THE ROLES OF HIPPO SIGNALING PATHWAY IN MOUSE OVARIAN FUNCTION

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by

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ABSTRACT

Mammalian follicular development is regulated by various secreted and gap-junction-mediated intercellular communication signals. This complicated network of regulatory and metabolic mechanisms is not completely known. The Hippo signaling pathway has been recognized as a highly conserved pathway among species that controls the organ size, stem cell functions and is also profoundly implicated in numerous human cancers and other diseases. Lats1 mutant mice exhibit follicle depletion and develop ovarian stromal tumors, while its effector YAP1 is not only recognized as an ovarian oncoprotein, but also found to promote follicular growth when actin polymerization is initiated by ovarian physical disruption. These studies demonstrated the potential importance of Hippo signaling in ovarian function, but little is known about what roles this pathway plays during early follicular development and ovulation. Our study first utilized Lats1 mutant mice and the YAP1 inhibitor verteporfin to study the roles of Hippo pathway in early follicular development and found that LATS1 is required for maintaining the primordial germ cell pool and both LATS1 and YAP1 are essential for follicle activation. Then we established a new mechanistic model for the regulation of ovulation, by demonstrating that oocytes induce granulosa cell proliferation by inhibiting the expression of Hippo components before ovulation, while ovulatory signals promote granulosa cell differentiation by activating the Hippo signaling activity in a timely manner during ovulation. Lastly, we accentuated the importance of Hippo pathway during ovulation by showing that blocking YAP1 leads to spindle deformation during oocyte maturation. More importantly, pYAP1 at T119 site is induced through meiotic progression and was shown to be a target of PP2A phosphatase. However, it’s unclear if this regulation of pYAP1 plays important roles in actin cytoskeleton-mediated spindle assembly and migration during oocyte meiosis. Together, our findings contributed to our understanding of
the impact of Hippo pathway in early follicular development and how it mediates the ovulatory transition in cumulus granulosa cells and oocytes.
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ABBREVIATIONS

AMH---Anti-Müllerian Hormone
ASPP---Apoptosis Stimulating Protein of P53
ARP---Actin Related Protein
ALK---Activin receptor-Like Kinase
AREG---Amphiregulin
BMP---Bone Morphogenetic Protein
bFGF---basic Fibroblast Growth Factor
CL---Corpus Luteum
CCND2---Cyclin D2
COCs---Cumulus Oocyte Complexes
CEEF---Cumulus Expansion Enabling Factor
COX2---Cyclooxygenase 2
Crb---Crumbs (cell polarity protein)
CTGF---Connective Tissue Growth Factor
CB---Cytochalasin B
CDK---Cyclin Dependent Kinase
DNMTs---DNA Methyltransferases
Dpp---Decapentaplegic
DAB---3,3’-Diaminobenzidine
EGF---Epidermal Growth Factor
ERK---Extracellular Signal Regulated-Kinases
EX---Expanded (an upstream regulator of Hippo kinases)
ESC---Embryonic Stem Cells
ECM---Extracellular Matrix
FOXL2---Forkhead Box L2
FOXO3---Forkhead Box O3
FIGLA---Factor in the Germline Alpha
FSH(R)---Follicle-Stimulating Hormone (Receptor)
FGO---Fully Grown Oocytes
FBS---Fetal Bovine Serum
FMN2---Formin 2
GDF9---Growth Differentiation Factor 9
GCNF---Germ Cell Nuclear Factor
GnRH---Gonadotropin-Releasing Hormone
GVBD---Germinal Vesicle Break Down
GSK---Glycogen Synthase Kinase
GPCR---G Protein Coupled Receptor
Hpo---Serine/Threonine Kinase Hippo
IL---Interleukin
IVF---*In vitro* Fertilization
JASP---Jasplakinolide
KL---Kit Ligand
KIBRA (WWC1)---Kidney and Brain Expressed (WW domain-containing) Protein
LATS---Large Tumor Suppressor
LHX8---LIM Homeobox Protein 8
LH---Luteinizing Hormone
LIF---Leukemia Inhibitory Factor
Lgl---Lethal Giant Larvae
mTORC---mammalian Target of Rapamycin Complex
MOB---Mps One Binder (adaptor protein in Hippo signaling)
MATS---MOB as Tumor Suppressor
MPF---Maturation Promoting Factor
MI---Meiosis I
MII---Meiosis II
MASTL--- Microtubule-Associated Serine/Threonine Kinase-Like
MAPK--- Mitogen-Activated Protein Kinases
MER---Merlin (an upstream regulator of Hippo kinases)
MST (STK)---Mammalian Sterile20-like Kinase (Serine/Threonine-Protein Kinase)
NOBOX--- Newborn Ovary Homeobox
NPPC---Natriuretic Peptide Precursor type C
NSN--- Non-Surrounded Nucleolus
OCT4---Octamer-binding Transcription Factor 4
OA--- Okadaic Acid
OOX---Ooctectomized complexes
OSFs---Oocyte Secreted/Specific Factors
PP2A--- Phosphatase protein 2A
PGC---Primordial Germ Cell
PARP--- Poly ADP Ribose Polymerase 1
PDE--- Phosphodiesterase
PDGF--- Platelet-derived growth factor
PGR---Progesterone Receptor
PGF2α--- Prostaglandin F2 alpha
PTEN---Phosphatase and Tensin Homolog
PARs---Protease-Activated Receptors
POI---Primary Ovarian Insufficiency
POF---Premature Ovarian Failure
PCOS—Polycystic Ovarian Syndrome
PMSG (eCG)—Pregnant Mare’s Serum Gonadotropin
PLC—Phospholipase C
RPTK—Receptor Protein Tyrosine Kinase
RASSF—Ras-Association domain Family
RUNX—Run-related transcription factor
SOX9—SRY (Sex Determining Region Y)-Box 9
SOHLH1/2—Spermatogenesis and Oogenesis Specific Basic Helix-Loop-Helix 1/2
SDF-1—Stromal cell-Derived Factor 1
SMAD—portmanteau of SMA (from C elegans) and mothers against decapentaplegic (MAD)
SAV—Salvador (adaptor protein in Hippo signaling)
SN—Surrounded Nucleolus
TGFβ—Transforming Growth Factor β
TNFα—Tumor Necrosis Factor α
βTRCP—Beta-Transducin Repeat Containing E3 Ubiquitin Protein
TZPs—Transzonal Projections
TEAD—TEA Domain Family Member
VP—Verteporfin
VGLL—Vestigial-Like Family Member
WNT—Wingless-Type MMTV Integration Site Family
WTS—NDR family kinase Warts
WWTR (TAZ)—WW domain-containing transcription regulator protein
YAP—Yes-Associated Protein
ZP—Zona Pellucida
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Chapter 1

Introduction

A mammalian ovary is a highly organized reproductive and endocrine organ that is responsible for producing fertilizable oocytes and steroid hormones. A follicle is a basic functional unit of the ovary. It consists of an oocyte and its surrounding granulosa cells and theca cells. Between follicles are the inter-follicular stromal cells, endothelial cells and fibroblasts. Starting in embryos, primordial germ cells migrate to the genital ridge to form germline cysts. Then cysts break down and individual primordial follicles are assembled with a squamous layer of granulosa cells. The follicle activation is represented by the appearance of cuboidal granulosa cells. After leaving the resting pool, follicles continue to grow through pre-antral and antral stages. Follicles finally undergo ovulation and luteinization. In the mammalian ovarian environment, various secreted and gap-junction-mediated intercellular communication signals regulate follicular development and ovulation so that competent oocytes are released at the time of ovulation, but this complicated network of regulatory and metabolic communications is not completely known.

The Hippo pathway was discovered over a decade ago and is a highly conserved pathway that regulates organ growth in many species. Its deregulation has been found in many human diseases including lung, breast, colorectal cancers and brain, liver, pancreatic and renal carcinomas [1]. More and more cellular mechanisms involving the Hippo signaling have been uncovered since its discovery. Particularly in the ovaries, Lats1 knockout mice have fewer ovarian follicles and can develop ovarian stromal tumors. LATS1 is an upstream kinase in the Hippo pathway and its downstream
transcriptional coactivator YAP1 is recognized as an ovarian cancer oncoprotein. Moreover, recent studies on the fragmentation of both human and mice ovaries showed that disrupting ovarian Hippo signaling leads to stimulation of follicular growth. These studies demonstrate the potential importance of Hippo signaling in ovarian function, but little is known about how this pathway is regulated and the role it plays during follicular development and ovulation. The broad hypothesis that will be tested in this dissertation is that the Hippo signaling pathway plays an indispensable role in early follicular development, granulosa cell proliferation and differentiation and oocyte maturation during ovulation. To test this hypothesis, the following specific aims were formed.

1. Determine the expression patterns of the Hippo pathway components during normal mouse follicular development and during ovulation.
2. Determine the importance of Hippo pathway kinase LATS1 in early follicular development, using Lats1 global knockout mice.
3. Interrogate the importance of YAP1 in early ovarian development, using a small molecule inhibitor verteporfin and tamoxifen-induced Yap-Cre mouse model.
4. Investigate the roles the Hippo pathway plays in modulating granulosa cell proliferation and differentiation during the peri-ovulatory transition.
5. Determine the possible roles of YAP1 during oocyte meiotic maturation.
Chapter 2

Literature Review

Primordial Germ Cell Development and Follicle Assembly

Originating from proximal epiblast cells, mouse primordial germ cell (PGC) precursors first appear at the posterior rims of embryonic disc at E6.25 after the onset of gastrulation. Around E7.25, PGCs appear at the visceral mesoderm around the base of allantois. From there, PGCs proliferate and migrate via the primitive mesentery and hindgut, to the developing “indifferent” gonad (identical at this time in males and females) [2]. Around E10.5, germline cysts start to form in ovigerous cords, surrounded by basement membrane [3, 4]. Kit ligand, its receptor c-kit, extracellular matrix protein fibronectin and OCT4 (Octamer-binding transcription factor 4), all play important roles in the maintenance of the pluripotent properties of migrating germ cells [5-7]. Gonadal sex differentiation occurs around E12.5 [8]. The classical theory states that the female sex differentiation is a default/passive process. However, recent studies demonstrated that embryonic female sex determination is in fact an active process [9]. Factors that promote female sexual differentiation by maintaining the Müllerian duct and that suppress male sexual differentiation by restraining the expression of SOX9 (germ cell specific gene) include WNT4 (a wingless type MMTV integration site protein), its regulator R-spondin (a thrombospondin protein, WNT pathway adaptor protein), FOXL2 (forkhead box L2, one of the earliest markers of ovarian differentiation) [9] and FIGLA (factor in the germ cell α) [10]. In fact, female mice lacking both WNT4 and FOXL2 have complete reversal phenotype of the ovary to a testis [11]. While the gonad differentiates into an ovary, PGCs continue to divide by mitosis and reaches a peak level around E13.5 [12] when
germ cell cyst formation is complete. The PGC mitosis is under the regulation of TGF-β (transforming growth factor) family members such as BMP 2, 4, 7 (bone morphogenetic protein) [13]. Oogonia and pre-granulosa cells in clusters are isolated from the stroma by a basal lamina [14]. It has been recognized that pre-granulosa cells are derived from surface epithelium through two waves. The first wave (embryonic wave) occurs just before sex differentiation and the second wave (neonatal wave) occurs right after birth during cyst breakdown [15-17]. While follicles containing granulosa cells recruited in the first wave were thought to be largely anovulatory [17], a recent study using a tamoxifen-induced Foxl2 knock-in mouse model showed that there are first-wave follicles in ovaries up to 3-month of age, indicating that the first-wave follicles contribute to growing follicle pool both before puberty and immediately after puberty [18]. The authors also showed functional differences between these two waves, in that the development of the first wave follicles to reach antral stage is twice as fast as the second [18]. Once the germ cell cysts are formed around E13.5, germ cells start to enter meiosis and at E17.5 they start to be arrested at diplotene phase of prophase I. The cyst structures remain intact until soon after birth, when individual oocytes become enclosed by a layer of squamous somatic cells, a process named germ cell syncytia breakdown or follicle assembly. Improper syncytia breakdown could lead to polyovular follicles, such as in mice lacking activin, BMP15 and having altered NOTCH signaling [19-21]. The syncytia breakdown lasts from shortly after birth until around day 5 in mice. During this time, as many as two thirds of germ cells undergo developmentally regulated apoptotic process, which is controlled by TNF-α (tumor necrosis factor α) signaling [22, 23] and other factors such as anti-apoptotic Bcl-x and pro-apoptotic Bax [22, 24]. However, this process is not
positively associated with nuclear condensation, activation of caspase 3, cleavage of PARP1 (poly ADP ribose polymerase 1) or fragmentation of DNA, therefore is distinct from adult follicular atresia [25, 26]. Instead of undergoing programmed cell death, the majority of the dying germ cells are still associated with their sister cyst cells during the breakdown [25]. Some cyst cells act as nurse cells and control the development of their sister oocytes, by selectively transferring cellular components such as mitochondria and vesicles through intercellular bridges [27]. The endowed primordial follicle pool serves as a source for developing follicles and fertilizable oocytes for the entire reproductive lifespan.

Follicle Activation

The dormant primordial follicles are activated continuously to join the growing follicle pool. The initial follicle activation starts just after follicle formation and continues throughout the reproductive life while the rest of the primordial follicles remain dormant [28]. When primordial follicles are activated and become primary follicles, squamous granulosa cells become cuboidal, after which, the oocyte quickly increases in size. The process of follicle activation is regulated by oocyte-specific transcription factors and transcription factors from pre-granulosa cells, and pathways activated in both oocytes and pre-granulosa cells by growth factors and cytokines. The oocyte-specific transcription factors include FIGLA, NOBOX (newborn ovary homeobox-encoding gene), LHX8 (LIM homeobox protein 8), SOHLH1/2 (Spermatogenesis and oogenesis-specific basic helix-loop-helix) [29, 30]. FIGLA plays a key regulatory role not only in the initial organization of primordial follicles but also the survival of the germ cells. It acts by regulating the expression of NALP (NACHT, leucine-rich repeat and PYD-containing)
family [10] and oocyte specific genes that encode zona pellucida proteins [31]. NOBOX directly regulates the transcription of GDF9 (growth differentiation factor 9) and OCT4 and deletion of Nobox accelerates postnatal oocyte loss and abolishes the primordial-primary transition [29, 32]. Sohlh1 mRNA is present in oogonia and primordial oocytes but absent from primary stages onward. Deletion of Sohlh1 leads to no primary follicles in post-natal ovaries [30] and the downregulation of Nobox, Figla and Lhx8 [30, 33]. Sohlh2/- ovaries form morphologically normal primordial follicles but they fail to develop into primary follicles. In these follicles, oocytes mis-express germ cell specific genes (Sohlh1, Nobox, Figla, Gdf9, Zp1, Zp3, Kit, etc) and pre-granulosa cells remain flat and undifferentiated [34]. Although it is not quite clear how those essential transcription factors interact with each other to control oocyte growth, studies have uncovered that mutations in Nobox and Figla lead to POF (premature ovarian failure) in both mice and human [29, 35, 36]. In pre-granulosa cells, the transcription factor FOXL2 is essential for mediating the squamous-to-cuboidal transition and as well as oocyte growth. Without FOXL2, oocytes undergo premature growth and lose their synchronization with the proper differentiation of surrounding granulosa cells [37, 38]. Furthermore, FOXL2 continues to play important roles in maintaining ovarian phenotypes by suppressing testis-specific genes throughout adult life [39].

The PI3K pathway plays essential roles during the development of immature follicles [40]. KL (kit ligand) was shown to activate PI3K pathway in oocytes which results in phosphorylation of Akt and FOXO3a (Forkhead Box O3) [41]. Phosphorylation of FOXO factors by Akt exports them from the nucleus to the cytoplasm and inhibits their transcriptional activity. In ovaries, FOXO3a is a suppressor of primordial follicle
activation and its global ablation led to premature primordial follicle activation followed by their rapid depletion and POF [42]. Oocyte-specific constitutive activation of FOXO3 caused a dramatic decrease of GDF9, BMP15 and FSHR (follicle stimulating hormone receptor) expression which suppresses normal granulosa cell proliferation and blocks follicle activation [43]. PTEN (phosphatase and tensin homolog) negatively regulates PI3K, therefore blocking PTEN enhances PI3K activity, leading to enhanced phosphorylation of Akt that inhibits nuclear transcriptional activity of FOXO3. As a result, similar to Foxo3a−/− ovaries, oocyte specific knock-out of Pten leads to overgrowth of immature oocytes and premature follicle activation followed by atresia [44]. However, granulosa cell specific knock-out of Pten leads to phosphorylation and degradation of FOXO1 and enhanced cell proliferation, more ovulated oocytes and corpora lutea (CL) that persisted longer [45]. Acting downstream of PI3K pathway, p27 potently inhibits CDK2 kinase activity which is necessary for the cell cycle progression. In ovaries, p27 is expressed in both oocytes and pre-granulosa cells and is important for negative regulation of follicular activation [46]. The depletion of p27 leads to premature activation of primordial follicle pool once endowed and follicular depletion later on. Also acting downstream of PI3K pathway, the tumor suppressor tuberous sclerosis complex 1 (TSC1) negatively regulates mTORC1 in preventing precocious follicle activation [47]. More importantly, PTEN/PI3K and TSC1/mTORC1 act synergistically to regulate the normal follicle activation and prevent POF syndromes in mice [47].

Follicular activation is also under the tight regulation of several growth factors and cytokines, such as the bFGF (basic fibroblast growth factor) and PDGF (platelet-derived growth factor) that activate the RPTKs (receptor protein tyrosine kinase), LIF
(Leukaemia inhibitory factor) that activates cytokine type 1 receptor and SDF-1 (stromal-derived factor-1) that activates the CXCR4 (C-X-C chemokine receptor type 4). The bFGF, produced by oocytes, stimulates both theca and stroma growth [48, 49]. PDGF, also produced by oocytes, acts on the surrounding somatic granulosa cells to enhance their KL production which promotes follicular activation [50, 51]. Chemokine SDF-1 and its receptor, CXCR4, maintain the size and the longevity of the primordial follicle pool by retaining follicles in un-activated form [52]. LIF is produced by granulosa cells. Through an autocrine pathway, LIF induces the expression of KL [53] and KL binds to oocyte expressed cognate receptors c-kit and regulates the expression of BMP15. The TGF-β family and steroid hormones also play indispensable roles during follicle activation. For example, unlike oocyte-expressed BMP15, BMP-4 is produced by mesenchymal cells, stromal and thecal cells [54, 55] and is required for oocyte survival [55]. BMP-7 is also stroma derived and was shown to play a role in primordial-primary transition [56]. AMH, produced by activated (cuboidal) granulosa cells, inhibits the activation of granulosa cells in the surrounding primordial follicles [57]. The steroid hormone estradiol has also been suggested to exert inhibitory roles in primordial follicle activation. Aromatase ablation decreases the primordial follicle number and increases the size of primordial oocytes [58]. Consistently, estradiol treatment both in vivo and in vitro increases the primordial follicle quantities in neonatal ovaries [59]. Although primordial follicles don’t possess functional FSHR [60], they respond to cAMP analogues by increasing expression of FSHR and aromatase [61].
Pre-antral Follicular Growth

KL from granulosa cells acts as a primary link between the growth of oocytes and the proliferation of granulosa cells in the follicular development upon the follicular activation [62]. KL induces activin production in granulosa and theca cells through autocrine and paracrine pathways [63, 64]. Activin mainly acts locally through an autocrine and/or paracrine manner, since circulatory activin binds with follistatin that keeps it in an inactive form [65]. Meanwhile, locally produced IGF-1 (insulin-like growth factor) not only is essential for the basal granulosa cell proliferation [66], but also enhances FSH responsiveness by inducing the expression of FSHR in mural granulosa cells which mediates the growth-promoting effect of FSH [67]. Therefore, unlike primordial follicles, follicles at primary stage and onward possess functional FSHR and follicles become increasingly gonadotropin-responsive as they grow. On the other hand, granulosa cells produce inhibin to suppress the production of FSH and also produce follistatin to indirectly inhibit the FSH activity by binding with activin [63, 64]. Importantly, KL expression in granulosa cells is modulated by two oocyte specific factors GDF-9 and BMP15, which play critical roles in granulosa cell proliferation in primary, secondary and antral follicles. GDF-9 mainly exerts its function from early primary follicle phase by inducing the proliferation of granulosa cells and recruitment of theca cells [62, 68]. BMP15 functions mainly in more advanced phases [20]. These two OSFs (oocyte secreted factors) have opposite effects on KL expression in granulosa cells in that GDF9 promotes KL while BMP15 inhibits KL [69]. In addition, the BMP15-mediated inhibition of KL in granulosa cells in turn suppresses BMP15 expression in oocytes through a negative feedback loop after binding with the c-Kit receptor [70]. Classical
studies stated that GDF-9 or BMP15, upon forming homodimers respectively, signal through either SMAD2/3 or SMAD1/5/8 pathway to regulate granulosa cell function [71, 72]. However, a very recent study demonstrated that compared to the homodimers, GDF-9: BMP-15 heterodimers are the most bioactive form that signals through a unique receptor complex, mediating SMAD2/3 phosphorylation in granulosa cells [73]. The paracrine regulation by GDF9 and BMP15 can be negatively regulated by GCNF (germ cell nuclear factor) during pre-antral follicular development [74]. GCNF, only expressed in primary, secondary and pre-ovulatory oocytes, [75] binds with promoter regions of GDF9 and BMP15 and represses their reporter activity [74].

