JAGGED-NOTCH SIGNALING BETWEEN OOCYTE AND GRANULOSA CELLS IS ACTIVE DURING FOLLICULAR GROWTH

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Abstract

Oocyte and follicular development depend on bi-directional communication between the oocyte and neighbouring somatic granulosa cells. The specific pathways that mediate this crucial communication are, however, only partly understood. The Jagged-Notch pathway is a highly conserved signaling pathway, where membrane-bound Jagged binds to membrane-bound Notch, triggering cleavage of Notch, releasing its intracellular domain, which migrates to the cytoplasm and ultimately to the nucleus. Previous studies have shown that Jagged-Notch signaling is required in early stages of ovarian development, namely for the formation of primordial follicles. Furthermore, being a cell-contact dependent pathway, Notch signaling in the mammalian ovary occurs between two cell types, the oocyte and granulosa cells. This raises our hypothesis that Jagged-Notch signaling pathway is one of the signaling pathways that mediates oocyte-granulosa cell communication. RT-PCR confirmed that Jagged1 mRNA is expressed at all stages of oocyte growth. Furthermore, using an antibody specific for cleaved Notch2, immunofluorescence of granulosa cell-oocyte complexes (GOCs) isolated at mid-follicular growth revealed strong cytoplasmic staining in the granulosa cells. Unexpectedly, anti-Jagged1 immunoblotting of oocytes revealed a band at ~20 kDa instead of the predicted 150 kDa. To identify the origin of the ~20kDa band, oocytes were incubated either as intact GOCs or in the absence of granulosa cells. Whereas the ~20 kDa band was retained by oocytes incubated as GOCs, the 150 kDa band appeared in oocytes incubated without granulosa cells. This suggests that activation of Jagged-Notch signaling in the ovarian follicle leads not only to cleavage of the Notch receptor in the granulosa cells, but also to cleavage of the Jagged ligand in the oocyte. The sole site of physical contact between the oocyte and granulosa cells is at the tips of specialized filopodia, termed transzonal projections (TZPs), that extend from the granulosa cells to the oocyte. To test whether

the ~20 kDa band depended on intercellular contact, we incubated cumulus-cell oocyte complexes (COCs) with epidermal growth factor (EGF), which triggers retraction of TZPs and thus loss of granulosa cell-oocyte contact. Following an 8-hour incubation in the presence of EGF, the 150 kDa Jagged1 species became detectable. These results suggest that activation of Jagged-Notch signaling in the ovarian follicle is dependent on the presence of granulosa cell contact, leading to cleavage of the Jagged ligand in the oocyte. Future work will address the potential role of the Jagged cleavage product. Understanding cell communication pathways such as Notch signaling could help us better understand the mechanisms involved in the healthy development of the ovarian follicle.

Résumé

Le développement ovocytaire et folliculaire dépend de la communication bidirectionnelle entre l'ovocyte et les cellules granuleuses somatiques voisines. Les voies spécifiques qui assurent la médiation de cette communication cruciale ne sont cependant que partiellement comprises. La voie Jagged-Notch est une voie de signalisation hautement conservée, où Jagged lié à la membrane se lie à Notch lié à la membrane, déclenchant le clivage de Notch, libérant son domaine intracellulaire, qui migre vers le cytoplasme et, finalement, vers le noyau. Des études antérieures ont montré que la signalisation Jagged-Notch est nécessaire aux premiers stades du développement ovarien, notamment pour la formation des follicules primordiaux. De plus, étant une voie dépendante du contact cellulaire, la signalisation Notch dans l'ovaire de mammifère se produit entre deux types de cellules, les cellules ovocytaires et granuleuses. Cela soulève notre hypothèse selon laquelle la voie de signalisation Jagged-Notch est l'une des voies de signalisation qui intervient dans la communication des cellules ovocytes-granulosa. La RT-PCR a confirmé que l'ARNm de Jagged1 est exprimé à tous les stades de la croissance des ovocytes. À l'inverse, en utilisant un anticorps spécifique de Notch2 clivé, l'immunofluorescence des complexes cellules-ovocytes de la granulosa (GOC) isolés à la croissance folliculaire moyenne et tardive a révélé une forte coloration cytoplasmique dans les cellules de la granulosa. De manière inattendue, l'immunoempreinte anti-Jagged1 des ovocytes a révélé une bande à ~ 20 kDa au lieu des 150 kDa prévus. Pour identifier l'origine de la bande ~ 20 kDa, les ovocytes ont été incubés sous forme de GOC intacts ou en l'absence de cellules de la granulosa. Alors que la bande de ~ 20 kDa était retenue par les ovocytes incubés sous forme de GOC, la bande de 150 kDa est apparue dans les ovocytes incubés sans cellules de granulosa. Cela suggère que l'activation de la signalisation Jagged-Notch dans le follicule ovarien conduit non seulement au clivage du récepteur Notch dans

les cellules de la granulosa, mais également au clivage du ligand Jagged dans l'ovocyte. Le seul site de contact physique entre l'ovocyte et les cellules de la granulosa se trouve aux extrémités de filopodes spécialisés, appelés projections transzonales (TZP), qui s'étendent des cellules de la granulosa à l'ovocyte. Pour tester si la bande ~ 20 kDa dépendait du contact intercellulaire, nous avons incubé des complexes d'ovocytes à cellules cumulus (COC) avec un facteur de croissance épidermique (EGF), ce qui déclenche la rétraction des TZP et donc la perte de contact cellule-ovocyte granulosa. Après une incubation de 8 heures en présence d'EGF, l'espèce Jagged1 de 150 kDa est devenue détectable. Ces résultats suggèrent que l'activation de la signalisation Jagged-Notch dans le follicule ovarien dépend de la présence d'un contact avec les cellules de la granulosa, conduisant au clivage du ligand Jagged dans l'ovocyte. Les travaux futurs porteront sur le rôle potentiel du produit de clivage Jagged. Comprendre les voies de communication cellulaire telles que la signalisation Notch pourrait nous aider à mieux comprendre les mécanismes impliqués dans le développement sain du follicule ovarien.

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Contribution of Authors

The experimental research presented in this thesis was carried out by Herthana Kandasamy under the supervision of Dr. Hugh Clarke. Herthana Kandasamy carried out the majority of the experiments, participated in experimental design, analyzed data, wrote the first draft of the thesis, and actively participated in the revision of the thesis. Dr. Hugh Clarke supervised the work, participated in experimental design, analyzed data, and was involved in thesis editing. The contribution of the remaining authors to each chapter are described below.

For **Chapter 3**, Dr. Qin Yang assisted with setting up the in vitro oocyte culture and immunoblot experiments. Dr. Sofia Granados Aparici, Karen Carvalho and Wusu Wang participated in experimental design as well as data analysis.

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List of Abbreviations

ADAM	disintegrin and metalloproteinase domain-containing protein
AKT	protein kinase B
ALK	activin receptor-like kinase
ATP	adenosine triphosphate
bHLH	basic helix-loop-helix
BMP	bone morphogenetic protein
BMPRII	BMP receptor Type II
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CDK1	cyclin-dependent kinase 1
cDNA	complementary DNA
cGMP	cyclic guanosine monophosphate
COC	cumulus cell-oocyte complex
Cx37	connexin 37
Cx43	connexin 43
DAPI	4’,6-diamidino-2-phenylindole
DLL	delta-like protein
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dpc	days post conception
ECL	enhanced chemiluminescence
EGF	epidermal growth factor

EGFR	EGF receptor
FSH	follicle stimulating hormone
FSHR	FSH receptor
GDF9	growth differentiation factor 9
GJA	gap junction protein, alpha
GOC	granulosa cell-oocyte complex
GVBD	germinal vesicle breakdown
hCG	human chorionic gonadotrophin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IU	international unit
KIT	Mast/stem cell growth factor receptor
KITL	KIT ligand
LH	luteinizing hormone
LHR	LH receptor
MAML	mastermind-like protein
MEM	minimal essential medium
mRNA	messenger ribonucleic acid
mTORC	mechanistic target of rapamycin complex
NICD	Notch intracellular domain
PBS	phosphate-buffered saline
PBST	PBS with Tween-20
PD	postnatal day
PDE5	phosphodiesterase 5

PDK1	phosphoinositide-dependent kinase
PGC	primordial germ cell
PI3K	phosphoinositide 3-kinase
PKA	protein kinase A
PMSG	pregnant mare serum gonadotrophin
PTEN	phosphatase and tensin homolog
PVDF	polyvinylidene fluoride
RBP-Jκ	recombination signal binding protein for immunoglobulin kappa j region
RNA	ribonucleic acid
RT-PCR	reverse-transcriptase polymerase chain reaction
SMAD	sma (*C. elegans Sma* genes) - and mad (*Drosophila Mad* genes)-related protein
TGF-β	transforming growth factor beta
TSC1/2	tuberin/tuberous sclerosis complex 1 or 2
TZP	transzonal projections

Chapter 1 - Introduction

Reproduction is a fundamental characteristic of all known life. Each organism exists as a result of reproduction. In mammals, sexual reproduction involves the fusion of a haploid female gamete, the oocyte, with a haploid male gamete, the sperm. Both sperm and oocytes emerge from highly specialized cells known as primordial germ cells (PGCs).

During female embryonic development, PGCs migrate to the genital ridges, where they proliferate and colonize the ovary (Monk and McLaren, 1981). These germs cells, now called oogonia, undergo several mitotic divisions with incomplete cytokinesis giving rise to clusters of primary oocytes, also known as germ cell cysts (Figure 1) (Pepling 2006). At the same time, these germ cell cysts become surrounded by proliferating somatic cells termed pre-granulosa cells. At this point in the cell cycle, oocytes cease to proliferate and enter meiosis, reaching the first prophase and remain arrested until post ovulation (Borum, 1961). Near the time of birth, oocyte clusters break down to form primordial follicles by a process known as follicle histogenesis (Pepling, 2012). A single layer of squamous somatic pre-granulosa cells encapsulates individual oocytes into primordial follicles. Each primordial follicle is enclosed by a basement membrane (Hirshfield, 1991). At this stage, the oocyte is meiotically arrested and the pre-granulosa cells are mitotically arrested (Maatouk et al. 2013). The cell cycle only resumes once the follicles are activated and undergo follicular growth and maturation.

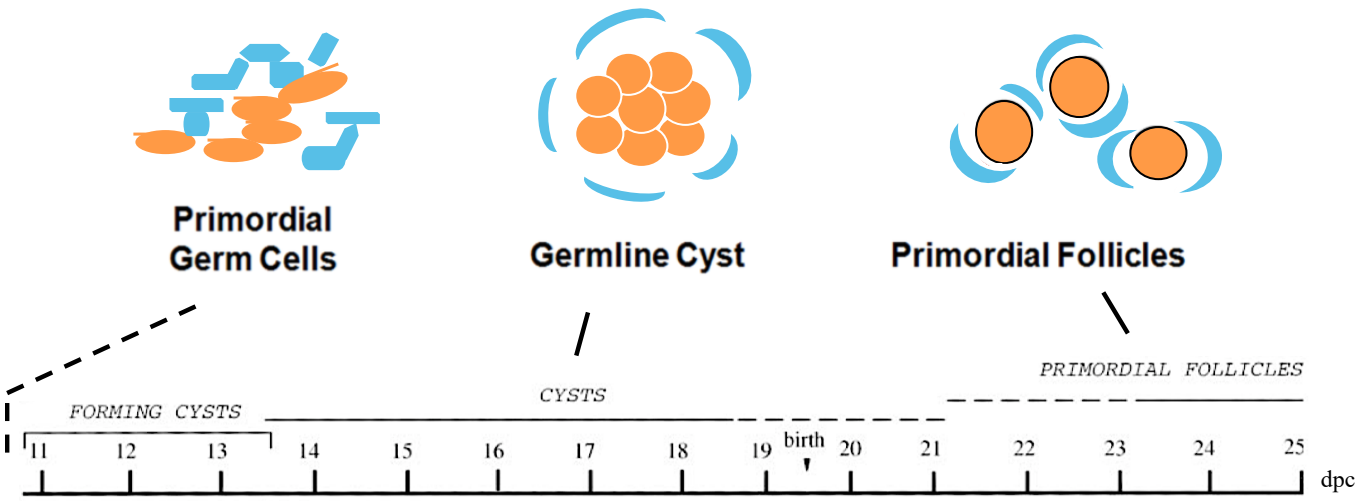

Figure 1. Follicle formation in mouse. PGCs (orange) and somatic cells (blue) arrive at the genital ridges at approximately 10.5 days post conception (dpc) and undergo several mitotic divisions giving rise to germline cysts surrounded by pre-granulosa cells (blue cells). At 13.5 dpc, primary oocytes within germline cysts enter meiosis and eventually arrest at prophase I by 17.5 dpc. Near the time of birth, germline cysts begin to break down and pre-granulosa cells encapsulate individual oocytes to form primordial follicles. (Adapted from Pepling and Spradling 2001 and Pepling 2006).

1.1 Folliculogenesis

1.1.1 Follicle Activation

At the time of birth, the mammalian ovary contains a finite number of primordial follicles which are non-renewable (Picton, 2001). These primordial follicles remain quiescent for a prolonged period of time until some become recruited to enter the growth phase. One visible event that occurs in follicle activation is the change of pre-granulosa cells from squamous to a cuboidal morphology and their proliferation as they enter the cell cycle after an extended period in G0 (Picton, 2001). Subsequently, a portion of these activated follicles will undergo further growth in preparation for ovulation or degenerate by atresia. This occurs at each estrus cycle throughout the female reproductive life span (Matsuda et al. 2012).

The mechanism of follicle activation remains, however, complex and unclear. The initial recruitment or follicle activation of primordial follicles is mediated through various signaling factors. Several lines of evidence point towards KIT ligand as the initial signal for oocyte growth (El-Hayek and Clarke, 2016). KIT ligand is expressed by granulosa cells and interacts with c-KIT, a receptor tyrosine kinase (RTK) localized to the oocytes. Following ligand binding, RTK signals through phosphoinositide 3-kinase (PI3K) and phosphoinositide-dependent kinase (PDK1) to activate serine/threonine kinase (AKT) via phosphorylation. Phosphorylated AKT then, in turn, phosphorylates the forkhead transcription factor, FOXO3A located in the nucleus, which relocates to the oocyte cytoplasm, thus suppressing its transcriptional activities such as cell apoptosis and cell cycle arrest (Arden and Biggs, 2002; Brunet et al. 1999). PTEN, a known antagonist of the PI3K/AKT pathway becomes inactive with active PDK1 (Reddy et al. 2008). PI3K/AKT pathway

activation ultimately leads to an increase in protein synthesis necessary for oocyte growth. Experiments in which KIT ligand binding to c-KIT is repelled using an anti-c-kit (ACK2) antibody prevents the resumption of growth of primordial follicles in vivo and in ex vivo models across various species (Yoshida et al. 1997). Similarly, addition of KITL to ex vivo cultured ovaries induces primordial follicle activation (Hutt et al. 2006). Moreover, FOXO3A, a transcription repressor localized in the nucleus of oocytes of primordial follicles, shows reduced nuclear activity with oocyte growth (Li et al. 2010). In fact, female mice lacking FOXO3A in oocytes, causes primordial follicles to begin to grow shortly after birth (Castrillon et al. 2003). In a similar fashion, conditional deletion of *Pten* in oocytes, the negative regulator of the PI3K/AKT pathway, leads to the activation of the entire primordial follicle pool resulting in its premature depletion (Reddy et al. 2008). Thus, activation of primordial follicles is repressed under normal conditions by repressors such as PTEN and FOXO3A, while follicle activation is induced by signaling factors such as KIT ligand, and subsequently by PI3K/AKT signaling.

