

# The role of Notch signaling in the mammalian ovary

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## Abstract

The Notch pathway is a contact-dependent, or juxtacrine, signaling system that is conserved in metazoan organisms and is important in many developmental processes. Recent investigations have demonstrated that the Notch pathway is active in both the embryonic and postnatal ovary and plays important roles in events including follicle assembly and growth, meiotic maturation, ovarian vasculogenesis and steroid hormone production. In mice, disruption of the Notch pathway results in ovarian pathologies affecting meiotic spindle assembly, follicle histogenesis, granulosa cell proliferation and survival, corpora lutea function and ovarian neovascularization. These aberrations result in abnormal folliculogenesis and reduced fertility. The knowledge of the cellular interactions facilitated by the Notch pathway is an important area for continuing research, and future studies are expected to enhance our understanding of ovarian function and provide critical insights for improving reproductive health. This review focuses on the expression of Notch pathway components in the ovary, and on the multiple functions of Notch signaling in follicle assembly, maturation and development. We focus on the mouse, where genetic investigations are possible, and relate this information to the human ovary.

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## Introduction

The mammalian ovary is a female organ critical for reproductive function that contains and supports the development of the female gamete, the oocyte. The functional unit of the ovary, the ovarian follicle, forms a specialized niche necessary for the development of a mature oocyte and is also essential for the production of steroid hormones that drive reproductive function and support female health. During embryonic development, primordial germ cells (PGCs), which arise within the proximal epiblast, migrate to the genital ridges of the bipotential gonad, at that time, under the influence of signals from surrounding somatic cells, they develop into either sperm or oocytes (McLaren 1984, Saitou & Yamaji 2012). During ovarian development, PGCs undergo several rounds of synchronous mitotic division without complete cytokinesis to form clusters of interconnected germ cells called germ cell syncytia, also known as cysts or nests (Pepling & Spradling 1998). Though the function of germ cell syncytia has not been established, these structures provide a shared local environment to respond to stimuli and to allow the exchange of macromolecular resources (Pepling & Spradling 1998). Following the induction of stimulated by retinoic acid gene 8 (*Stra8*) expression by retinoic acid, germ cells within the developing female gonad cease mitotic division and enter meiosis in an anterior to posterior

wave across the ovary, arresting at the diplotene stage of meiotic prophase I (Menke *et al.* 2003, Koubova *et al.* 2006, Anderson *et al.* 2008).

Interactions between germ cells and somatic pregranulosa cells are critical for the formation of ovarian follicles. Perinatally in mice, and embryonically in humans, somatic pregranulosa cells send cellular projections around individual germ cells within fragmenting syncytia to encapsulate and form primordial follicles through the coordination of syncytial breakdown and follicle assembly in a process known as follicle histogenesis. This process involves bidirectional communication between germ cells and somatic support cells and it has been shown to be regulated by steroid hormone signaling, neutrophins, KITL/KIT signaling and members of the transforming growth factor-beta (TGF $\beta$ ) superfamily. Follicle histogenesis is also associated with significant levels of germ cell loss (Pepling 2012), which may serve to eliminate defective germ cells or provide a means to fragment syncytia (Pepling & Spradling 2001). Aberrations in follicle histogenesis can result in the formation of multi-oocytic follicles, which are a consequence of incomplete fragmentation of syncytia, and in oocyte or follicle loss due to errors in follicle assembly (Silva-Santos & Seneda 2011). Notably, follicle histogenesis results in the formation of the primordial follicle pool, which represents the entirety of oocytes available during a female's reproductive life.

**Table 1** Expression of Notch signaling components in the mammalian ovary.

Gene	Oocyte	Prim	1°	2°	Antral	CL	Theca	Vasc	OSE
<i>Notch1</i>	1, 2	1				3–5		3, 6–9	10
<i>Notch2</i>	4, 11–13	4, 11, 13, 14	4, 11–14	4, 6, 8, 12–14	4, 6, 8, 12–14	4	4		
<i>Notch3</i>			4	4, 6	4, 6	4, 6, 15	4		
<i>Notch4</i>		7	7	7	7	3, 7		3, 6, 7	7
<i>Jag1</i>	4, 6, 11–14, 16	4	4	4, 13		4, 15		7	
<i>Jag2</i>	1			6	6				
<i>Dll1</i>				4		4			
<i>Dll3</i>									
<i>Dll4</i>	4	4	4	4	4	3, 4		3, 9	
<i>Hes1</i>	4, 11	4, 11	4	4		4	4		10
<i>Hes5</i>	4	4	4	4, 6	4, 6	4			10
<i>Hes7</i>									
<i>Hey1</i>	12, 17	17	12	6, 12	6, 12				
<i>Hey2</i>	11, 12	11	12	6, 12	6, 12				
<i>Heyl</i>				6	6				
<i>Lfng</i>				18	18			18	
<i>Mfng</i>								18	
<i>Rfng</i>					18				

References: (1) Guo *et al.* 2012, (2) Feng *et al.* 2014, (3) Hernandez *et al.* 2011, (4) Murta *et al.* 2014, (5) Accialini *et al.* 2015, (6) Johnson *et al.* 2001, (7) Vorontchikhina *et al.* 2005, (8) Jovanovic *et al.* 2013, (9) Garcia-Pascual *et al.* 2013, (10) Pan *et al.* 2015, (11) Trombly *et al.* 2009a,b, (12) Zhang *et al.* 2011, (13) Wang *et al.* 2014, (14) Vanorny *et al.* 2014, (15) Wang *et al.* 2015, (16) Dorfman *et al.* 2011, (17) Manosalva *et al.* 2013, and (18) Hahn *et al.* 2005.

1°, primary follicles; 2°, secondary follicles; CL, corpora lutea; OSE, ovarian surface epithelium; Prim, primordial follicles; vasc, vasculature.

Following puberty, and during each estrous cycle in rodents or menstrual cycle in humans, a cohort of follicles is recruited to undergo further growth and maturation. Early stages of follicle activation and development are generally considered to be gonadotropin-independent, while later stages, including antrum formation, cumulus expansion and ovulation, require follicle stimulating hormone (FSH) and luteinizing hormone (LH). Following the release of a meiotically competent oocyte, remaining granulosa and theca cells of a follicle luteinize to form a corpus luteum, which is essential for the production of steroid hormones needed to prepare the uterus for pregnancy. In addition, there is rapid neovascularization of blood vessels within the theca layer to support this process.

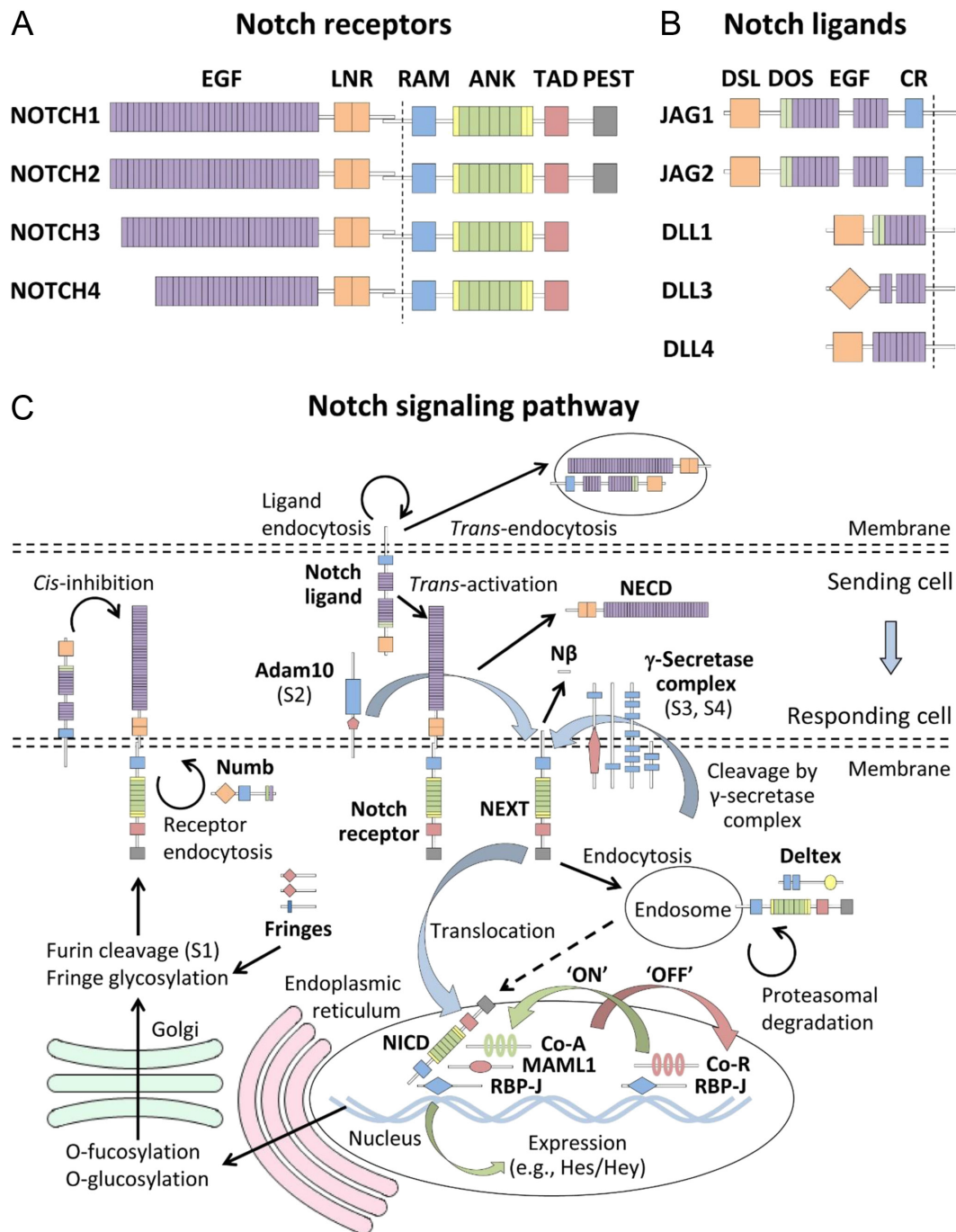
The Notch pathway is one of the most conserved signaling systems in multicellular organisms, and perturbations in Notch signaling are responsible for a number of inherited human diseases and cancers (Maillard & Pear 2003, Leong & Karsan 2006, Penton *et al.* 2012). While the function of the Notch pathway in ovarian development has been extensively studied in model organisms such as *Drosophila* and *C. elegans* (Andersson *et al.* 2011, Greenwald & Kovall 2013), there is limited information available on the function of Notch signaling in the mammalian gonad, and only recently have reports become available implicating Notch signaling in ovarian (Manosalva *et al.* 2013, Xu & Gridley 2013, Vanorny *et al.* 2014) or testicular (Tang *et al.* 2008, Garcia & Hofmann 2013, Huang *et al.* 2013) development. Within the mammalian ovary, Notch receptors, ligands, modulators and target/effector genes are expressed and dynamically regulated