Antral Follicular Development

A fluid-filled antrum appears in a follicle when the total granulosa cell population reaches around 2000 in mice [76]. The functional diverging of the granulosa cells is under the control of both FSH and oocytes [77, 78]. FSH acts on granulosa cells and activates pathways such as cAMP/PKA [79] which stimulates granulosa cell proliferation and differentiation into mural cells that line the follicle wall and cumulus cells that are in proximity of the oocytes. When the antrum forms, the oocytes stimulate the cumulus cell phenotype which resembles granulosa cells in pre-antral follicle [78, 80]. By secreting paracrine factors such as GDF9, BMP15 and FGF8b, oocytes control the expression of cell cycle proteins and DNA synthesis in granulosa cells to promote their proliferation [81] and oocytes also control metabolic activities in cumulus cells including encoding amino acids transporters and essential metabolic enzymes for oocytes metabolism [82]. FOXL2, a transcriptional factor from granulosa cells themselves, through regulation of *Inhibin βb, Cyp11a1, Star*, regulates metabolic aspects for granulosa cell proliferation
and differentiation [83, 84]. In addition, as an important microenvironment for all the follicular cells, the follicular fluid in the antrum provides numerous factors to support proper follicular functions and oocyte quality, such as granulocyte-colony stimulating factor, interleukin-6 (IL-6), 8, 12, BMP2, AREG (amphiregulin) and GDF-9 [85].

The cyclic recruitment wave that recruits early antral follicles and/or pre-antral follicles to grow further starts at the onset of puberty, and is the result of increasing circulatory levels of FSH and LH (luteinizing hormone). At the beginning of follicular phase, low frequency GnRH pulse slightly stimulates FSH level, which enhances the overall growth of a follicular cohort. Here the follicles remain largely FSH-dependent. As the follicular phase progresses, increased estrogen produced by granulosa cells of those growing follicles inhibits hypothalamic GnRH production, leading to the drop of FSH to the basal level and the growth of additional follicles is somewhat inhibited. Meanwhile, inhibin B also acts to suppress FSH secretion during this phase [86]. However, more importantly, through an unknown mechanism, granulosa cells in some follicles start to acquire LH receptors [87, 88] and these follicles are selected as dominant follicles and continue growing in spite of the basal FSH level. From this point on, these growing follicles become largely LH dependent. However, the cumulus cells don’t acquire LH receptors due to inhibitory influence of oocyte secreted factors [89, 90]. As the follicles grow further and produce increasing amount of estrogen, the hypothalamus-pituitary axis changes its responsiveness to estrogen from negative feedback to positive feedback. This switch is partly because while estrogen inhibits GnRH production, it elicits elevated GnRH pulses. Therefore, as more estrogen is produced, GnRH pulses become more and more frequent. Furthermore, when the increased estrogen reaches a threshold level, it
sensitizes the responsiveness of pituitary gonadotrophs to GnRH so that LH surge and FSH surge occur and cause ovulation [86]. During this short period, locally produced estrogen and activin both enhance the action of FSH in pre-ovulatory follicles [91, 92]. Estrogen acts on estrogen receptor β (ER-β) to inhibit PDE1C (phosphodiesterase 1 C) expression, leading to increased cAMP production that facilitates FSH action [91]. Activin acts on ALK4 and ActRII receptors, through SMAD2/3/4 to regulate CCND2 (cyclin D2) and steroidogenic enzymes that are involved in granulosa cell proliferation and differentiation. Those genes are also FSH target genes [88] and activin acts synergistically with FSH to modulate their expression [93]. Mechanisms other than adenylyl cyclase (AC) and protein kinase A (PKA) also mediate FSH action in granulosa cells. FSH activates small GTP-binding protein RAS in a PKA-independent manner but the activation of RAS requires both EGFR activity and the activity of SRC family tyrosine kinase (SFK) that converge downstream to activate PKB/Akt and ERK1/2 [94]. In addition, downstream of PKB, glycogen synthase kinase 3 (GSK3) phosphorylates β-catenin, and β-catenin was shown to be essential for FSH/cAMP regulated Cyp19a1 expression [95], indicating the importance of WNT signaling as an alternative downstream mediator of FSH action. The transcriptional factors FOXO1 and 2 expressed in granulosa cells of antral follicles also act downstream of PKB. Constitutively expressing FOXO1 was shown to suppress proliferation related genes such as cyclin D2 and genes that are critical for lipid, sterol and cholesterol biosynthetic pathways in granulosa cells [93, 96].

Ovulation
Several important events take place during ovulation, including cumulus expansion, active remodeling of extracellular matrix, granulosa cell differentiation into luteal cells, local microcirculatory vasomotion and contraction of the smooth muscle to extrude the COCs. Here we mainly discuss the functional changes of COCs during ovulation. Upon ovulatory signals, LH binds to LHR and activates the adenylyl cyclase (AC), followed by an increase in cAMP that leads to activation of phospholipase C (PLC) [97]. These intracellular messengers activate and regulate various downstream protein kinases including cAMP-dependent PKA, RAS, ERK1/2, etc, leading to rapid curtailment of granulosa cell proliferation and stimulation of terminal differentiation [98-100]. For example, the LH surge, while it does not last long, leads to rapid expression of EGF-LP (EGF like peptides) such as EREG, AREG and BTC in a PKA-dependent manner in mural granulosa cells [101]. These EGF-LPs bind with the EGF receptor on mural and cumulus cells, lead to receptor dimerization and auto-phosphorylation, activation of RAS and downstream target genes for cumulus expansion and oocyte meiotic resumption, such as Tnfaip6 (TNF-α-induced protein 6), Has2 (hyaluronan synthase 2) and Ptgs2 (prostaglandin-endoperoxide synthase 2) [102]. Additionally, EGF signaling, through MAPK, upregulates steroidogenesis by enhancing the expression of key steroidogenic enzymes for progesterone as well as estradiol synthesis [103]. At the same time, EGF network in granulosa cells also reinforces their expression of EGFR and thereby further facilitates the propagation of the LH signaling [94]. Ovulation is also associated with innate immune responses, indicated by changes of cytokines such as IL-6, RUNX1/2 (runt-related transcription factor), LIF and many more [104, 105]. Recent studies have already indicated some clinical significance of the inflammatory aspects of ovulation.
The observation that IL-6 stimulates cumulus expansion as well as expression of expansion related genes [104] indicates that in PCOS (and other chronic diseases) patients, the elevated IL-6 level may interfere with supposedly normal ovulatory functions. Particularly, IL-6-treated COCs exhibited higher successful rate at IVM, which emphasized the contribution of IL-6 and related cytokines on oocyte quality and its potential in improving IVF procedures [104, 106]. Mechanistically, AREG and PGR (progesterone receptor) were both shown to regulate induction of IL-6 during ovulation [104, 107] but it is still largely unclear how IL-6 and other inflammatory cytokines participate in this complex process.

After ovulation, the luteal phase follows, where progesterone production remains relatively high. High progesterone level sends negative feedback to inhibit more production of FSH and LH while inhibin A mainly acts to inhibit FSH secretion during this phase [86]. Interestingly, in rodents, without a successful fertilization and implantation, there is only partial luteinization (and low progesterone) induced by LH surge and the partially functional corpora lutea eventually undergo regression and a new cycle begins with the selection of dominant follicles. However, if the animal is mated and becomes pregnant, then there is a prolactin surge and CL becomes fully functional and lasts for the entire pregnancy, which is followed by the regression of CL and the immediate drop of prolactin and prolactin receptor at parturition due to the action of PGF2α [108-110].

Oocyte-Cumulus Cell Communication
Between oocytes and cumulus cells, the secreted and gap-junction-mediated bi-directional communications forms a complicated network of both regulatory and metabolic pathways which are essential for the development and function of both cells. With the thickening of zona pellucida (ZP) structure around growing oocytes, trans-zonal cytoplasmic projections (TZPs) within ZP originated from cumulus cells are constantly modified to meet the need of the cargo transferring between oocytes and cumulus cells through gap junctions [111, 112]. The gap junction protein Connexin 37 is localized to oocytes whereas Connexin 43 is localized to granulosa cells and to the outer part of zona pellucida to connect the cumulus-trans-zonal projections [113].

The oocyte stimulates proliferation of granulosa cells at all stages of follicular development. The re-aggregation experiment combining 14 days old oocytes and newborn ovarian somatic cells and showing accelerated formation of antral follicles clearly demonstrated that oocytes play central roles in promoting the growth of granulosa cells [89]. Co-culturing OOX (oocytectomized cumulus cells) with FGOs (fully grown oocytes) for short term (20h) is sufficient to rescue the transcriptional changes in the granulosa cells that are caused by oocytectomy [114]. A few other co-culture studies of oocytes and granulosa cells uncovered that oocytes stimulate DNA synthesis and expression of the cell cycle transcript, Ccnd2, in both mural and cumulus cells [81, 115]. OSFs also control cumulus cell metabolism including cholesterol biosynthesis and glycolysis before ovulatory surge [114, 116]. The mitotic effect of OSFs is induced in granulosa cells through TGF-β receptors BMPR-II and ALK 4,5,7 that are SMAD2/3-stimulatory [81]. As stated before, GDF-9: BMP-15 heterodimers are likely to be the most bioactive form that mediates SMAD2/3 activation [73].
The growth-promoting activity of oocytes is greatly diminished after ovulation [117]. During this time, LH signaling mediates the activation of EGF network in granulosa cells, leading cells to exit from the cell cycle and terminal differentiation such as cumulus expansion [101]. Oocytes help to stabilize the mucified matrix by attenuating its degradation and inhibiting the proteases and proteolytic enzymes such as urokinase-type plasminogen activator uPA. Meanwhile, oocytes produce CEEF (cumulus expansion enabling factor) composed of TGF super family members and signals through SMAD2/3 to enable cumulus expansion synergistically with ovulatory signals [118, 119]. Stimulation from both oocytes and ovulatory signals result in activation of MAPK (ERK1/2 and p38) and increased Has2, Ptgs2, Tnfaip6 and Ptx3 mRNA levels, which are all required for extracellular matrix formation and cumulus expansion [78]. The HA-rich viscoelastic matrix is critical to facilitate the follicle rupture prior to luteinization. HAS2 (hyanuronan synthase 2), crosslinking protein TNFαIP6, PTX3 (pentraxin 3), proteoglycan versican and PGE2 (rapidly produced upon the induction of COX2 expression) serve to maintain and stabilize the structure of the matrix.

Although oocytes play central roles in defining granulosa cell function, granulosa cells and especially cumulus cells, play indispensable roles in supporting oocyte growth, maintaining meiotic arrest [120], transcriptional silencing and chromatin remodeling [121] and also for achieving full developmental potential in early embryos by regulating intracellular zinc [122, 123]. For example, mouse oocytes are deficient in the enzymes in de novo cholesterol synthesis pathway, therefore they rely on cumulus cells for the products of cholesterol synthesis for normal oocyte growth [114]. During oocyte development, the chromatin configuration undergoes dynamic changes. Companion
granulosa cells potentiate oocyte chromatin remodeling and actively modulate the subsequent transcriptional activity of oocyte genome [121]. DNA methylation accomplished by DNMTs (DNA methyltransferases), increases in accordance with oocyte growth and reaches the peak levels in FGO (fully-grown oocytes) [124]. Cumulus cells suppress free zinc in the oocytes, through production of a secreted product of unknown identity [122] and dietary zinc deficiency before ovulation was shown to disrupt chromatin methylation in oocytes [123]. Those studies presented another regulatory role of granulosa cells on oocyte functions. Mural granulosa cells are also involved in regulating oocyte functions. Mural cells produce NPPC that binds to its receptor NPR2 (Guanylyl Cyclase) on cumulus cells and increases cGMP levels in cumulus cells, which causes the induction of cGMPs in oocytes by transfer of cGMP through gap junctions [125]. FSH, cooperatively with estrodial, stimulates the expression of Nppc in mural cells and Npr2 in cumulus cells [125, 126]. Oocytes also play a role in that OSFs such as GDF9 and BMP15 induce the expression of not only Npr2, but also inosine monophosphate dehydrogenase gene in cumulus cells, which encodes the key enzyme for cGMP production [127]. High cGMP inhibits the hydrolysis of cAMP by the phosphodiesterase, PDE3A, and high levels of cAMP ensure the meiotic arrest by regulating MPF (maturation promoting factor) [128, 129]. MPF, composed of a regulatory component cyclin B and a catalytic component CDK1 kinase, remains at an inactive form before ovulation [130]. High cAMP activates PKA, which phosphorylates the dual specific phosphatase CDC25 and kinases WEE1B and MYT1, causing the inactivation of the former and the activation of the latter [131]. When the tyrosine and threonine residues of CDK1 are phosphorylated and inactivated, MPF activity is
repressed, therefore GVBD is inhibited. At ovulation, the NPPC expression in mural granulosa cells and the follicular cGMP level both are decreased [132]. The reduction of cGMP is the result of the upregulation of PDE5 and the downregulation of guanylyl cyclase activity of NPR2 to convert GTP to cGMP [133]. Also in cumulus cells, the activation of MAPK-dependent pathways leads to the phosphorylation of serine residues of connexin-43 and therefore the suppression of gap junctions which prevent the transfer of cGMP into the oocytes [134]. Low cGMP level in oocytes releases the inhibition of PDE3A activity which allows the hydrolysis and reduction of cAMP level, activation of the phosphatase CDC25 and inactivation of the two kinases WEE1B and MYT, eventually leading to the activation of MPF that are required for oocyte meiotic resumption [135, 136].

Figure 2-1: Mammalian folliculogenesis.

Hippo Signaling Pathway Introduction
The Hippo signaling pathway was recently discovered and is defined as a highly conserved pathway that controls organ size during development and when disrupted can lead to tumor formation in many species.

**Hippo Signaling Pathway in *Drosophila***

The Hippo pathway was first discovered in *Drosophila* through mosaic genetic screens for tumor suppressor genes, the genetic inactivation of which led to overgrowth of tissues, such as eyes, wings and legs. These identified genes include: Wts [137]; Sav [138, 139]; Hpo [140-143] and Mats [144]. Early studies uncovered that HPO protein, a STE20 family protein kinase, directly interacts with WW domain-containing protein SAV and promotes SAV and WTS phosphorylation [140], meanwhile, MATS interacts physically with WTS as an activating subunit and is also phosphorylated by HPO [144-146]. Those studies thus established the core cascade of *Drosophila* Hippo pathway. Sd and Yki were later identified by yeast two-hybrid as targets of the Hippo pathway and are transcriptional activator and co-activator [147-150]. They mediate transcriptional activation of *Cyclin E, Diap1, bantam* microRNA, etc to modulate cell proliferation and apoptosis [151]. The upstream regulating network is believed to be quite complex. Although not entirely clear, some factors found to feed into Hippo core cascade in *Drosophila* involve cell-cell contact, cell polarity cues, behavior of actin cytoskeleton and patterning molecules. For cell-cell contact, atypical cadherins Fat and Dachsous (Ds), act as ligand-receptor pair [152, 153], that when activated lead to the sequesters of Yki in the cytoplasm by inhibiting atypical myosin Dachs, promoting abundance of Wts and shifting FERM domain-containing protein EX to the apical surface [152, 154-156]. In the subcellular compartment close to cell surface, Ex, together with a WW domain-
containing protein KIBRA and another FERM domain-containing protein MER were reported to physically interact with each other and play somewhat similar roles in activating Hippo pathway [157-160]. They were both shown to modify Hippo pathway activity by directly or indirectly recruiting WTS [161] or HPO [157-159] to the apical membrane to facilitate Hippo activation. While KIBRA, MER and EX all serve as part of negative feedback loop mediated by Yki, EX has been postulated to repress Yki directly, independent of the Hippo core cascade [162, 163]. EX itself can also be regulated by Crumbs in a contact-dependent way [164, 165]. In addition to Crumbs (Crb), several other adheren junction proteins such as Ajuba and Echinoid also regulate Hippo pathway activity [166, 167]. Crb is also one of the well-defined proteins that regulate apicobasal polarity in epithelial cells and is linked with Hippo pathway activity. Another example of is Lethal giant larvae (Lgl): the balance between Lgl and αPKC determines the localization of HPO and RASSF [168, 169]. Hippo pathway activity has also been shown to be sensitive to changes of the actin cytoskeleton. For instance, actin cytoskeletal tension was found to modulate Wts activity through recruiting of various inhibitor [170], as well as influencing binding and membrane recruitment of Mer and Wts [161]. On the other hand, Hippo kinase Wts influences border cell migration in the Drosophila ovary by negatively regulating the activity of actin regulator protein Ena [171]. Patterning factors such as Dpp are also known to have profound influence on tissue and organ growth. The crosstalk between Hippo signaling and patterning factors lies mainly in the upstream signaling network. For example, in developing wings and many other organs, the gradients of Ds and Fj (kinase that modulates the binding of Fat-Ds) have critical
roles in modulating cell polarity [172]; and they are regulated not only by Dpp, but also NOTCH and Wingless pathways [173-175].