The mTORC signaling pathway is one more pathway known to contribute to oocyte growth (Adhikari and Liu, 2009). mTORC signaling is activated by AKT as it inactivates mTORC1 suppressors tuberin/tuberous sclerosis complex 1 or 2 (TSC1/2) through its phosphorylation. Phosphorylated TSC1/2 cannot suppress mammalian target of rapamycin (mTOR) and its downstream factors/proteins can directly control the protein translation needed for cell growth (Adhikari et al. 2009; 2010). Conditional deletion of TSC1 or TSC2 in oocytes leads to a global activation of primordial follicles (Adhikari et al. 2009). Moreover, granulosa cells of primordial follicles with Tsc1-/- ovaries express high levels of KIT ligand, which leads to a hyperactivation of the c-KIT mediated PI3K/AKT pathway in oocytes (Zhang and Liu, 2015).

To sum up, the initial recruitment of primordial follicles can be seen as a balance between activating and maintenance signals. While c-KIT, PI3K/AKT and mTORC signaling factors actively recruit primordial follicles from their resting pool, negative regulators such as FOXO3A, PTEN and TSC1/2 repress follicle recruitment, thus maintaining the dormant primordial follicle population (Prasasya and Mayo, 2019).

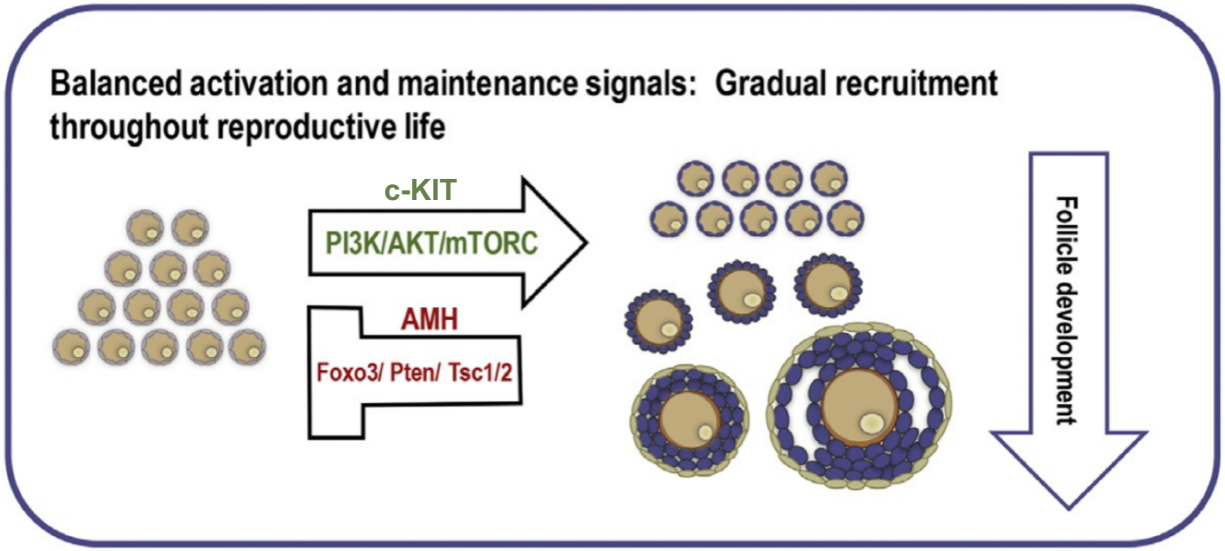

Figure 2. Initiation of growth in primordial follicles. Primordial follicles are recruited for follicular development through activation signals. While c-KIT, PI3K/AKT and mTORC pathways promote the exit of primordial follicles from quiescence, molecules such as FOXO3, PTEN and TSC1/2 complex restrict their activation. A balanced activation and maintenance signals allow primordial follicles to be activated in a cyclic manner, and this throughout the reproductive lifespan. (Adapted from Prasasya and Mayo, 2019)

1.1.2 Follicular Growth

Once primordial follicles are activated, they undergo a prolonged period of growth, which lasts about 3-4 months in humans and 3 weeks in mice (Figure 3) (Clarke, 2018). The first sign that a primordial follicle has entered the growth phase is the transition of the granulosa cells, from squamous to a cuboidal morphology, thus becoming a primary follicle. (Demeestere et al. 2012). Early signals derived from the oocyte may be necessary to promote proliferation of pre-granulosa cells in primordial follicles. Apart from c-KIT, other potential candidates may include members of the TGF-β superfamily such as growth differentiation factor 9 (GDF9) (Picton, 2001). GDF9 binds to activin receptor-like kinase-5 (ALK5) and bone morphogenetic protein type-II receptor (BMPRII) which activates SMAD signaling, leading to the nuclear translocation of SMAD, regulating the expression of target genes (Heldin et al. 1997; Vitt et al. 2002). Furthermore, GDF9 appears to be essential for early follicle development since female mice lacking GDF9 have oocytes with no more than a single layer of granulosa cells, arresting at the primary stage. (Dong et al. 1996). However, mutation of GDF9 does not prevent activation of primordial follicles (Picton, 2001).

During growth, the oocyte expands rapidly, up to 100 times its original size, while still being arrested in meiosis I (El-Hayek and Clarke, 2016). As the oocyte grows, the granulosa cells need to proliferate to cover the expanding surface area of the oocyte, thus creating 2 or more layers of granulosa cells (Da Silva-Buttkus et al. 2008). In addition to that, another cell type appears during follicular growth. A layer of stromal cells known as thecal cells forms around the granulosa layers, with both cell types separated by a basement membrane (Li and Albertini, 2013). As a result, the primary follicle now becomes a secondary follicle. Both thecal cells and granulosa cells work

together to produce estradiol, which is necessary for oocyte and granulosa cells survival (Billig et al. 1993; Matsuda et al. 2012). This is in response to the follicle stimulating hormone (FSH) released by the anterior pituitary, whose secretion is stimulated by hypothalamus-derived gonadotropins (Hunzicker and Maizels, 2006). FSH interacts with follicle stimulating hormone receptors (FSHRs) expressed in granulosa cells to regulate granulosa cells proliferation and differentiation, at later stages of growth (O'Shaughnessy et al. 1996). However, preantral follicular development is considered FSH-independent, while late antral follicles become FSH-dependent (Palermo, 2007). As shown through genetic models lacking FSH or FSHR, follicles can develop up until the early antral stage but fail to develop further (Kumar et al. 1997; Dierich et al. 1998).

With increasing oocyte volume comes changes in the ovarian follicle. Oocytes begin to accumulate messenger RNAs (mRNAs), proteins and organelles required for early embryonic development (El-Hayek and Clarke 2016). Fluid-filled cavities appear within the follicle as the oocyte reaches later stages of growth defining antral follicles. This cavity known as the antrum causes granulosa cells to separate and differentiate into two different cell populations, known as mural and cumulus granulosa cells (El-Hayek and Clarke, 2016). Cumulus cells immediately surround the oocyte and continue to regulate oocyte development while mural cells, as their name suggests, are found in the outer layers and regulate the production of steroid hormones (Jamnongjit and Hammes, 2005). The fully-grown Graafian follicle is now ready to ovulate. By this stage, the majority of the follicles become atretic, and only a few are selected for ovulation (Hirshfield, 1991).

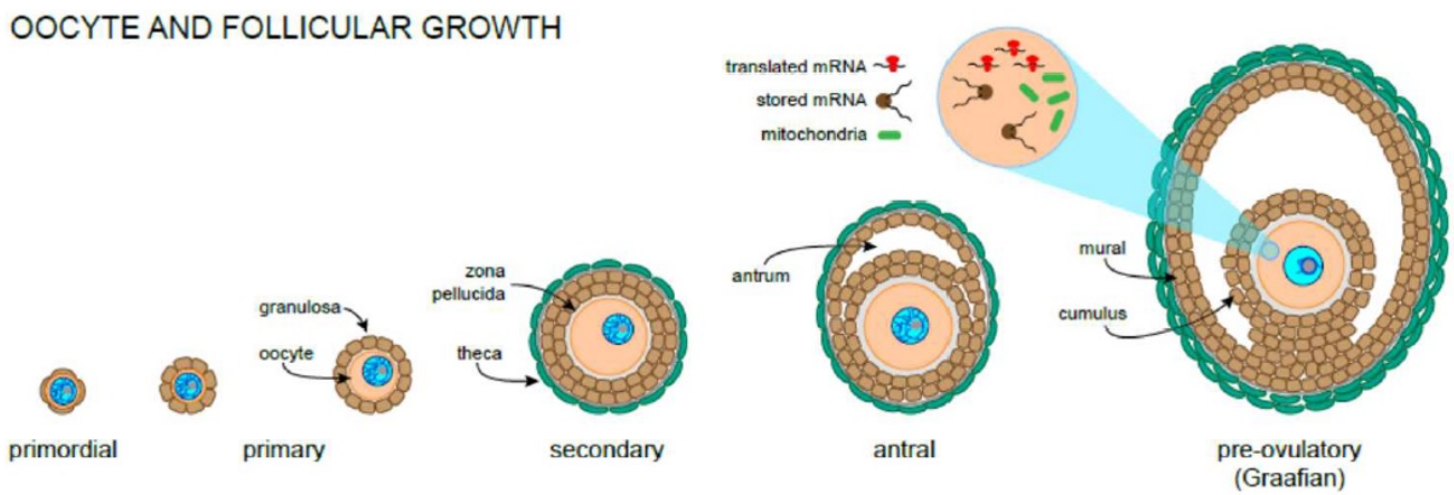

Figure 3. Folliculogenesis of the mouse ovarian follicle. The ovarian follicle undergoes a prolonged period of growth, 3-4 months in humans and ~3 weeks in mice. Primordial follicles have an oocyte enclosed by a single layer of flattened granulosa cells which cuboidalize and proliferate with follicle activation, transitioning to a primary follicle. At the same stage, oocytes begin to secrete a zona pellucida that separates the oocytes from the granulosa cells. Secondary follicles form when growing follicles acquire more than two layers of granulosa cell. Later, the follicle reaches the preantral stage, where fluid-filled cavities form and fuse to form an antrum. As the antrum enlarges, granulosa cells are separated into two distinct cell populations known as mural and cumulus granulosa cells. A fully-grown follicle, termed the Graafian follicle, becomes ready for ovulation (Clarke, 2018).

1.1.3 Oocyte Meiotic Maturation

Up until the pre-ovulatory stage, the follicle remains arrested at prophase I of meiosis, with its germinal vesicle, the nuclear envelope of the oocyte nucleus, intact (Li and Albertini, 2013). Oocytes overcome meiotic arrest and proceed through meiotic maturation, the final stage of oocyte development, in response to LH surge (Neal and Baker 1975; Lei et al. 2001).

Under normal conditions, meiotic arrest is maintained when cyclic adenosine monophosphate (cAMP) concentrations are high in oocytes. The elevated intracellular cAMP level produced by the oocyte continuously activates protein kinase A (PKA) and keeps cyclic dependent kinase 1 (CDK1) in an inactive state, preventing the progression of meiosis to metaphase II (Pan and Li, 2019). Furthermore, cAMP is metabolized in the oocyte by phosphodiesterase, PDE3A. Its breakdown is prevented by cyclic guanosine monophosphate (cGMP), a secondary messenger synthesized by mural and cumulus granulosa cells that diffuses to the oocyte to inhibit PDE3A, thereby maintaining high cAMP levels (Shuhaibar et al. 2015; Jaffe and Egbert, 2017). Thus, both the oocyte and granulosa cells play an essential role in maintaining oocyte meiotic arrest. It is worth mentioning however, that mid-grown oocytes taken out of their follicle are capable of meiotic maturation, which suggests that the follicular environment actively represses meiotic maturation during growth (Cho et al. 1974; Dekel and Beers 1978).

As the oocyte reaches the pre-ovulatory stage, meiosis resumes in response to LH surge. Upon binding of LH to its receptor LHGCR, found on mural granulosa cells, EGF-like proteins are secreted by the mural granulosa cells and activate EGF receptors (EGFR) on both mural and cumulus granulosa cells. Activation of EGFR results in the phosphorylation of PDE5A, a

phosphodiesterase of cGMP (Egbert et al. 2016). Active PDE5A degrades cGMP in the granulosa cells and causes cGMP levels in the oocytes to drop as well (Norris et al. 2010). With low cGMP concentration in the oocyte, PDE3A is no longer inhibited and in turn degrades cAMP, rendering PKA inactive and thereby activating CDK1 (Jaffe and Egbert, 2017). Active CDK1 stimulates the germinal vesicle to break down and triggers meiotic maturation (Li and Albertini, 2013).

With germinal vesicle breakdown (GVBD) and meiosis resumed in the oocyte, the chromosomes condense and align along the meiotic spindle on the metaphase plate. The spindle migrates to the periphery of the oocyte where it extrudes its first polar body containing a set of homologous chromosomes. The mature oocyte is now arrested at metaphase II of meiosis until after fertilization (Li and Albertini, 2013). During maturation, cumulus cell layer expansion occurs, where cumulus cells secrete a type of matrix that allows the cells to separate from the oocyte and from each other. The oocyte is now ready to ovulate. The remains of the follicle become the corpus luteum, which produces hormones like progesterone to sustain the endometrium during pregnancy (Wassarman and Litscher, 2018).

Over time, the ovary essentially becomes devoid of follicles because they have been depleted through the processes of both ovulation and atresia. Once the ovary is devoid of follicles, the female becomes infertile and is said to have entered menopause or reproductive senescence (Hirshfield, 1991; Broekmans et al. 2007).