during follicular development (Table 1). Activation of the Notch pathway has been described in both germline and somatic cell populations, and new studies involving the use of transgenic reporter mice and conditional knockout mouse models suggest that productive Notch signaling occurs between germ cells and granulosa cells, between adjacent granulosa cells, and between cells of the ovarian vasculature.

Morphogenesis of the mammalian ovary requires the precise spatial and temporal organization and function of multiple cell types, and is coordinated by endocrine, paracrine, autocrine and juxtacrine signaling mechanisms. The actions and regulation of paracrine and endocrine hormone signaling within the ovary has been extensively studied (Albertini *et al.* 2001, Knight & Glister 2006, Edson *et al.* 2009, Conti *et al.* 2012); however, the roles of juxtacrine signaling within the ovary remain largely unexplored. This review will focus on the Notch pathway, a juxtacrine or contact-dependent signaling system, during ovarian and follicular development.

### Notch pathway signal transduction and regulation

The Notch signaling pathway (Fig. 1) is a highly conserved and broadly used molecular transduction system for processes including cell-fate specification (Lindsell *et al.* 1996, Weijzen *et al.* 2002, Chau *et al.* 2006, Raetzman *et al.* 2006, Hayashi & Kume 2008, Doetzlhofer *et al.* 2009), cell migration (Jordan *et al.* 2000), mesenchymal/epithelial transition (Li *et al.* 1998), cell survival/death (Nickoloff *et al.* 2002, Sainson *et al.* 2005, Choi *et al.* 2008), cell division (Sainson *et al.* 2005, Kolev *et al.* 2008, Monahan *et al.* 2009) and



**Figure 1** The Notch signaling pathway. In mammals, there are (A) four Notch receptors and (B) five Notch ligands. Extracellular interactions between receptors and ligands expressed on juxtaposed cells are mediated by several conserved domains. Among these are epidermal growth factor (EGF) repeats found on both ligands and receptors, LIN-12/Notch repeat (LNR) domains that are found on all receptors, and a highly conserved *Delta-Serrate-Lag2* (DSL) domain found on ligands. Notably, the DSL domain of DLL3 is structurally divergent from that of other Notch ligands and does not promote Notch receptor activation. JAG1 and JAG2 also contain a cysteine-rich (CR) domain, while DLL3 and DLL4 lack a Delta and OSM-11 (DOS) domain composed of atypical EGF repeats. (C) Productive interactions between juxtaposed cells lead to the cleavage of Notch receptors at the juxtamembrane region by the  $\gamma$ -secretase complex. Nuclear localization signals (yellow bars) within the intracellular domain of Notch receptors (NICD) allow for nuclear translocation following receptor cleavage. The RBP-J associated molecule (RAM) domain facilitates interactions between the intracellular domain and the DNA-binding factor RBP-J. Binding of the NICD with RBP-J displaces corepressors (Co-R) and allows for the recruitment of transcriptional coactivators (Co-A) via interactions with CDC10/ankyrin (ANK) repeats and a *trans*-activation domain (TAD). Target or effector genes of the Notch pathway include the Hes and Hey families of transcriptional repressors. Notch signaling is terminated by ubiquitination of a proline–glutamate–serine–threonine (PEST) domain that targets the intracellular domain for proteasomal degradation.

cell adhesion (Choi *et al.* 2008). Disruption of Notch signaling results in multiple human diseases including Alagille syndrome (Oda *et al.* 1997), familial aortic valve disease (Garg *et al.* 2005), Adams–Oliver syndrome (Stittrich *et al.* 2014), Hajdu–Cheney syndrome (Simpson *et al.* 2011), spondylocostal dysostosis (Bulman *et al.* 2000), cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) (Joutel *et al.* 1996), Alzheimer's disease (Kopan & Goate 2000), and cancers including T-cell acute lymphoblastic leukemia (Weng *et al.* 2004) and pancreatic (Mullendore *et al.* 2009), colon (Reedijk *et al.* 2008) and ovarian cancers (Groeneweg *et al.* 2014).

Notch signaling is frequently used to select among pre-existing cellular potentials to specify cell-fate decisions. The two classical modes by which Notch signaling functions to mediate cell-fate decisions include lateral inhibition and inductive signaling (Flores *et al.* 2000, Haines & Irvine 2003). Lateral inhibition is a mechanism by which a cell that adopts a particular fate inhibits neighboring cells from developing in a similar manner. By contrast, inductive signaling is a mechanism by which one cell, or a group of cells, remains in its current state, and signals to and causes a neighboring cell, or group of cells, to differentiate.

In mammals, the Notch pathway (Artavanis-Tsakonas *et al.* 1999, Mumm & Kopan 2000, Kopan 2002, Schweisguth 2004, Ehebauer *et al.* 2006) involves the interaction of one of four hetero-oligomer (Blau Mueller *et al.* 1997) single-pass type I transmembrane receptors (NOTCH1, NOTCH2, NOTCH3 and NOTCH4) with one of five single-pass type I transmembrane ligands (JAG1, JAG2, DLL1, DLL3 and DLL4). Notch receptors contain 29–36 N-terminal epidermal growth factor (EGF) repeats, of which EGF repeats 11–12 are essential for ligand binding, while Notch ligands contain a conserved N-terminal *Delta-Serrate-Lag2* (DSL) domain, which is important for mediating interactions with Notch receptors. Notably, transmission of Notch signaling utilizes a unique mechanism mediated through a series of sequential proteolytic cleavage events that does not involve amplification by classical intracellular secondary messengers.

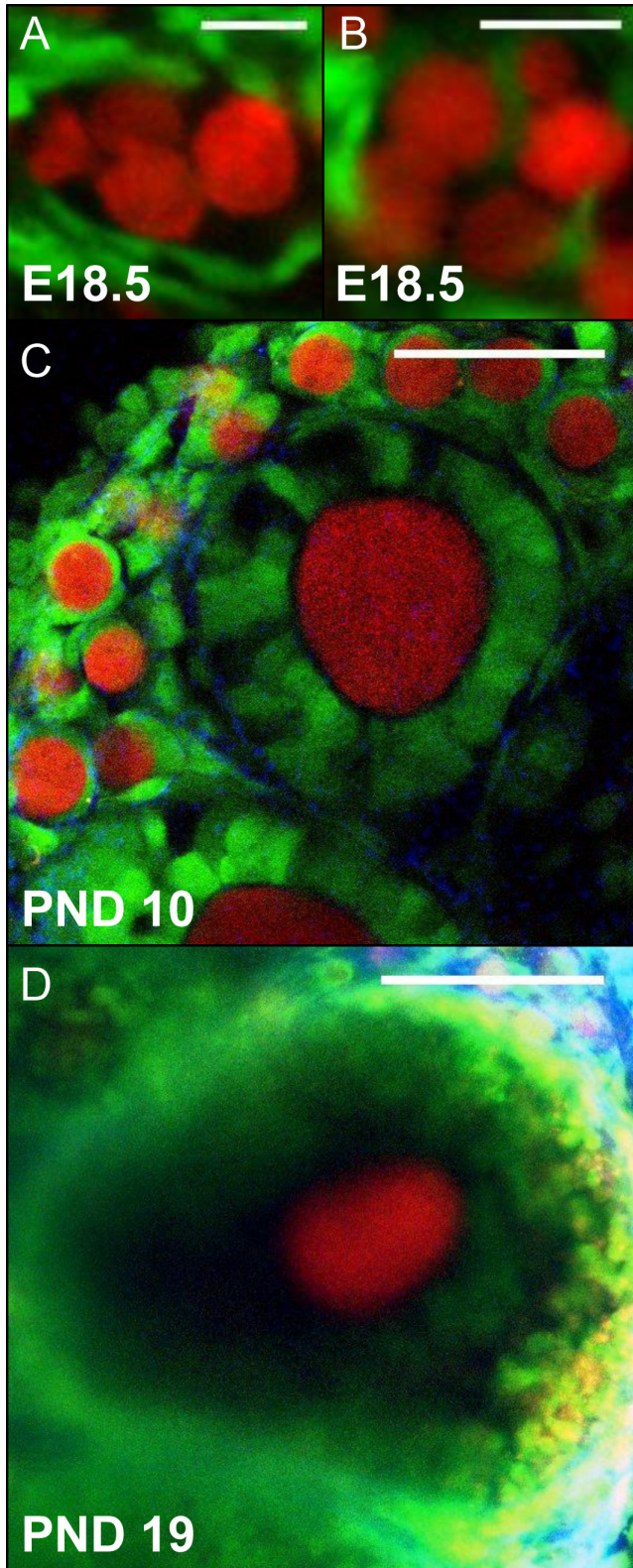
Following their synthesis, Notch receptors are post-translationally modified through fucosylation by protein O-fucosyltransferase 1 (POFUT1) within the endoplasmic reticulum. The receptors are further modified within the Golgi by the GlcNAc-transferase proteins lunatic (LFNG), manic (MFNG) and radical fringe (RFNG), which function to modulate receptor–ligand interactions. During their transit through the Golgi, Notch receptors are cleaved by the furin-like protein proprotein convertase subtilisin/kexin type 5 (PCSK5) at site 1 (S1) during exocytosis, which regulates receptor trafficking and signaling activity

