Richards, unpublished observation). Furthermore, ovulation can be restored in the ER $\alpha$ KO mice by controlling pituitary secretion of LH (Couse and Korach, 1999; Couse *et al.*, 1999). Two regions of the PR promoter previously thought to be involved in mediating LH induction of this gene – namely, the GC-rich region of the distal promoter and the estrogen response element (ERE) $_3$  site of the proximal promoter (Clemens *et al.*, 1998) – also do not seem to be required in the context of the intact promoter (S.C. Sharma and J.S. Richards, in preparation). Specifically, deletion or mutation of the GC-rich region in the context of the intact murine promoter does not alter functional activity of a luciferase reporter construct when transfected into primary cultures of granulosa cells. Likewise, deletion or mutation of the ERE $_3$  site in the context of the intact promoter does not alter activity. Additional deletional studies and mutational studies have indicated that, in granulosa cells, the critical region of the PR promoter resides downstream relative to the putative transcriptional initiation site. In fact, the putative transcriptional start site can be deleted without affecting PR promoter activity. This critical downstream region contains one of many numerous putative cap sites scattered in the promoter, as indicated by computer-generated sequence homology searches of the PR promoter. In addition, numerous RNA transcripts are expressed in granulosa cells (Natraj and Richards, 1993). Thus, transactivation of the PR promoter in these cells appears to utilize a specific region or can use many different cap sites. Therefore, although nuclear factor Y (NF-Y), GATA, and Sp-1 binding sites have been characterized by electrophoretic mobility shift assays (EMSA), none of these binding sites is obligatory for LH activation of PR-promoter-luciferase reporter constructs in granulosa

cells (S.C. Sharma and J.S. Richards, in preparation). Thus, the key transcription factor(s) remain to be identified.

Mice null for PR fail to ovulate, even when stimulated by exogenous hormones. These findings support other studies that implicated progesterone as a key player in the ovulatory process (Lydon *et al.*, 1995; Rose *et al.*, 1999; Pall *et al.*, 2000). More specifically, mice null for PRA but not PRB exhibit impaired ovulation, indicating the subtype specificity of PR action in the ovulation process (Mulac-Jericevic *et al.*, 2000; Conneely *et al.*, 2001). Despite the failure of ovulation to occur in PRKO/PRAKO mice, the expression of COX-2, cumulus expansion, and luteinization proceed normally (Robker *et al.*, 2000). Recently, two targets of PR action were identified. These are ADAMTS-1 (for a disintegrin and metalloproteinase with thrombospondin-like repeats) that is known as METH-1 in human (Vazquez *et al.*, 1999; Espey *et al.*, 2000b), and cathepsin L (Robker *et al.*, 2000). Expression of ADAMTS-1 mRNA and protein is markedly reduced in granulosa cells of PR null mice (Figure 5).

## 2. ADAMTS-1

ADAMTS-1 is selectively induced by LH in granulosa cells and cumulus cells, with the peak level of mRNA and protein being produced 8–12 hours after exposure of ovaries to an ovulatory dose of hCG (a gonadotropin that is functionally analogous to LH) (Espey *et al.*, 2000b; Robker *et al.*, 2000). The peak in ADAMTS-1 transcription occurs after the peak of PR expression but before ovulation, which is usually observed at 14–16 hours after exposure to ovulatory hormones in mice and rats. Quite significantly, there are clear data to show that the induction of ADAMTS-1 is drastically reduced in rats when the preovulatory synthesis of progesterone is inhibited with epostane (Espey *et al.*, 2000b) or in mice that are null for PR (Robker *et al.*, 2000). Thus, the temporal pattern of these events indicates that ADAMTS-1 has a critical downstream role in mediating the PR-regulated ovarian activity that culminates in the rupture of a follicle. Whether or not PR acts directly or indirectly to control the expression of these two distinct proteases remains to be determined. To date, no consensus PR response element (PRRE) has been identified in the 1.6-kb ADAMTS-1 promoter and evidence for a direct effect of PR has not been observed in culture. Therefore, we propose at the moment that PR controls expression of an intermediary step that may be a specific signaling pathway that impacts transcription factor activity at the ADAMTS-1 promoter (Figure 5).

ADAMTS-1 is a multifunctional protein and, as such, could exert more than one function in the ovary. Which, if any, specifically impacts ovulation needs to be defined. Three major sites of action are likely (Figure 5). ADAMTS-1 is a potent active protease that cleaves, among other substrates, the bait region of  $\alpha$ 2-macroglobulin (Kuno *et al.*, 1999). As an active secreted protease, it is

likely to initiate one or more proteolytic cascades that account for the observed phenotype of the mice null for PR. As a protease, ADAMTS-1, like ADAMTS-4 (also expressed in the ovary), may also control the amount and the cellular location of various proteoglycans. Brevican and versican are present in follicular fluid, perlican is present in the thecal compartment, and the cell surface proteoglycans such as syndecan or glypican may be on either granulosa cells or theca cells (Ishiguro *et al.*, 1999; MacArthur *et al.*, 2000). Both ADAMTS-1 and ADAMTS-4 have been shown to degrade aggrecan and brevican (Kuno *et al.*, 2000; Nakamura *et al.*, 2000; Tortorella *et al.*, 2000). The action of ADAMTS-1 on proteoglycans present in ovarian follicles is highly likely. By altering the local concentrations of proteoglycans, ADAMTS-1 could also regulate the activity of specific growth factors, such as GDF-9, FGF-2 and FGF-7, EGF, TGF- $\alpha$ , or Wnts, whose activity is known to be blocked by proteoglycans (Park *et al.*, 2000). Thus, a lack of ADAMTS-1 might prevent the activation of one or more potent bioactive factors in the follicular fluid by preventing their release from the proteoglycans.