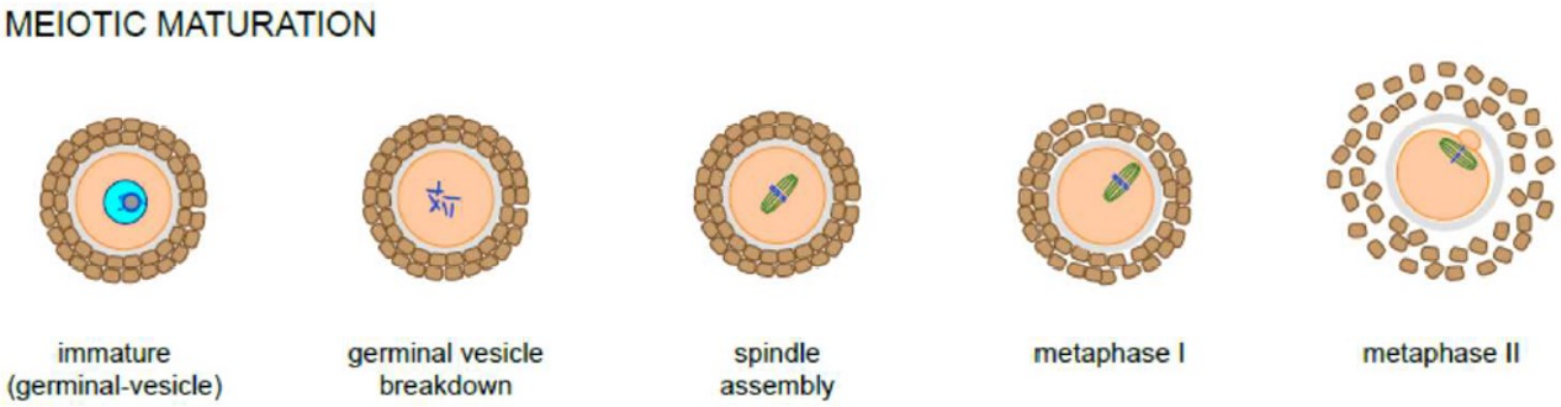

Figure 4. Oocyte meiotic maturation. During meiotic maturation, the oocyte, arrested at prophase I of meiosis during growth, resumes meiosis. The germinal vesicle breaks down, allowing the chromosomes to condense and align along the spindle. The spindle moves to the periphery of the oocyte and undergoes polar body extrusion, where a set of homologous chromosomes is discarded. The oocyte now remains arrested at metaphase II of meiosis until fertilization. Moreover, the cumulus cell layer expands, terminating its communication with the oocyte (Clarke, 2018).

1.1 Zona Pellucida and Transzonal Projections

Mammalian oocytes are surrounded by an extracellular matrix called the zona pellucida. This layer appears as soon as the oocyte reaches the primary follicle stage (El-Hayek and Clarke, 2016). The mouse zona pellucida is composed of three glycoproteins, ZP1, ZP2 and ZP3 which are synthesized and secreted by growing oocytes. The zona pellucida provides structural support to the oocyte and plays an important role in fertilization by preventing polyspermy (Wassarman and Litscher 2013; 2018). Experiments in which mice fail to synthesize ZP2 or ZP3 lack a zona pellucida and are unfertile (Liu et al. 1996; Rankin et al. 1996; 2001). Moreover, both mouse ZP2 and ZP3 serve as receptors to sperm to allow binding and initiate acrosome reaction at the time of fertilization (Bleil et al. 1988). Once the egg is fertilized, the zona pellucida degenerates and becomes replaced by the underlying layer of trophoblastic cells (Red-Horse et al. 2004).

At the beginning of folliculogenesis, oocytes are in direct contact with their somatic environment. Nutrients and signaling factors are exchanged between them inevitably (Clarke, 2017). As follicles enter the growth phase and oocytes secrete an extracellular coat, a barrier forms between the oocyte and the surrounding somatic cells. To allow contact between both structures, the granulosa cells send out long cytoplasmic projections resembling filopodia through the zona pellucida to reach the plasma membrane of the oocyte (Anderson and Albertini, 1976). These actin-rich filopodia are called transzonal projections, or TZPs for short. Transzonal projections allow communication between the oocyte and granulosa cells, despite the presence of the zona pellucida. These projections mainly originate from the innermost layer of granulosa cells, immediately adjacent to the oocyte, but it has also been observed that they arise from granulosa cells found in distal layers

(Jaffe and Egbert 2017). It has been shown that oocyte-derived GDF9 may promote the formation of new TZPs. Granulosa cell-oocyte complexes (GOCs) incubated for five days in the presence of GDF9 increased the number of TZPs as well as its density (El-Hayek et al. 2018). On the other hand, mice lacking GDF9 have TZPs with abnormal morphology and their oocytes fail to develop normally (Dong et al. 1996; Carabatsos et al. 1998).

TZPs allow the transfer of essential nutrients, metabolites and signaling factors from the granulosa cells to the oocyte. They anchor to the oocyte plasma membrane with adherens and gap junctions, found at the tips of TZPs (Li and Albertini, 2013). These gap junctional proteins easily allow the transfer of pyruvate, nucleotides and amino acids to the oocyte necessary for its development (Clarke, 2018).

During oocyte maturation, TZPs retract to release the oocyte from its follicle for ovulation (Motta et al. 1994). In fact, loss of TZPs and oocyte maturation spontaneously occur when cumulus cell-oocyte complexes (COCs) are cultured in vitro. Furthermore, TZP loss is accelerated when COCs are incubated in the presence of epidermal growth factor (EGF). Retraction can be seen as early as 6 hours of incubation.

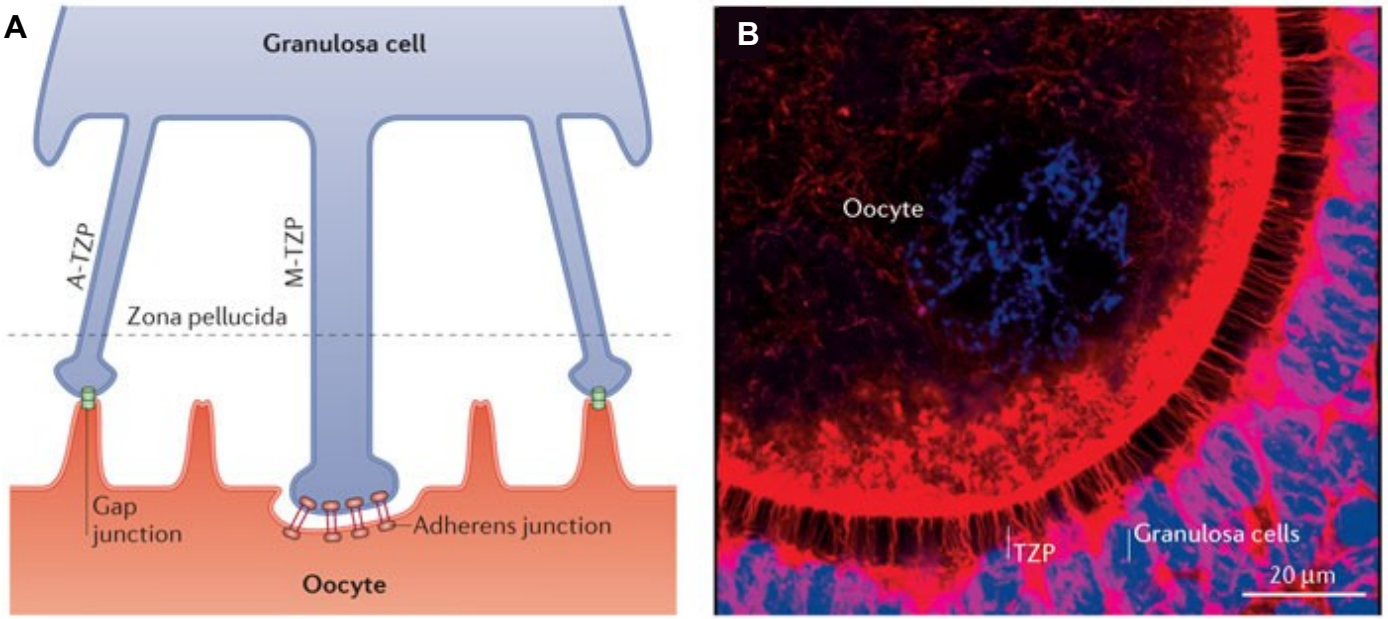

Figure 5. A) Transzonal projections from granulosa cells extend through the zona pellucida to make contact with the oocyte plasma membrane via gap and adherens junctions. B) A cow oocyte with actin filaments stained with red phalloidin shows the presence actin-rich TZPs, emanating from the surrounding granulosa cells (Li and Albertini, 2013).

1.3 Communication between the Oocyte and Granulosa Cells

The interaction between oocyte and its surrounding somatic granulosa cells is essential for proper oocyte development. Experiments have demonstrated this by culturing either intact granulosa cell-oocyte complexes or granulosa cells co-cultured with oocytes. When intact granulosa cell-oocyte complexes are cultured, oocytes continue to grow whereas oocytes co-cultured with granulosa cells fail to grow (Eppig, 1979). Therefore, granulosa cells play a crucial role in the development of a healthy oocyte, and this requires direct cell-contact.

This direct cell-contact is maintained at the primordial follicle stage but is eventually lost with the appearance of the zona pellucida. With the formation of transzonal projections, the surrounding somatic cells can maintain their communication with the oocyte. Gap junctions are intercellular channels that allow various molecules to pass through between two adjacent cells (Kidder and Mhawi, 2002). These gap junctions are also found in TZPs, specifically at their tips where they contact the oocyte plasma membrane. Made of transmembrane proteins called connexins, both connexin37 (Cx37) and connexin43 (Cx43) play important roles in folliculogenesis (Veitch et al. 2004).

Cx37 localizes to the oocyte surface while Cx43 localizes between granulosa cells (Kidder and Mhawi, 2002). Targeted deletion of *Gja4*, the gene encoding Cx37, in mouse ovaries causes disruption of folliculogenesis (Simon et al. 1997; Carabatsos et al. 2000). For instance, ovaries from Cx37-deficient mice contained few or no follicles in the late preantral or antral stages (Carabtsos et al. 2000). Similarly, in the absence Cx43, dye transfer experiments indicated that intercellular coupling between granulosa cells is reduced. As a result, oocytes of Cx43-/- mice are

poorly developed and are incompetent for fertilization (Ackert et al. 2001; Gittens et al. 2003). Thus, gap junctional communication between granulosa cells and with the oocyte is essential for the production of a healthy oocyte.

ATP production in the oocyte depends on the supply of pyruvate by its surrounding granulosa cells. Since oocytes cannot metabolize glucose and require energy, granulosa cells break down glucose molecules into pyruvate, which is transferred to the oocyte through TZPs, via gap junctions (Brinster 1971; Tsutsumi et al. 1990; Clarke, 2018).

A bidirectional communication also exists in mouse ovarian follicles. At the initiation of oocyte and follicular growth, KIT ligand secreted from pre-granulosa cells diffuses out to reach the c-KIT receptor located on oocytes, activating the PI3K/AKT pathway, as previously described. This form of paracrine signaling is present in mouse ovarian follicles enabling a communication between both cell types. Although TZPs serve as physical bridges to allow contact-dependent communication between the growing oocyte and its somatic environment, paracrine signaling still plays an important role in growing follicles. Therefore, the bidirectional communication between oocyte and granulosa cells is not limited to TZPs (Clarke, 2018).

Once the oocyte reaches the preovulatory stage, EGF-like proteins from the granulosa cells are required for the oocyte to resume meiotic maturation. With LH surge, EGF-like proteins secretions lead to a decrease in cGMP levels in the granulosa cells. The difference in cGMP levels between oocyte and granulosa cells creates a gradient causing a cGMP flux from the oocyte to the granulosa cells through the gap junctions (Clarke, 2018; Li and Albertini, 2013). Thus, gap junctional communication is required for meiotic maturation.

Altogether, communication between oocyte and granulosa cells occurs through a bidirectional communication and intra-follicular cell-contact. The oocyte alone is not capable of growth and is highly dependent on its follicular environment. After all, both means of communication are present throughout oocyte development, from follicle activation to follicular growth until oocyte meiotic maturation.

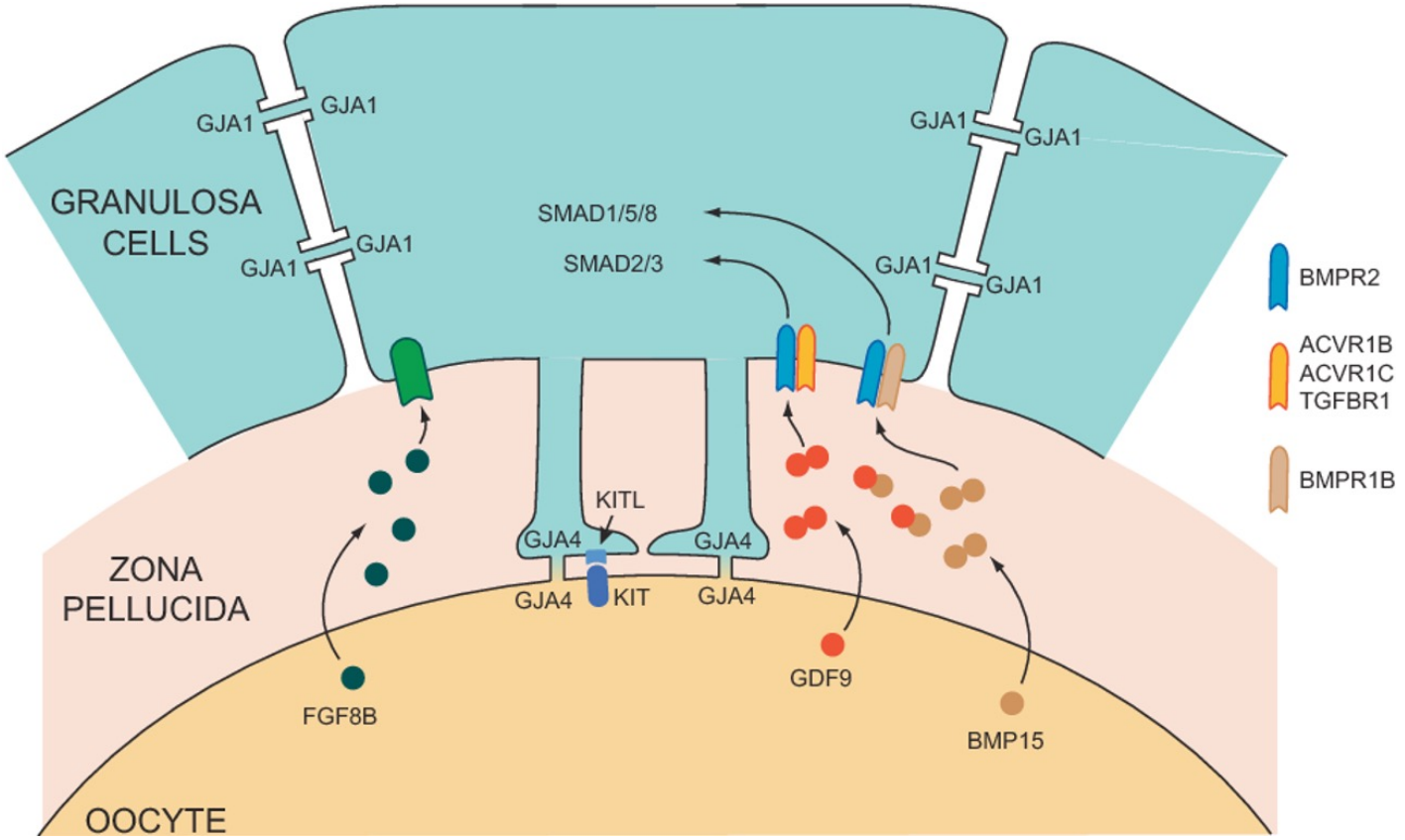

Figure 6. Oocyte-granulosa cell communication during growth. Oocytes and granulosa cells communicate using gap junctions, which are found between adjacent granulosa cells (GJA1 encoding Cx34) and between oocytes and their immediately adjacent granulosa cells (GJA4 encoding Cx37). Paracrine signaling factors secreted from the oocyte such as KITL and GDF9 mediate a bidirectional communication between both cell types and are required for oocyte development and granulosa cell proliferation and differentiation (El-Hayek and Clarke, 2016; Clarke 2017).