(Logeat *et al.* 1998). The two receptor cleavage products remain associated at the cell surface as a heterodimer through non-covalent, calcium-dependent interactions (Gridley 2003). Importantly, the S1 cleavage site is contained within a critical negative regulatory region (NRR) of the receptor that is comprised of three highly conserved Lin-12/Notch repeat (LNR) domains and a heterodimerization domain (HD), which cooperate to prevent premature signaling in the absence of a ligand (Gordon *et al.* 2009).

Productive interactions between the extracellular domains of Notch ligands and receptors occur intercellularly in *trans* (i.e., *trans*-activation) between juxtaposed cells, whereas inhibitory interactions occur between receptors and ligands coexpressed along the membrane of the same cell in *cis* (i.e., *cis*-inhibition). As a consequence of *trans*-activating interactions, Notch receptors undergo a conformational change that exposes the site 2 (S2) cleavage moiety that is recognized by a disintegrin and metalloproteinase domain-containing protein 10 (ADAM10). The remaining membrane-anchored Notch extracellular truncation (NEXT) fragment is recognized by the  $\gamma$ -secretase complex, an enzymatic assemblage composed of presenilin 1 (PSEN1), nicastrin (NCSTN), presenilin enhancer 2 (PEN2) and anterior pharynx-defective 1 (APH1) (Saxena *et al.* 2001). Further proteolytic processing at the juxtamembrane region of the Notch receptor by the  $\gamma$ -secretase complex at site 3 (S3) and site 4 (S4) results in the release of the Notch intracellular domain (NICD) and N $\beta$  peptide, which allows the NICD to translocate to the nucleus where it acts as a modulator of transcription (Fortini 2002).

Within the nucleus, the NICD interacts with the DNA-binding transcriptional repressor recombination signal binding protein for immunoglobulin kappa j region (RBP-J), also known as CBF1/Su(H), through its RBP-J associated molecule (RAM) domain (Lubman *et al.* 2007). Subsequently, this interaction positions CDC10/ankyrin repeats within the NICD to recruit transcriptional activators, such as mastermind-like proteins (MAMLs), to the promoters of Notch target genes. Classic target genes of Notch signaling include the hairy/enhancer-of-split (Hes) and hairy/enhancer-of-split related with YRPW motif protein (Hey) genes, which are members of the basic helix-loop-helix (bHLH) family and function as transcriptional repressors. The ankyrin repeats of Notch receptors also facilitate interactions with NUMB and *Deltex* homologs, which are important cytosolic factors that regulate the Notch pathway. Downregulation of Notch signaling requires phosphorylation of NICD on a C-terminal proline, glutamic acid, serine and threonine (PEST) domain, and further modification by E3 ubiquitin ligases, such as F-box/WD repeat-containing protein 7 (FBW7), which promote proteasomal degradation of NICD. In the absence of NICD, RBP-J is associated with co-repressor (Co-R) proteins and histone deacetylases (HDACs), which act to repress the transcription of target





**Figure 2** Notch activity during follicle assembly, growth, and maturation. A Notch-responsive GFP reporter mouse line, used to label Notch active cells (green), was crossed with a *Vasa-Cre* mouse and conditional *tdTomato* reporter line, to label germ cells (red). (A) Notch active pregranulosa cells surround clusters of germ cells in

genes; thus, activation of the Notch pathway involves a switch from a repressed state to an activated state.

Emerging evidence also indicates that the Notch pathway can be regulated through several non-canonical mechanisms (D'Souza *et al.* 2010, Heitzler 2010, Andersen *et al.* 2012). These include activation by membrane ligands such as delta-like 1 homolog (DLK1) (Baladron *et al.* 2005) and delta- and notch-like epidermal growth factor-related receptor (DNER) (Eiraku *et al.* 2005), GPI-linked ligands including F3/contactin 1 (CNTN1) (Hu *et al.* 2003) and NB3/contactin 6 (CNTN6) (Cui *et al.* 2004) and secreted ligands such as connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed gene 3 (CCN3) (Sakamoto *et al.* 2002), microfibril-associated glycoprotein family 1 (MAGP1) and 2 (MAGP2) (Miyamoto *et al.* 2006), thrombospondin 2 (TSP2) (Meng *et al.* 2009), Y-box protein 1 (YB1) (Rauen *et al.* 2009) and EGF-like domain 7 (EGFL7) (Schmidt *et al.* 2009). Some of these ligands impact Notch signaling by functioning as activators or inhibitors in either *trans* or *cis*, and some of them can also regulate Notch activation independent of  $\gamma$ -secretase proteolysis by causing dissociation of the Notch receptor heterodimer, as with MAGP2, or by facilitating interactions with other downstream modulators at the cell surface, including *Deltex* and  $\beta$ -Arrestin (Mukherjee *et al.* 2005). Importantly, many of the observations regarding non-canonical Notch signaling have yet to be proven within *in vivo* systems; thus, the significance of these findings is still unknown, and non-canonical signaling has not been well-studied within the ovary.

### Notch pathway in the developing ovary

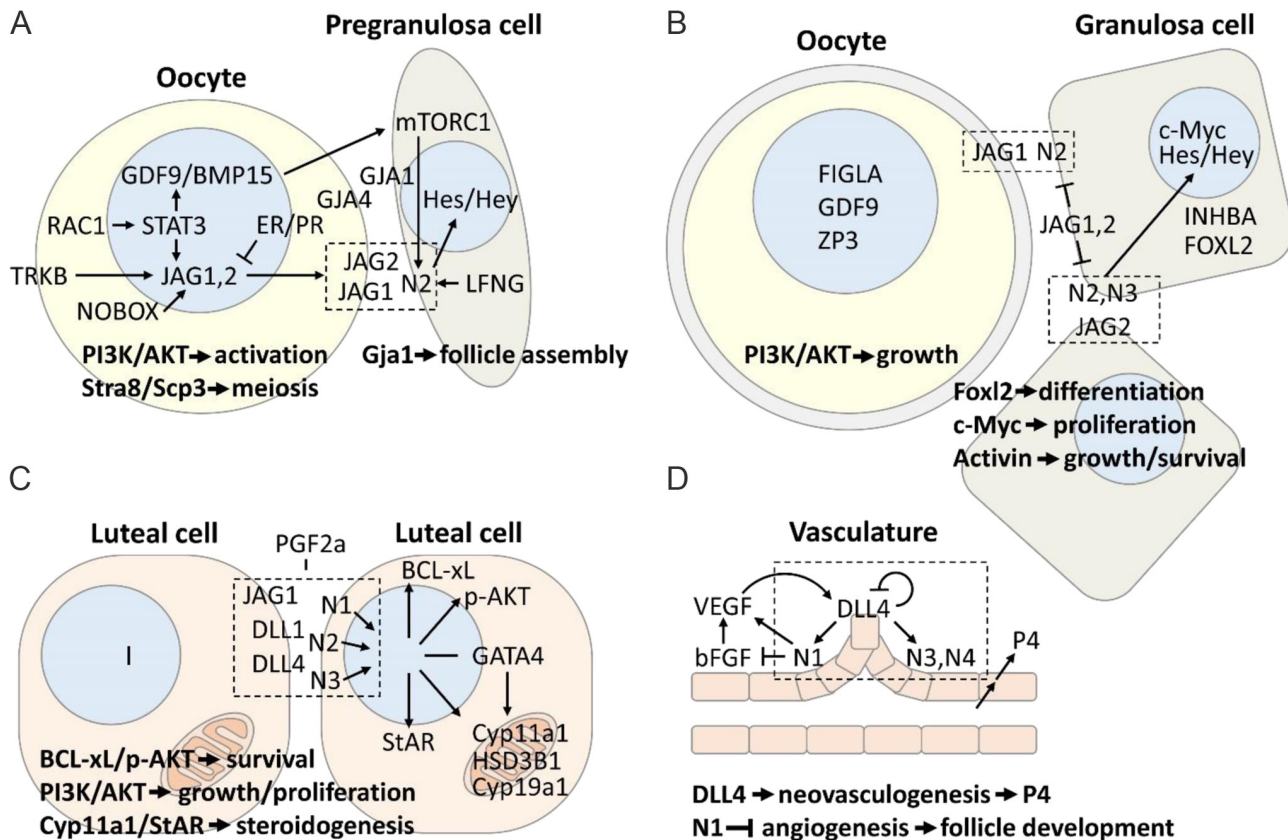
During embryonic ovarian development, Notch receptors, ligands and target genes are expressed (Table 1). NOTCH2, which is localized to somatic pregranulosa cells, is the most abundantly expressed receptor within the embryonic ovary, while the Notch ligands JAG1 and JAG2, which are found in the germ cells of the embryonic ovary, are the most abundantly expressed ligands (Vanorny *et al.* 2014). The use of a summative Notch-responsive fluorescent reporter mouse line demonstrates that activation of the Notch pathway in the embryonic ovary is first observed