Mammalian Hippo Signaling Pathway

The Hippo pathway is highly conserved from *Drosophila* to mammals, particularly the core components: MST1/2 (Hpo homolog), SAV1, LATS1/2 (Wts homolog), MOB1B (Mats homolog), YAP/WWTR (Yki homolog), TEAD1/4 (Sd homolog). Their importance was shown by both knock-out mice models and reported human diseases. *Lats1*−/− mice showed partial postnatal lethality with defects in overall growth retardation and mammary gland defects in survivors [176]. *Lats2*−/− mice are lethal at E10, with the embryos displaying centrosome amplification and genomic instability [177]. The hypermethylation and low mRNA expression levels of both *Lats1* and *Lats2* were associated with large tumor sizes and poor prognosis in human breast cancers [178]. *Yap*−/− mice are lethal at E8 due to defects in development of extraembryonic structures. YAP is upregulated in various human cancers [179]. Unlike *Yap*−/− mice, *Taz*−/− (*Wwtr*−/−) mice are partially lethal at birth with survivors having defects in kidney and lung development. *Taz*−/− mice develop polycystic kidney disease and emphysema [180]. Similar to the *Drosophila* Hippo pathway, the canonical mammalian core cascade starts with a series of phosphorylation events which include the phosphorylation of kinases MST1/2 and the adaptor protein SAV. Then the kinases LATS1/2 and adaptor protein MOB are phosphorylated by activated MST1/2 which leads to the phosphorylation of YAP (at S127, S61, S109, S164) and WWTR (at S89, S66, S117, S311) [181, 182] and
cytoplasmic retention of both YAP and WWTR via interaction with 14-3-3 proteins. The binding of 14-3-3 proteins to YAP1 can also be generated by Akt phosphorylation at S127, despite the fact that S127 is an atypical Akt phosphorylation site [182]. Without the activation of the core cascade, YAP/WWTR in their un-phosphorylated and active form, move to the nucleus and induce transcriptional activity. Therefore, activation of the core cascade generally results in the inhibition of the downstream transcriptional output.

Each level of the canonical Hippo axis (MST-LATS-YAP/TAZ) is under multifaceted regulation. In the canonical Hippo axis, the phosphorylation of LATS1/2 requires activation of MST1/2; however, another kinase, MAP4K, was found to be fully capable of phosphorylating LATS2 via an MST1/2-independent but cell-type dependent way [183]. At the level of the transcriptional co-activators YAP/WWTR, there are sites in addition to serine 127 that are phosphorylated by LATS and other kinases. In most cases, these modifications lead to the SCFβ-TRCP-dependent ubiquitination and degradation of YAP and WWTR [180]. In addition, the N-terminal phosphodegron domain of WWTR can be phosphorylated by LATS which then induces phosphorylation by GSK3 also leading to SCFβ-TRCP-dependent ubiquitination and degradation [184]. However, just like MST is not the only kinase that phosphorylates LATS, LATS is not the only kinase that directly phosphorylates YAP/WWTR. Several other kinases were also found to regulate YAP/WWTR by phosphorylation. For instance, CDK1 phosphorylates YAP1 at T119, S289 and S367 to maintain G2/M mitotic arrest [185]. Yes kinase phosphorylates YAP1 to mediate the YAP1-TEAD2 dependent transcription induced by LIF (Leukemia inhibitory factor) in embryonic stem cells [186]. Src kinase phosphorylates YAP to regulate the repression of Runx2 [187]. Meanwhile, YAP and WWTR are not the only
substrates that LATS kinase phosphorylates. YAP1 was reported to block the binding and phosphorylating action of LATS2 on ASPP1 (Apoptosis-stimulating protein of p53-1). Due to the tumor suppressive nature of the ASPP1-p53 path when ASPP1 is un-phosphorylated, this interference of ASPP1 phosphorylation by YAP1-LATS2 adds another pathway for controlling pro- and anti-apoptosis [188]. Other modification of YAP/WWTR at the post-translational level include: acetylation [189], methylation [190] and sumoylation [191].

As transcription co-activators, YAP and WWTR interact with various transcription factors. Among these, some interact with both YAP and WWTR, while others are distinctive for interacting with YAP or WWTR. The TEAD family has been recognized as the main transcription factors that directly interact with YAP/WWTR. Physical binding of YAP-TEAD and WWTR-TEAD are similar overall but bear some differences [192]. TEAD proteins have DNA-binding domain at the N-terminal and transactivation domain at the C-terminal which is used to bind with not only YAP/WWTR, but also VGLL (vestigial like family). VGLL has been shown to be necessary to mediate the function of the default repressor TEAD and it competes with YAP/WWTR for binding with TEAD [193, 194]. SMAD family is recognized as another main TF family that interacts with YAP/WWTR. BMP signaling induces the CDK8/9-mediated phosphorylation of SMAD1 at the linker region which enhances the interaction between WW domain of YAP1 and the PPxY domain on the linker region, and overall YAP1 promotes SMAD1-mediated signals [195]. Moreover, without YAP1, airway epithelial progenitors are unable to respond to TGFβ-induced cues properly [196]. SMAD3 was also shown to directly interact with YAP1 in human mesothelioma cells by forming a
complex with TEAD4 and p300 [197]. Interaction between WWTR and SMADs is very important for embryonic stem cells (ESCs) function. For example, WWTR is indispensable for mediating TGF-β signaling as well as maintaining self-renewal markers of stem cells [198]. Moreover, WWTR dominantly controls the nuclear shuttling and coupling of SMAD2/3-4 complex to the transcriptional machinery [198]. However, since the SMADs family includes several SMADs (1,2,3,4,5,8,9) that positively mediate TGF and BMP signaling and inhibitory SMADs (6,7) that antagonize them, it is not surprising that YAP/WWTR may interact with different SMADs in different ways, which may have contradictory effects on transducing TGF and BMP signals to the interior of the cell. In fact, YAP1 was shown to interact with SMADs in a context-dependent manner. Unlike the stimulatory effect on TGF-β signaling upon YAP1-SMAD1/3 interactions, YAP1-SMAD7 interaction was reported to potentiate the inhibitory effect of SMAD7 in a fibroblast-like cell line COS-7 [199]. In addition to the effect on induction or inhibition of SMADs-TGF/BMP signaling, YAP/WWTR can also modulate SMAD signaling by controlling cellular localization of SMADs in response to cell density cues and during early embryogenesis [200]. In early mouse embryos, through the activity of LATS1/2 and TEAD4, YAP1 is localized to the nucleus of trophectoderm cells but excluded from the nucleus of cells in the inner cell mass [201]. Crumbs polarity complex relays cell density information by enhancing the phosphorylation of YAP/WWTR which drives the cytoplasmic localization of SMAD2/3; because of the retention of these mediators, TGF-β signaling is suppressed [202].

However, it is important to notice that YAP/WWTR don’t always induce the transcription of pro-proliferative and anti-apoptotic genes. Although not entirely clear, in
response to DNA damage, c-ABL phosphorylates YAP1 to facilitate the interaction of YAP1 and p73, which is required for inducing pro-apoptotic transcription [203]. In response to UV radiation, however, JNK-phosphorylated YAP1 promotes p73-dependent apoptosis in skin cancer cells while protects normal skin cells from p73-dependent apoptosis [204, 205].

With many new findings uncovered in the past decade, the upstream regulatory network of Hippo pathway in mammals is believed to be even more complex than in invertebrates, though many of the regulators are functionally conserved with their invertebrates’ counterparts. GPCRs (G protein coupled receptors) were recently discovered to impinge on Hippo signaling in a receptor-dependent way [206]: Serum borne LPA (lysophophatidic acid) and S1P (sphingosine 1-phosphophate) act through G12/G13-coupled receptors and Rho to induce the transcriptional activity of YAP/WWTR [207, 208]. In contrast, glucagon and epinephrine act through Gs-coupled receptors to activate LATS1/2 and therefore inhibit nuclear activity of YAP/WWTR. Thrombin is another signal that triggers Hippo signaling [209]. It acts upon protease-activated receptors (PARs) which also recruit G12/13 subunit and inhibit LATS1/2 kinases [209]. The regulation of G12/13 and other G coupled machineries are mediated by Rho and actin cytoskeleton [210, 211]. The modification and activation of Rho is required for nuclear YAP1 and WWTR localization, as reported in [212, 213]. Actin cytoskeleton is one of the major upstream mediators of Hippo signaling, upon the induction of mechanical signals, see review [214, 215]. Cells read ECM elasticity, cell shape and cytoskeletal forces as levels of YAP/WWTR activity; furthermore,
experimental manipulations of YAP/WWTR levels could even override the mechanical inputs, dictating cell behaviors and fates [212].

Hippo and Reproduction

As a potent regulator of cell proliferation, differentiation, programmed cell death and thus tissue homeostasis, the Hippo signaling pathway has quickly drawn scientists’ attention in various physiological and pathological contexts in the past decade [1, 216-218]. However, the role of the Hippo signaling in mammalian reproduction is still poorly understood. An early study using a global knockout mouse model reported that the deletion of the Lats1 gene in mice is associated with fewer ovarian follicles, no corpora lutea and development of ovarian stromal tumors [176]. In ovaries, YAP1 has been reported to be expressed at high level in ovarian serous cystadenocarcinoma and ovarian clear cell carcinoma, compared to normal ovarian tissues and its expression is highly correlated with poor prognosis in cancer patients [179, 219]. Recently, Dr. Hsueh’s group contributed to our understanding of the mechanisms underlying treating POI (primary ovarian insufficiency) and PCOS (polycystic ovarian syndrome) using physically disruptive procedures such as ovarian wedge resection or laser drilling. They reported that the fragmentation in both human and mice ovaries leads to stimulation of follicular growth, through promoting actin polymerization, disrupting ovarian Hippo signaling and upregulation of YAP1 [220]. More recently, the same group applied short-term treatment of mouse ovaries with actin-polymerization enhancing drugs JASP (Jasplakinolide) and S1P (sphingosine-1-phosphate), further demonstrated that increased formation of
filamentous actin results in increased nuclear YAP1 accumulation and increase of the YAP1 target gene CCN2 during follicular development [221]. These observations demonstrated the potential importance of the Hippo signaling in ovarian function, but still little is known about how this pathway is regulated and the role it plays during specific stages of ovarian development, especially the ovulatory transition.

Overall Objectives

Our overall goal in this study is to uncover how the Hippo signaling pathway regulates cellular proliferation and differentiation during different stages of follicular development. To achieve this goal, we first investigated the roles of LATS1 and YAP1 on defining the follicle organization during early follicular development. Then we examined how Hippo activity determines cell fate by mediating oocyte-originated effects before ovulation and by mediating ovulatory signal-induced effects during ovulation. Lastly, we explored some possible novel roles of YAP1 in oocyte maturation by linking it with cell cycle regulators and actin polymerization. It’s fundamentally important to further understand the mechanisms underlying the follicular development, granulosa cell fate determination and oocyte maturation dynamics in normal situations. Furthermore, the improved understanding of Hippo signaling in our follicular system will potentially provide invaluable clues on various pathological conditions such as POF (premature ovarian failure), POI (primary ovarian insufficiency), PCOS (polycystic ovarian syndrome), ovarian cancer, and any infertility or subfertility that’s caused by poor oocyte quality.
Chapter 3

*Lats1 Deletion Causes Increased Germ Cell Apoptosis and Follicular Cysts in Mouse Ovaries*

Abstract

The Hippo signaling pathway is essential for regulating proliferation and apoptosis in mammalian cells. The LATS1 kinase is a core member of the Hippo signaling pathway that phosphorylates and inactivates the transcriptional co-activators YAP1 and WWTR1. Deletion of Lats1 results in low neonate survival and ovarian stromal tumors in surviving adults, but the effects of Lats1 on early follicular development are not understood. Here, the expression of Hippo pathway components including Wwtr1, Stk4, Stk3, Lats2 and Yap1 transcripts were decreased by 50% in mouse ovaries between 2 and 8 days of age while expression was maintained from 8 days to 21 days and after priming with eCG. LATS1, LATS2, and MOB1B were localized to both germ and somatic cells of primordial to antral follicles. Interestingly, YAP1 was predominantly cytoplasmic, while WWTR1 was nuclear in oocytes and somatic cells. Deletion of Lats1 caused an increase in germ cell apoptosis from 1.7% in control ovaries to 3.6% in Lats1 mutant ovaries and a 58% and 32% decrease in primordial and activated follicle numbers in cultured mutant ovaries. Surprisingly, there was an increase in Bmp15, but not Gdf9, Figla, Nobox transcripts or somatic-specific transcripts Amh and Wnt4 in cultured Lats1 mutant ovaries. Lastly, Lats1 mutant ovaries developed ovarian cysts at a higher frequency (43%) compared to heterozygous (24%) and control ovaries.
(8%). The results show that the Hippo pathway is active in ovarian follicles and that LATS1 is required to maintain the pool of germ cells and primordial follicles.
Introduction

The production of fertile gametes is an essential biological process important for species’ survival. Among vertebrate species, there is tremendous variation in estrus cycle length, follicular dynamics and ovulation patterns, but the basic sequence of follicle assembly, activation, growth and ovulation is highly conserved. Mammalian follicular development is controlled by multifaceted regulatory mechanisms including intra-ovarian paracrine signaling and endocrine regulation by systemic hormones such as FSH (follicle stimulating hormone) and LH (luteinizing hormone). The paracrine communication between oocytes and somatic cells forms a complicated network of regulatory and metabolic pathways that are critical for proper ovarian function. For example, mammalian oocytes orchestrate follicular development by potently promoting granulosa cell proliferation and differentiation [222-224]. In turn, granulosa cells promote oocyte developmental competence [225, 226], partly by supplying the oocytes with needed nutrients [114, 116, 227] and regulating oocyte maturation [121, 226]. Although we know that these paracrine effects are important for ovarian function, the signals responsible for follicle activation and growth are still not fully defined.

The Hippo pathway is a key regulator of cellular proliferation. First discovered in Drosophila in early the 1990s, Hippo signaling is highly conserved among different species and has emerged as an important signaling pathway for controlling organ size by regulating the cell fate decision to remain quiescent, divide or undergo cell death [228]. Although the upstream signals that regulate Hippo signaling are not precisely known, the activation of the Hippo signaling pathway converges on the activation of the Hippo kinases, STK4 (MST1) and STK3 (MST2). STK3/4 will then phosphorylate the adaptor
protein SAV1 and form a complex, which phosphorylates and activates the DBF family kinases LATS1/2. Activated LATS proteins then associate with the adaptor protein MOB1A/B and phosphorylate the transcriptional co-activators YAP1 and WWTR1 (TAZ). Phosphorylated YAP1/WWTR1 associate with 14-3-3 proteins and are retained in the cytoplasm and therefore inactivated. Conversely, inactivation of Hippo signaling leads to hypo-phosphorylation of YAP1 and WWTR1 followed by translocation to the nucleus where in conjunction with binding partners, such as TEAD1-4, induce cellular proliferation and/or survival [228, 229].

Genetic studies in mice have provided a detailed understanding of Hippo pathway function and how Hippo signaling is regulated. For example, liver-specific Mst1−/−Mst2−/− deletion causes upregulation of YAP1 protein and increased liver size in mice [230]. However, the role of Hippo signaling in mammalian reproduction is poorly understood. Recently, Kawamura and colleagues have shown that fragmentation of mouse and human ovaries caused an increase in nuclear YAP1 localization in somatic cells that was required for increased cellular proliferation and follicular growth [220]. Also, deletion of the Lats1 gene in mice is associated with fewer ovarian follicles, no corpora lutea and development of ovarian stromal tumors [176]. However, the Lats1−/− mouse strain has a high perinatal mortality [176] and an analysis of early follicular development is difficult. These observations demonstrate the potential importance of Hippo signaling in ovarian function, but little is known about how this pathway is regulated and the role it plays during specific stages of ovarian development. To begin addressing these issues and to examine the role of Hippo signaling during early follicular development, we examined the expression and distribution of key Hippo pathway components in mouse ovaries.
Moreover, we investigated the early ovarian phenotype of \textit{Lats1} mutant mice using organ culture. The findings indicate that Hippo pathway components are highly expressed in both somatic and germ cells and that \textit{Lats1} is required to prevent oocyte apoptosis and for maintenance of the primordial follicle pool. Deletion of \textit{Lats1} also caused frequent ovarian cyst formation \textit{in vitro} suggesting deregulated somatic cell proliferation. Collectively, the results indicate that Hippo signaling participates in a complex paracrine mechanism regulating early follicular development and survival.

\textbf{Methods}

\textbf{Animals}

Female CD1 mice were maintained in the research colony of the investigators. Heterozygous (B6; 129S2-\textit{Lats1}^{tm1Tx/J}) young adult males and females were purchased from Jackson laboratory and maintained as heterozygous mating pairs. Progeny were genotyped by PCR with specific primers (m\textit{Lats1} common-AAAGACGTTCTGCTCCGAAA; m\textit{Lats1} wildtype-GCACAAGGCCAAAGTCAGTCA; m\textit{Lats1} mutant-GCCAGAGGCCACTTGTGTAG). Animals were maintained according to the Guide for the Care and Use of Laboratory Animals (Institute for Learning and Animal Research). All animal use was reviewed and approved by the IACUC committee at The Pennsylvania State University.

\textbf{Newborn ovary culture}

Due to the low survival rate of \textit{Lats1}^{-/-} mice, ovaries were isolated from newborn \textit{Lats1}^{+/+} and \textit{Lats1}^{-/-} mice on the first postnatal day and cultured \textit{in vitro}, as described previously [231]. Briefly, surrounding tissues, including the ovarian bursae were removed and 3-4 ovaries were placed on Costar Transwell Col membrane inserts (3.0
µm, 24 mm; Corning, NY). Ovaries were cultured in Waymouth 752/1 medium supplemented with 0.23 mM pyruvic acid, 10 µg/ml streptomycin sulfate, 75 µg/ml penicillin G, and 10% fetal bovine serum (FBS). The organ cultures were maintained for either 7 days (for the observation of cyst development) or 11 days (for the counting of primordial follicles and activated follicles) at 37°C in an incubator infused with a gas mixture of 5% CO2, 20% O2 and medium was replaced every other day.

**Histology**

11 day-cultured and newborn Lats1 ovaries were fixed in 4% paraformaldehyde and embedded in paraffin. Ovarian sections (5µm for cultured ovaries, 4µm for newborn) were stained with hematoxylin and eosin using standard methods. Brightfield images were captured for assessment of follicular morphology of cultured ovaries.

**TRA98 and cleaved PARP immunohistochemistry**

Ovaries were fixed in 5% electron microscopy-grade paraformaldehyde (Electron Microscopy Sciences) overnight at 4°C and stained as previously described [27]. Whole ovaries were permeated with PT (PBS+0.1% Triton X-100) and incubated with TRA98 antibody (B-Bridge) diluted 1:100 to label germ cells [232, 233] and cleaved PARP (E51, Abcam) diluted 1:100 to label cells undergoing apoptosis overnight and then with goat anti-rat secondary Alexa 488 and goat anti-rabbit Alexa 568, respectively. Nuclei were labeled with TOTO-3 (Invitrogen). A Zeiss LSM 710 confocal microscope was used to image the ovaries.

**Germ cell counting**

Total oocytes (TRA98 positive) and apoptotic oocytes (cleaved PARP-positive) were determined by counting the number of cells found within each of 8 optical sections.
per ovary (N=12). The numbers were averaged and reported as number of oocytes per section. The number of oocytes per section was used as a proxy for the total oocytes per ovary; therefore, only ovaries that appeared to be of similar size and depth were used for counting oocyte numbers.

**Follicle counting**

Primordial and activated follicles were examined in \textit{Lats1}^{+/+} and \textit{Lats1}^{-/-} newborn ovaries cultured for 7 days and stained with H&E. Either 3 or 4 sections, 8-10 sections apart, from each ovary were selected for counting. Follicles were independently counted by two persons, who were blinded to the two genotypes. The follicles with a single squamous layer of granulosa cells were counted as primordial follicles. The follicles with a layer of cuboidal granulosa cells and follicles beyond this stage were counted as activated follicles. The area of each section was measured using DP2-BSW (Olympus, Tokyo, Japan). Follicle numbers were normalized to the area of each section.