1.4 Notch Signaling Pathway

The Notch signalling pathway is a highly conserved signaling system amongst species. Notch signaling has been heavily studied in model organisms such as *Drosophila* and *C. elegans* (Greenwald, 1998). It is known to be involved in many cellular processes which are implicated in early embryonic development, namely, to mediate important interactions between germ line cyst cells and surrounding somatic cells (Vanorny et al. 2014). A few of them are cell-fate specification, cell migration, mesenchymal to epithelial transition, cell survival or death and cell division (Artavanis-Tsakonas et al. 1999). For instance, the absence of Notch pathway during egg chamber formation in the *Drosophila* gonad results in fused egg chambers (Trombly et al. 2009). Moreover, Notch activity in the gonad of *C. elegans* is required for germ cell proliferation and differentiation (Kadyk et al. 1997; Lai, 2004). An essential feature of the Notch signalling pathway is that both receptor and ligand are membrane-bound. In other words, Notch signaling is a contact-dependent signaling system or juxtracrine signalling (Dumortier et al. 2005).

In mammals, the Notch pathway involves the interaction with one of the four transmembrane receptors (NOTCH1, NOTCH2, NOTCH3 and NOTCH4) with one of the transmembrane ligands (JAG1, JAG2, DLL1, DLL3 and DLL4) (Trombly et al. 2009). Notch receptors contain 29-36 N-terminal epidermal growth factor (EGF) repeats of which EGF repeats 11-12 are necessary for ligand binding. Notch ligands on the other hand include a conserved N-terminal *Delta-Serrate-Lag2* (DSL) domain which is vital for mediating interactions with Notch receptors (Trombly et al. 2009).

1.4.1 The Intracellular Cascade

Interactions between the extracellular domains of Notch receptors and ligands initiate the Notch signaling pathway (Vanorny and Mayo, 2017). As a consequence of ligand-receptor interaction, Notch receptor undergoes a conformational change exposing the site 2 (S2) cleavage moiety, triggering its cleavage by a disintegrin and metalloproteinase domain-containing protein 10 (ADAM10) (Vanorny and Mayo, 2017) (Figure 6). This cleavage releases the extracellular portion of Notch receptor, which is trans-endocytosed by the ligand-expressing cell (Park et al. 2000; Yavropoulou and Yovos, 2014). The remaining transmembrane-anchored Notch exposes another cleavage moiety at sites 3 and 4, (S3, S4) which is recognized and cleaved by the γ-secretase complex. This final cleavage releases the Notch intracellular domain (NICD) into the cytoplasm, which translocates to the nucleus, to act as a modulator of transcription, activating Notch target genes (Fortini, 2002; Lewis et al. 2009). Within the nucleus, NICD interacts with the DNA-binding transcriptional repressor, recombination signal binding protein for immunoglobulin kappa j region (RBP-Jκ), to displace co-repressors and recruit transcriptional activators such as mastermind-like proteins (MAMLs). Well-known Notch target genes 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. These are transcriptional repressors that regulate cellular differentiation and embryonic patterning (Davis and Turner, 2001).

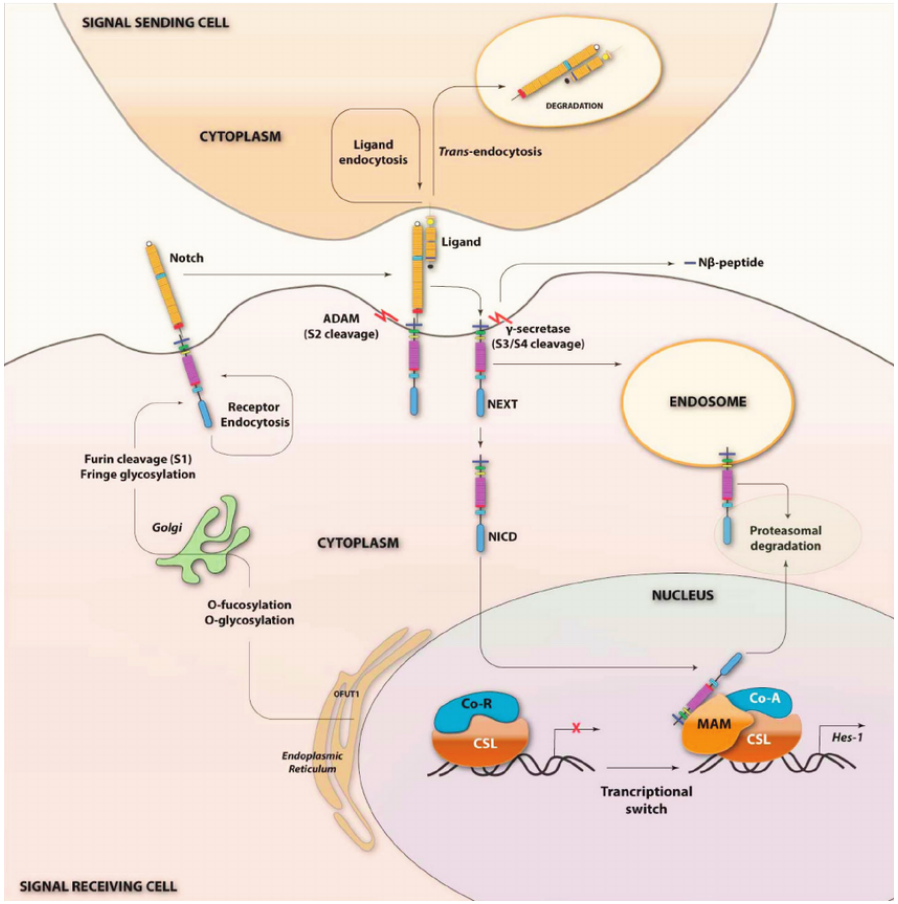

Figure 7. The Notch signaling pathway. Ligand-receptor interactions leads to two consecutive proteolytic cleavages. The first cleavage occurs close to the transmembrane domain and is mediated by ADAM10. The cleaved extracellular portion of Notch is trans-endocytosed by the ligand-expressing cell. The second cleavage is mediated by γ-secretase, releasing the inner portion of Notch, the NICD, allowing its translocation into the nucleus. Within the nucleus, NICD interacts with transcriptional repressors and activators to activate Notch target genes such as Hes and Hey (Dumortier et al. 2005; Yavropoulou and Yovos, 2014).

1.4.2 Notch Signaling in the Mammalian Ovary

In the mammalian ovary, Notch signaling is present very early in development. During embryonic ovarian development, NOTCH2 is the most abundantly expressed receptor in somatic pre-granulosa cells while JAG1, JAG2 are the most expressed ligands in germ cells (Vanorny et al. 2014). Given that JAG1 is known to interact with NOTCH2, it is hypothesized that JAG1-expressing germ cells signal through NOTCH2-expressing pre-granulosa cells to facilitate the assembly of primordial follicles (Shimizu et al. 1999; Vanorny et al. 2014). With the use of transgenic Notch-responsive green fluorescence protein (GFP) reporter mice, it was demonstrated that NOTCH2 active pre-granulosa cells (green) surround and invade germ cell nests (red) to form primordial follicles (Vanorny et al. 2014) (Figure 8).

Moreover, knock-out experiments have demonstrated the importance of Notch signaling in coordinating follicular growth. Conditional deletion of JAG1 in germ cells (J1KO) or NOTCH2 in pre-granulosa cells (N2KO) create abnormal follicles with enlarged oocytes and with lack of granulosa cell growth (Vanorny et al. 2014). Disruption of Notch signaling the ovary also results in the formation of multi-oocytic follicles, a result of incomplete fragmentation of the germ cell nest. Decrease in granulosa cells proliferation and increase in cell apoptosis are observed as well in J1KO and N2KO mice (Vanorny et al. 2014).

Interestingly, during mid and late follicular growth, once follicles become responsive to gonadotrophins, JAG1 ligand becomes localized to granulosa cells that are steroidogenically active. Following hormone stimulation by human chorionic gonadotrophin in PD 19 CD-1 mice,

JAG1 expression became more robust and co-localized with steroid-producing somatic cells (Prasasya and Mayo, 2018).

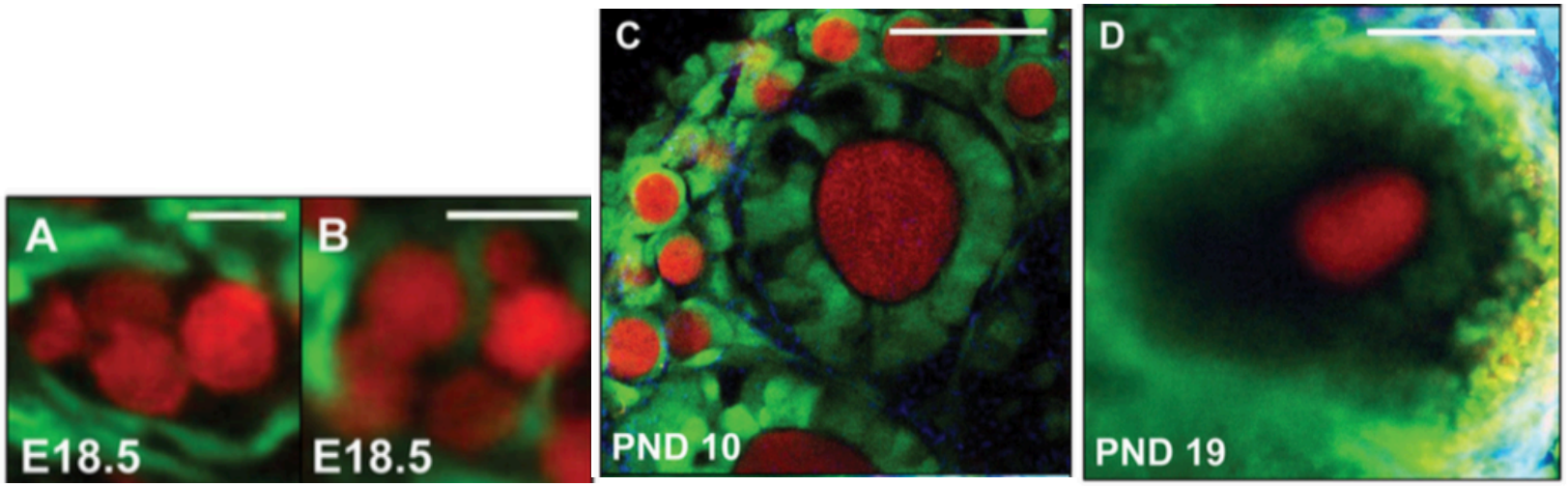

Figure 8. Notch signaling during follicle assembly, growth and maturation. A) Notch-responsive GFP reporter mouse line labels Notch active pre-granulosa cells (green) that surround clusters of germ cells (red), labelled with conditional tdTomato reporter line, driven by *Vasa-Cre*. in embryonic day 18.5 (E18.5) ovary. B) Notch active pre-granulosa cells are seen invading the germ cell nest, sending projections around individual germ cells to form primordial follicles. C) Notch active pre-granulosa cells are observed in primordial follicles found at the ovarian cortex of a postnatal day (PND) 10 ovary, while granulosa cells within primary follicles show variable Notch reporter activity. D) Antral follicles show Notch activity in both cumulus and mural granulosa cells (Vanorny et al. 2014; Vanorny and Mayo, 2017).

1.5 Hypothesis and Objectives

Given that oocyte and follicular development depend on bi-directional communication, and that Jagged-Notch signaling occurs through an intercellular communication between two cell types, we hypothesize that Jagged-Notch signaling pathway is one of the signaling pathways that mediates oocyte-granulosa cell communication. The objectives of this thesis are to establish the expression patterns of NOTCH2 and JAG1 in mouse ovarian follicles as well as the gene and protein expression of JAG1 in growing oocytes.

Chapter 2 –Materials and Methods

2.1 Mice

All experiments were performed using CD-1 mice from Charles River Canada (St. Constant, QC, Canada). Experiments were approved by the Animal Care Committee of the Research Institute of McGill University Health Centre (RI-MUHC) and complied with the Canadian Council on Animal Care. For some experiments, 19-day-old female mice were given 5 international units (IU) of pregnant mare serum gonadotropin (PMSG) injections intraperitoneally.

2.2 Collection and culture of cells

To collect granulosa-oocyte complexes (GOCs), ovaries were removed from postnatal day (PD) 10 female mice. Ovaries were dissected into fragments in 37 ºC minimal essential medium (MEM, Life Technologies) buffered at pH 7.2 with HEPES (MEM-H), supplemented with sodium pyruvate (0.28 mM; Sigma), bovine serum albumin (BSA, 3 mg/ml; Sigma), streptomycin (50 µg/ml; Sigma) and penicillin G (63 µg/ml; Sigma). The ovary fragments were incubated in MEM-H along with collagenase (2µg/ml; Worthington) and deoxyribonuclease I (DNase I, 10µg/ml; Sigma) at 37°C in air for 10 minutes. Following incubation, the fragments were gently pipetted to disrupt them. Individual GOCs were collected with a mouth-controlled micropipette. To obtain granulosa cell-free oocytes, the cells were mechanically stripped off from GOCs by mouth pipetting. GOCs were incubated in MEM-$NaHCO_3$ containing pyruvate, BSA, and antibiotics overnight. Following the incubation period, the granulosa cells were mechanically stripped off by mouth pipetting to isolate the oocytes.