an embryonic day 18.5 (E18.5) ovary (scale bar=20 microns). (B) Notch active cells can also be seen invading germ cell synctia and sending projections around individual germ cells to form primordial follicles (scale bar=20 microns). (C) Notch active pregranulosa cells are observed within primordial follicles at the ovarian cortex of a postnatal day 8 (PND8) ovary, while granulosa cells within primary follicles demonstrate variable Notch reporter activity. Collagen fibrils (blue) were detected by second harmonic generation (scale bar=50 microns). (D) Within antral follicles, both cumulus and mural granulosa cells are Notch active (scale bar=100 microns).

around embryonic day 15.5 (E15.5) and is restricted to somatic cells (Vanorny *et al.* 2014). Furthermore, Notch activation within the embryonic mouse ovary is concomitant with a significant increase in the expression of the ligands, *Jag1* and *Jag2*, the receptor, *Notch2*, and the Notch target genes *Hes1* and *Hey2* (Vanorny *et al.* 2014). At E18.5, Notch active pregranulosa cells can be observed undergoing dramatic reorganization to form an intricate network around ovigerous cords containing partially fragmented germ cell nests (Fig. 2A and B). Additionally, Notch active pregranulosa cells, which are

organized along collagen fibrils, begin to invade nests and can be seen sending cellular projections around and encapsulating individual germ cells to form primordial follicles (Vanorny *et al.* 2014). With these data, a model begins to emerge in which upregulation of *Jag1* and *Jag2* in the oocyte, signals through *Notch2* expressed in pregranulosa cells to facilitate the resolution of germ cell syncytia and assembly of primordial follicles.

Functional studies in embryonic ovaries demonstrate that the Notch pathway is important for germ cell syncytia formation and meiotic entry. The inhibition of



**Figure 3** Regulation and function of Notch signaling during ovarian and follicle development. The Notch pathway has multiple roles during follicle assembly and growth, steroidogenesis and ovarian vascular development. (A) Bidirectional communication between germ cells and somatic support cells is critical for follicle assembly. In the mammalian ovary, this process appears to be regulated in large part by the modulation of *Jag1*, and possibly *Jag2*, transcription in germ cells, which is regulated by NOBOX, STAT3, TRKB and steroid hormone action. Upregulation of Notch ligand expression in oocytes facilitates interactions with adjacent pregranulosa cells expressing Notch receptor that is supported by growth factors (i.e., GDF9 and BMP15) from the oocyte. Notch signaling between germ cells and somatic pregranulosa cells promotes follicle assembly and has non-cell autonomous roles on oocyte development including the regulation of meiosis and oocyte activation. (B) Notch signaling supports follicular growth and maturation by regulating granulosa cell differentiation, proliferation, and survival. Formation of zona pellucida in oocytes may impede productive Notch signaling between oocytes and granulosa cells; thus, within multi-laminar follicles, continued *trans*-activating interactions between Notch receptors and ligands likely occur between juxtaposed granulosa cells. The expression of Notch receptors and ligands in these granulosa cells therefore, must facilitate both bidirectional inductive signaling yet overcome *cis*-inhibition due to receptor–ligand co-expression. Functionally, continued Notch signaling in growing follicles promotes *Foxl2* expression, activin action, and has non-cell autonomous effects on oocyte growth and maturation. (C) Steroid production in luteal cells is modulated by Notch signaling through transcriptional regulation of enzymes involved in steroid biosynthesis. Bidirectional inductive signaling and *cis*-inhibitory mechanisms likely coordinate these processes. Notch signaling also functions to prevent luteolysis by regulating the expression of the anti-apoptotic factor BCL-xL and the pro-survival factor p-AKT. (D) Notch signaling, mediated predominately through the actions of *Notch1*, *3* and *4*, regulates bFGF-induced VEGF-mediated angiogenesis and the differentiation and activity of endothelial tips cells that express DLL4. These events modulate neovascularization and vascular density to support follicle growth and progesterone (P4) production. (A, B and C) The schematic representations of cells used in these figures were spatially separated for clarity.



Notch signaling in embryonic ovarian explants using the pharmacological  $\gamma$ -secretase inhibitor N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) results in the suppression of retinoic acid induced stimulation of *Stra8* expression and a reduction in downstream targets deleted in azoospermia-like (*Dazl*), disrupted meiotic cDNA protein 1 (*Dmc1*) and meiotic recombination protein 8 (*Rec8*) (Feng *et al.* 2014). Furthermore, siRNA knockdown of *Notch1* leads to markedly reduced expression of both *Stra8* and synaptonemal complex protein 3 (*Scp3*) (Fig. 3A). Reduction in *Stra8* expression following Notch inhibition is correlated with increased methylation of the *Stra8* proximal promoter region, suggesting that Notch signaling regulates the epigenetic status of the *Stra8* promoter. These changes cause delayed meiotic progression, defective oocyte growth and aberrant primordial follicle assembly resulting in the formation of multi-oocytic follicles within renal grafts of embryonic ovarian tissue exposed to  $\gamma$ -secretase inhibitors (Feng *et al.* 2014), consistent with a relationship between meiotic progression and follicle assembly.

Pharmacological inhibition of Notch signaling with the  $\gamma$ -secretase inhibitors DAPT and L-685,458 in neonatal ovaries using an *ex vivo* ovary culture system results in delayed syncytial breakdown (Trombly *et al.* 2009b, Chen *et al.* 2014), fewer primordial follicles (Trombly *et al.* 2009b, Chen *et al.* 2014), decreased granulosa cell proliferation (Terauchi *et al.* 2016), increased numbers of degenerative oocytes (Terauchi *et al.* 2016), and decreased expression of the germ cell markers newborn ovary homeobox protein (*Nobox*), factor in the germline alpha (*Figla*), LIM/homeobox protein 8 (*Lhx8*), and spermatogenesis and oogenesis specific basic helix-loop-helix 1 (*Sohlh2*) (Chen *et al.* 2014). Interestingly, the deletion of *Nobox* in mice causes markedly delayed syncytial breakdown, accelerated postnatal oocyte loss and decreased ovarian expression of *Jag1* (Fig. 3A) (Rajkovic *et al.* 2004), while *Figla* null mice display aberrant follicle assembly and postnatal oocyte loss due to the failure of pregranulosa cells to associate properly with germ cells (Soyal *et al.* 2000). The disruption of Notch signaling using DAPT is also associated with decreased recruitment of granulosa precursors and aberrant follicle assembly, which is similar to the phenotype observed with pharmacological inhibition or conditional knockout of the cell surface sheddase *Adam10* (Feng *et al.* 2016). In addition, RNAi-mediated knockdown of *Jag2* or *Notch1* also leads to disrupted follicle assembly in cultured ovarian explants (Guo *et al.* 2012).