**Total RNA isolation and real-time PCR**

Total RNA was isolated from fresh or cultured ovaries using RNeasy microkit (QIAGEN, Valencia, CA). Total RNA was isolated from individual \textit{Lats1}^{+/+} and \textit{Lats1}^{-/-} ovaries (N=6) and from ovaries (N=4) collected from CD-1 mice on 2, 8 and 21 days after birth. Quantitect cDNA synthesis kit (QIAGEN) was used to reversely transcribe total RNA into cDNA. The quantification of transcripts for the Hippo pathway was normalized to the house-keeping gene \textit{Rpl19}, and gene-specific primers used in real-time PCR are shown in Table 3-1. The relative fold change in transcript was measured using the 2^{\text{dCt}} method as described previously [234].

**Immuno-histochemistry**
Ovaries from 2, 8, and 21 day old CD1 females were fixed in electron microscopy-grade 4% paraformaldehyde for 1-4 hours at room temperature and embedded in paraffin. The following steps were the same for freshly collected or cultured ovaries. Sections (4-5µm) were de-waxed in histoclear (EMS, Hatfield, PA) 3 times for 5 min each and then incubated for 5 min in each of the following: 100% ETOH, 100% ETOH, 95% ETOH, 85% ETOH, 70% ETOH and ddH2O, ddH2O. Next, samples were incubated in 1X antigen retrieval solution (Dako S2367) at 94°C for 30 min. After cooling to room temperature, slides were washed 3 times with ddH2O for 5 min. Then the sections were incubated in 3% hydrogen peroxide for 10 min, followed by two washes in ddH2O and two washes in wash buffer (1 X TBST, 0.1% Tween-20), 5 min each followed by incubation in goat blocking buffer (TBST, 5% goat serum) for 1 h at room temperature. Slides were then immediately incubated with anti-YAP1 (Proteintech, 13584-1-AP); anti-TAZ (Abcam, ab84927); anti-pYAP1 (Ser 127) (Cell signaling, D9W2I); anti-LATS1 (Abcam, ab85893); anti-LATS2 (Abcam, ab70765) at 1:800 dilution; and with MOB1B (Proteintech, 12790-1-AP) antibody at 1:400 dilution. After primary antibody incubation, slides were washed in 1 X TBST 3 times for 5 min each. Then 1-3 drops of room temperature-equilibrated SignalStain Boost Detection Reagent was used to cover sections, incubated at room temperature for 30 min in a humidified chamber. Then slides were washed 3 times in 1 X TBST, after which, 100-400ul SignalStain DAB reagent was applied to each section and monitored closely for color development for 1.5-5 min depending on the antibody. Slides were then immersed in ddH2O to stop the reaction. For MOB1B, pYAP1, LATS1, LATS2 staining, slides were counterstained with hematoxylin for 20 seconds and then incubated twice in 95%, 100% ethanol and twice in histoclear for
10 seconds. The normal rabbit IgG antibody applied with corresponding dilutions (1:400 for MOB and 1:800 for others) was used for negative controls. Finally, slides were mounted with cytoseal and covered with glass coverslips.

**Statistical analyses**

The gene expression was analyzed by one-way ANOVA followed by Tukey’s post-hoc test. Follicle and germ cell counting data were presented as means±SEM. The number of total germ cells activated and primordial follicles as well as apoptotic germ cell ratios were analyzed using General Linear Model or Student t-test. The cyst frequencies of cultured ovaries were analyzed by Chi-square test. Microsoft excel or JMP 9 (SAS) software was used for all analyses. A p-value <0.05 was considered statistically significant.

**Results**

**Expression of Hippo pathway components in ovaries**

The relative concentrations of transcripts encoding Hippo pathway components were analysed in freshly isolated ovaries collected from CD1 mice of different ages. Ovaries from postnatal day 2 contain mostly primordial follicles, day 8 primordial and primary follicles and day 21 ovaries contain large preantral/early antral follicles. Priming with eCG caused development of large antral follicles. Overall, the expression of all Hippo transcripts decreased as ovaries developed from 2 to 21 days. With the exception of Yap1 mRNA, all transcripts decreased 30-60% from 2 to 8 days after birth (Figure 3-1). Transcripts for Wwtr1, Wwc1, Sav1, Stk4, Stk3, Lats2 and Yap1 mRNA decreased by 10-70% from 8 to 21 day old mice. Priming animals with eCG did not result in any changes in mRNA levels for any transcripts (Figure 3-1).

**Immunolocalization of Hippo pathway proteins in the ovary**
To begin investigating the possible regulation of Hippo signaling in ovarian follicles, the protein localization of LATS1, LATS2, MOB1B, YAP1, WWTR1 (TAZ) and pYAP1 were examined in ovaries from 2-day, 8-day and 21-day old mice. LATS1 and LATS2 proteins were strongly localized to the cytoplasm of oocytes from primordial, primary, secondary and antral follicles (Figure 3-11). In the somatic cells, both LATS1 and LATS2 were most prominently expressed in granulosa cells of small and antral follicles, with much lower expression in stromal cells, including theca of large antral follicles (Figure 3-11). Similar to LATS1 and LATS2, MOB1B was localized to germ cells and somatic cells, but was notably absent from ovarian stromal cells between follicles (Figure 3-2). The downstream transcriptional co-activators YAP1 and WWTR1 were differentially localized in the ovary. WWTR1 was strongly nuclear in both oocytes and granulosa cells from primordial to antral follicles, with the exception of oocytes in large follicles (Figures 3-3). On the other hand, YAP1 was localized predominantly in the cytoplasm of oocytes from primordial to fully grown oocytes (Figure 3-4). In the somatic cells, YAP1 was ubiquitously expressed and predominantly cytoplasmic (Figure 3-4). Localization of pYAP1 changed during follicular development. In primordial follicles, pYAP1 was present in granulosa cells, but less evident in oocytes (Figures 3-5). As follicles were activated, pYAP1 became more prominent in the cuboidal granulosa cells of primary (arrow in Figure 3-5F) and secondary follicles (Figure 3-5B,C) compared to squamous granulosa cells in primordial follicles (arrowhead in Figure 3-5F). In antral follicles, pYAP1 was localized to granulosa cells and the fully grown oocyte (Figure 3-5). In adult ovaries, WWTR1 was localized strongly to the nucleus of both germ cells, granulosa cells and the corpora lutea (Figure 3-6A,B), while YAP1 (Figure 3-6D,E) and
pYAP1 (Figure 3-6G,H) localized more strongly to follicles than the corpora lutea, but did not appear to differ between healthy and atretic follicles (Figure 3-6J,K).

**Germ cell apoptosis in $Lats1^{-/-}$ ovaries**

To determine if $Lats1$ mutant pups have fewer germ cells and/or higher incidence of cell death at birth, newborn ovaries were stained with germ cell-specific TRA98 antibody and the cleaved PARP (poly ADP-ribose polymerase) antibody which marks apoptotic cells. Image analysis of ovarian sections immunostained with TRA98 antibody to mark germ cells showed similar number of germ cells in $Lats1$ mutant ovaries compared to control ovaries ($Lats1^{+/+}$: 56±3.8, $Lats1^{-/-}$: 54±4.0 germ cells/section) (Figure 3-7ABD). However, the proportion of germ cells undergoing apoptosis was higher in $Lats1$ mutant ovaries compared to control ($Lats1^{+/+}$: 1.7±0.19%, $Lats1^{-/-}$: 3.6±0.46% of total germ cells; P<0.05) (Figure 3-7ABE). Brightfield images of H&E sections showed no apparent morphological difference between $Lats1^{+/+}$ and $Lats1^{-/-}$ ovaries (Figure 3-7C).

**Morphological changes in cultured $Lats1^{+/+}$ and $Lats1^{-/-}$ ovaries.**

To determine changes in morphology during early ovarian development, newborn $Lats1^{+/+}$ and $Lats1^{-/-}$ ovaries were cultured for 11 days. During this time primordial follicles assemble and some are activated and begin growth. In control ovaries, there were abundant numbers of primordial follicles as well as primary and secondary follicles at the end of culture (Figure 3-8A-B). In contrast to control ovaries, $Lats1^{-/-}$ ovaries exhibited abnormal follicle development. In many cases oocytes underwent growth in groups without a complete layer of granulosa cells surrounding each oocyte (Figure 3-8A). In addition, $Lats1^{-/-}$ ovaries had more than a 50% decrease in the number of primordial follicles (control: 122.86±25.51, $Lats1$ mutant: 52.00±9.01 primordial
follies/mm², P<0.05) (Figure 3-8B). Likewise, the number of activated follicles was also decreased in \textit{Lats1}⁻/⁻ ovaries compared to control ovaries (control: 117.98±11.85, \textit{Lats1} mutant: 79.98±9.25 primordial follicles/mm², P<0.05) (Figure 3-8C). To determine if the abnormal follicle distribution results in altered transcript abundance, the expression of key transcripts was examined in cultured \textit{Lats1} control and mutant ovaries by quantitative PCR (Figure 3-9). Low expression of \textit{Lats1} mRNA confirmed that the \textit{Lats1} gene was deleted in these cells. Surprisingly, among the genes for oocyte specific factors, \textit{Bmp15} mRNA showed significantly higher expression in \textit{Lats1} mutant ovaries compared to control, whereas \textit{Gdf9, Figla} and \textit{Nobox} were not different (Figure 3-9). Likewise, the somatic cell specific genes, \textit{Amh} and \textit{Wnt4} did not differ between control and \textit{Lats1} mutant ovaries (Figure 3-9).

**Development of ovarian cysts**

A novel observation in cultured ovaries was the development of cystic structures in a large proportion of \textit{Lats1}⁻/⁻ ovaries (Figure 3-10). By day 7 of culture, 43% of \textit{Lats1}⁻/⁻ and 23% of \textit{Lats1}⁺/⁺ ovaries developed ovarian cysts (Figure 3-10). In contrast, only 8% of control ovaries developed cysts (Figure 3-10). The cysts that developed were lined by a single inner layer of palisading cuboidal epithelial (granulosa) cells or in some cases squamous cells, with an outer layer of spindled cells (Figure 3-10).

**Discussion**

Mammalian follicular development is controlled by complex paracrine and endocrine mechanisms. The ovary contains follicles at all developmental stages and at any given time, multiple processes including follicular activation, growth, and atresia are occurring simultaneously in the ovary. However, the mechanisms controlling the fate of
specific follicles (activation, growth or atresia) are not completely understood. The Hippo pathway is emerging as a key mediator of the cell fate decision to proliferate, remain quiescent or undergo apoptosis. A recent finding showed that increased follicular development resulting from ovarian fragmentation is due to increased activation of the Hippo pathway target, YAP1 [220]. In this study, we examined the expression of the core components of the Hippo pathway in mouse ovaries during follicular development and examined the effect of ablating a core kinase, LATS1, on early follicular development. The findings show that Hippo transcripts are decreased as follicles progress from primordial to antral follicles and that the proteins for several members of the core Hippo pathway are widely expressed in mouse ovarian follicles of different developmental stages. Interestingly, ablation of LATS1 caused increase germ cell apoptosis with subsequent primordial follicle loss, but also caused development of ovarian cysts. These findings demonstrate the complex and essential nature of the Hippo pathway during early follicular development.

Genetic ablation studies have shown that disruption of the Hippo signaling pathway causes proliferation defects and in some cases defects in fertility. Global ablation of Mst1/Mst2, Yap1, Lats2 and Nf2 are embryonic lethal in mice [235-238]. However, tissue-specific deletion of Mst1 and Mst2 showed that these proteins act as robust tumor suppressors in liver and colon where ablation of both kinases leads to YAP1 up-regulation and increased cellular proliferation [230, 239]. Yap1 and Wwtr1, on the other hand, act as oncogenes and stimulate cellular proliferation [229] and are upregulated in different types of cancers [179, 240], including ovarian cancer [219]. Thus far, Wwtr1, Lats1 as well as Sav1(Ww45) global knock-out mice exhibit fertility defects. Wwtr1" and
Sav1−/− mice are severely subfertile and suffer from decreased placenta vascularization, but the overall effect on fertility and ovarian function remain uncharacterized [180, 241]. Mice with a deletion in the Lats1 gene exhibit hyperplastic changes in the pituitary, decreased in serum levels of LH, growth hormone and prolactin, lack of mammary gland development, and overall growth retardation [176]. Specifically in the ovary, Lats1 ablation causes ovarian stromal cell tumors and soft tissue sarcoma by 4 months of age in the few pups that survive to adulthood [176]. Consistent with this previously described phenotype, we now show that Lats1 mutant ovaries develop cystic structures after just 7 days in culture. The more frequent occurrence of cyst formation in cultured Lats1−/− ovaries compared with Lats1+/- suggests that Lats1−/- ovaries are more prone to proliferation defects in vitro, but how this relates to the in vivo development of ovarian stromal tumors is not yet clear. The cystic structures appear to be lined by a cuboidal or squamous epithelium, but it is unclear if these are derived from follicles or the surface epithelium. The rapid growth of cystic structures and the single epithelial cell layer lining the inside of the cyst suggests that these structures might be different from the stromal cell tumors described previously [176].

Shortly after birth in mice, germ cell cysts start to break down into individualized germ cells and become surrounded by flattened granulosa cells to form primordial follicles [25]. Germ cell nest breakdown involves apoptosis of as many as two thirds of oocytes in a process that is hormone and growth factor dependent [25, 242]. Several oocyte transcription factors including FIGLA, FOXO3, NOBOX, SOHLH1, SOHLH2, and LHX8 are essential for follicle activation and early growth [243]. While the signaling pathways regulating these transcription factors are largely unknown, some candidate
factors include KitL, LIF, FGFs and CTGF [243, 244]. The increase in germ cell death in *Lats1* mutant ovaries at birth could be caused by either accelerated cyst breakdown or defects in somatic cells that impact germ cell survival. It will be important to determine how *Lats1* deletion leads to increased germ cell death. In cultured *Lats1* mutant ovaries primary follicular structure was poorly organized with morphologically normal oocytes, but an incomplete layer of granulosa cells (Figure 3-8). This observation is surprising since ablation of LATS1 would be expected to cause an increase in YAP1 and/or WWTR1 activity (i.e. nuclear localization) in granulosa cells which would then stimulate proliferation. Clearly, the roles of LATS1, YAP1 and WWTR1 in the ovary are more complicated than this simple model would suggest. The strong localization of pYAP1 in somatic cells in ovarian sections suggests that LATS1/2 may be more active in the somatic cells than in the germ cells. Although LATS1/2 are ubiquitously expressed, pYAP1 is highly localized to the somatic cells of primordial, primary and early pre-antral follicles rather than the oocytes suggesting that the activity of LATS1/2 may be regulated differently in germ cells and somatic cells. In future experiments, it will be important to determine the relative role of LATS1 deletion in germ cells versus the somatic cells. Conceivably, lack of LATS1 activity in the somatic cells could lead to a decrease in YAP and/or WWTR phosphorylation and an uncoupling of germ cell growth and somatic cell proliferation, but this concept remains speculative and requires further investigation.

Although the paralogs YAP1 and WWTR1 are structurally similar and share similar functions, they do not overlap completely. For example, knock-out studies revealed that *Yap1* deletion causes an embryonic lethal phenotype, while *Wwtr1* mutant mice are viable but suffer from kidney defects [180, 245]. In the ovary, we found a striking
difference in the localization of YAP1 and WWTR1 proteins. YAP1 was mostly cytoplasmic, while WWTR1 was strongly nuclear. Thus, it is possible that these effectors of the Hippo signaling pathway may play different roles in the ovary. In other mammalian cell models, YAP1 is known to interact with hnRNP and SMAD1/5, while WWTR1 is known to bind to PPARɤ and SMAD2/3, though both were shown to share transcriptional factor binding partners such as TEADs and RUNX proteins [229, 246, 247]. Both YAP1 and WWTR1 are oncoproteins and their activities are mostly associated with over proliferation phenotypes [179, 248], but how and why WWTR1 is localized to the nucleus in ovarian cells, while YAP1 is cytoplasmic is not clear, but may involves interactions with distinct binding partners.

The upstream signals that regulate Hippo signaling in the ovary are not known. Ovarian fragmentation leads to suppression of Hippo signaling and thus an increase in nuclear localization of YAP1 in preantral follicles [220]. This suggests that intra-ovarian signals activate Hippo in the un-fragmented ovary to regulate the rate of follicular development. Some of these signals could include changes in cell-cell contact, cell density, or cell-extracellular matrix (ECM) interactions [249-251]. The Hippo pathway could also interact with well-known regulators of follicular development such as FOXL2 [252] and members of the TGF-β family through interaction with SMAD signaling proteins [253-255]. Hippo signaling could also be regulated by secreted paracrine or endocrine factors that act through G-protein coupled receptors: Studies using multiple mammalian cell lines suggest that GPCR-mediated diffusible signals can either up-regulate or down-regulate the activity of YAP1/WWTR1 by acting through Rho GTPase and actin cytoskeleton [206, 207, 209]. Moreover, G-protein coupled receptors also
acutely regulate LATS kinases and thus YAP1/WWTR1 [206]. Given the strong interaction between GPCRs and Hippo signaling it seems very likely that signaling through FSH and LH receptors could also modulate Hippo signaling in the follicle.