To collect cumulus-oocyte complexes (COCs), PD 19 female mice were administered 5 IU of PMSG intra-peritoneally, and the ovaries were collected 44 hours later. To isolate COCs, the collected ovaries were punctured with a 30G1/2 needle in MEM-H medium to release the COCs from their follicles. To obtain cumulus cell-free oocytes, the cells were mechanically stripped off from COCs by mouth pipetting. COCs were transferred to MEM-NaHCO3 with epidermal growth factor (EGF, 10ng/ml; BD Biosciences) to induce oocyte meiotic maturation in vitro. The cells were incubated for 8 hours at 37°C in 5% CO2 in air. After 8 hours of incubation, COCs were denuded in MEM-H medium with the presence of hyaluronidase to digest the extracellular matrix and release the oocytes. The cumulus cell-free oocytes or granulosa cell-free oocytes were also incubated overnight in MEM-NaHCO3. The next day, oocytes were retrieved from the culture and used in the following experiments. All collected samples were cultured in 4-well dishes with 400μl of MEM-NaHCO3 per well.

2.3 Immunofluorescence

GOCs were fixed in a solution of 2% paraformaldehyde (Fisher Scientific, 30525-89-4) in phosphate-buffered saline (PBS) containing 0.1% Triton X-100 for 20 minutes. After fixation, the samples were washed twice in blocking buffer (PBS containing 0.1% Triton X-100 and 3% BSA) for 15 minutes removing any traces of paraformaldehyde. Samples were incubated overnight at 4°C on a shaker in primary antibodies (anti-NOTCH2, dilution 1:400 or anti-JAG1, dilution 1:100) diluted in blocking buffer. The next day, the samples were washed twice in blocking buffer for 15 minutes at room temperature and then incubated in secondary antibodies (Alexa 488-conjugated goat anti-rabbit, dilution 1:100 or Alexa 488-conjugated donkey anti-mouse, dilution 1:100) diluted in blocking buffer for 1 hour at room temperature. 4',6-diamidino-2-phenylindole (DAPI,

Roche, 236-276, dilution 1:100) and Phalloidin Alexa 555 (Invitrogen, A34055, dilution 1:100) were added to the secondary antibody solution to stain the nucleus and the actin cytoskeleton, respectively. The samples were then washed twice in blocking buffer and plated on a glass-bottomed dish, inside droplets of PBS, covered with mineral oil. The samples were imaged using a Zeiss LS M880 laser scanning confocal microscope. The antibodies and their appropriate dilutions are listed in Table 1.

2.4 Reverse transcription-polymerase chain reaction (RT-PCR)

Oocytes and granulosa cells were isolated in separate microtubes. RNA was extracted from oocytes and granulosa cells using the ARCTURUS® Picopure® RNA isolation Kit (KIT0204; Life Technologies Applied Biosystems) and following the manufacturer's protocol. The isolated RNA samples underwent complementary DNA (cDNA) synthesis, again following the manufacturer's protocol. Finally, the obtained cDNA underwent PCR amplification with specific primers. The samples were separated using a 2% agarose gel. The bands were visualized in a gel doc, in the presence of a UV light. Primers used for PCR are listed in Table 2.

2.5 Immunoblotting

Collected oocytes or granulosa cells were transferred to the bottom of 0.7ml microtubes containing 10µl of 2X Laemmli buffer (161-0737, Bio Rad) and denatured at 95°C for 5 minutes. A precast 4-15% polyacrylamide gel (456-1086, Bio Rad) was used to separate the proteins. After the gel was run for 2 hours at 120V, the proteins from the gel were transferred onto a polyvinylidene fluoride (PVDF) membrane for 90 minutes at 100V. The PVDF membrane with the transferred proteins was blocked in 5% skimmed milk, in 0.1% Tween-20 phosphate-buffered saline (PBST)

for 1 hour. After blocking, the membrane was incubated in a primary antibody solution – anti-JAG1, anti-ACTB or anti-β-TUBULIN– diluted to 1:1000 in 3% BSA in PBST overnight at 4°C with gentle shaking.

Following overnight incubation, the membrane was washed three times in PBST then incubated with the corresponding horseradish peroxidase conjugated secondary antibodies diluted to 1:5000 in 0.1% milk with PBST at room temperature for 1 hour. The membrane was then washed three times in PBST and incubated with enhanced chemiluminescence (ECL) plus substrate for 5 minutes. To visualize the bound antibody signals, the membrane was imaged using a Storm 860 phosphorimager.

Table 1. Antibody Table

Peptide/Protein Target	Name of Antibody	Manufacturer; Catalog No.	Species Raised In; Monoclonal or Polyclonal	Dilution used
JAG1	Rabbit anti-JAG1	Abcam; ab109536	Rabbit; monoclonal	1:1000 for immunoblot
JAG1	Mouse anti-JAG1	SantaCruz; (E-12): sc-390177	Mouse; monoclonal	1:100 for immunofluorescence
NOTCH2	Rabbit anti-NOTCH2	Abcam; ab8926	Rabbit; polyclonal	1:400 for immunofluorescence
ACTB	Mouse anti-ACTB	Abnova; H00000060-M01	Mouse; monoclonal	1:1000 for immunoblot
β -TUBULIN	Mouse anti-β-TUBULIN	Sigma; T4026	Mouse; monoclonal	1:1000 for immunoblot
IgG rabbit	Alexa Fluor 488 Goat anti-Rabbit IgG	Life Technologies; A32731	Goat; polyclonal	1:100 for immunofluorescence
IgG mouse	Alexa Fluor 488 Donkey anti-Mouse IgG	Invitrogen; A-21202	Donkey; polyclonal	1:100 for immunofluorescence

Table 2. PCR primer sequences

Transcript	Sequence
Jagged1	F: 5’-TGGACATTATGCCTGTGACC-3’ R: 5’-CAACCGTACTGGCACCTG-3’
Notch2	F: 5’-GACCCTATCCTACCCTCTAGTG-3’ R: 5’AGCAGGATGAAGAACAGGATG-3’
Actb	F: 5’-GGCTGTATTCCCCTCCATCG-3' R: 5’-CCAGTTGGTAACAATGCCATGT-3’

Chapter 3 – Results

3.1 NOTCH2 and JAG1 Expression Patterns in GOCs

First, we looked at the presence of NOTCH2 receptor and JAG1 ligand in GOCs. In order to determine the presence of NOTCH2 receptor, we used the whole-cell immunofluorescence technique on GOCs isolated at mid-follicular growth. Before staining, these GOCs were partly denuded to allow the antibodies to penetrate into the cells. At least ten complexes were stained for NOTCH2. The anti-NOTCH2 antibody used in this experiment recognizes the epitope exposed after γ-secretase cleaves NOTCH2 at the S3, S4 cleavage sites. This cleavage releases the intracellular portion of NOTCH2, the NICD, which is no longer tethered to the membrane and freely translocates to the cytoplasm and to the nucleus. NOTCH2 (green) was detected in the granulosa cells and was abundantly expressed in the cytoplasm, shown by the white arrows (Figure 9A). However, it was not detected in the nucleus. The DNA staining in blue clearly delineates the nucleus from the cytoplasm with no overlap between DNA and NOTCH2 staining in the granulosa cells. Thus, the staining of cleaved-NOTCH2 receptor seen in GOCs demonstrates the presence of active Notch signaling in mouse ovarian follicles. Notch signaling is presumably initiated through Notch ligands found in close proximity.

Having looked at the presence of NOTCH2 in GOCs, we then, in turn, looked at the presence of its ligand, JAG1. Whole-cell immunofluorescence technique was applied to GOCs isolated at mid-follicular growth. Denuding GOCs was not necessary as the antibody gave clear staining results. Fifteen complexes were stained for JAG1. The anti-JAG1 antibody used in this experiment

recognizes the C-terminal of the protein. JAG1 is detected in the oocyte (white arrows) and in granulosa cells' cytoplasm (Figure 9B). Actin cytoskeleton staining in red outlines granulosa cell membrane with the emanating TZPs as well as the oocyte cortex (Figure 9C). JAG1 is not detected in the nucleus of both the oocyte and granulosa cells, clearly seen by the DNA staining in blue. Moreover, both JAG1 and NOTCH2 are detected in similar locations, in granulosa cells' cytoplasm. Along with oocyte-derived JAG1, granulosa cell-derived JAG1 may be initiating Notch signaling in the GOC. Thus, JAG1 staining confirms the presence of a Notch ligand in mouse ovarian follicles midway through growth and could be assumed to initiate Notch signaling via cell contact.

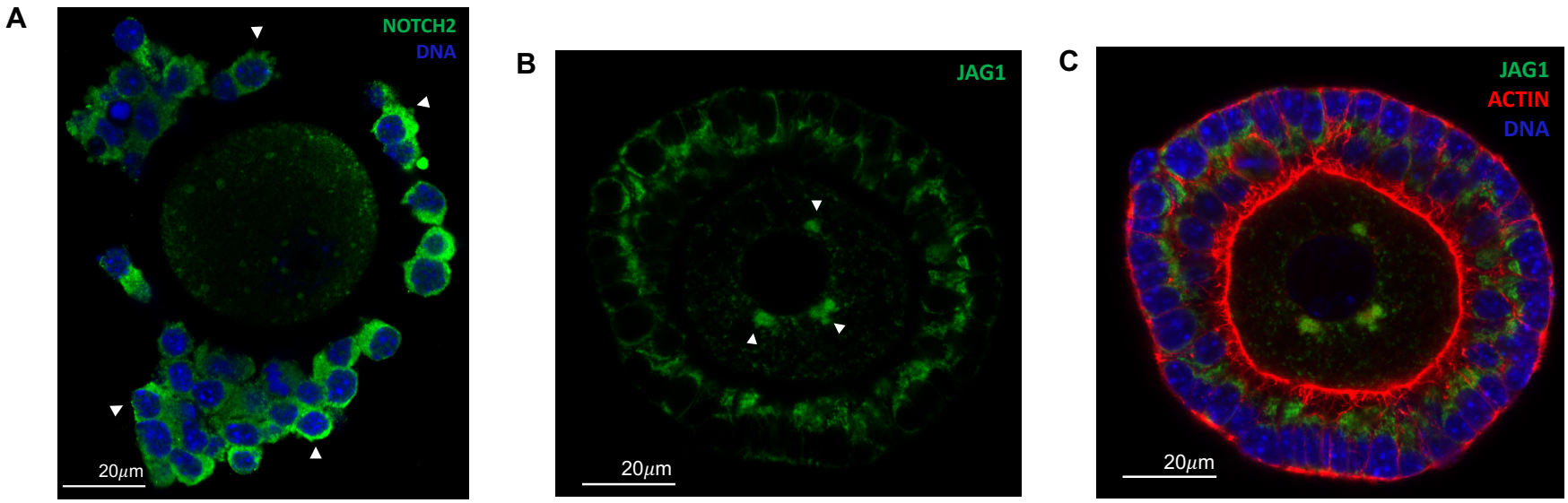

Figure 9 – Localization of NOTCH2 and JAG1 in granulosa-oocyte complexes. **(A)** A partly denuded GOC with NOTCH2 (green) abundantly expressed in granulosa cells' cytoplasm (white arrows) but not in the nucleus. DNA staining (blue) clearly delineates the nucleus from the cytoplasm. (**B)** A GOC with JAG1 (green) detected in the oocyte cytoplasm (white arrows) and in granulosa cell cytoplasm. **(C)** The same GOC stained for actin (red), DNA (blue) and JAG1. DNA staining of the granulosa cell nucleus permits visualization of JAG1 in the cytoplasm, and actin cytoskeleton staining delineates the cell membrane of each granulosa cells, as well as the TZPs and oocyte cortex.

3.2 *Jagged1* and *Notch2* mRNA presence in oocytes and granulosa cells

Having established the expression patterns of NOTCH2 and JAG1 in GOCs, we next looked at the presence of mRNA for both genes. To determine the presence of *Jagged1* mRNA in oocytes and granulosa cells, we isolated GOCs at mid-follicular growth. We then stripped off the granulosa cells from the oocytes and ran a reverse-transcriptase polymerase chain reaction (RT-PCR) on both samples separately. The chosen *Jagged1* primers amplify a band of 150 base pairs (bp). The PCR gel in Figure 10A reveals a band migrating at the expected position, between 100 and 200 bp, in both oocytes and granulosa cells. Two replicates for both samples were run and produced similar and consistent results. RT-PCR on a mouse tail tissue was also performed to confirm that *Jagged1* is tissue-specific. Thus, the *Jagged1* primers do amplify a band of the expected size and is specifically expressed in oocytes and granulosa cells.

To determine the presence of *Notch2* mRNA in oocytes and granulosa cells, we isolated mid-grown GOCs, stripped off the granulosa cells from the oocytes and ran a RT-PCR on both samples separately. The chosen *Notch2* primers amplify a band of 226 bp. The PCR gel in Figure 10B reveals a band migrating at the expected position, between 200 and 300 bp, in granulosa cells only. Non-specific lower bands are detected in both oocytes and granulosa cells. Two replicates for both samples were run and produced similar and consistent results. RT-PCR was also performed on similar samples with *Actin* primers as a loading control (Figure 10C). Thus, the *Notch2* primers do amplify a band at the expected size and is expressed only in granulosa cells, confirming that its location is cell specific.