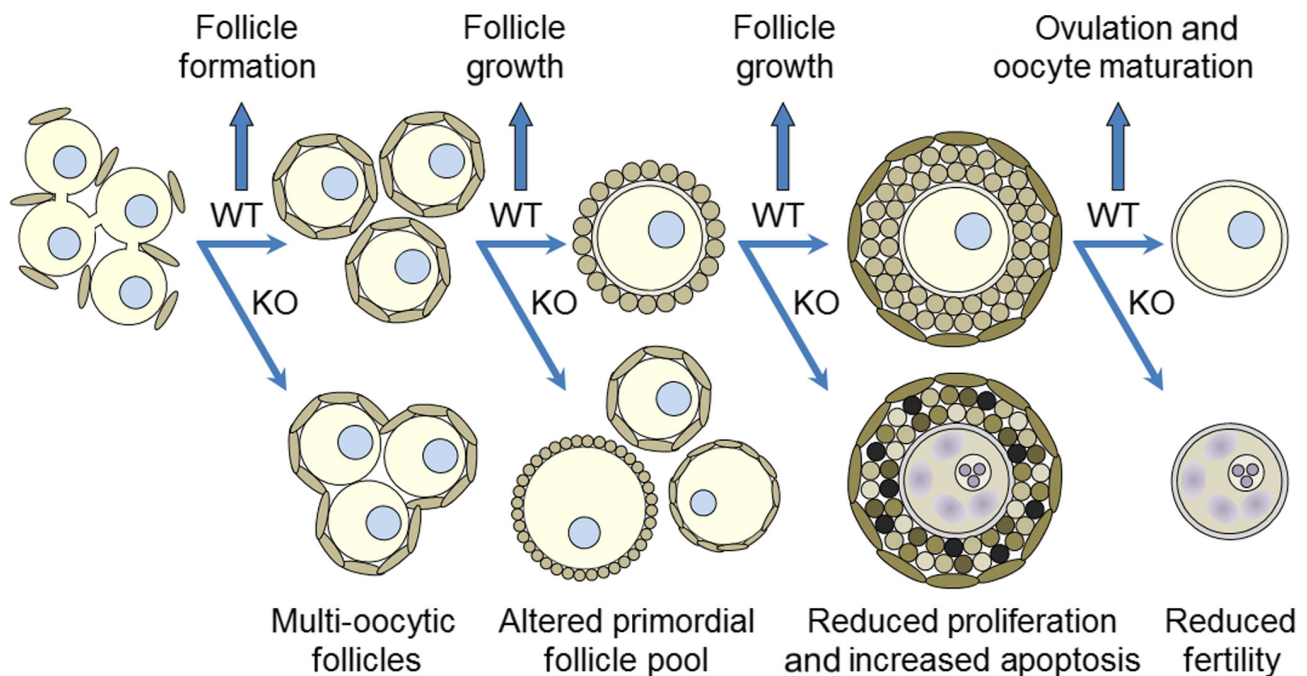
Multiple observations suggest that maternally-derived steroid hormones are important for the maintenance of germ cell syncytia in the ovaries of fetal rodents, and that the subsequent loss of exposure to these hormones at the time of birth leads to the initiation of follicle histogenesis. Additionally, treatment with

estrogens (Iguchi *et al.* 1990, Chen *et al.* 2007, 2009) or progestins (Iguchi *et al.* 2001, Nilsson *et al.* 2006) leads to an increased incidence of multi-oocytic follicles. Progesterone has also been shown to directly disrupt follicle histogenesis (Iguchi 1992, Kezele & Skinner 2003, Chen *et al.* 2007) and result in the suppression of *Jag2*, *Notch1* and *Hey2* expression in both *in vivo* and *in vitro* systems (Guo *et al.* 2012). Surprisingly, the treatment of neonatal ovaries with the progesterone antagonist RU486 also leads to the suppression of *Jag2*, *Notch1* and *Hey2* expression (Guo *et al.* 2012), suggesting that an appropriate steroid hormone environment is required for the accurate regulation of Notch components. Despite cross-talk between steroid hormone signaling and the Notch pathway, the formation of multi-oocytic follicles in models of altered steroid hormone action may have distinct etiologies from those resulting from disrupted Notch signaling. Unlike treatment with DES, exposure of neonatal ovaries to DAPT does not result in increased numbers of multi-oocytic follicles when examined histologically after renal grafting (Terauchi *et al.* 2016). This is in contrast to similar studies that did report the formation of multi-oocytic follicles following treatment with DAPT using embryonic ovaries (Feng *et al.* 2014). These findings indicate that alternative mechanisms or windows of sensitivity may exist in which DES exposure or Notch inhibition can cause perturbations to follicle assembly.

### Genetic models of Notch pathway disruption in the ovary

Given that *Notch1* and *Notch4* expression is largely restricted to the ovarian vasculature (Table 1), and that mice with global deletion of *Notch3* (Krebs *et al.* 2003), *Notch4* (Krebs *et al.* 2000) or both *Notch3* and *Notch4* (Xu 2011) have no overt reproductive phenotype, the expression of *Notch2* in granulosa cells has the greatest potential to mediate the actions of Notch signaling during follicle assembly and growth. Moreover, JAG1 has been shown to productively interact with NOTCH2 (Shimizu *et al.* 1999, 2000a,b); thus, it is hypothesized that JAG1, and possibly JAG2, expressed in germ cells of perinatal ovaries signals through NOTCH2 expressed in granulosa cells to facilitate germ and somatic cell interactions that are important for follicle formation and development. However, importantly, since global deletion of either *Jag1* or *Notch2* results in embryonic lethality (Xue *et al.* 1999, McCright *et al.* 2006), tissue-specific approaches are required to investigate the role of these genes within the developing ovary.

Conditional deletion of the Notch ligand, *Jag1*, in germ cells (J1KO) (Vanorny *et al.* 2014) or the Notch receptor, *Notch2*, in somatic pregranulosa cells (N2KO) (Xu & Gridley 2013, Vanorny *et al.* 2014) produces a phenotype characterized by decreased numbers of



**Figure 4** Summary of observed phenotypes in Notch pathway knockout mice during ovarian follicle development. Disruption of Notch signaling between oocytes and somatic pregranulosa cells, as observed in J1KO and N2KO mice, leads to perturbations in follicle assembly characterized by the presence of multi-oocytic follicles, which represent a failure to completely resolve germ cell syncytia. Loss of Notch signaling also leads to changes in follicle dynamics that adversely impact the primordial follicle pool and development of antral follicles, which is accompanied by decreased proliferation and increased apoptosis of granulosa cells. As a consequence of these defects, female Notch knockout mice are subfertile. These mouse models demonstrate important functions for Notch signaling in the resolution of germ cell syncytia, coordination of somatic and germ cell growth within follicles, and oocyte maturation.

primordial follicles, persistence of germ cell syncytia at the ovarian cortex, uncoordinated follicle growth and aberrant folliculogenesis (Fig. 4). The most striking feature of these genetic models is the presence of numerous multi-oocytic follicles, which is evidence of significantly disturbed ovigerous cord fragmentation or syncytial breakdown. Interestingly, in the *Drosophila* ovariole, disruption of the ligand *Delta* in the germ line, the receptor *Notch* in follicle cells or the *Lfng* homolog, *Fringe* in somatic polar cells, results in the production of fused and enlarged egg chambers containing multiple oocytes (Grammont & Irvine 2001, Torres et al. 2003), suggesting functional conservation of Notch signaling across metazoan ovarian development.

In postnatal day 1 (PND1) N2KO ovaries, decreased germ and pregranulosa cell apoptosis is observed (Xu & Gridley 2013), possibly due to delayed syncytial breakdown, which is a process that normally results in massive germ cell loss. In contrast, at PND19, there is a significant increase in somatic cell apoptosis and a decrease in granulosa cell proliferation within growing follicles of both J1KO and N2KO ovaries (Fig. 4) (Vanorny et al. 2014). Both knockout models also contain follicles in which there is oocyte expansion in the absence of granulosa cell growth, which is consistent with a role for Notch signaling in somatic cell proliferation and maturation. N2KO ovaries also contain

increased numbers of atretic follicles and follicles that become cystic, hemorrhagic and contain trapped oocytes (Xu & Gridley 2013), similar to mouse genetic models of disturbed estrogen (Couse & Korach 1999, Britt et al. 2000), activin (McMullen et al. 2001, Bristol-Gould et al. 2005, Pangas et al. 2007) and gonadotropin signaling (Risma et al. 1995, 1997, Kumar et al. 1999, Keri et al. 2000, Matzuk et al. 2003, Meehan et al. 2005). In addition, both conditional knockout models contain reduced numbers of antral follicles, while J1KO ovaries also contain fewer corpora lutea, consistent with the reduction in ovarian follicle growth and ovulation capacity (Vanorny et al. 2014).

Interestingly, the transcript levels of *Notch2* are elevated in J1KO ovaries, and conversely, the levels of *Jag1* are elevated in N2KO ovaries, signifying a mechanism of compensatory expression between *Jag1* and *Notch2* (Vanorny et al. 2014). In addition, the expression of *Jag2* and *Notch1* are increased in J1KO ovaries. These data indicate that the effects of *Jag1* or *Notch2* deletion might be masked through compensation or functional complementation by the expression of other Notch ligands or receptors. Transcript levels of growth differentiation factor 9 (*Gdf9*), zona pellucida glycoprotein 3 (*Zp3*) and *Figla* are elevated in both J1KO and N2KO ovaries, consistent with the observation of follicles with uncoordinated growth between the oocyte



and somatic layer (Fig. 3B). Additionally, within J1KO ovaries, the expression of inhibin beta A (*Inhba*) and B (*Inhbb*) is decreased, consistent with the phenotype of altered granulosa cell proliferation and decreased ovarian reserve respectively (Fig. 3B).