Follicular activation, growth and atresia are controlled by paracrine and endocrine signals that regulate the balance between quiescence, proliferation and apoptosis during follicle activation or between continued proliferation and concurrent atresia during follicular development. The Hippo signaling pathway is intimately involved in the cell fate decision to proliferate, remain quiescent or undergo apoptosis. In the present study, we show that Hippo signaling is active in the ovary and that the LATS1 protein has different roles in germ cells versus somatic cells. In germ cells, \textit{Lats1} is required for maintenance of germ cell pool at birth, while in somatic cells \textit{Lats1} deletion leads to an over proliferation phenotype and formation of ovarian cystic structures. However, there is much yet to learn regarding the role of the Hippo pathway in controlling follicular development. Future studies will identify the functional differences between YAP1 and WWTR1 in different compartments of the ovary and the possible upstream regulators of Hippo signaling, including gonadotropin receptors. This new information will fill critical gaps in our knowledge of follicular development.
Figure 3-1. Expression of Sav1, Mob1b, Wwc1, Nf2, Stk4, Stk3, Last1, Lats2, Yap1 and Wwtr1 mRNAs in ovaries collected on postnatal day 2, 8, 21 and 21 days (primed with eCG (5 IU) 44-48 hours before harvesting). Values are mean±SEM, N=4, different letters indicate significant differences by ANOVA followed by Tukey’s post-hoc test, P<0.05.
Figure 3-2. Immunostaining for MOB1 protein in tissue sections from ovaries collected on postnatal day 2 (A, E), 8 (B, F) and 21 (C, G) days (primed with eCG (5 IU) 44-48 hours before harvesting). Negative control sections (D, H) were incubated with normal rabbit IgG. All slides were counterstained with hematoxylin for 20 seconds. Scale = 100 µm (A-D); 50 µm (E-H).
Figure 3-3. Immunostaining for WWTR1 (TAZ) protein in tissue sections from ovaries collected on postnatal day 2 (A, E), 8 (B, F) and 21 (C, G) days (primed with eCG (5 IU) 44-48 hours before harvesting). Negative control sections (D, H) were incubated with normal rabbit IgG. Scale = 100 µm (A-D); 50 µm (E-H).
Figure 3-4. Immunostaining for YAP1 protein in tissue sections from ovaries collected on postnatal day 2 (A, E), 8 (B, F) and 21 (C, G) days (primed with eCG (5 IU) 44-48 hours before harvesting). Negative control sections (D, H) were incubated with normal rabbit IgG. Scale = 100 μm (A-D); 50 μm (E-H).
Figure 3-5. Immunostaining for phosphorylated YAP1 protein (Ser127) in tissue sections from ovaries collected on postnatal 2 (A, E, I), 8 (B, F, J) and 21 (C, G, K) days (primed with eCG (5 IU) 44-48 hours before harvesting). Negative control sections (D, H, L) were incubated with normal rabbit IgG. Arrow and arrowhead in panel F indicate flattened and cuboidal granulosa cells of primordial and primary follicle. All slides were counterstained with hematoxylin for 20 seconds. Scale = 100 µm (A-D); 50 µm (E-H); 25 µm (I-L).
Figure 3-6. Immunostaining for WWTR1 (TAZ) protein (A-C), YAP1 protein (D-F), phosphorylated YAP1 protein (Ser127) (G-L) in tissue sections from ovaries collected from 2 month old mice. Negative control tissue sections were stained with normal IgG (C, F, I, L). Panels A-I were counterstained with hematoxylin for 20 seconds. Panels J-L were not counterstained to better visualize pYAP1 localization in healthy and atretic follicles. *Asterisk denotes atretic follicles based on the absence of cumulus cells around the oocyte and disorganized granulosa cell layer, CL = corpus luteum, Scale = 100 µm.
Figure 3-7. Overlay of TRA98 and cleaved PARP (A), DNA (B), and H&E (C) staining in newborn ovaries from Lats1+/+ and Lats1−/− mutant animals. TRA98 and PARP staining and germ cell counting were performed by Dr. Melissa Pepling from Syracuse University. The white circles denote PARP positive cells (apoptotic oocytes). D. Number of germ cells (oocytes/section) in newborn Lats1+/+ and Lats1−/− ovaries. E. Proportion of apoptotic germ cells (% PARP positive) in Lats1+/+ and Lats1−/− ovaries (N=12). Values are mean±SEM. *Indicates significant differences using General Linear Model, P<0.05. Scale = 50 µm (Panels A-B), Scale = 100µm (Panel C).
**Figure 3-8.** A. Brightfield images of H&E stained sections from \( Lats1^{+/+} \) and \( Lats1^{-/-} \) ovaries cultured for 11 days. B. Primordial follicle number (follicles/mm\(^2\)) in tissue sections from control (\( Lats1^{+/+} \)) and \( Lats1 \) mutant (\( Lats1^{-/-} \)) ovaries cultured for 11 days. C. Number of activated follicles (follicles/mm\(^2\)) in tissue sections from control (\( Lats1^{+/+} \)) and \( Lats1 \) mutant (\( Lats1^{-/-} \)) ovaries cultured for 11 days. Values are mean±SEM, N=6. *Indicates significant differences by Student’s t-test, P<0.05. Scale = 100 μm.
Figure 3-9. A. Expression of *Lats1*, *Bmp15*, *Gdf9*, *Figla*, *Nobox*, *Amh* and *Wnt4* mRNAs in *Lats1*<sup>+/+</sup> and *Lats1*<sup>-/-</sup> ovaries cultured for 11 days on collagen membrane. Values are mean±SEM, N=6. *Indicates significant differences by Student’s t-test, P<0.05.
Figure 3-10. A. Representative brightfield images of $Lats1^{+/+}$ and $Lats1^{-/-}$ ovaries cultured for 7 days showing development of cysts on $Lats1^{-/-}$ ovary, Scale = 500 µm. B. Cyst frequency (%) in $Lats1^{+/+}$, $Lats1^{+/+}$ and $Lats1^{-/-}$ ovaries after 7 days in culture. C. Representative images of H&E sections showing cysts formation from cultured $Lats1^{-/-}$ and $Lats1^{+/+}$ ovaries, scale = 50 µm. Arrow indicates spindled cells. *Indicates significant differences by Chi squared test, P<0.05.
Figure 3-11. Immunostaining for LATS1 (A-C) and LATS2 (D-F) proteins in tissue sections from ovaries collected on postnatal day 21 primed with eCG (5 IU) 44-48 hours before harvesting. Negative control sections (G, H) were incubated with normal rabbit IgG. Scale = 100 µm.


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<td><em>Wwc1 (Kibra)</em></td>
<td>ACAGGGTGCTACAAAAGTGATGA</td>
<td>ATCCTTCAGCATGGCGAGAATCTTT</td>
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<tr>
<td><em>RPL19</em></td>
<td>TTCAAAAAAAGCGCATCCT</td>
<td>CTTCGTGCTTCCTTGGTCT</td>
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Chapter 4

YAP1 is Essential in Germ Cell Survival and Follicle Activation During Early Follicular Development

Abstract

The Hippo signaling is important in regulating cell fate of various mammalian systems. YAP1 is a transcriptional coactivator of the Hippo signaling pathway that mediates the upstream activity of Hippo kinases. Little was known about the functions of YAP1 during early follicular development until a recent report showed the activation of YAP1 downstream of ovarian fragmentation or induction of actin polymerization in mice and human ovarian tissue. Our present study examined the possible roles of YAP1 during early follicular development in intact mouse ovaries. We uncovered that blocking the nuclear binding of YAP1 predisposes germ cells to apoptosis and impedes the follicle activation in short-term cultured ovaries. Counterintuitively, the deletion of YAP1 by inducible YAP1-Cre knockout exhibited an unexpected stimulation of follicle activation, indicating the existence of complicated regulatory mechanisms on YAP1 interference in heterogeneous follicular environment that requires more sophisticated examination.
Introduction

Mammalian follicular development is under the coordinated regulation of endocrine hormones from the hypothalamus-pituitary axis and paracrine/autocrine factors produced in the ovary. This complicated regulatory network involves multiple organs and cell types and is highly stage-specific along the path of follicular development. Around the time of gonadal sex differentiation, PGCs undergo incomplete cell division and form gem cell cysts [2]. This process is regulated by locally produced factors such as BMPs, FOXL2, and WNT4 [9, 11, 13]. After germline cysts formation, the oocytes begin to enter meiosis and arrest at the diplotene stage of prophase I until ovulation. Soon after birth, cyst breakdown takes place and individual oocytes become surrounded by somatic cells to form primordial follicles [256]. At the end of the cyst breakdown, only a small portion of germ cells survive and contribute to the primordial follicle pool [256]. Soon after follicle assembly, primordial follicles are activated and become primary follicles in a continuous manner. This activation is modulated by oocyte-produced factors (cFGF, PDGF) [49, 50], or oocyte-expressed transcription factors (FIGLA, NOBOX, LHX8, SOHLH1/2, FOXO3, OCT4) [29, 30, 32], and factors from pre-granulosa cells (FOXL2, KL, LIF) [38, 53]. The PI3K/PTEN signaling pathway in oocytes is especially important for suppressing follicle activation [40]. After activation and growth beyond primary stage, follicles become increasingly FSH-responsive as they grow into antral follicles. Follicular cell-produced factors act either in a paracrine/autocrine manner, interact with the action of FSH locally, or feed back to the H-P axis to modify the secretion of the gonadotropins.

Our current understanding of early follicular development in mammals has mostly been derived from genetically modified mouse models where researchers are able to
unveil the roles of various factors and some signaling cascades in controlling follicular physiology. However, the mechanisms regulating cell-cell communication and the different signaling cascades that contribute to activation and early follicular development are still not very well understood. The Hippo signaling pathway discovered recently, has been recognized as a key pathway for determining cell fate and controlling organ size among species. The step-by-step phosphorylation and activation of its core cascade leads to the cytoplasmic retention of its downstream effectors YAP/WWTR while the inhibition of the core cascade induces nuclear transcriptional activity of YAP/WWTR, through binding of YAP/WWTR with TEAD1/4, which eventually result in cell proliferation and survival in many mammalian cell types. Deletion of *Lats1* in mice leads to impaired folliculogenesis, progressive ovarian stromal tumors and complete lack of corpora lutea [176]. YAP1 was reported as an onco-protein in several types of ovarian tumors and cancers, as its expression, especially its nuclear accumulation, is highly associated with poor survival rate in ovarian cancer patients [179, 219, 257]. However, until recently, very little was known about how the Hippo pathway is regulated in follicular cells during follicular development. Reports by the Hsueh group proposed a model in which the ovarian fragmentation in both mice and human stimulates their follicular growth, through actin polymerization and disruption of Hippo signaling leading to upregulation of YAP1 [221, 258]. This improved our understanding of the subcellular mechanisms behind the physically disruptive procedures when treating POI or PCOS patients. The same group then further addressed the relation between Hippo activity and the actin cytoskeleton, utilizing actin-polymerization enhancing drugs JASP (Jasplakinolide) and S1P (sphingosine-1-phosphate) to treat ovaries from immature mice.
They reported that increased filamentous actin leads to not only increased YAP1 expression, but also the expression of growth-promoting YAP1 target genes such as Ccn2 [221].

Our main objective in this study was to investigate the possible roles of YAP1 during follicular activation and early follicular development. Two different approaches were used. First, the small molecule inhibitor Verteporfin (VP) which directly inhibits the binding of YAP1 and TEAD [259], was used to treat newborn ovaries in vitro to examine the roles of YAP1-TEAD mediated Hippo signaling during cyst breakdown and early follicular activation. Secondly, with a tamoxifen-induced Yap1 knock-out model approach, we were also able to investigate the role of YAP1 in early folliculogenesis. For both approaches, we determined the changes on follicle organization, and oocyte numbers that were caused by ablating YAP1.

In addition, using ex vivo culture of newborn ovaries, we also investigated how modulators of filamentous actin may affect the expression of YAP1 in young postnatal mouse ovaries that might impinge on the cyst breakdown and follicle activation later. We applied either actin polymerization enhancer JASP or inhibitor cytochalasin B to investigate the impact of filamentous actin on YAP1 localization in day 3 ovaries.

**Methods**

**Animals**

Female CD1 newborn mice were produced in the research colony of the investigators. Offsprings of Yapi^{tm1.1Dup} [260] and B6.Cg-Tg(CAG-Cre/Esr1*)5Amc/J [261] were kind gifts from Dr. Robert Paulson at Penn State University and Dr. Duojia Pan from Johns Hopkins University School of Medicine and maintained as heterozygous mating pairs. Newborn progeny were used and genotyped by PCR with specific primer
set \( P1:5'\text{-CCATTGTGTCCTCATCTCTTACTAAC-3'}, P2:5'\text{-GATTGGGCACTGTCAA}
TTAATGGGCTT -3', P3: 5'\text{-CAGTCT GTAACAACCAGTCAGGGATAC-3'}. \) \( P1/P2 \) primer pair was used for examining the inducible Cre; \( P1/P3 \) primer pair was used for examining \( \text{Yap/flox} \) deletion. Breeding pairs were maintained according to the Guide for the Care and Use of Laboratory Animals (Institute for Learning and Animal Research). All animal use was reviewed and approved by the IACUC committee at The Pennsylvania State University.

**Ovary culture**

Ovaries were isolated from newborn CD1 mice or \( \text{Tm-Yap} \) mice on the first postnatal day and cultured \textit{in vitro} as described previously [231]. Briefly, surrounding tissues, including the ovarian bursae were removed. Ovaries were cultured in Waymouth752/1 medium supplemented with 0.23 mM pyruvic acid, 10 \( \mu \)g/ml streptomycin sulfate, 75 \( \mu \)g/ml penicillin G, and 10% fetal bovine serum (FBS) at 37°C in an incubator infused with a gas mixture of 5% CO2, 20% O2. For CD1 mice, 3-4 ovaries were placed on one Costar Transwell Col membrane insert (3.0 \( \mu \)m, 24 mm; Corning, NY) with or without Verteporfin (1\( \mu \)M) for 0 days, 2days, 4 days or 8 days before harvesting. Every mouse served as its own control. One side of the ovary was treated with control medium, the other side was treated with medium containing 100nM hydroxytamoxifen (Sigma). To examine the effect of actin modulators on YAP1 expression in young ovaries in short term culture, we treated the 3d CD1 ovaries with 2.5\( \mu \)M cytochalasin B for 4h before harvesting, or with 5 \( \mu \)M JASP for 1h, washed it out and continued to culture in control medium for 3 more hours. In all cases, medium was replaced every other day.
Histology

Cultured ovaries were fixed in 4% electron microscopy-grade paraformaldehyde (Electron Microscopy Sciences) for 4 hours in 4°C refrigerator and embedded in paraffin. Ovarian sections (4 µm) were stained with hematoxylin and eosin (H&E) using standard methods. Brightfield images on H&E stained sections were captured for assessment of follicular morphology of cultured ovaries.

TRA98 and cleaved PARP immunohistochemistry

Ovaries were fixed in 4% electron microscopy-grade paraformaldehyde (Electron Microscopy Sciences) for 4 hours in 4°C refrigerator and stained as previously described [27]. Whole ovaries were incubated with TRA98 antibody (B-Bridge) diluted 1:100 to label germ cells [232, 262] and cleaved PARP (E51, Abcam) diluted 1:100 to label cells undergoing apoptosis overnight and then with goat anti-rat secondary Alexa 488 and goat anti-rabbit Alexa 568, respectively. Nuclei were labeled with DAPI (Invitrogen). A Zeiss LSM 710 confocal microscope was used to image the ovaries.

Germ cell counting

Total oocytes (TRA98 positive) and apoptotic oocytes (cleaved PARP-positive) were determined by counting the number of cells found within each of 8 optical sections per ovary (N=5). The numbers were averaged and reported as number of oocytes per section. The number of oocytes per section is used as a proxy for the total oocytes per ovary; therefore, only ovaries that appeared to be of similar size and depth were used for counting oocyte numbers.

Follicle counting
Primordial and activated follicles were examined for CD1 ovaries cultured for 7 days and for Tm-Yap mice treated with 1µM verteporfin. Two middle sections, with 6~8 sections apart, from each ovary were selected for counting. Follicles were independently counted by two persons, who were blinded to the two genotypes. The follicles with a single squamous layer of granulosa cells were counted as primordial follicles. The follicles with a layer of cuboidal granulosa cells and follicles beyond this stage were counted as activated follicles. The area of each section was measured using DP2-BSW (Olympus, Tokyo, Japan). Follicle numbers were normalized to the area of each section.

Immuno-histochemistry

Cultured ovaries were fixed in electron microscopy-grade 4% paraformaldehyde for 4 hours at 4°C and embedded in paraffin. Sections (4µm) were de-waxed in histoclear (EMS, Hatfield, PA) 3 times for 5 min each and then incubated for 5 min in each of the following: 100% ETOH, 100% ETOH, 95% ETOH, 85% ETOH, 70% ETOH and ddH2O, ddH2O. Next, samples were incubated in 1X antigen retrieval solution (Dako S2367) at 94°C for 30 min. After cooling to room temperature, slides were washed 3 times with ddH2O for 5 min. Then the sections were incubated in 3% hydrogen peroxide for 10 min, followed by two washes in ddH2O and two washes in wash buffer (1 X TBST, 0.1% Tween-20), 5 min each followed by incubation in goat blocking buffer (TBST, 5% goat serum) for 1 h at room temperature. Slides were then immediately incubated with anti-YAP1 antibody (1:200, Cell Signaling Technology) at 1:800 dilution. After primary antibody incubation, slides were washed in 1 X TBST 3 times for 5 min each. Then 1-3 drops of room temperature-equilibrated SignalStain Boost Detection Reagent was used to cover sections, incubated in room temperature for 30 min in a
humidified chamber. Then slides were washed 3 times in 1 X TBST, after which, 100-400ul SignalStain DAB reagent was applied to each section and monitored closely for color development for 2.5 min. Slides were then immersed in ddH2O to stop the reaction. The normal rabbit IgG antibody applied with corresponding concentration was used for negative controls. Finally, slides were mounted with cytoseal and covered with glass coverslips.

**Statistical analyses**

Follicle and germ cell counting data are presented as means±SEM. The activated and primordial follicles and proportion of apoptotic germ cells were analyzed using Student t-test or one way ANOVA followed by Dunnett’s post-hoc test. A p-value <0.05 was considered statistically significant.

**Results**

**Verteporfin predisposed germ cells to apoptosis and impeded follicle activation in cultured ovaries.**

VP acts to directly block the binding of YAP1 and TEAD [259]. To investigate the role of YAP1 in germ cells during cyst breakdown and early follicular development, we used the small molecule inhibitor VP to treat cultured newborn ovaries for 7 days. Primordial and activated follicles were counted and recorded as follicle numbers per unit area with or without VP (1µM). Our results showed that there wasn’t a significant difference between primordial follicle numbers per unit area between control and VP treatment (Figure 4-1A, B). Nevertheless, follicle activation was significantly impeded in VP treated ovaries by 80%, from 117.55±33.53 to 23.82±5.25 follicles/mm², P<0.05) (Figure 4-1C). To further investigate the progression of the VP effect during cyst breakdown in culture ovaries, we then cultured newborn ovaries for 2, 4, 8 days. Our
control group showed that the germ cell number decreased dramatically after the first two
days of culture (P<0.05, Figure 4-2A-E) but the continuous decrease of germ cells till day 8 was not statistically significant. Despite the similar sizes of germ cell populations between control and VP treatment of day 8 ovaries, the primordial follicle and activated follicle populations were significantly different (Figure 4-2G, H). VP treated day 8 ovaries exhibited significantly larger primordial follicle pools while fewer follicles became activated, compared to control ovaries (Figure 4-2G, H). On the other hand, the percentage of apoptotic germ cells in total germ cell population of the control group was consistent throughout the culture (Figure 4-2A-D, F). The comparison between control and VP treated groups showed that although VP didn’t cause a significant decrease in germ cell numbers per section in any of the time points observed (Figure 4-2A-E), it actually led to 1.9 and 2.8 fold increase in apoptotic germ cell percentages on day 2 and day 4, respectively (P<0.05, Figure 4-2A-D, F).

**Tamoxifen-inducible YAP1 knockout advanced follicle activation**

In addition to directly interfering with YAP/TEAD binding, we also utilized a tamoxifen-inducible YAP1 conditional knockout model, to investigate the role of YAP1 in early folliculogenesis, by inducing the YAP1 knockout in 11d cultured ovaries. Tamoxifen induction didn’t lead to significant changes in primordial follicle pool for either *Yap F/-* or *Yap F/- Cre* genotype (Figure 4-3A, B). For activated follicles, the effect caused by tamoxifen was not significant in *Yap F/-* group (Figure 4-3C). However, the tamoxifen-induced YAP1 knockout in *Yap F/- Cre* ovaries led to an 1.82 fold increase in activated follicle numbers per unit area (Figure 4-3C).
Actin modulators JASP and cytochalasin B didn’t exhibit apparent impact on YAP1 expression in cultured young ovaries

To characterize if either actin polymerization enhancer JASP or actin polymerization inhibitor CB had an immediate effect on the YAP1 expression in young CD1 ovaries, 3d CD1 ovaries were treated with 2.5µM cytochalasin B for 4h before fixing, or with 5 µM JASP for 1h, washed out and cultured in control medium for 3 more hours, before fixing. CB treated ovarian sections showed what appeared to be an overall lighter staining of YAP1 (Figure 4-4B), compared to control (Figure 4-4A). JASP, on the other hand, seemed to cause a shift of YAP1 from granulosa cells of activated follicles to the growing oocytes (Figure 4-4C). However, the data were not quantified in the current study.