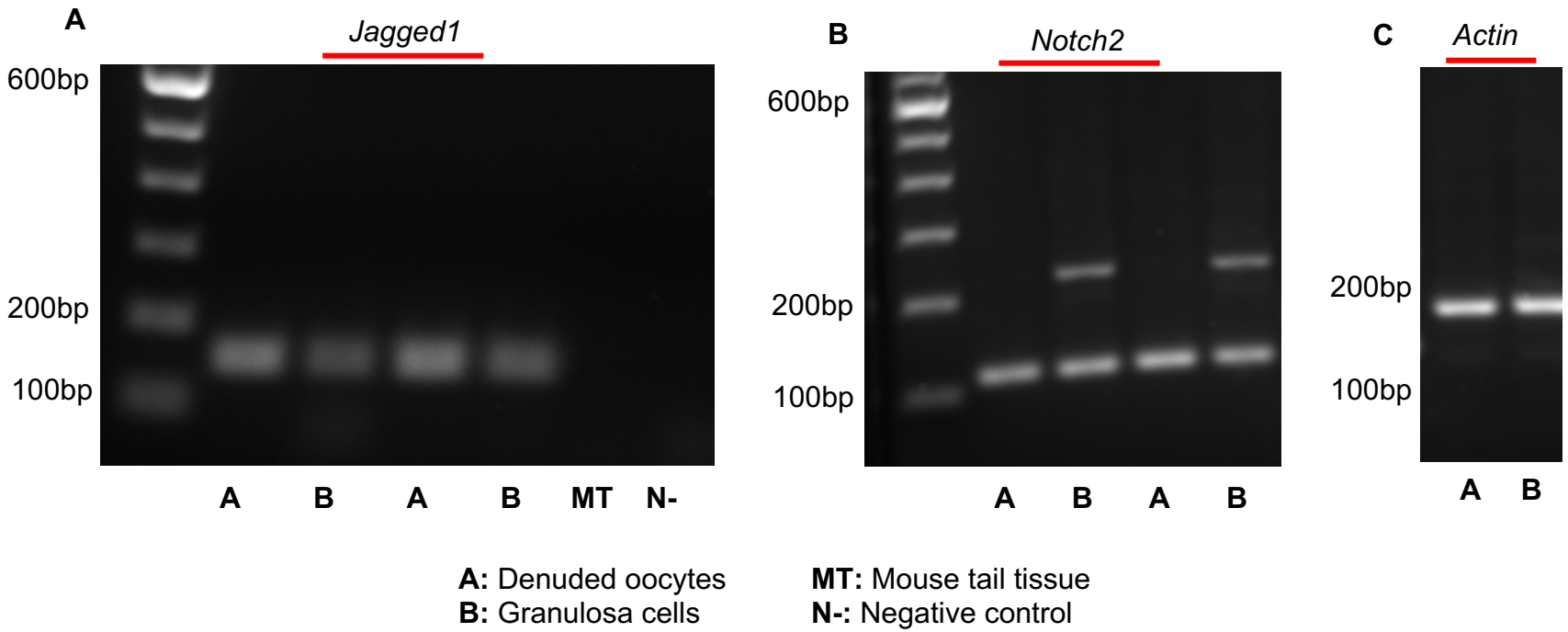

Figure 10 – *Jagged1* and *Notch2* mRNA expression in oocytes and granulosa cells. **(A)** *Jagged1* mRNA is present in both oocytes and granulosa cells (lane A and B). The chosen *Jagged1* primers amplify a 150 bp product. The bands in the gel migrate at the expected position, between 100 and 200 bp in both samples. Similar results are seen in both replicates. **(B)** *Notch2* mRNA is present in granulosa cells only (lane B). The Notch2 primers amplify a 226 bp product. The bands in the gel migrate at the expected position between 200 and 300 bp in granulosa cells. Similar results are observed in both replicates. **(C)** RT-PCR for *Actin* was performed on both oocytes and granulosa cells as a loading control.

3.3 JAG1 protein expression in partly grown and fully-grown oocytes

3.3.1 Unexpected JAG1 protein size detected in oocytes

Having established the expression pattern of JAG1 in GOCs and its mRNA presence in both oocytes and granulosa cells, we wanted to study the protein expression of JAG1, specifically in oocytes. Since JAG1-expressing oocytes interact with NOTCH2-expressing granulosa cells at early stages of follicular development, we wanted to investigate the role oocyte-derived JAG1 plays in follicular growth.

To detect JAG1 protein in oocytes, we first collected GOCs and COCs from PD 10 and 19 (44 hours after PMSG injection) mice to span the follicular growth phase. The complexes were stripped off of granulosa or cumulus cells to obtain entirely denuded oocytes. 100 partly grown oocytes obtained from GOCs, and 100 fully-grown oocytes obtained from COCs, were isolated separately and an immunoblot for anti-JAG1 was performed on both samples. The anti-JAG1 antibody used for western blots detects the C-terminal domain of JAG1 as well. The molecular weight of JAG1 is 134 kDa which migrates around 150 kDa in western blots. However, the immunoblot in Figure 11A reveals an unanticipated strong band at ~20 kDa in both partly grown and fully-grown oocytes. A band is perceivable at 150kDa, but it is rather faint. Anti-ACTIN immunoblot was also run on those samples as a loading control (Figure 11B). These results indicate that oocyte-derived JAG1 may exist in different forms in oocytes.

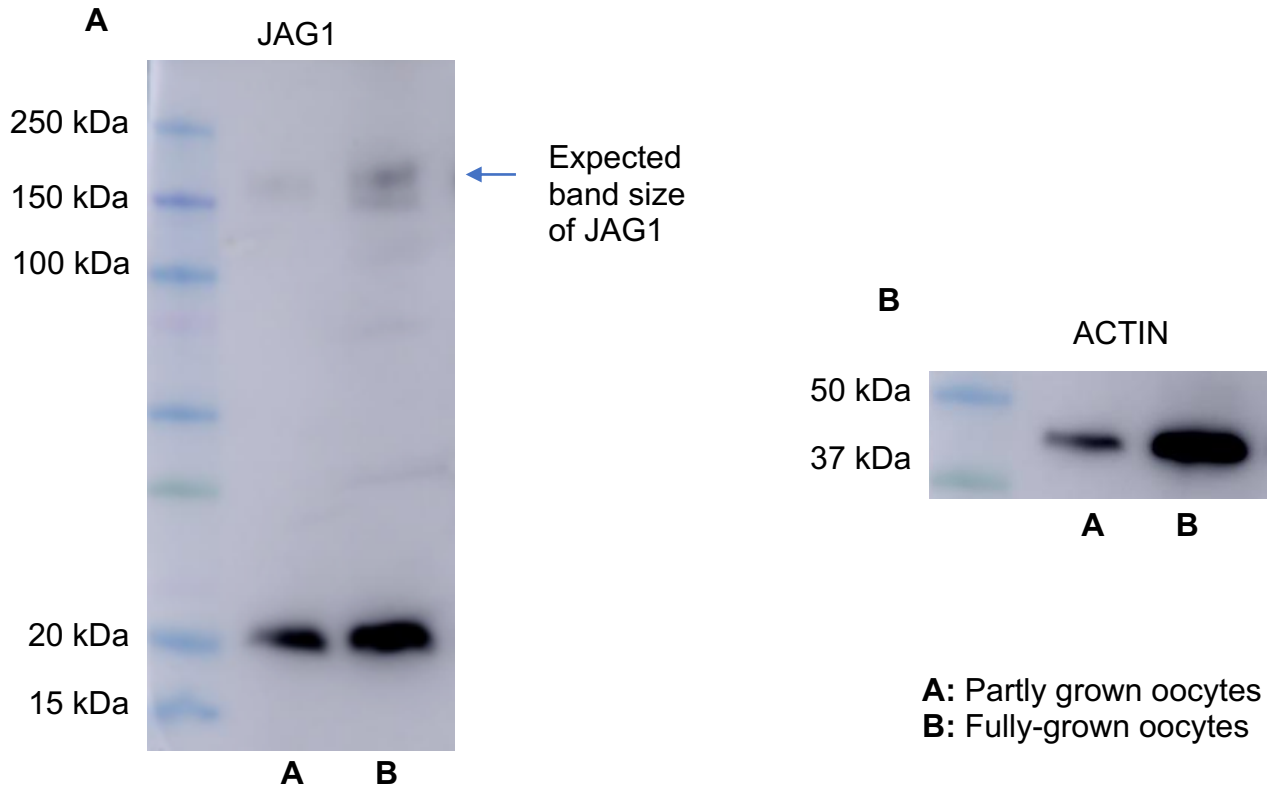

Figure 11 – Unexpected JAG1 protein size detected in partly grown and fully-grown oocytes. **(A)** 100 partly grown granulosa-free oocytes (lane A) and 100 fully-grown cumulus cell-free oocytes (lane B) were subjected to anti-JAG1 immunoblotting. The expected molecular weight of JAG1 is 134 kDa, which migrates around 150 kDa in western blots. A strong band is revealed around 20 kDa, while the 150 kDa band is rather faint. **(B)** Immunoblot of partly grown oocytes (lane A) and fully-grown oocytes (lane B) detecting ACTIN as loading control.

3.3.2 JAG1 ligand undergoes processing similarly to NOTCH2 receptor

Jagged1 protein is known to undergo processing in the developing mammalian lens (Azimi and Brown, 2019). The possibility that JAG1 ligand could be cleaved similarly to NOTCH2 receptor was investigated in the developing mammalian lens. Predicted forms and molecular weights of JAG1 were established based on the consensus cleavage sites of NOTCH2. According to their results, three forms of JAG1 exist, a 134 kDa full-length JAG1 (FL-Jag1), a 24 kDa JAG1 C-terminal fragment (Jag1-CTF) and a 14 kDa JAG1 intracellular domain (JICD) (Figure 12A). All three forms of JAG1 are detected in embryonic lens tissue, shown in the western blot in Figure 12B. They each appear at their respective sizes in the blot, as postulated in panel A. All three forms were detected using a JAG1 C-terminal specific antibody, which can detect all forms of JAG1 and can be differentiated with their molecular weights.

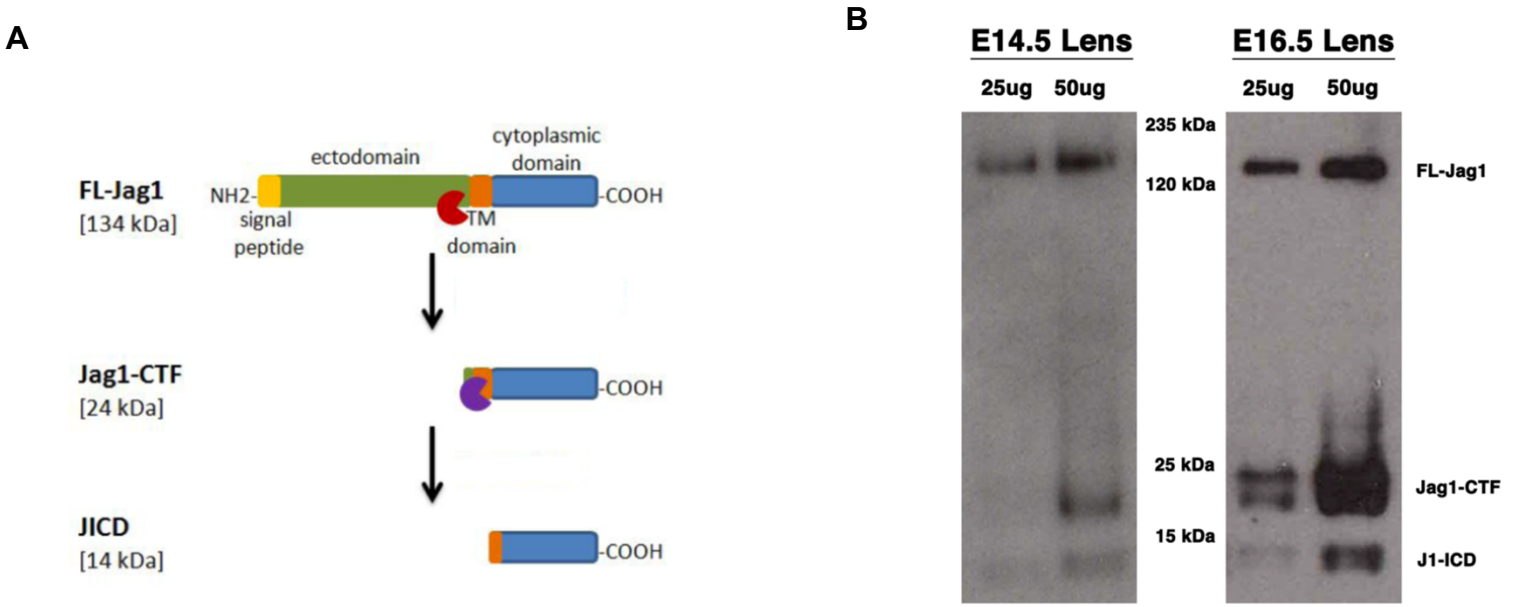

Figure 12 – Three forms of JAG1 ligand exist in embryonic lens tissue**. (A)** A schematic depicts the three different forms JAG1 ligand assumes after undergoing proteolytic cleavages by ADAM10 (red symbol) and γ-secretase (purple symbol), FL-Jag1 (134 kDa), Jag1-CTF (24 kDa) and JICD (14 kDa). **(B)** Western blot analysis using a C-terminal specific JAG1 antibody recognizes all three forms of JAG1 in the developing lens, distinguished by their distinct molecular weights (Azimi and Brown, 2019).

3.3.3 Establishing Model 1

Keeping in mind the lower band seen in the immunoblot and the new information gained from the publication above, a model is proposed to understand the possible JAG1 ligand processing in mouse ovarian follicles (Figure 13). Given the location of JAG1 ligand and NOTCH2 receptor in granulosa-oocyte complexes, and that Jagged-Notch signaling is a contact-dependent signaling, the complex can be easily manipulated to separate both ligand and receptor. In Model 1, two cases are shown to demonstrate the different cleaved forms of JAG1 ligand in oocytes. First, in the case where the oocyte lies within its complex, JAG1 ligand in oocytes has access to NOTCH2 receptors located in the surrounding granulosa cells. Jagged-Notch signaling will initiate causing cleavage of JAG1 ligand and creating two cleavage products, JAG1-CTF and JICD (Figure 13A). Second, in the case where the oocyte does not reside within its complex and the granulosa cells are absent, JAG1 ligand in oocytes would not have access to NOTCH2 receptors. Thus, Jagged-Notch signaling would not activate, leaving the oocyte retaining the full-length form of JAG1 ligand, FL-JAG1 (Figure 13B).