Histological examination of ovaries in which *Jag1* and *Notch2* are both deleted in the germ and somatic cell compartments reveals that the effect of combined deletion of *Jag1* and *Notch2* produces an ovarian phenotype similar to the conditional deletion of either gene individually (Vanorny *et al.* 2014). This finding is consistent with a model in which *Jag1* in germ cells directly signals through *Notch2* in granulosa cells. As a consequence of the multiple defects resulting from disrupted Notch signaling between germ cells and granulosa cells, both J1KO (Vanorny *et al.* 2014) and N2KO (Xu & Gridley 2013) models display reduced fertility, as measured by a reduction in litter size and total progeny, and some female J1KO and N2KO mice appear to undergo premature reproductive senescence (Vanorny *et al.* 2014). Furthermore, preliminary studies suggest that the impaired fertility observed in J1KO females may result from a combination of reduced oocyte quality and a trend toward reduced ovulatory capacity, whereas N2KO females have reduced oocyte quality, but preserved ovulatory potential (Vanorny 2016).

Constitutive expression of *Notch1* intracellular domain (*NICD1*) through induction with *Amhr2-Cre* leads to multiple abnormalities within the female reproductive tract of mice (Manosalva *et al.* 2013, Ferguson *et al.* 2016). Importantly, *NICD1* has been shown to fully complement the actions of the *Notch2* intracellular domain (*NICD2*) (Liu *et al.* 2015); thus, the overexpression of *NICD1* is functionally equivalent to constitutive activation of *Notch2*. The predominant ovarian phenotype displayed in these animals includes the presence of hemorrhagic blood vessels and cystic lesions (Ferguson *et al.* 2016), and an increased number of mature oocytes and a decreased number of pregranulosa cells (Manosalva *et al.* 2013). Importantly, these mice have significant extra-ovarian pathologies consisting of aberrant coiling and canalization of the oviducts, and the presence of hemorrhagic blood vessels and cystic lesions in the uteri and oviducts. These features, which are fully penetrant in older females (Ferguson *et al.* 2016), are similar to the phenotypes observed in mouse models with disruption of Wnt family members 4 (*Wnt4*) and 7a (*Wnt7a*),  $\beta$ -catenin and *Dicer* (Vainio *et al.* 1999, Nagaraja *et al.* 2008, Hernandez Gifford *et al.* 2009). Although gain-of-function *NICD1* female mice are subfertile (Manosalva *et al.* 2013, Ferguson *et al.* 2016), follicular development appears to be largely normal in these mice (Ferguson *et al.* 2016), suggesting that the altered fertility observed in these animals is possibly extra-ovarian in origin.

Ovaries from traditional or conditional *Hes1* knockout mice contain germ cells with decreased cellular

diameters and fewer total germ cells, similar to those treated with DAPT, due to increased apoptosis resulting from the failure to suppress the pro-apoptotic gene *Inhbb* (Manosalva *et al.* 2013). In addition, *Hes1* knockout ovaries contain germ cells with reduced markers of oocyte maturity, as measured by the expression of *Kit*, which has been shown to promote primordial follicle activation and survival of late-stage follicles (Hutt *et al.* 2006). Interestingly, despite the presence of fewer germ cells, there are increased numbers of pregranulosa cells in *Hes1* knockouts, which is correlated with increased levels of forkhead box protein L2 (*Foxl2*) and follistatin (*Fst*) expression (Manosalva *et al.* 2013). Notably, in either *Hes1* conditional or global knockouts, female mice are subfertile; thus, suppression of the Notch effector gene within granulosa cells leads to impaired follicular function that impacts female fertility.

Disruption of the fucose-specific glycosyltransferase *Lfng* leads to complete infertility in a large subset of female mice that survive into adulthood (Hahn *et al.* 2005), although some survivors can have productive matings (Xu *et al.* 2006). Mutation of *Lfng* is also associated with abnormal folliculogenesis characterized by the presence of multi-oocytic follicles and follicles with disorganized thecal layers. Unexpectedly, given the poor fertility of *Lfng* knockouts, follicles of all developmental stages are present within mutant ovaries at roughly equivalent numbers and percentages in comparison to controls (Hahn *et al.* 2005). Additionally, corpora lutea are observed in *Lfng* null ovaries, indicating that mutant mice are capable of cycling and ovulating naturally; however, many of the corpora lutea contain trapped oocytes indicative of defective ovulation. Interestingly, despite altered folliculogenesis and diminished fertility, no difference is observed in the number of oocytes ovulated following gonadotropin stimulation. Analysis of ovulated oocytes however demonstrates significantly impaired meiotic maturation resulting in the failure of oocytes to complete meiosis due to aberrant meiotic spindle assembly (Hahn *et al.* 2005). Notably, ovarian expression of both *Jag1* and *Notch2* is reduced in *Lfng* null mice, and mutant ovaries share many of the phenotypic features observed in J1KO and N2KO ovaries.

### Regulation of Notch pathway expression and action during follicle development

Suppression of Notch signaling in *ex vivo* cultured ovarian explants using the  $\gamma$ -secretase inhibitors DAPT or L-685,458 results in the presence of fewer primordial follicles and delayed follicle recruitment, culminating in fewer growing and total follicles (Wang *et al.* 2014). This reduction in total follicle numbers is associated with decreased granulosa cell proliferation, increased germ cell apoptosis and decreased expression of

germ cell-specific transcription factors. Interestingly, Notch inhibition appears to be coupled with increased expression of PTEN, suggesting that suppression of Notch signaling in pregranulosa cells may disrupt follicle development by preventing PI3K/AKT mediated germ cell activation (Fig. 3A), consistent with studies demonstrating a role of pregranulosa cells in primordial follicle activation (Zhang & Liu 2015).

Secondary ovarian follicles cultured in a Notch suppressive environment with the  $\gamma$ -secretase inhibitors DAPT and L-685,458 have been shown to display arrested growth, exhibit granulosa cell detachment and contain degenerative oocytes (Zhang *et al.* 2011). Furthermore,  $\gamma$ -secretase inhibitor treatment leads to a reduction in granulosa cell proliferation that is concomitant with decreased *c-Myc* expression. Surprisingly, gain-of-function experiments using lentiviral-mediated expression of *NICD2* within cultured granulosa cells show a restoration of *c-Myc* expression and rescue of granulosa cell proliferation in the presence of DAPT (Zhang *et al.* 2011). This demonstrates that the Notch effector *c-Myc* plays a critical role in the maintenance and function of granulosa cells.

Ovarian follicles require the formation of gap junctions through the expression of gap junction protein alpha 1 (*Gja1*) in granulosa cells and gap junction protein alpha 4 (*Gja4*) in oocytes to prevent premature meiotic resumption (Simon *et al.* 1997, Juneja *et al.* 1999). Pharmacological disruption of gap junction complexes in cultured ovarian explants leads to a marked decrease in the expression of *Notch2* in pregranulosa cells resulting in reduced somatic cell proliferation (Teng *et al.* 2016). In addition, germ cells remain in syncytial structures and fail to appropriately assemble into follicular units. Inhibition of Notch signaling using DAPT results in a reciprocal decrease in the expression of multiple gap junction proteins and the key granulosa cell transcription factor *Foxl2*, supporting an important and complementary relationship between productive Notch signaling and gap junction formation that is required for primordial follicle formation and maintenance (Fig. 3A).

The small GTPase, *Rac1*, has been shown to play an indispensable role in primordial follicle formation by controlling nuclear import of STAT3 in germ cells, which facilitates the transcription of the Notch ligand, *Jag1*, and the expression of *Gdf9*, bone morphogenetic protein 15 (*Bmp15*) and *Nobox* (Fig. 3A) (Zhao *et al.* 2016). Increased expression of the oocyte-specific growth factors leads to increased translation of NOTCH2 in granulosa cells, via mammalian target of rapamycin complex 1 (mTORC1) modulation (Fig. 3A), which is subsequently activated by juxtaposed germ cells expressing JAG1. Similar to models of suppressed Notch signaling, disruption of *Rac1* action through pharmacological inhibition or siRNA knockdown leads to delayed syncytial breakdown, reduced numbers of

primordial follicles and increased numbers of multi-oocytic follicles. Conversely, overexpression of *Jag1* in oocytes rescues *Rac1* inhibition and leads to accelerated syncytial breakdown and follicle assembly through the activation of *Notch2* in juxtaposed pregranulosa cells.

The tyrosine kinase receptor tropomyosin-related kinase receptor B (*TrkB*), which supports the actions of neurotrophin 4/5 (*Nt-4*) and brain-derived neurotrophic factor (*Bdnf*) during folliculogenesis, has also been shown to impact Notch signaling within the developing ovary (Dorfman *et al.* 2011). Similar to *Rac1*, *TrkB* expression within the ovary is specific to germ cells where it is suspected of being a direct regulator of *Jag1* transcription (Fig. 3A). In addition to *Jag1*, the expression of *Hes1* and *Hey2* are reduced in *TrkB* null ovaries, suggesting that the inability to express *Jag1* within the oocyte leads to suppressed Notch signaling within adjacent granulosa cells expressing *Notch2*. The deletion of *TrkB* also leads to a reduction in granulosa cell proliferation, delayed follicular growth and reduced ovarian follicle survival, consistent with findings from other reports of disrupted Notch signaling within the ovary and studies using blocking antibodies against BDNF and NT-4 (Spears *et al.* 2003). Interestingly, lentiviral-mediated expression of *Jag1* targeted to germ cells of *TrkB* null ovaries restores Notch activation and rescues the expression of the downstream Notch effector *c-Myc* (Fig. 3B). Notably, although lacking in *TrkB* null ovaries, mice with disruption of the neurotrophin nerve growth factor (*Ngf*) exhibit multi-oocytic follicles in addition to reduced numbers of primary and secondary follicles (Disson *et al.* 2001).