Discussion

From oogenesis, germ cell cyst formation to cyst breakdown and follicle activation, the process of early follicular development is largely under the regulation of locally produced autocrine, paracrine factors and transcription factors that mediate critical signaling pathways such as PI3K/PTEN pathway. The Hippo signaling pathway, discovered more recently, has shed light on our understanding of organ size control and cell fate determination in many organ systems including ovaries. For example, YAP1 was reported by several studies as an onco-protein in multiple types of ovarian tumors and cancers, and its nuclear accumulation is highly associated with poor prognosis in ovarian cancer patients [179, 219, 257]. Additionally, the deletion of *Lats1* gene which encodes the upstream kinase LATS1 that regulates YAP1 activity, leads to impaired folliculogenesis, progressive ovarian stromal tumors and complete absence of corpora lutea in mice [176]. However, little was known about how YAP1 regulates ovarian functions, the lack of which may be linked to early developmental defects in ovarian
folicles or later ovarian malignancies. Our findings show that while germ cell populations in postnatal ovaries experienced a significant drop only after 2 days in culture, extended culture till 8 days didn’t result in further loss of germ cells (Figure 4-2E). Blocking YAP1/TEAD binding with a small molecule inhibitor verteporfin didn’t seem to shrink the existing germ cell pool size by the end of the culture, but VP caused more germ cells to undergo apoptosis after 2 days and 4 days of culture (Figure 4-2F) which presumably will result in a significant loss of germ cell populations under more extended VP treatment. By the end of the culture, even though total germ cell numbers remained unchanged, VP apparently impeded the process of recruiting primordial follicles to leave the primordial follicle pools and become activated (Figure 4-2G, H, Figure 4-1B, C).

YAP1 has been recognized as an oncoprotein and a growth promoter in a number of functional studies in mammalian somatic cells. Our current study is the first to show quantitatively that interfering with YAP1 signaling actually led to more apoptosis in germ cells. During cyst breakdown which lasts till postnatal day 5, a large portion of germ cells undergo apoptosis that is regulated by TNF-α, Bcl-x and Bax [22, 23]. However, as a unique process that’s different from a classic programmed cell death, it is not positively associated with nuclear condensation, activation of caspase 3, cleavage of PARP, or fragmentation of DNA [25, 26]. Instead, the majority of dying germ cells are associated with their sister cyst cells during the breakdown [25]. Our study uncovered that YAP1 is essential in germ cell survival during cyst breakdown. But how YAP1 is involved in germ cell survival and interacts with other factors still remains unclear. It has been reported that YAP1 blocks the binding and LATS2-induced phosphorylation of the tumor
suppressor, ASPP1 (Apoptosis-stimulating protein of p53-1) in human cell line WI-38 [188]. Also, apart from the canonical pro-proliferative and anti-apoptotic effect of YAP1, in response to DNA damage, c-ABL phosphorylates YAP1 to facilitate its interaction with p73 which induces pro-apoptotic transcription [203]. Whether the interference of ASPP1 by YAP1-LATS2 and the modulation of YAP1 by c-ABL play similar functions in follicular cells during cyst breakdown requires further investigation. Another finding of this study is that VP impeded follicle activation in cultured ovaries. Among numerous factors that regulate this process, LIF (Leukaemia inhibitory factor) produced by granulosa cells, induces the expression of KL [53] which binds to oocyte expressed receptor c-kit and regulates the oocyte expression of BMP15. In embryonic stem cells, Yes kinase phosphorylates YAP1 to mediate the YAP1-TEAD2 dependent transcription induced by LIF (Leukemia inhibitory factor) [186]. It is possible that in our system, blocking YAP1-TEAD2 binding blunted YAP1-TEAD2 dependent transcription induced by LIF so that any downstream effectors that are regulated by LIF were potentially attenuated, for example, the oocyte expression of BMP15. But further studies are required for testing this hypothesis.

On the other hand, the inducible YAP1 knockout model allowed us to investigate the importance of YAP1 from a slightly different angle. Due to the difficulty of obtaining pups with Yap F/F Cre and Yap F/F genotypes, instead, we investigated the ovarian phenotypes of Yap F/- Cre pups with or without the tamoxifen induction, using Yap F/- as a control for dissecting out the side effect of tamoxifen treatment. Surprisingly, in contrast to what we observed when YAP1 was blocked by VP, our findings with the inducible knockout approach suggested that tamoxifen induced YAP1 knockout led to a
significant increase in activated follicle numbers. Though this paradoxical observation raised many unanswered questions, it could partly be explained by the following reasons. Firstly, the ideal control genotype for inducible knockout study would be \( \text{Yap } F/F \) where both alleles of YAP1 remain present. In our study, \( \text{Yap } F/- \) with one YAP1 allele deleted globally, might already predispose the prenatal ovarian development to certain defects, prior to the treatment of inducible tamoxifen. Differently, the interference of YAP/TEAD binding by VP began only after birth, which means that the prenatal ovarian development was not influenced by any manipulation of YAP1 expression or activity. Secondly, though the effect caused by tamoxifen examined was not significant here, it shall be thoroughly investigated in future studies whether tamoxifen could potentially complicate the phenotypes for all available genotypes investigated such as \( \text{Yap } F/F \) and \( \text{Yap } F/F \) \( \text{Cre} \). Also, it remains unknown how efficient the inducible \( \text{Yap-Cre} \) was for ablating YAP1 expression in the \textit{in-vitro} culture system. Furthermore, besides optimizing the inducible knockout model, other transgenic models such as oocyte specific or granulosa specific YAP1 conditional knockout mice might be utilized in future studies. Also mechanistically, though VP has been widely used in studies in other organ systems that require the inhibition of YAP/TEAD(s) [263], mechanistic studies on VP blocking YAP/TEAD binding only were documented on TEAD2 [264]. Therefore, it is still not quite clear how efficient the other members of TEAD family are impacted by VP interference and whether this results in counteracting the growth-promoting effect of YAP1 in ovarian follicular context.

A recent study was conducted to link Hippo activity with the actin cytoskeleton, utilizing actin-polymerization enhancing drugs JASP and S1P to culture ovaries from 10
day-old immature mice [221]. They reported that acute treatment of those actin
enhancing drugs leads to increased YAP1 expression those ovaries [221]. Here in our
study, we examined both actin polymerization enhancer JASP and inhibitor cytochalasin
B in even younger ovaries, from 3 day-old mice, during the cyst breakdown. Though
further investigation is required to draw a solid conclusion, JASP didn’t seem to cause an
overall increase of YAP1, as reported before [221]. Instead, YAP1 expression seemed
lower in the granulosa cells while the growing oocytes exhibited more abundant YAP1.
CB treated ovarian sections, on the other hand, showed what an overall lighter staining of
YAP1 than control. It is possible that during cyst breakdown, early follicles do not
respond to actin modulator drugs as strongly as what was reported by others [221], where
older ovaries with significant amount of secondary follicles were utilized. At the same
time quantitative assays are required to validate our current result, it will also be
appealing to examine the long term effect of actin modulating drugs JASP and CB on
follicular organization after the acute treatment. For example, ovaries may be treated for
a few hours and then subjected to continuous culture in control medium for 10 days,
before fixation and quantitative assays.

In summary, our present study discovered that by blocking the transcriptional
binding of YAP1, verteporfin predisposes germ cells to apoptosis and impedes the
follicle activation in short-term cultured ovaries. However, the deletion of YAPI by
inducible YAPI-Cre knockout exhibited an unexpected stimulation of follicle activation.
This intriguing observation raised the question of whether it was the lack of efficiency of
the inducible YAPI knockout, or the possibly much complicated regulatory and
compensatory mechanisms on YAPI interference in heterogeneous follicular
environment. In addition, the actin polymerization enhancer JASP and cytochalasin B didn’t exhibit apparent impact on YAP1 expression in young postnatal ovaries, which requires further investigation.
Figure 4-1. A. Brightfield images of H&E stained sections from ovaries cultured in control or VP (1 µM) medium for 7 days. B. Primordial follicle number (follicles/mm²) in sections from ovaries cultured in control or VP (1 µM) medium for 7 days. C. Number of activated follicles (follicles/mm²) in sections from ovaries cultured in control or VP (1 µM) medium for 7 days. Values are mean±SEM, N=4. *Indicates significant differences by Student’s t-test, P<0.05. Scale = 100 µm.
Figure 4-2. Overlay of TRA98, cleaved PARP, DAPI, next to H&E staining (A-D) in newborn, day2, day4 and day8 ovaries cultured with control or VP (1 μM) medium. TRA98 and PARP immunostaining and germ cell counting were generated by Dr. Melissa Pepling from Syracuse University. The white circles denote PARP positive cells (apoptotic oocytes). E. Number of germ cells (oocytes/section) in newborn and cultured ovaries. F. Proportion of apoptotic germ cells (% PARP positive) in newborn and cultured ovaries (N=5). G. Number of primordial follicles (follicles/section) in cultured ovaries for 8 days with control or VP (1 μM) medium (N=5). H. Number of activated follicles (follicles/section) in cultured ovaries for 8 days with control or VP (1 μM) medium (N=5). Values are mean±SEM. Lower case letter a,b indicate significant differences between newborn group and cultured groups, using one-way ANOVA followed by Dunnett’s post-hoc test. *Indicates significant differences between control and VP treatments at certain time point using students’ t test, P<0.05. Scale = 50 μm.
A. H & E

Yap F/Cre in control medium
Yap F/Cre in medium with Tamoxifen
Yap F/ in control medium
Yap F/ in medium with Tamoxifen

B. Primordial Follicles

C. Activated Follicles
**Figure 4-3.** A. Brightfield images of H&E stained sections from *Yap F/-* or *Yap F/- Cre* ovaries cultured in control or medium containing Tamoxifen (100nM) for 7 days. B, C. Primordial follicle number (follicles/mm²) and the number of activated follicles in sections from *Yap F/-* or *Yap F/- Cre* ovaries cultured in either control or medium containing Tamoxifen (100nM) for 7 days. Values are mean±SEM, N=4. *Indicates significant differences by Student’s t-test, P<0.05. Scale = 100 μm.
Figure 4-4. Immunostaining for YAP1 protein in tissue sections from ovaries collected on postnatal day 3, cultured for 4h with cytochalasin B (2.5 µM) (Figure 4-4B) or with 5 µM JASP for 1h, washed out and continued to be cultured in control medium for 3 more hours (Figure 4-4C) before harvesting. Negative control sections (Figure 4-4D) were incubated with normal rabbit IgG. Scale = 100 µm.
Chapter 5

Hippo Signaling Regulates Proliferation and Differentiation of the Cumulus-oocyte Complex During the Peri-ovulatory Transition in Mice

Abstract

The Hippo signaling pathway plays critical roles in regulating cell proliferation, differentiation and apoptosis in many species. Hippo pathway proteins are expressed in the ovary and are involved in ovarian function. Deletion of Lats1 causes germ cell loss, ovarian stromal tumors and reduced fertility. Ovarian fragmentation induces nuclear YAP1 accumulation and is required for increased follicular development. At ovulation, follicular cells stop proliferating and terminally differentiate. However, the mechanisms controlling this transition are not completely known. Findings in this study show that before ovulation oocytes inhibit expression of Hippo transcripts, partly through a SMAD2/3-mediated pathway, and stimulate granulosa cell proliferation through a YAP1-mediated signal. Moreover, inhibition of YAP1 signaling with the small molecule inhibitor, verteporfin, triggers premature differentiation as indicated by cumulus expansion and progesterone production from cumulus-oocyte complexes in vitro. The findings also show that hCG-stimulated ovulation causes an increase in Hippo transcripts and stimulates Hippo pathway activity as indicated by increased phosphorylation of the Hippo targets YAP1 and WWTR1. In vitro, EGF stimulation of COC causes a transient increase in YAP1 phosphorylation followed by degradation of YAP1 protein with only modest effects on WWTR1. Surprisingly, the findings also show that inhibition of YAP1 activity disrupts oocyte maturation. Overall, our results uncovered a YAP1-mediated
mechanism that controls proliferation and differentiation of granulosa cells and oocyte maturation. These findings establish a new mechanistic model for the control of ovulation.
Introduction

The ovarian follicle undergoes a dramatic transformation during the peri-ovulatory transition. Before ovulation, oocyte secreted factors define the cumulus cell phenotype by promoting glycolysis [116], cholesterol synthesis [114], proliferation [223, 265] and survival [266], while suppressing luteinization [267-269] and opposing FSH-stimulated gene expression [224]. Many of the effects of oocytes on cumulus cells are mediated, in part, by oocyte-specific factors, such as GDF9 and BMP15, which activate the SMAD2/3 and SMAD1/5/9 signaling pathways [72, 116, 224, 270, 271]. Cumulus cells, in turn, promote developmental competence of the oocyte [225, 226, 272-281]. The cumulus cell-derived factors that promote oocyte development are less well-understood, but include factors that regulate transcriptional silencing [121], meiotic arrest [125, 267] and intracellular zinc [122].

The LH surge initiates a signaling cascade that profoundly transforms the cellular phenotype of theca, mural granulosa and cumulus cells. The LH surge leads to a self-reinforcing increase in EGF-like peptides in the mural and cumulus granulosa cells [282-285]. In the cumulus cells, activation of the EGF signaling pathway initiates the process of cumulus expansion [224], causes cells to exit from the cell cycle and increases resistance to apoptosis [286-289]. In addition, EGF causes the up-regulation of progesterone from the cumulus cells [284] which may serve as a sperm chemoattractant factor during fertilization and/or may be important for oocyte nuclear maturation [290-293]. Thus, granulosa cells transition from highly proliferative and un-differentiated cells into terminally differentiated cells with little capacity to proliferate. The oocyte also undergoes a dramatic transition at ovulation. Oocyte meiotic arrest is maintained by
cumulus cells through the production and transfer of cGMP to the oocyte where it inhibits phosphodiesterase (PDE) activity and thus, maintains high levels of cAMP before ovulation. Stimulation by EGF-like peptides prompts oocyte maturation by causing both a reduction in cGMP and the closure of gap junctions which results in activation of PDE, reduction of cAMP, activation of MPF and resumption of meiosis [125, 294]. These dramatic transformations of the cells in the pre-ovulatory follicle are critical for optimal fertility and ovarian function, but the downstream intra-follicular mechanisms mediating these responses are not completely known.

The Hippo pathway is a key regulator of the cell fate decision to proliferate, remain quiescent or undergo cell death [295]. Activation of the Hippo kinases (STK3/4) leads to a kinase cascade including phosphorylation and activation of the kinases LATS1 and LATS2, which in turn, phosphorylate and inactivate the transcriptional co-activators, YAP1/WWTR1 (also known as TAZ). Phosphorylated YAP1 and WWTR1 proteins are sequestered in the cytoplasm and are prevented from acting as transcriptional co-activators. When the Hippo pathway is suppressed, YAP1 and WWTR1 are dephosphorylated and move into the nucleus where they activate genes involved in proliferation [229, 296]. Given its role in proliferation, it is not surprising that the Hippo pathway is important for the regulation and maintenance of various stem cell populations [297, 298]. Early studies found that disruption of various Hippo pathway components caused increased organ size in *Drosophila* [299]. In mammals, liver-specific deletion of the Hippo kinases, *Stk4*<sup>-/-</sup>*Stk3*<sup>-/-</sup> (*Mst1*<sup>-/-</sup>*Mst2*<sup>-/-</sup>), causes up-regulation of YAP1 protein and increases liver size in mice [230, 300, 301]. Deletion of several Hippo pathway components also results in ovarian defects, including decreased follicular development,
germ cell loss, follicular cysts and ovarian stromal tumors in Lats1 mutant mice [176, 302] and reduced fertility in Wwtr1 (Taz) mutant mice [303]. More recently, a study by Kawamura and colleagues shows convincingly that fragmentation of mouse and human ovaries altered the actin cytoskeleton and stimulated nuclear YAP1 accumulation in somatic cells that was required for increased proliferation and follicular development [220].

Follicles undergo a dramatic transition during ovulation. In this study we uncover a novel role for the Hippo signaling pathway in mediating the peri-ovulatory transition. The findings indicate that oocyte-secreted factors, signaling partly through the SMAD2/3 pathway, suppress Hippo signaling in cumulus cells. Oocyte-mediated signals also activate YAP1 signaling which is necessary to stimulate cell proliferation and inhibit differentiation before ovulation. However, ovulatory signals transiently activate Hippo signaling to allow terminal differentiation of follicular cells. Interestingly, the findings also show that YAP1 activity is necessary for oocyte maturation in vitro. Thus, the Hippo pathway is essential for regulating the ovulatory transition in mice.

Methods

Animals

Female CD1 mice were produced and raised in the research colony of the investigators. Animals were maintained according to the Guide for the Care and Use of Laboratory Animals (Institute for Learning and Animal Research). All animal use was reviewed and approved by the IACUC committee at The Pennsylvania State University. Mice were weaned at 18 days old and primed with PMSG (5 IU) for 48 hours before
euthanasia and tissue collection. In some experiments, mice were primed with PMSG for 48 hours, followed by hCG (5 IU) for 6 or 24 hours before tissue collection.

**In-vitro cell culture of cumulus-oocyte complexes**

Cumulus oocyte complexes (COC) were collected from mice (18 days old) primed with PMSG for 48 hours, as described previously [224]. Briefly, fresh ovaries were placed in bicarbonate-buffered MEM-α medium (Life Technologies, Grand Island, NY), supplemented with 75 mg/L penicillin G, 50 mg/L streptomycin sulfate, 0.23 mM pyruvate and 2mg/ml BSA, unless otherwise noted. COCs were released from antral follicles by gentle puncture with 25 gauge needles. In some cases, COC were oocytectomized (OOX) using a narrow bore glass pipet. For co-culture experiment (Figure 5-1), the following groups were used: 1) Control: 20 COCs cultured for 20 hours, followed by harvesting of the cumulus cells; 2) OOX: cumulus cells from 20 COCs cultured for 20 hours; 3) Co-culture: Cumulus cells from 20 COCs co-cultured with 40 denuded oocytes (2 oocytes/µl). To test the effect of SMAD2/3 inhibitor, SB431542, 20 intact COCs were cultured for 0, 4, 8 or 16 hours with 10µM SB431542. In the EGF experiment (Figure 5-9), 20 intact COCs per group were cultured for 0, 4, 8, 12 or 16 hours with EGF (10 ng/ml). To determine the effect of YAP1 inhibition with verteporfin (VP) on cumulus cell steroidogenesis, 20 intact COCs per group were cultured in BSA-free MEM-α with 5% charcoal stripped serum alone (control) or in medium containing 200 nM or 1µM VP for 16 hours. Cells were then collected for analysis of gene expression and progesterone content as described below.

**In-vitro cell culture (monolayer)**
Fresh ovaries from unprimed 18-day old mice were placed in MEM-α medium, mural cell clumps were released from the antral follicles by gentle puncture with syringes and needles. Mural cell clumps were collected and pipetted gently to create a single cell suspension. Approximately 5.0×10⁴ cells were plated in medium containing 10% FBS in a 384-well plate (Corning CLS 3985) overnight (25 µl/well). The next day, the medium was replaced with medium containing low serum (0.5% FBS) and cells were co-cultured with or without denuded oocytes (2 oocytes/µl) and with or without VP (200 nM) for 48 hours. Cell number was determined using the CellTiter 96 Aqueous One Cell proliferation Assay (Promega, Madison, WI). Absorbance was measured on a FLUOstar Omega Microplate Reader at 490nm.