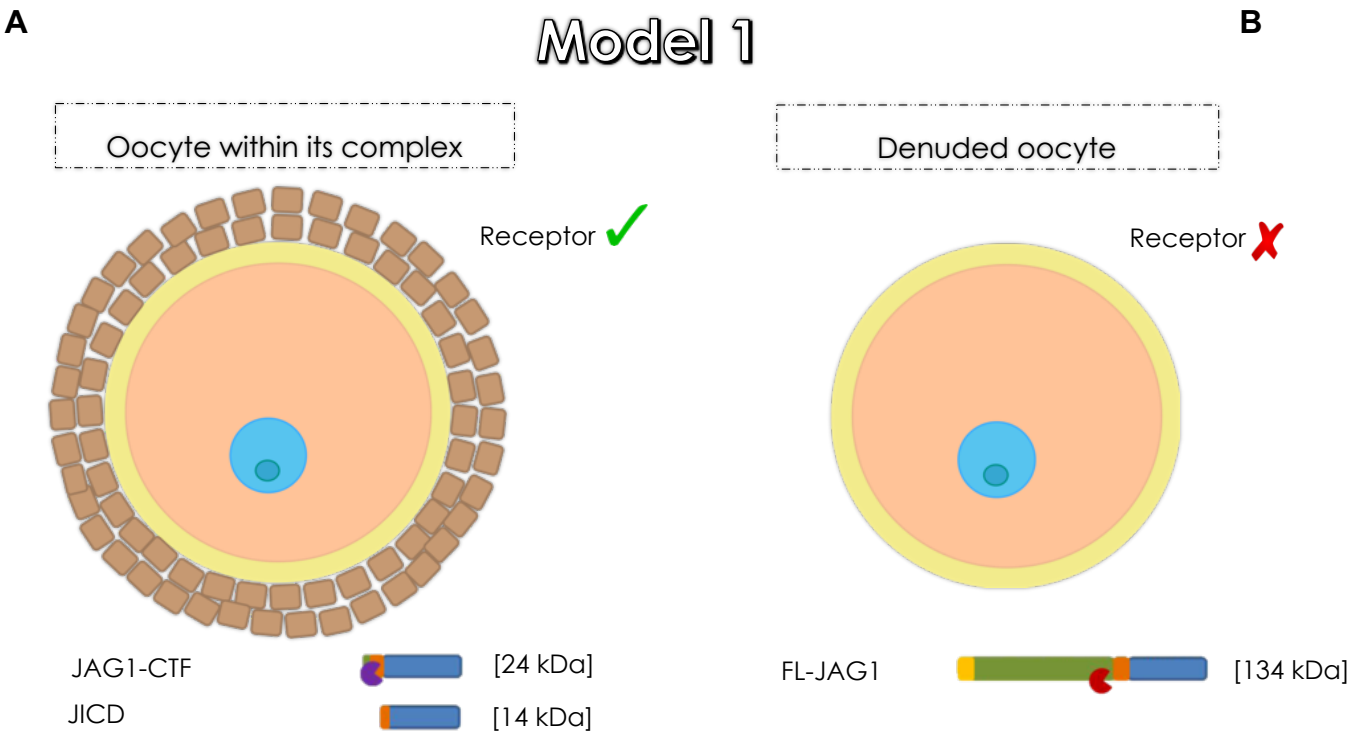

Figure 13 – A schematic showing the different cleaved forms of JAG1 depending on the state of the oocyte. **(A)** Oocyte residing within its complex has direct contact with granulosa or cumulus cells. The NOTCH2 receptor in granulosa cells will contact JAG1 ligand in oocytes and cleave it, yielding two cleavage products, JAG1-CTF and JICD. **(B)** Oocyte deprived of granulosa cells or cumulus cells has no contact with its surrounding somatic cells. The NOTCH2 receptor being absent cannot bind to JAG1 in the oocyte and cleave it, leaving JAG1 ligand in its full-length form (FL-JAG1).

3.3.4 A low and a high molecular weight of JAG1 ligand are detected in oocytes

Having proposed a model demonstrating the possible JAG1 ligand processing in oocytes, we then tested whether JAG1 ligand undergoes cleavages in the presence and absence of granulosa or cumulus cells. To experimentally approach this idea, we collected GOCs and COCs from PD 10 and 19 (44 hours after-PMSG injection) mice. The granulosa and cumulus cells were removed to isolate partly grown and fully-grown oocytes. The cell-free oocytes were immediately prepared for cell lysis and immunoblotting. During both collections, the oocytes were in direct contact with their respective somatic cells. A similar collection was performed except the partly grown and fully-grown oocytes were incubated overnight in the absence of granulosa or cumulus cells. As shown in the previous experiment, in Figure 11A, the anti-JAG1 immunoblot reveals a ~20kDa band in the partly grown and fully-grown oocytes that were in immediate contact with the granulosa cells. The same blot is shown in Figure 14A (lane A and B) for reference purposes. The partly and fully-grown oocytes that were deprived of the granulosa cells for an overnight period reveal a 150kDa band in the immunoblot, seen in lane C and D. Thus, anti-JAG1 immunoblot reveals a low molecular weight of JAG1, corresponding to JAG1-CTF (24kDa), and a high molecular weight of JAG1, corresponding to FL-JAG1 (134 kDa) in oocytes. These data suggest that loss of contact between the oocyte and granulosa cells leaves JAG1 ligand uncleaved, in its full-length form, while cell-contact between oocyte and granulosa cells allows cleavage of JAG1.

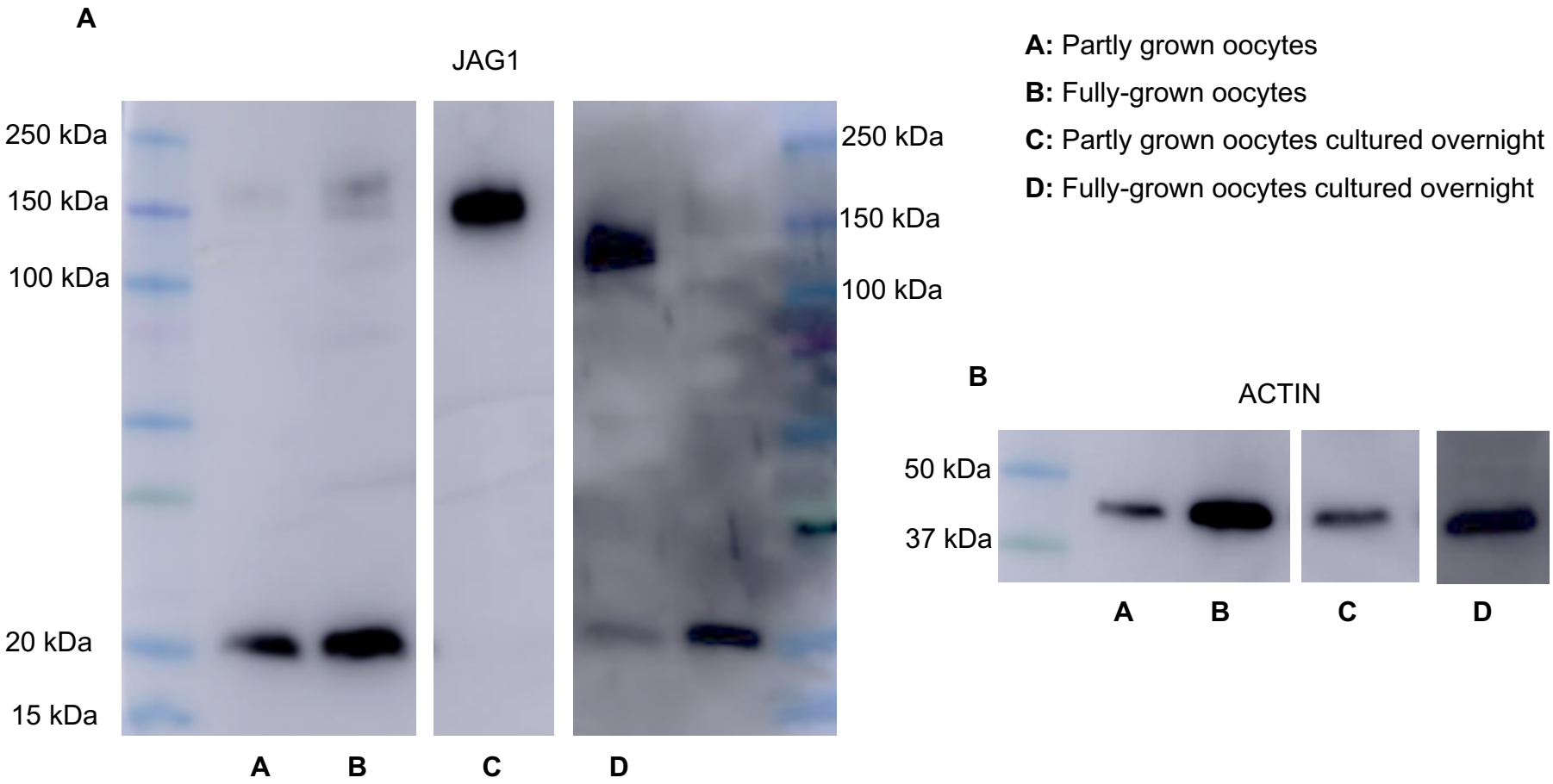

Figure 14 – Anti-JAG1 immunoblot detects two molecular forms of JAG1 in partly and fully-grown oocytes. **(A)** 100 partly grown granulosa-free oocytes (lane A) and 100 fully-grown cumulus-free oocytes (lane B) reveal a lower molecular weight of JAG1 with bands migrating at ~20 kDa. 100 partly grown granulosa-free oocytes cultured overnight (lane C) and 100 fully-grown cumulus-free oocytes cultured overnight (lane D) reveal a higher molecular weight of JAG1 with bands migrating around 150 kDa. **(B)** Anti-ACTIN immunoblot was performed on these samples as a loading control.

3.3.5 Establishing Model 2

Having demonstrated that JAG1 ligand in oocytes undergoes cleavage in a cell-contact dependent manner, we next focused on the specific point of contact that mediates this communication between oocyte and granulosa cells, the transzonal projections. A second model is proposed to understand the role TZPs play in JAG1 ligand processing, between both cell types. In Model 2, two cases are shown to demonstrate the different cleaved forms of JAG1 in oocytes, in the presence and absence of TZPs. First, in the case where the oocyte resides within its complex, while the granulosa cells and TZPs are present, the JAG1 ligand in oocytes has access to the granulosa cell-derived NOTCH2 receptors nearby. This interaction would lead to the cleavage of JAG1 yielding two cleavage products, JAG1-CTF and JICD (Figure 15A). Second, in the case where the oocyte resides within its complex but the TZPs have undergone retraction after cumulus cell layer expansion, JAG1 ligand does not have access to NOTCH2 receptors located in the surrounding cumulus cells. This would leave JAG1 in its full-length and uncleaved form, FL-JAG1 (Figure 15B).

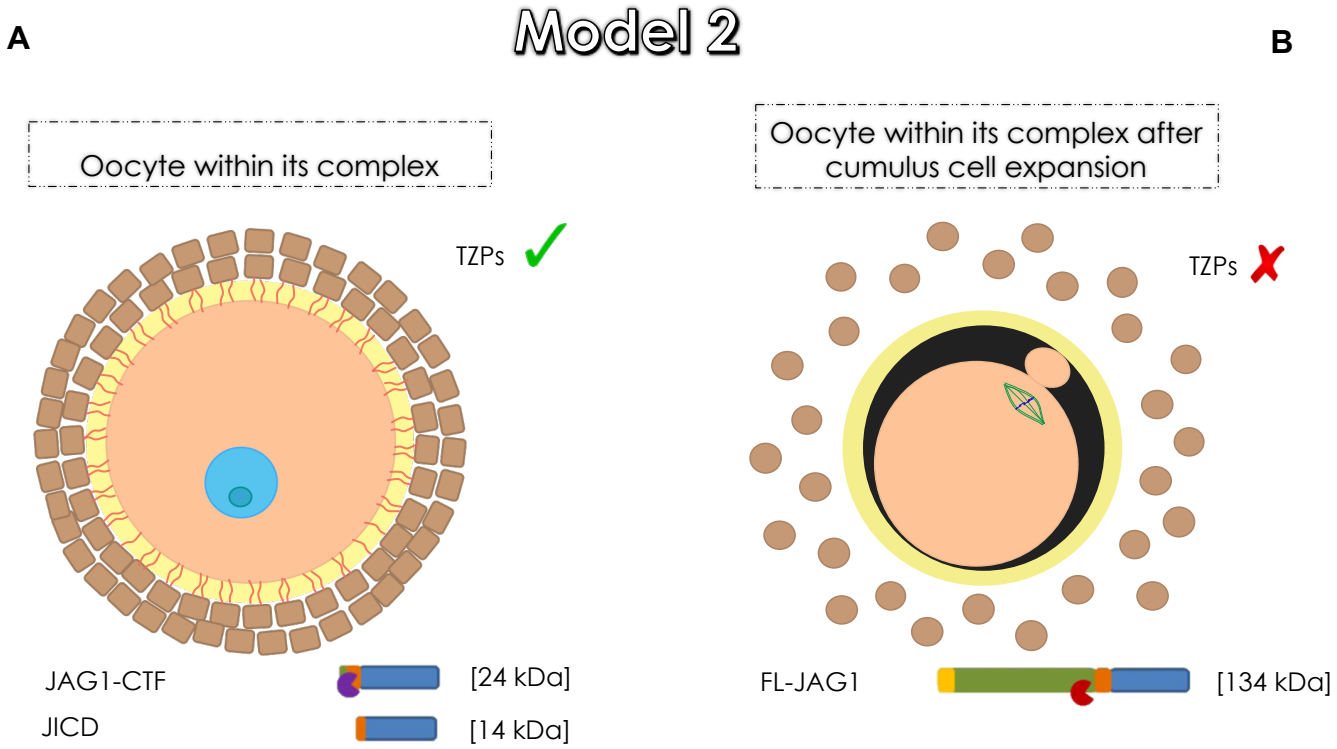

Figure 15 – A schematic showing the different cleaved forms of JAG1 depending on the state of the oocyte. **(A)** Oocyte residing within its complex has direct contact with granulosa cells through the TZPs. The NOTCH2 receptor will contact JAG1 ligand and cleave it, giving two cleavage products, JAG1-CTF and JICD. **(B)** Oocyte within its complex after cumulus cell layer expansion. The cumulus cells are present around the oocyte but the TZPs have retracted. The NOTCH2 receptor cannot bind to JAG1 and cleave the ligand, leaving JAG1 ligand in its full-length form, FL-JAG1.

3.3.6 Communication between oocyte and granulosa cells provides cleavage of JAG1

Having proposed the second model, we then tested whether JAG1 ligand undergoes cleavage in the presence of TZPs. We collected GOCs from PD 10 mice and cultured them for an overnight period. The following day, the GOCs were retrieved from the culture and the granulosa cells were stripped off to isolate the partly grown oocytes for cell lysing and immunoblotting. The anti-JAG1 immunoblot reveals a very strong ~20 kDa band in the partly grown oocytes that were within granulosa cell-oocyte complexes overnight, seen in lane A and lane D (Figure 16A). The granulosa cells and the TZPs were present and intact throughout the culture. Thus, the data suggest that JAG1 ligand in oocytes is cleaved by NOTCH2 receptors in granulosa cells with the presence of TZPs, giving the cleavage product of JAG1-CTF.