Once formed, primordial follicles remain quiescent until select cohorts are recruited each reproductive cycle when they undergo a transition from primordial to primary follicles. This process, termed follicle activation, results in morphological and physiological changes in both the oocyte and somatic cells. Oocytes within activated follicles begin to grow, while squamous pregranulosa cells transition into cuboidal granulosa cells, become proliferative, and later steroidogenic and responsive to pituitary gonadotropins. Within this growing pool of follicles, components of the Notch pathway are dynamically expressed (Table 1) (Johnson *et al.* 2001, Hahn *et al.* 2005, Trombly *et al.* 2009a, Vanorny *et al.* 2014). The ligand *Jag2* and the receptors *Notch2* and *Notch3* are localized to granulosa cells, the ligand *Jag1* is found in oocytes, and the target genes *Hes1* and *Hey2* are expressed in granulosa cells. Interestingly, *Hes1* is localized to both somatic and germ cells of embryonic and postnatal ovaries (Trombly *et al.* 2009b, Zhang *et al.* 2011, Manosalva *et al.* 2013), suggesting an important bidirectional role for the Notch pathway during folliculogenesis. However, the deletion of *Rbp-j* in oocytes under the direction of *TNAP-Cre* does not have any impact on follicle development or fertility (Manosalva *et al.* 2013), confirming that canonical

Notch activity within the oocyte is dispensable for normal ovarian function and that the actions of Notch signaling are mediated through its function in somatic cell types.

Within a developing follicle, physical contact between the inner layer of granulosa cells and oocyte is maintained via the formation of transzonal projections (TZPs), which are thin cellular extensions that emanate from granulosa cells and penetrate the zona pellucida (Li & Albertini 2013). However, as granulosa cells proliferate to form multi-laminar follicles, they consequently lose direct contact with the oocyte, which may preclude continued juxtacrine signaling between the two cell types. Accordingly, continued Notch activation within the granulosa cells of developing follicles requires the interactions of juxtaposed granulosa cells. Examination of a Notch fluorescent reporter demonstrates continued, though variable, Notch activity within granulosa cells of secondary follicles (Fig. 2C) and within both mural and cumulus granulosa cells of antral follicles (Fig. 2D) that persists through ovulation and luteinization (Vanorny *et al.* 2014, Vanorny 2016). It is interesting to consider whether a change in the source of Notch ligand during this transition may play a role in follicle activation and in the new cellular processes acquired by granulosa cells.

Change in the source of ligand used for continued Notch activation following follicle recruitment also requires the conversion from *Jag1*, in oocytes, to *Jag2*, in granulosa cells. Although the specific role of *Jag2* in the ovary has not been determined, a naturally occurring hypomorphic allele for *Jag2* reportedly has poor female fertility (Gruneberg 1956), signifying that this ligand may be critical for ovarian function. Additionally, the expression pattern of *Jag2* resembles that of the Notch ligand *Delta*, which is expressed in the germline cells and later in somatic follicle cells of *Drosophila* ovarioles (Lopez-Schier & St Johnston 2001, Torres *et al.* 2003), again suggesting an evolutionarily conserved role for Notch signaling in metazoan follicle development.

### Notch signaling during luteinization

Following stimulation by pituitary gonadotropins, granulosa and thecal cells that remain within follicles that ovulate undergo luteinization to form corpora lutea, which provide a source of steroid hormones throughout pregnancy. The Notch ligands *Jag1*, *Dll1* and *Dll4* and the Notch receptors *Notch1–4* have all been shown to be expressed within small and large luteal cells (Table 1) (Johnson *et al.* 2001, Vorontchikhina *et al.* 2005, Hernandez *et al.* 2011, Murta *et al.* 2014, Accialini *et al.* 2015, Wang *et al.* 2015), consistent with a role for Notch signaling in their function. Following *in vivo* treatment with prostaglandin F2 alpha (PGF2a), a uterine-derived factor that initiates luteolysis in rodents, transcripts for

*Notch1*, *Notch4* and *Dll4* are reduced, as are cleaved forms of the receptors (Fig. 3C) (Hernandez *et al.* 2011). Additionally, the suppression of Notch signaling through intrabursal injection with DAPT leads to decreased progesterone production and increased luteal cell apoptosis. These findings suggest that the luteolytic effects of PGF2a may be in part an effect of decreased Notch activation, which is consistent with a luteotropic role for Notch signaling.

Treatment of cultured mouse luteal cells with DAPT or L-658,458 results in a reduction in both basal and hCG-induced progesterone production (Wang *et al.* 2015). In addition, the inhibitory effects on progesterone levels observed in luteal cells treated with DAPT have been shown to arise from the suppression of cytochrome P450 family 11 subfamily A member 1 (*Cyp11a1*) expression. In contrast, overexpression of the intracellular domain of *Notch3* (*NICD3*) causes an increase in both basal and hCG-induced progesterone secretion through the regulation of *Cyp11a1* and steroidogenic acute regulatory protein (*StAR*), which controls the rate-limiting step in steroid biosynthesis (Fig. 3C). These results indicate that the activation of Notch signaling and stimulation with hCG have synergistic effects on progesterone production in luteal cells. Interestingly, overexpression of *NICD3* has no observable effect on estrogen levels despite the regulation of upstream enzymes in the steroid synthesis pathway.

Mechanically isolated rat corpora lutea cultured in the presence of DAPT also have decreased levels of progesterone production as a consequence of the reduced expression of *Cyp11a1* (Accialini *et al.* 2015). Furthermore, suppression of Notch signaling is able to reverse progesterone-induced expression of *StAR*. Consistent with *in vivo* studies, cultured corpora lutea treated with DAPT have increased markers of apoptosis, as a consequence of decreased levels of the anti-apoptotic protein BCL-xL and the pro-survival factor phosphorylated AKT (Fig. 3C). Moreover, suppression of Notch activation is able to reverse the survival effects that progesterone has on luteal cells. Similar to treatments with DAPT, corpora lutea incubated with an inactivating DLL4 antibody have decreased progesterone production. These findings support a novel role for the Notch pathway in the regulation of luteal function in response to progesterone stimulation, and indicate an important relationship between the actions of progesterone and Notch signaling.

In contrast to the findings above, transfection of a gonadotropin-responsive mouse cell line with *NICD1* or *NICD2* leads to a decrease in progesterone production, implying that Notch activation normally functions to suppress steroidogenesis (George *et al.* 2015). In addition, the expression of *StAR*, *Cyp19a1* and *HSD3b1* are significantly upregulated following pharmacological suppression of Notch signaling in cultured preantral follicles and downregulated with transient transfection



of *NICD1–3*. Furthermore, the activation of the Notch pathway represses the ability of GATA4 to induce the expression of these genes through Notch target gene expression and promoter binding, rather than through dimerization of Notch target genes with GATA4 (Fig. 3C) (George *et al.* 2015). These findings, together with studies employing DLL4 and  $\gamma$ -secretase inhibitors discussed above, indicate that alteration in the levels of basal Notch activity negatively impact progesterone production in steroidogenically active cells, suggesting complex regulatory effects of steroid hormone production by Notch signaling.

### Notch pathway in the ovarian vasculature

The Notch pathway has previously been shown to be critical for multiple aspects of vasculogenesis and angiogenesis including arteriovenous specification, endothelial and vascular smooth muscle cell differentiation, and the regulation of vessel sprouting and branching (Fig. 3D and Table 1) (Krebs *et al.* 2000, Gridley 2007, 2010). Several Notch components are expressed within the ovarian vasculature including *Dll4* located at the tip of endothelial cells within the thecal layer (Jovanovic *et al.* 2013) and growing luteal vessels (Garcia-Pascual *et al.* 2013); *Notch3* within endothelial cells and vascular smooth muscle cells (Jovanovic *et al.* 2013); and *Notch1*, *Notch4* and *Jag1* within thecal layer endothelial and vascular smooth muscle cells (Vorontchikhina *et al.* 2005, Jovanovic *et al.* 2013).

The Notch ligand, *Dll4*, is primarily localized to vascular endothelial cells where it acts to regulate the differentiation and activity of tips cells, which are located at the growing edge of the ovarian neovasculature. The treatment of marmosets during the periovulatory period with a neutralizing DLL4 antibody demonstrates that *Dll4* acts as a negative regulator of vascular endothelial growth factor (VEGF)-mediated sprouting and branching within the ovarian vasculature (Fig. 3D) (Fraser *et al.* 2012). In addition, the inhibition of *Dll4* induces luteal hypervascularization, including increased angiogenesis and vascular density, which is associated with a decrease in progesterone production. Furthermore, primate ovaries in which *Dll4* is perturbed are reduced in size and weight, and corpora lutea undergo enhanced degeneration due to increased cellular apoptosis. Interestingly, the inhibition of *Dll4* during the mid-luteal phase has only a minimal impact on progesterone production and does not affect follicular development.