The function for ADAMTS-1 in the follicle may be mediated by its ability to interact with specific cellular signaling molecules through disintegrin or thrombospondin motifs at the carboxy terminus of the protein (Kuno *et al.*, 1999). Like some other ADAM proteins, ADAMTS-1 may be a signaling protein that regulates some aspect of granulosa cell function via interactions with specific cell surface G protein-coupled receptors (GPCRs), integrins, and tetraspan proteins (Bigler *et al.*, 2000; Le Naour *et al.*, 2000). ADAMTS-1, like thrombospondin 1 (TS-1), is also a potent antiangiogenic factor that may interact with integrins (Vazquez *et al.*, 1999). Although TS-1 and -2 are expressed in ovarian follicles (Bagavandoss *et al.*, 1998), the specific roles of thrombospondins and ADAMTS-1 have not been clearly delineated. Based on our limited understanding of ADAMTS-1 in mammalian cells, it is difficult to predict which of its multiple functions might be critical for impacting the process of ovulation. Mutations of ADAMTS-1, ADAMTS-4, and ADAMTS-9 genes are clearly needed to resolve this important area of ovarian cell function and ovulation (Figure 5).

### 3. *Cathepsin L*

Cathepsin L is another LH- and PR-regulated gene in the ovary that was identified by cDNA array technology (Robker *et al.*, 2000). Cathepsin L is a member of the papain family of enzymes. It is commonly a lysosomal protease but it is also secreted from certain endocrine cells such as Sertoli cells of the testis and placental trophoblasts and from certain tumors (Ishidoh and Kominami, 1998). In the cat uterus, cathepsin L is also regulated by progesterone (Jaffe *et al.*, 1989). The function of cathepsin L in the ovary appears to be complex. This

enzyme is expressed in granulosa cells of follicles at several different stages of development in response to both FSH and LH. In addition, its expression in ovulatory follicles is impaired in PR null mice (Robker *et al.*, 2000). A functional link between PR-regulated expression of ADAMTS-1 and cathepsin L is not immediately obvious but this issue will be clarified as more information is gained about the specific roles of these proteases in the ovulation process. Cathepsin L, like cathepsin G, may activate protease-activated receptors (PARs) (Sambrano *et al.*, 2000).

#### 4. PACAP and the Type-1 PACAP Receptor (PAC1)

Pituitary adenylate cyclase-activating peptide (PACAP) and PAC1 are also LH-inducible genes that have been shown to be responsive to regulation by progesterone and PR-antagonists *in vivo* and *in vitro* (Gras *et al.*, 1999; Ko *et al.*, 1999; Ko and Park-Sarge, 2000; Park *et al.*, 2000). PACAP has been shown to stimulate progesterone production as well as meiotic maturation in follicle-enclosed, cumulus-enclosed oocytes (Gras *et al.*, 1999; Ko and Park-Sarge, 2000). Thus, PACAP seemed to be a potential and attractive candidate that might act downstream of PR to regulate transcriptional activation of ADAMTS-1. However, expression of PACAP mRNA in ovaries of mice null for PR is identical to that observed in ovaries of wild-type mice, indicating that PR is not essential for LH-induced expression of PACAP (K.H. Doyle and J.S. Richards, unpublished observations). Therefore, other signaling cascades may be regulated by PR that impact the expression of ADAMTS-1 and cathepsin L.

### IV. Summary

The number of factors now known to control the embryonic development of the gonad as well as the regulated progression of follicular growth, ovulation, and luteinization is steadily increasing. Knockout studies have shown that members of the TGF $\beta$  family, the FGF family, and the Wnt/Frizzled pathway are among the newer players to emerge on the scene. In addition, new actions for some of the older players have been identified, such as the ability of FSH to impact the PI3-kinase cascade in a manner independent of PKA. The multiplicity of LH actions in specific microenvironments during ovulation has now documented how complex and finely tuned this process really is. Although many proteases are expressed in the ovary and are hormonally regulated, the novel protease ADAMTS-1 and cathepsin L, rather than the MMPs, have gained particular recognition. New members and new functions for steroid receptors are being unraveled. The roles of ER subtypes ER $\alpha$  and ER $\beta$  in the ovary as well as PR are critical for ovarian function. The identification of SF-2 is intriguing and demands that the specific roles of SF-1 and SF-2 be reanalyzed at later stages of

follicular growth. The future will clearly bring more excitement and resolution to the dynamics of ovarian follicular development, ovulation, and luteinization.

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