To test whether JAG1 ligand undergoes cleavage in the absence of TZPs, COCs were collected from PD 19 mice (44 hours post PMSG injection) and cultured in the presence of EGF for an 8-hour period. Following the 8-hour incubation, the COCs were retrieved and denuded of cumulus cells to isolate the oocytes for cell lysis and immunoblotting. The anti-JAG1 immunoblot reveals a strong band at 150 kDa and a slightly weaker band at ~ 20kDa band in lane E (Figure 16A). The high molecular form of JAG1 or FL-JAG1 could have appeared when the TZPs retracted completely, losing its contact with the oocyte, in COCs. The lower molecular form of JAG1 or JAG1-CTF could have appeared when TZPs were still in contact with the oocyte at the beginning of the culture and may have not undergone complete degradation in the oocyte, thus revealing a band in the blot. These data suggest that cleavage of JAG1 ligand in oocyte is mediated by TZPs that provide the NOTCH2 receptors from the granulosa cells.

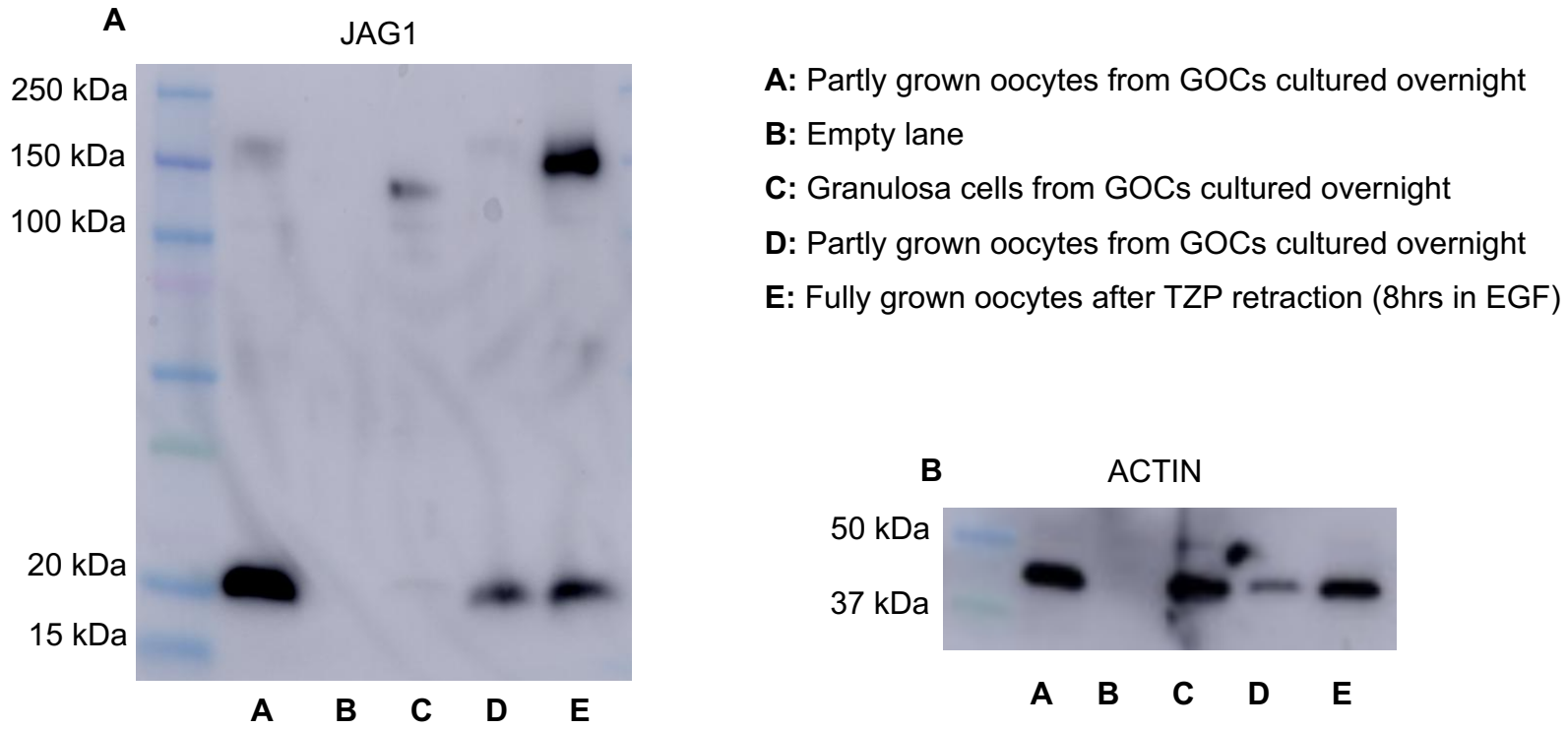

Figure 16 – Anti-JAG1 immunoblot detects two molecular forms of JAG1 in oocytes with the presence and absence of TZPs. **(A)** 100 partly grown oocytes from GOCs cultured overnight (lane A) reveal a low molecular weight of JAG1 with a band at ~20 kDa. Empty lane B. Granulosa cells from GOCs cultured overnight reveal a weak band migrating between 100 and 150 kDa. (lane C). 50 partly grown oocytes from GOCs cultured overnight also reveal a band at ~20 kDa (lane D). 100 fully-grown oocytes from COCs after cumulus cell layer expansion reveal bands at 150 kDa and ~20 kDa (lane E). **(B)** Anti-ACTIN immunoblot was performed on these samples as a loading control.

Chapter 4 – Discussion and Conclusion

4.1 Discussion

Putting all the results together, it is perceivable that JAG1 ligand undergoes processing in oocytes. The presence of a high and low molecular weight JAG1 in the immunoblots are important indications that ligand cleavage occurs in the oocyte. Figure 17 shows a diagram with the existing Notch signaling that occurs between oocyte and granulosa cells. NOTCH2 receptor undergoes proteolytic cleavages in the granulosa cells following ligand binding and activates Notch transcription genes. With the results obtained above, we show that JAG1 ligand also undergoes cleavages in the oocyte following ligand binding, giving rise to a cleavage product. Thus, two forms of JAG1 exist of which FL-JAG1 (~150kDa) and JAG1-CTF (~20kDa) show detection in murine oocytes.

Our results indicate that Jagged-Notch signaling is active in mouse ovarian follicles. Staining for cleaved-NOTCH2 in partly denuded GOCs not only show the presence of a NOTCH2 receptor in the granulosa cells but also indicate activation of Notch signaling in granulosa cells. However, it is unclear as to why NOTCH2 is undetected in the nucleus. The anti-NOTCH2 antibody detects the receptor after γ-secretase cleavage, which release the NICD into the cytoplasm and nucleus. It is possible that the epitope of the Notch receptor becomes masked once it enters the nucleus and interacts with other transcription factors. It is also possible that something limiting its translocation to the nucleus in the first place.

The expression of JAG1 in GOCs confirms the presence of a NOTCH2-specific ligand in mouse ovarian follicles and is presumably the source of activation of Notch signaling in the follicle. It is interesting to see that both NOTCH2 and JAG1 localize in granulosa cells' cytoplasm. Perhaps, granulosa cell-derived JAG1 activates Notch signaling in granulosa cells that are not in contact with the oocyte. However, we were interested in the presence of JAG1 observed in the oocyte, which could play a role in initiating Notch signaling in the cells immediately adjacent to the oocyte.

The mRNA expression of both *Jagged1* and *Notch2* genes not only confirms their presence in oocytes and granulosa cells but also validates the expression patterns seen in the immunofluorescence images described above. Non-specific bands are detected in oocytes and granulosa cells in *Notch2* RT-PCR. However, the bands detected in granulosa cells migrating between 200 and 300 bp are specific to this cell type only. Moreover, it is not surprising to see *Jagged1* expression in granulosa cells of growing follicles since it has been shown to localize in somatic cells later in growth. However, it remains possible that oocyte-derived *Jagged1* plays an important role in Notch signaling during follicular growth.

Cell-contact between oocytes and granulosa cells plays an important role in JAG1 ligand processing in the oocyte. Just as the results from the immunoblots indicate, in the presence of granulosa or cumulus cells around oocytes, ligand-receptor interaction takes place and allows cleavage of JAG1 in oocytes by NOTCH2 in granulosa cells. The absence of these somatic cells, however, leaves the oocyte deprived of the receptor preventing ligand-receptor interaction and JAG1 cleavage.

Moreover, the presence of the low (JAG1-CTF) and high (FL-JAG1) molecular forms of JAG1 could be an indicator of Jagged-Notch signaling activation. When the contact is present between oocyte and granulosa cells, JAG1 in oocytes can bind to NOTCH2 receptor in granulosa cells. This interaction would cause JAG1 cleavage into JAG1-CTF and the activation of Jagged-Notch signaling. Loss of contact between oocyte and granulosa cells, however, causes JAG1 ligand to be left uncleaved, in its full-length form, in the absence of NOTCH2 receptor, and not activating Jagged-Notch signaling.

While in the presence of granulosa-oocyte contact, JAG1 underwent cleavage and gave rise to a cleavage product of ~20 kDa. However, the last cleavage product of JAG1, the JICD, was not detected in the immunoblots. No band lower than ~20 kDa was detected in the blots. It is possible that the endogenous level of JICD is low in oocytes, rendering it undetectable by the antibody, or that such cleaved form does not exist in the mammalian oocyte. Future work will address the potential role of the JAG1 cleavage products.

Not only is cell-contact important in mediating Jagged-Notch signaling and ligand processing in mouse ovarian follicles but so are the sites of physical contact between oocytes and granulosa cells, the TZPs. Our results clearly show two different forms of JAG1 ligand in the presence and absence of TZPs. In fact, oocytes that underwent cumulus cell layer expansion demonstrate a good example of TZP-mediated communication between both cell types. Though the cumulus cells are still present, the gradual loss of cell-contact between oocyte and cumulus cells, caused by TZP retraction, prevents ligand-receptor interaction, as shown by the presence of the full-length form of JAG1 in oocytes. Put in another way, TZPs could be physically providing NOTCH2 receptors

from the granulosa cells to the oocyte under normal conditions. Thus, these results reveal a new role for TZPs in regulating essential ligand-receptor signaling between the oocyte and its somatic environment. Importantly, activation of Jagged-Notch signaling in the ovarian follicle leads not only to cleavage of the NOTCH2 receptor in the granulosa cells, but also to cleavage of the JAG1 ligand in the oocyte.

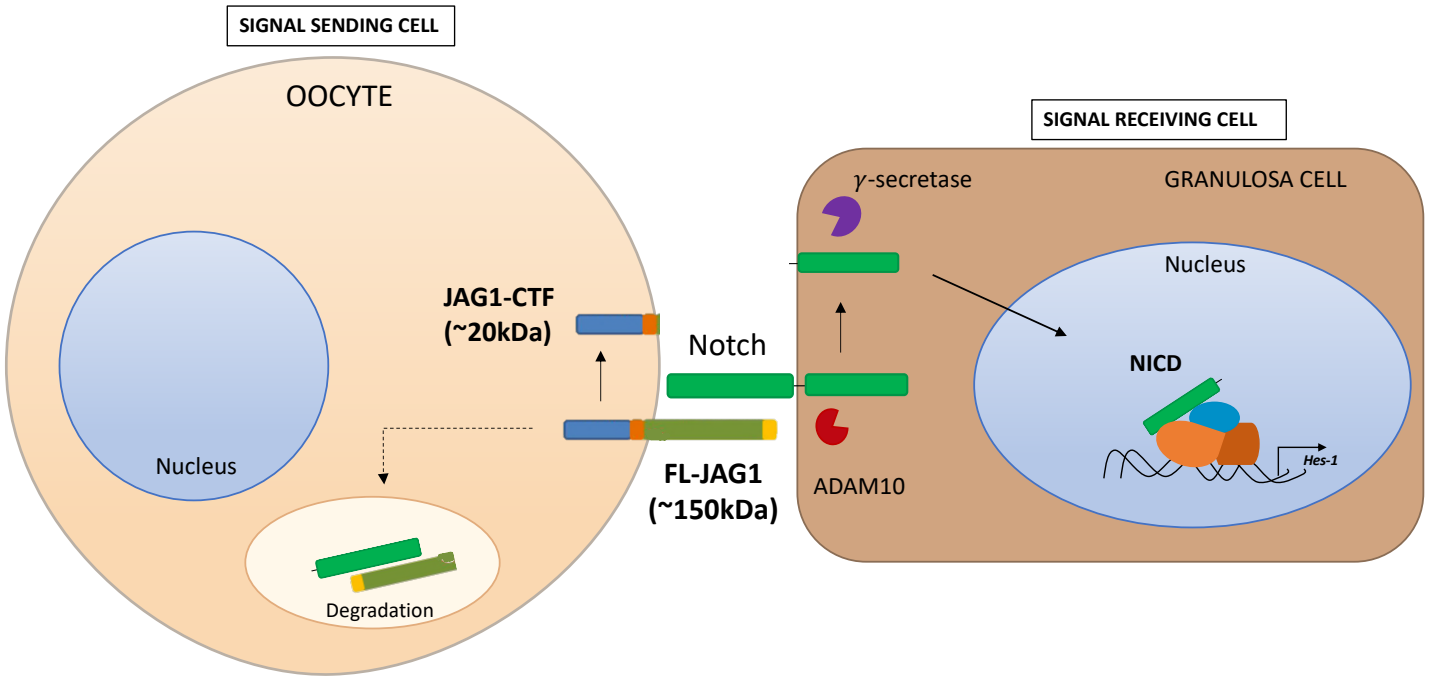

Figure 17 – JAG1 ligand processing in the oocyte. The existing Notch signaling between oocytes and granulosa cells is depicted in this diagram along with JAG1 processing in oocytes. NOTCH2 receptor located in granulosa cells binds to JAG1 in oocytes and undergo proteolytic cleavages by ADAM10 and γ-secretase, ultimately leading to the activation of Notch target genes by NICD in the granulosa cell nucleus. In the oocyte, following ligand binding, JAG1 also undergoes cleavages. Two forms of JAG1 exist of which FL-JAG1 and JAG1-CTF show detection in murine oocytes.

4.2 Conclusion

Taken together, these data reveal an important role for the oocyte in JAG1 ligand processing with the activation of Notch signaling. They support a model in which Notch ligands such as JAG1 from the oocyte act on Notch receptors, mainly NOTCH2, on granulosa cells by cell-contact between oocyte and granulosa cells and TZPs. This interaction not only leads to the activation of Notch signaling but also to the cleavage of JAG1 ligand in oocytes. The results also reveal a new function for TZPs in mediating contact-dependent signaling between the oocyte and its somatic cells.

Much work remains to establish whether cleaved-JAG1, notably JICD, can influence gene transcription in oocytes, as NICD does in granulosa cells. Investigating the potential roles of JAG1 cleavage products could further inform on the process of bidirectional Notch signaling. Understanding cell communication pathways such as Jagged-Notch signaling could help us better understand the mechanisms involved in the healthy development of the ovarian follicle.

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