In immature female mice treated with PMSG and hCG, to stimulate follicle development, injection of an inhibitory DLL4 antibody promotes promiscuous expression of *Dll4* leading to increased, but non-functional, vascularization that is associated with decreased progesterone production (Fig. 3D) (Garcia-Pascual *et al.* 2013). In contrast, suppression of Notch

signaling using a pan-Notch inhibitor, compound E ((s,s)-[2-(3,5-Difluorophenyl)-acetylamino]-N-(1-methyl-2-oxo-5-phenyl-2,3-dihydro-1H-benzo[e][1,4]diazepin-3-yl)-propionamide), causes a reduction in ovarian weight and estrogen production as a result of uninhibited, but highly disorganized, vascular proliferation that culminates in blocked follicle development at the preovulatory stage (Jovanovic *et al.* 2013). Thus, while the blockade of Notch signaling using a DLL4 inhibitory antibody has an effect on the organization of the follicular vasculature, it appears to have little or no impact on gonadotropin-dependent folliculogenesis, suggesting the involvement of other Notch ligands in this process. These studies demonstrate a specific role for *Dll4* in suppressing vascular overgrowth and in organizing ovarian neovasculature during luteal formation.

The role of Notch signaling within the ovarian vasculature of mice has also been examined using a gain-of-function genetic model (Liu *et al.* 2014). Constitutive activation of the Notch pathway in vascular endothelial cells by the induction of *NICD1* expression under the control of a tamoxifen-inducible *Tie2-Cre* allele results in severely altered female fertility, and ovaries from these mice lack mature ovarian follicles. The specific mechanism behind the interrupted development of advanced follicles is attributed to the inhibition of bFGF-induced angiogenesis (Fig. 3D), supporting a critical role for Notch signaling in vascular development during ovarian follicle maturation.

### Role of Notch signaling in the human ovary

Transcriptional profiling of human preantral follicles demonstrates that Notch components are expressed and dynamically regulated during follicle growth in humans (Kristensen *et al.* 2015). Abundantly expressed Notch pathway transcripts in human preantral follicles include *Jag1*, *Hes1* and *Hey2*, whereas low to moderately expressed transcripts include *Notch1*, *Notch2*, *Notch3*, *Jag2* and *Hes5*. Notch signaling components with no discernable expression in human preantral follicles include *Notch4*, *Dll1*, *Dll3*, *Dll4* and *Hes7*. Additionally, the expression of the Notch target gene *Hey1* is significantly reduced with increasing preantral follicle size. In human cumulus granulosa cells obtained by follicle aspiration during IVF, all four Notch receptors and the Notch ligands JAG1 and JAG2 are expressed (Tanriverdi *et al.* 2013). When comparing poor and normal responders to IVF treatments, there is a significant reduction in the expression of NOTCH2 in the cumulus cells of the poor responders and the expression of both NOTCH2 and NOTCH3 are positively correlated with IVF response.

Polycystic ovarian syndrome (PCOS) is the most common endocrine disorder in females of reproductive

age and is characterized by hyperandrogenism, insulin resistance, chronic anovulation and infertility (Fauser *et al.* 2012). Ovarian follicles in PCOS patients frequently exhibit arrest of follicle growth at the antral stage leading to the appearance of ovaries containing multiple cysts on diagnostic imaging studies. In addition, there is a reduction in granulosa cell proliferation, hyperplasia of the theca interna, and disrupted ovulation. Profiling of miRNA transcripts in women with PCOS demonstrates that *Notch3* is a target of miRNAs that are differentially expressed in the cumulus cells of these patients (Xu *et al.* 2015). The miRNA *hsa-miR-483-5p* has been specifically shown to regulate the expression of both *Notch3* and mitogen-activated protein kinase (*Mapk*), suggesting that women with PCOS may have alterations in granulosa cell proliferation and function in part through decreased Notch action.

Each year more than 20,000 women in the United States are diagnosed with, and approximately 14,000 die from, ovarian cancer, making it the leading cause of death from gynecologic malignancy (Group 2014). The overall five-year survival rate for ovarian cancer is approximately 31% (Jemal *et al.* 2009), and this high rate of mortality can be largely explained by the frequent occurrence of patients with an advanced-stage of disease at the time of diagnosis (Koonings *et al.* 1989). Notably, genomic profiling has revealed that as many as 22% of high-grade serous ovarian carcinomas contain mutations in components of the Notch pathway with more than 50% of those cases involving *Notch3* (Cancer Genome Atlas Research Network 2011), which is highly associated with poor clinical outcomes (Hu *et al.* 2014). In addition, the Notch ligands *Jag1* (Choi *et al.* 2008, Cancer Genome Atlas Research Network 2011) and *Jag2* (Euer *et al.* 2005, Cancer Genome Atlas Research Network 2011) are also commonly amplified in these cancers, resulting in enhanced Notch signaling, likely through a *Notch3* juxtacrine loop, which supports ovarian tumor growth and cellular adhesion (Choi *et al.* 2008). Though *Notch3* is the receptor most frequently involved in ovarian carcinogenesis, the activity of *Notch1* has also been shown to be elevated in a subset of these cancers (Rose *et al.* 2010).

Alterations in the expression of Notch pathway genes has also been correlated with ovarian cancers that develop resistance to treatment (Groeneweg *et al.* 2014); however, it is unclear by what means aberrant Notch signaling may facilitate the survival of cancer cells. Difficulty in treating ovarian tumors has at least in part been attributed to the formation or maintenance of cancer stem cells (Jordan *et al.* 2006, Shah *et al.* 2013, Walters Haygood *et al.* 2014). Stem cell factors such as *Nanog* and *Oct4* have been shown to be upregulated by the overexpression of *NICD3* in cells of the ovarian surface epithelium, suggesting a potential role in supporting an undifferentiated cellular state (Park *et al.* 2010). In addition, aberrant Notch signaling may cause

the loss of an epithelial phenotype through an epithelial to mesenchymal transition; thus, allowing tumor cells to acquire a stem cell-like phenotype and resistance to certain therapies (Espinoza & Miele 2013).

Notch signaling has also been shown to play important roles in tumor angiogenesis of other cancers (Li *et al.* 2007, Benedito *et al.* 2009), though only a few studies have specifically examined an angiogenic role in ovarian cancer. In one report, *Jag1* was shown to be upregulated in ovarian tumor epithelial cells, and disruption of the gene led to a reduction in blood vessel formation and endothelial migration (Lu *et al.* 2007). In a second study (Hu *et al.* 2011), *Dll4* was found to be upregulated in more than half of the ovarian cancer samples that were analyzed, and increased levels of *Dll4* expression were correlated with overall poor survival. New treatments designed to target the Notch pathway will hopefully provide additional means to counter the various mechanisms by which altered Notch signaling promotes the establishment and maintenance of tumors, especially those that are resistant to current therapeutic options (Rose 2009, Domingo-Domenech *et al.* 2012, Sahebjam *et al.* 2013, Groeneweg *et al.* 2014).

## Conclusions

The Notch pathway supports juxtacrine signaling between families of membrane-bound ligands and receptors, and plays a fundamental role in numerous cellular processes during metazoan development. Notch signaling is active in the embryonic mouse ovary and is strongly upregulated at birth in somatic cells that surround germ cell syncytia, coincident with the resolution of these syncytia and establishment of the primordial follicle pool. Communication between juxtaposed germ cells and somatic support cells of the ovary is critical for the formation of follicles and for the establishment of the follicular niche in which the oocyte will develop through ovulation. In addition, the Notch pathway promotes the growth and maturation of ovarian follicles through interactions with juxtaposed follicular cells. Cellular interactions between other populations of cell types within the developing ovary also facilitate productive Notch signaling that is critical for ovarian processes including luteinization and vascular development, which are central to the production and delivery of critical secreted hormonal factors that support pregnancy and female health.

The phenotypes observed in models of disrupted Notch signaling are consistent with the broad functions ascribed to Notch signaling in the regulation of cellular processes that include proliferation, cell death or survival, adhesion and migration, and differentiation and cell-fate specification. These diverse roles for Notch signaling are highly context-dependent and consistent with the concept that the ovary and its functional unit,

the follicle, represent a highly complex and dynamic continuum of developmental stages. To address the pathophysiology underlying the considerable histological derangements observed in ovaries with disrupted Notch signaling, additional studies are needed to identify new genes and interactions with other signaling pathways involved in the downstream regulatory actions of the Notch pathway. Furthermore, new strategies to culture and visualize ovarian tissues should help to elucidate the dynamic cellular behaviors and relationships involved in the coordination of ovarian and follicle development.

This review and emerging evidence indicates that the Notch pathway is critical for the establishment of a microenvironment where the female germ cell develops and has distinct regulatory roles throughout the development of the ovarian follicle to support female reproductive function. Understanding the juxtacrine signaling mechanisms by which ovarian follicles serve as a critical environmental niche for the maturation of female germ cells is vital for addressing human infertility and for establishing new reproductive technologies to improve oocyte quality and preserve reproductive function and fertility. Furthermore, examination of the interplay between multiple signaling modalities within the ovary and reproductive axis should allow for a greater recognition of essential signaling and regulatory networks for the development of new therapeutic paradigms for the improvement of women's health.

### Declaration of interest

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

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