**Total RNA isolation and real-time PCR**

Total RNA was isolated from 20 intact COC (N=4, for EGF and SB experiments) or cumulus cells from 20 COCs (N=5, for oocyte co-culture experiments) using RNeasy Microkit (QIAGEN, Valencia, CA). Quantitect Reverse Transcription Kit (QIAGEN) was used to reverse-transcribe total RNA into cDNA. The quantification of transcripts for the Hippo pathway was normalized to the house-keeping gene *Rpl19*, and gene-specific primers used in real-time PCR are shown in Table 5-1. The relative fold changes in transcripts were measured using the 2^ddCt_ method as described [234].

**Progesterone assay**

COCs were homogenized in 5% TCA (trichloroacetic acid), and subjected to ELISA for quantification of progesterone, modified from a previous hormonal assay [304]. Briefly, a 96-well Nunc-Immuno MicroWell-plate was incubated with 100 µl secondary antibody (Goat anti-Mouse IgG, Calbiochem) at 2 µg/ml, and coated overnight at 4°C.
with shaking. After washing 5 times with wash buffer (4.175g/L MOPS, 0.05% Tween 20, PH 7.2), 100 µl primary antibody (monoclonal progesterone, Eastcoast, 2.89 mg/ml, diluted by 1:50,000 as working antibody) was added (except for negative control wells) and incubated at room temperature for 1.5 hours. Each COC sample was vortexed and homogenized in 10 µl 5% TCA which was then diluted by 1:20 in assay buffer (0.04M MOPS, 0.12M NaCl, 0.01M EDTA, 0.05% Tween 20, 0.005% Chlorhexidine Digluconate, 0.1% gelatin, PH 7.4). Both samples and progesterone standards (10, 5, 2.5, 1.25, 0.625, 0.3125, 0.15625ng/ml, Cayman Chemical) were prepared in a disposable plate (Thermo Scientific) before the incubation of the primary antibody was complete. After washing for 5 times, samples and progesterone standards were transferred to washed plate in duplicates and incubated at room temperature for 1.5 hours, after which, 50µl P3-P4-HRP conjugate (Steraloids Inc.) was added to each well and incubated for 1.5 hours. The plate was washed 5 times and 125 µl freshly made substrate (0.05M sodium acetate, 0.5M H2O2, 20mg/ml tetramethyl benzidine) was added to each well and incubated at 37°C on shaker for 25 minutes. 50 µl of stop solution (0.5M sulfuric acid) was then added to each well. The absorbance was read by FLUOstar Omega Microplate Reader at a wavelength of 450nm.

**Immuno-histochemistry**

Ovaries were harvested from CD-1 mice (~3 weeks) that were primed with PMSG alone for 48 hours or PMSG followed by hCG for 6 or 24 hours. Ovaries were fixed in 4% electron microscopy-grade paraformaldehyde (PFA) for 4 hours at room temperature and embedded in paraffin. Sections (5µm) were de-waxed in histoclear (EMS, Hatfield, PA) 3 times for 5 min each and then incubated for 5 min in each of the following: 100%
ETOH, 100% ETOH, 95% ETOH, 85% ETOH, 70% ETOH and ddH₂O. Next, samples were incubated in 1X antigen retrieval solution (Dako, S2367) at 94°C for 30 min. After cooling to room temperature, slides were washed 3 times with ddH₂O for 5 min each. Then the sections were incubated in 3% hydrogen peroxide for 10 min, followed by 2 washes in ddH₂O and 2 washes in wash buffer (1 X TBST, 0.1% Tween-20), 5 min each followed by incubation in goat blocking buffer (TBST, 5% goat serum) for 1 h at room temperature. Slides were then immediately incubated with MOBKL1A (Proteintech, 12790-1-AP), LATS1 (Abcam, ab85893), LATS2 (Abcam, ab70565), YAP1 (Proteintech, 13584-1-AP), TAZ/WWTR1 (Abcam, ab84927), and pYAP1 (Ser 127) (Cell Signaling Technology, D9W2I) antibodies, all with 1:800 dilution. Normal rabbit IgG antibody (Cell Signaling Technology, 2729) was used as a negative control. After primary antibody incubation, slides were washed in 1 X TBST 3 times for 5 min each. Then 1-3 drops of room temperature-equilibrated SignalStain Boost IHC Detection Reagent (Cell Signaling Technology) was used to cover sections, incubated at room temperature for 30 min in a humidified chamber. Slides were then washed 3 times in 1 X TBST and 200 ul of SignalStain DAB substrate kit reagent (Cell Signaling Technology) was applied to each section and monitored closely for color development for 1.5-5 min depending on the antibody. For each antibody, samples (including the negative control) were incubated for the same amount of time. Slides were then immersed in ddH₂O to stop the reaction. For MOB1B, LATS1, LATS2 and pYAP1, slides were counterstained with hematoxylin for 20 seconds and all slides were then incubated twice in 95%, 100% ethanol and twice in histo-clear for 10 seconds. Finally, slides were mounted with cytoseal (Richard-Allan Scientific) and covered with glass coverslips.
**Immunoblotting**

Cumulus cells from 30 COCs were denatured by boiling for 5 min in Laemmli sample buffer (with 5% 2-Mercaptoethanol), followed by quenching on ice and prepared for immunoblotting as previously described [80]. Proteins were separated on a 4-12% Bis-tris gel (Novex NuPAGE) and transferred to PVDF membrane (0.2 µm). The membranes were blocked in TBST+5% BSA for 1 hour with shaking at room temperature, followed by incubation with 1:1000 diluted phospho-LATS1 (Ser 909) (Cell Signaling Technology, 9157), phospho-YAP1 (Serine 127) (Cell Signaling Technology, 13008), phospho-TAZ (Ser 89) (Santa Cruz, 17610), YAP1 (Cell Signaling Technology, 14074), or TAZ (Abcam, ab84927) antibodies with agitation at 4°C overnight. Following incubation, blots were washed 3~4 times, 10 min each with 1 X TBST, and incubated with HRP-labeled Goat anti-Rabbit secondary antibody (1:50,000) for 1 hour at room temperature in the dark. Blots were washed and Pierce ECL Plus substrate (Life Technologies, 80197) was added for 5 min before detecting signal in a phosphorimager STORM 860.

**Statistical analyses**

Data were analyzed by one-way ANOVA followed by Tukey’s post-hoc test (Figures 5-1, 5-3, 5-4, 5-8, 5-10 and 5-11) or Dunnett’s post-hoc test (Figures 5-2 and 5-9). Minitab 17.1 software was used for all analyses. A p-value <0.05 was considered statistically significant.

**Results**

**Oocytes suppress the expression of Hippo transcripts in cumulus cells**
The relative transcript level (fold change from control) of Hippo transcripts were compared among COC, OOX and OO groups after 20 hours of culture. Results showed that compared to COC group, the upstream regulator \textit{Nf2}, adaptor genes \textit{Sav1} and \textit{Mob1b} were significantly increased in OOX group, but levels returned to baseline after oocyte co-culture (P<0.05) (Figure 5-1). Similar expression patterns were observed for \textit{Stk4}, \textit{Lats1}, and \textit{Lats2} (Figure 5-1). However, expression of \textit{Stk3}, \textit{Yap1} and \textit{Wwtr1} (Taz) mRNA were not significantly different between any of the treatment groups.

\textbf{Inhibition of SMAD2/3 phosphorylation upregulates expression of Hippo transcripts}

The relative level (fold change from control) of Hippo transcripts at 4, 8, 16 hours after treatment with 10 \(\mu\)M SB431542 (pSMAD2/3 inhibitor) were compared to untreated cells (0 hours). \textit{Sav1}, \textit{Stk3} and \textit{Lats2} mRNAs all showed a significant increases by 16 hours (P<0.05) (Figure 5-2); whereas \textit{Mob1b}, \textit{Stk4} and \textit{Lats1} were not significantly different at any time-point examined. While the expression of \textit{Wwtr1} was not different at any time-point examined, the expression of \textit{Yap1} was significantly increased by 16 hours (P<0.05) (Figure 5-2).

\textbf{Verteporfin inhibits oocyte-stimulated granulosa cell proliferation}

Verteporfin (VP) acts as a small molecule YAP-TEAD inhibitor, directly inhibiting the binding of YAP and TEAD [259]. To examine the effect of VP on oocyte-induced cell proliferation, mural granulosa cells were plated overnight and cultured alone or with oocytes in the presence or absence of VP for 48 hours. As expected, VP (200 nM) alone caused a decrease in cell proliferation (P<0.01) compared to untreated cells. Also as
expected, oocyte co-culture significantly increased cell number compared to mural cells cultured alone (P<0.01) (Figure 5-3). However, VP (200nM) completely blocked the ability of oocytes to stimulate cell proliferation (P<0.01) (Figure 5-3). The population of granulosa cells plated overnight was not significantly different from that of VP treated group (data not shown), indicating that VP didn’t significantly deteriorate the live cells.

**Verteporfin induces premature differentiation of cumulus cells in vitro**

Bright field images (Figure 5-4A) showed that cumulus cells treated with VP (1µM) for 16 hours underwent what appeared to be normal cumulus expansion without any ovulatory signals. This phenomenon was absent in control groups or COC treated with a lower VP concentration (200 nM). Consistent with the morphological cumulus expansion caused by VP (1 µM), expansion transcripts (*Has2, Ptgs2, Ptx3, Tnfaip6*) increased 3-20 fold when treated with 1 µM, but not 200 nM VP (Figure 5-4B). In addition to cumulus expansion, 1 µM, but not 200 nM VP induced an increase in key steroidogenic transcripts including *Star* and *Cyp11a1* but not *Hsd3β2* mRNA (Figure 5-4C). Consistent with an increase in *Star* mRNA, cells treated with 1 µM VP contained significantly higher progesterone than the 200 nM VP or control groups (Figure 5-4D).

**The localization and expression of Hippo proteins in peri-ovulatory ovaries**

To investigate the possible role of Hippo signaling during the peri-ovulatory transition, the protein localization of kinases LATS1/2, adaptor protein MOB1B, transcriptional coactivators YAP1, WWTR1 as well as pYAP1 (Ser 127) were examined in ovaries collected from mice primed with PMSG (48h), or hCG (6h or 24 h). LATS1/2, MOB1B and pYAP1 were counterstained with hematoxylin. MOB1B was mainly
localized in the cytoplasm of somatic cells at all stages of follicular development and
luteal cells of the corpus luteum (Figure 5-5A-C). LATS1 and LATS2 proteins were also
strongly localized to the cytoplasm of somatic cells in follicles at all stages of follicular
development and in the corpus luteum (Figure 5-5E-G, I-K). Similar to LATS1/2, both
YAP1 and pYAP1 were strongly localized to the cytoplasmic compartment of the
granulosa cells (Figure 5-6A-B, and D-E and Figure 5-7A-B, and 5-7D-E). LATS1,
LATS2 and MOB1B were absent from ovarian stromal cells between follicles. In PMSG
treated ovaries, pYAP1 appeared to localize more to the cumulus and periantral granulosa
cells than the mural granulosa cells, but by 6 hours after hCG, pYAP1 was distributed
throughout the follicle and later in the corpus luteum (Figure 5-6D-F, and Figure 5-7D-
F). WWTR1 (TAZ), on the other hand, was strongly localized to the nuclear
compartment of the granulosa cells before ovulation (Figure 5-6G-H, and Figure 5-7G-H)
and to the luteal cells 24h after hCG (Figure 5-6I, and Figure 5-7I). Similar to LATS1/2
and MOB1B, WWTR1 was also absent from ovarian stromal cells between follicles. To
quantify possible changes in Hippo pathway activation, ovarian extracts from PMSG
(48h) and hCG (6h, 24h) injected mice were used for immunoblotting experiments.
Treatment with hCG for 24 hours, but not 6 hours, caused an overall decrease in total
YAP1 (Figure 5-8A), but a robust increase in both pYAP1 (Ser 127) and pWWTR1 (Ser
89) compared to PMSG (48h) injection alone (Figure 5-8). Expression of total WWTR1
remained unchanged between groups (Figure 5-8B).

**EGF causes a robust increase in the expression of Hippo transcripts**

To examine the possible regulation of Hippo transcripts during *in vitro* maturation,
COC were treated with EGF (10 ng/ml) for 4, 8, 12 and 16 hours. The transcripts for
$Sav1$, $Mob1b$, $Stk3/4$, $Lats1$, $Lats2$, $Yap1$ and $Wwtr1$ were all significantly increased by 8 hours of culture with EGF, with $Mob1b$ increasing by 4 hours after the treatment ($P<0.05$) (Figure 5-9). On the other hand, $Yap1$ mRNA did not change during the in vitro culture with EGF (Figure 5-9).

**Acute and sustained effects of EGF on the protein expression of Hippo components**

To investigate whether acute or prolonged EGF treatment causes changes in Hippo pathway activation, COC were treated with EGF for either 0, 10 min and 1.5 hours (Figure 5-10) or 0h, 4h, 8h, and 20 hours (Figure 5-11). After culture, COC were lysed and subjected to immunoblotting experiments. Acute EGF treatment caused an increase in pYAP between 10 minutes and 1.5 hours after treatment, while pWWTR1 did not change (Figure 5-10). Prolonged EGF treatment caused a decrease in the active form of LATS1 (pLATS1) by 4 hours after treatment which remained low at 8 and 20 hours (Figure 5-11A-B). Prolonged EGF treatment had divergent effects on YAP1 and WWTR1 levels. EGF significantly decreased total YAP1 by 8 hours and remained low until 20 hours. In contrast, WWTR1 was transiently increased by 4 hours, but returned to baseline by 20 hours after EGF treatment (Figure 5-11D), while pWWTR1 decreased modestly 20 hours after treatment (Figure 5-11E).

**Discussion**

The Hippo pathway is emerging as a key regulator of the cell fate decision to remain quiescent, divide or undergo apoptosis. Recent reports show that Hippo pathway proteins are expressed in the ovary and regulate follicular development [220, 302, 305]. Deletion of $Lats1$ results in germ cell loss, ovarian cysts and stromal tumors [176, 302], while
ovarian fragmentation leads to YAP1 upregulation and increased follicular development [220, 306]. These findings strongly suggest that the Hippo pathway is essential for ovarian follicular development and function. However, the possible involvement of the Hippo pathway during ovulation where granulosa cells transform from highly proliferative to highly differentiated has not been explored. In the present study, we uncovered a role of oocytes in modulating the Hippo pathway and YAP1 activation prior to and during ovulation. Before ovulation, the oocytes suppress the Hippo pathway activity leading to YAP1 activation and stimulating granulosa cell proliferation while inhibiting premature differentiation. During ovulation, these effects are reversed by signals that stimulate Hippo pathway activity to inhibit YAP1 and allow cells to terminally differentiate.

Granulosa cell development and function are regulated by intra-ovarian signals, including oocyte-secreted products and by endocrine signals such as gonadotropins. Oocytes play a central role in promoting the proliferation of granulosa cells throughout follicular development. An elegant study using re-aggregation of oocytes and somatic cells showed that 14 day-old oocytes dramatically accelerate formation of antral follicles from somatic cells of newborn ovaries compared to oocytes from newborn ovaries [89]. Clearly oocytes potently influence granulosa cell proliferation [223] and survival [5]. Two well-known oocyte-secreted products, GDF9 and BMP15, which recently were discovered to form heterodimers that potently activate the SMAD2/3 signaling pathway at concentrations that are much lower than either GDF9 or BMP15 homodimers [270]. In rodents, GDF9 is necessary for follicular development beyond the primary stage [68]. Although we know that oocyte-secreted products potently stimulate granulosa cell
proliferation, the underlying mechanism responsible for this response has not been completely defined. In this study, we found that oocytes negatively regulate the expression of Hippo pathway components in cumulus cells at the mRNA level. Moreover, when we blocked SMAD2/3 phosphorylation with a small molecule inhibitor, SB431542, the negative impact of oocytes on Hippo gene expression in cumulus cells was partly attenuated, suggesting that oocytes regulate the Hippo signaling, at least in part, through a SMAD2/3-mediated mechanism. Although we clearly show that oocytes suppress expression of Hippo pathway transcripts, it is not known if this regulation occurs at the level of transcription or perhaps through regulation of post-transcriptional events, such as changes in mRNA stability. The findings also show that Hippo transcripts are induced more robustly by oocytectomy compared to the pSMAD2/3 inhibitor (SB431542). Thus, there are likely other pathways involved in regulating expression of Hippo transcripts in response to oocyte factors in addition to the SMAD2/3 pathway. Nevertheless, it is clear from our results that oocytes stimulate proliferation of granulosa cells by inhibiting the Hippo pathway and stimulating activity of the transcriptional co-activator, YAP1. This is shown in experiments that inhibit YAP1 activity with verteporfin, a small molecule inhibitor that changes the conformation of YAP1 and inhibits the interaction of YAP1 with TEAD [259]. However, still to be determined are the molecular steps that link oocyte-secreted factors to YAP1 activation in cumulus cells. Either SMAD2/3 or SMAD1/5/9 proteins have been shown to physically interact with YAP1 and/or WWTR1 in other tissues [253, 254, 307, 308]. Thus, one attractive possibility is that oocyte-stimulated SMAD2/3 [224] and/or SMAD1/5/9 [116] proteins
bind to YAP1 and/or WWTR1 to regulate cellular proliferation and differentiation, but this remains to be shown experimentally.

In addition to promoting cellular proliferation, oocytes also prevent a premature increase in progesterone production before ovulation. This can be thought of as a block to terminal differentiation and was described years ago by the pioneering work of Pincus and Eczeman who demonstrated that oocytes secrete an “anti-luteinization” factor that blocks progesterone production from cumulus cells [267]. Similar effects also occur in other species [309, 310]. Recently, YAP1 has been shown to maintain pluripotency of embryonic stem cells [298, 311], and can also block differentiation of tissue specific progenitor cells such as myoblasts [312], pancreatic acinar cells [313] and neuronal cells [314-316]. Thus, we tested the effect of YAP1 inhibition with verteporfin on progesterone production and cumulus expansion, two hallmarks of terminal differentiation in cumulus cells. Consistent with a role for YAP1 in blocking cell differentiation, we found that treatment with VP induced premature differentiation of cumulus cells. Remarkably, VP induced morphological cumulus expansion and increased expression of expansion-related transcripts in the absence of any ovulatory signals. However, the induction of expansion transcripts was not as robust as observed during normal cumulus expansion [224], suggesting that ovulatory signals are required to fully induce cumulus expansion response. Another differentiation event, the increase in progesterone production that occurs at ovulation was also inhibited by YAP1 because treatment with VP induced the expression of transcripts involved in steroidogenesis and increased intracellular progesterone concentration in cumulus cells. Thus, it appears that oocyte-stimulated YAP1 activity is required to both promote cellular proliferation and
inhibit premature differentiation of cumulus cells. Identification of the oocyte-derived signal that activates YAP1 is an important area of investigation and could identify the anti-luteinizing factor previously described by Pincus and Eczeman [267].

Recent studies reported that both transcripts and proteins of the Hippo pathway are expressed in the ovary [220, 302, 305]. SAV1, MST1/2, LATS1/2, YAP1 and pYAP1 proteins are primarily localized to the cytoplasm in granulosa cells, theca cells and oocytes at all stages of follicular development. WWTR1, on the other hand, is strongly nuclear in granulosa cells of follicles of all sizes and in the corpus luteum. Supporting these recent findings, we found in this study that LATS1, LATS2, YAP1 and pYAP1 were strongly localized to the cytoplasm of granulosa cells. However, the adaptor protein, MOB1B, localized to somatic cells of all sizes of follicles but preferentially localized to luteal cells after ovulation. Premature differentiation of COC treated with VP is consistent with a model that YAP1 blocks cellular differentiation. If this model is correct, then it follows that ovulatory signals should upregulate Hippo signaling to terminate YAP1 activity and allow terminal differentiation. To test this we measured levels of total and phosphorylated YAP1 and WWTR1 before and after an ovulatory dose of hCG. Consistent with our model, both pYAP1 and pWWTR1 increased, while total YAP1 decreased in lysate of whole ovaries after hCG administration. Immunostaining experiments showed that pYAP1 became more widespread in the follicle, particularly in the mural granulosa cells after the ovulatory dose of hCG. WWTR1 remained strongly nuclear, underscoring the potential difference in action between YAP1 and WWTR1 in the follicle.
The Hippo pathway was regulated differently in the COC during *in vitro* maturation stimulated by EGF. The main difference was in the effects of EGF on Hippo pathway activity as reflected by phosphorylation of YAP1 and LATS1. However, at the level of transcript expression, EGF induced levels of all Hippo transcripts, except *Yap1*, between 2-5 fold within 16 hours. This is consistent with ovulatory signals inducing Hippo pathway activity to abolish YAP1 signaling in the COC. Also consistent with this model, acute treatment with EGF (1.5 hours) induced higher pYAP1 levels, indicating greater Hippo signaling activity. Unlike YAP1, phosphorylation of WWTR1 was not changed by EGF treatment up to 20 hours. This again suggests divergent roles of these paralogues during the ovulatory transition. However, unlike acute EGF treatment, prolonged EGF treatment actually decreased levels of phosphorylated LATS1, suggesting that there was a decrease in Hippo kinase activity. Even more surprising was the observation that there was a precipitous decrease in YAP1 protein after prolonged EGF treatment. This suggests that there may be post-translational mechanisms induced at the time of ovulation that degrade YAP1. For example, β-TRCP-dependent proteosomal degradation of both YAP1 and WWTR1 has been shown to occur in other tissues or cells [182, 317]. Unlike YAP1, levels of total WWTR1 increased modestly, while pWWTR1 decreased modestly after prolonged EGF treatment. The decrease of pWWTR1 by 20 hours, together with the decrease of pLATS1 suggests that sustained EGF treatment eventually leads to the downregulation of Hippo signaling during ovulation. In summary, *in vivo* ovulatory signals stimulated by hCG lead to an increase in Hippo pathway activity, but *in vitro* maturation of COC with EGF leads to a downregulation of Hippo signaling and degradation of YAP1 protein. Nevertheless, in both cases the activity of YAP1 is
curtailed, albeit through different mechanisms, which then removes a block to cellular differentiation.

The present study uncovers a previously unknown role of the Hippo pathway and in particular, the transcriptional co-activator, YAP1, during the periovulatory transition. Before ovulation, oocytes potently stimulate granulosa cell proliferation through a YAP1-dependent mechanism since blocking YAP1 activity with VP blocks oocyte-induced proliferation. A recent seminal study by Kawamura and colleagues showed that ovarian fragmentation increases F-actin formation and this is required to upregulate YAP1 activity and promote follicular development [220]. Other than ovarian fragmentation, oocyte factors are the only signals identified so far that also stimulate YAP1 activity in the ovary, but whether oocytes activate YAP1 through modulation of the actin cytoskeleton is not known. During ovulation, our findings indicate that Hippo signaling is upregulated leading to ablation of YAP1 signaling which may allow terminal differentiation of somatic cells. This is a new area of investigation and offers the tantalizing possibility that YAP1 serves as a master switch controlling proliferation and differentiation of follicular somatic cells. We have just begun to uncover how the Hippo pathway operates in the ovary to maintain dynamic growth and differentiation during the reproductive cycle.
Figure 5-1. Expression of *Nf2*, *Stk4*, *Stk3*, *Sav1*, *Lats1*, *Lats2*, *Mob1b*, *Yap1* and *Wwtr1* mRNA in cumulus cells from intact cumulus-oocyte complexes (COC), oocytectomized COC (OOX) and OOX co-cultured with fully-grown oocytes (OO) for 20 hours. Values are mean±SEM, N=5. A,B indicate significant differences by ANOVA followed by Tukey’s post-hoc test, P<0.05.
Figure 5-2. Expression of Sav1, Mob1b, Stk4, Stk3, Last1, Lats2, Yap1 and Wwtr1 mRNA in COCs cultured alone (control) or with the pSMAD2/3 inhibitor, SB431542 (10 µM) for 0, 4, 8, or 16 hours. Values are mean±SEM, N=4. *Indicates significant differences from control by ANOVA followed by Dunnett’s post-hoc test, P<0.05.
Figure 5-3. Optical density of granulosa cells cultured in medium containing low (0.5%) fetal bovine serum (control) or medium supplemented with oocytes (2 oocytes/µl) and/or verteporfin (200 nM) for 48 hours. Values are mean±SEM of background subtracted optical density readings. A,B,C Indicate significant differences by ANOVA followed by Tukey’s post-hoc test, P<0.01, N=3.
Figure 5-4. A. Representative bright field images of freshly collected COCs treated with control medium or medium containing verteporfin (200 nM or 1 µM) for 16 hours, N≥5, scale = 100 µm. B. Fold change of cumulus expansion markers (Has2, Ptgs2, Ptx3, Tnfaip6) of COCs treated with verteporfin (200 nM or 1 µM) for 16 hours and compared to control, N≥6. C. Fold change of steroidogenic genes (Star, Cyp11a1, Hsd3β2) of COCs treated with verteporfin (200 nM or 1 µM) for 16 hours, N=3-9. D. Progesterone concentrations (ng/ml) of COC lysates treated with medium only (control) or verteporfin (1µM) for 16 hours, N≥5. Values are mean±SEM. A,B,C Indicate significant differences by ANOVA followed by Tukey’s post-hoc test, P<0.05.
Figure 5-5. Immunostaining for MOB1B (A-C), LATS1 (E-G) and LATS2 (I-K) proteins in tissue sections (5 µm) of ovaries from mice primed with PMSG (5 IU) for 48 hours (A, E, I), and/or 6 (B, F, J) or 24 hours (C, G, K) after an ovulatory dose of hCG. Negative controls for MOB1B (D) and LATS1 and LATS2 (H) were incubated with normal rabbit IgG with corresponding concentration of each antibody. All sections were counterstained with hematoxylin. Scale = 100 µm.
Figure 5-6. Immunostaining for YAP1 (A-C), Ser 127 pYAP1 (D-F, counterstained with hematoxylin), WWTR1 (G-I) proteins in tissue sections (5 µm) of ovaries from mice primed with PMSG (5 IU) for 48 hours (A, D, G), or PMSG and hCG for 6 (B, E, H) or 24 hours (C, F, I). Negative control (J) was incubated with normal rabbit IgG. Scale = 100 µm.
Figure 5-7. High power images of ovarian sections immunostained for YAP1 (A-C), Ser 127 pYAP1 (D-F, counterstained with hematoxylin), or WWTR1 (G-I) proteins in tissue sections (5 µm) of ovaries from mice primed with PMSG (5 IU) for 48 hours (A, D, G), or PMSG and hCG for 6 (B, E, H) or 24 hours (C, F, I). Negative control (J) was incubated with normal rabbit IgG. Scale = 50 µm.
Figure 5-8. Immunoblotting for YAP1 (A), WWTR1 (B), Ser 127 pYAP1 (C) and Ser 89 pWWTR1 (D) of ovary extracts from mice primed with PMSG for 48 hours, and/or 6 or 24 hours after hCG injection. The band density of each sample was normalized to β-actin, N=3-4. Values are mean±SEM. ^A^B indicate significant differences by ANOVA followed by Tukey’s post-hoc test, P<0.05.
Figure 5-9. Expression of Sav1, Mob1b, Stk4, Stk3, Last1, Lats2, Yap1 and Wwtr1 mRNA in COCs cultured alone (control) or with EGF (10 ng/ml) for 0, 4, 8, 12 or 16 hours. Values are mean±SEM. *Indicates significant differences from control by ANOVA followed by Dunnett’s post-hoc test, P<0.05, N≥3.
Figure 5-10. A. Immunoblotting for pYAP1 (Ser127), pWWTR1 (Ser89) and β-actin in COCs treated with EGF (10 ng/ml) for 0, 10, 30 min and 1.5 hours. B-C. Densitometric ratio of each protein relative to β-actin, N≥3. Values are mean±SEM. Note: 30 minute time point was excluded from analysis because there were only 2 observations. A,B indicate significant differences by ANOVA followed by Tukey’s post-hoc test, P<0.05.
Figure 5-11. A. Immunoblotting for pLATS1 (Ser909), YAP1, WWTR1, pWWTR1 and β-actin in COCs treated with EGF (10 ng/ml) for 0, 4, 8, 20 hours. B-C. Densitometric ration of band density for pLATS1 (B), YAP1 (C), WWTR1 (D) and pWWTR (E) normalized to β-actin, N≥3. Values are mean±SEM. 

A,B,C indicate significant differences by ANOVA followed by Tukey’s post-hoc test, P<0.05.
**Figure 5-12.** Schematic figure on regulation of Hippo signaling pathway in a cumulus granulosa cell before and during ovulatory transition.
### Table 5-1. Primer Sequences used for qPCR.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
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<tbody>
<tr>
<td><em>Lats1</em></td>
<td>GAATGAGCCCGTGAAAGCAACA</td>
<td>TTCAGACTTCAGACGCTCCCATGCT</td>
</tr>
<tr>
<td><em>Lats2</em></td>
<td>TGAAGAGGGCCAAGATGGACAAGT</td>
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Chapter 6

YAP1 Plays a Critical Role in Oocyte Maturation

Abstract

As an effector and transcriptional coactivator of Hippo signaling pathway, YAP1 plays essential roles in modulating the balance of cell proliferation and differentiation in various mammalian contexts. However, our understanding on how Hippo-YAP1 participates in oocyte maturation during ovulatory transition is still lacking. In this study, we show that blocking YAP1/TEAD interactions with verteporfin causes decreased oocyte maturation and arresting the majority of oocytes at MI phase, with a disorganized spindle structure. By applying CDK1 inhibitor RO-3306 and PP2A inhibitor Okadaic Acid (OA), we revealed that the novel phosphorylation site T119 of YAP1 is the target of PP2A phosphatase in oocytes at the onset of meiotic resumption, while CDK1 may or may not exert apparent regulatory impact on the phosphorylation status of YAP1 T119. However, the protein expression of phosphorylated pYAP1 T119 presented a similar pattern as CDK1 activity throughout the meiotic maturation, which suggests the great potential of further investigating the relation between CDK1, pYAP1 and PP2A. Lastly, to investigate how oocytes respond to actin modulating factors during maturation, we interfered maturing oocytes with actin polymerization inhibitor cytochalasin B and enhancer JASP. Our findings suggested that either inhibiting or enhancing actin polymerization results in meiosis I arrest in oocytes. The interesting finding of spindle-accumulated pYAP1 T119 indicated a currently unknown role of Hippo signaling in spindle functions and meiotic progression. Together, our current investigation linked
Hippo-YAP1 with cell cycle regulators CDK1, PP2A and actin cytoskeleton, which shed light on our understanding of the mechanisms that regulate proper oocyte maturation.
Introduction

From dpc 17.5 in mice, oocytes become arrested at the diplotene stage of meiosis I, with an intact nuclear structure named GV (germinal vesicle). Upon follicle activation, while remaining in meiotic arrest, oocytes progressively increase in size, with the chromatin dispersed in the nucleus in a non-surrounded nucleolus (NSN) configuration. As follicles develop to the antral stage, oocytes grow concomitantly and some acquire a surrounded nucleolus (SN) configuration where the chromatin becomes arranged around the nucleolus. FGO (fully grown oocytes) resume meiosis in preovulatory follicles after the LH surge or after experimentally removing COCs from preovulatory follicles, as marked by GVBD (germinal vesicle breakdown). Meiosis progresses to metaphase II and is arrested till fertilization. Before ovulation takes place, high cGMP level in oocytes inhibits the hydrolysis of cAMP by the PDE3A, and high levels of cAMP inhibit the resumption of meiosis by repressing MPF activity [128, 129]. At ovulation, follicular cGMP production drops [132, 133], and along with the closure of gap junctions prevents the transfer of cGMP into the oocytes [134]. Low cGMP level in oocytes releases the inhibition of PDE3A activity which allows the reduction of cAMP level, the activation of MPF and meiotic resumption [135, 136].

During oocyte growth and maturation, mechanical cues and events such as GV positioning, remodeling of actin cytoskeleton, spindle assembly and changes of cortical stiffness all play pivotal roles for oocyte development. Eccentric GV positioning assures the minimal loss of cytoplasm after meiosis and maximal cytoplasmic support for early embryonic development. On the contrary, oocytes with centrally positioned GVs and MI spindle bear developmental risks due to higher rate of larger polar bodies [318] and
ooocyte aneuploidy [319]. In vivo, FGO in mice display an eccentric GV that is associated with a higher density of trans-zonal projections (TZP) after the administration of hCG. However, under in vitro conditions, FGOs released from preovulatory follicles exhibit a central GV and MI spindle that then migrates to the cortex; however, treatment with FSH maintains TZP and anchors the GV to the cortex [318]. The F-actin cytoplasmic meshwork was found to play key roles in gathering chromosomes and positioning spindles in maturing oocytes [320, 321], which don’t possess centrosomes and astral microtubules like somatic cells do. The meshwork of actin consists of thin filaments, crossing points and an actin cage around the spindle and can be detected with specific F-actin probes [322, 323]. Formation of actin filaments is dependent on actin-nucleating proteins Formin2 (FMN2) and Spire 1/2 [322, 323]. FMN2 is fundamental in repositioning the MI spindle to the cortex of the oocyte [324]. Shortly after GVBD, FMN2 first clusters around the spindle, co-localized with ER, inducing the nucleation and polymerization of F-actin and forces the spindle to move randomly within short range from the center of oocyte. Then, chromosomes move towards the cortex, with faster speed and more fixed trajectory, which is caused by a flow of cytoplasmic materials that generate a pushing force [325]. Under the orchestration of ARP2/3 (actin related protein) as well as FMN2, this cytoplasmic streaming continues to progress from meiosis I to II and remain at MII [325]. A recent study specifically showed that a concurrent drop of FMN2 is required for the destabilization of the actin meshwork for normal spindle assembly [326]. In addition, myosin II, located at the spindle poles as a motor protein, was proposed to anchor the leading spindle at the cortex and pull on it by an actomyosin-based contractile mechanism [323, 327]. The stiffness of the cortex that’s essential for
oocyte cytokinesis decreases dramatically throughout maturation [328], which differs from that in mitosis. The thickening of the actin cap takes place near the MI spindle [329] while the overall actin content of the oocyte reduces progressively during maturation [328]. The thickening of the cortical actin cap depends on Mos-MAPK pathways and Arp2/3 that acts downstream of Mos-MAPK [329]. Mos-MAPK also drives the change of localization of myosin II, from throughout the cortex to the area adjacent to the spindle poles [329]. Although these studies characterized the architecture of the actin meshwork and proposed possible models for its action and the roles of other cytoskeletal components in oocytes, the exact mechanisms are still under investigation.

Meanwhile, mechanical cues have been recognized as one of the major inputs that act upon the Hippo signaling pathway, a key pathway for determining cell fates. However, little is known about how the mechanical cues are transduced through Hippo pathway in regulating ovarian functions. Recently, a series of ovarian fragmentation assays in mice and human showed that an increase in filamentous actin leads to increased overall YAP1 expression that underlies the stimulation of follicular activation. These previous studies on the role of the Hippo pathway in ovarian functions were conducted to study somatic cell proliferation [258]. In oocytes, the actin cytoskeleton and spindle microtubules undergo such a dynamic remodeling during meiotic maturation, as stated above. However, the roles that Hippo pathway plays during such mechanically dynamic process in oocytes are thus far still poorly understood. Also, the observation that treatment of cultured ovaries with VP causes a decrease of oocyte numbers in activated follicles, leads us to speculate that both actin and YAP1 play essential roles in not only oocyte survival but also later growth and maturation.
In this study, we first documented the relative abundance the localization of YAP1 in maturing oocytes, following which we documented the possible functions of YAP1 in oocyte maturation by using verteporfin to block YAP1/TEAD interactions. We then examined the hypothesis that modulating actin polymerization in maturing oocytes results in alteration of YAP1 expression and/or localization and a disorganized spindle assembly during meiosis I and/or meiosis II. More importantly, we tested the hypothesis that MPF phosphorylates YAP1 at T119 in oocytes as it does during mitosis by applying CDK1 inhibitors (RO-3306 and Roscovitine) [185]. Lastly, to address the relationship between YAP1 and both PP2A activities that is essential for meiosis, we suppressed the activity of PP2A with okadaic acid to examine whether or not blocking PP2A alters CDK1-mediated phosphorylation of YAP1, as well as potential consequences on spindle assembly during meiosis I.

Methods

Animals

Female CD1 mice were produced and raised in the research colony of the investigators. Animals were maintained according to the Guide for the Care and Use of Laboratory Animals (Institute for Learning and Animal Research). All animal use was reviewed and approved by the IACUC committee at The Pennsylvania State University. Mice were weaned at 18 days-old and primed with PMSG (5 IU) 48 hours before euthanasia and cell collection. In some experiments, mice were primed with PMSG, 48 hours before hCG (5 IU) injection. Then after 13 hours, MII oocytes were collected.

Total RNA isolation and real-time PCR
Total RNA was isolated from freshly collected fully grown oocytes (FGO) from 20 COC or cumulus cells from 10 COC (N=3) using RNeasy Microkit (QIAGEN, Valencia, CA). Quantitect Reverse Transcription Kit (QIAGEN) was used to reverse-transcribe total RNA into cDNA. The quantification of transcripts for the Hippo pathway was normalized to the house-keeping gene *Rpl19*, and gene-specific primers used in real-time PCR are shown in Table 6-1. The relative fold changes in transcripts were measured using the $2^{\text{dCt}}$ method as described [234].

**In-vitro cell culture**

Cumulus oocyte complexes (COC) were collected from mice (18 days old) primed with PMSG for 48 hours, as described previously [224]. Briefly, fresh ovaries were placed in bicarbonate-buffered MEM-α medium (Life Technologies, Grand Island, NY), supplemented with 75 mg/L penicillin G, 50 mg/L streptomycin sulfate, 0.23 mM pyruvate and 2mg/ml BSA, unless otherwise noted. Mature COC were released from antral follicles by gentle puncture with 25 gauge needles. Cumulus cells were carefully removed by using mouth pipet to aspirate and release the oocytes a few times. For experiments using MII oocytes, mice were primed with PMSG for 48 hours, followed by hCG (5 IU) for 13 hours before the collection of ovulated COC from oviducts. To test the effect of VP on oocyte maturation, denuded oocytes from mature COC were cultured for 16 hours with or without VP (200nM, 1 µM) before being fixed and subjected to immunofluorescent staining. To examine possible effect of VP on spindles, denuded oocytes from mature COC were cultured for 5 hours with or without VP (1µM) before being fixed and subjected to immunofluorescent staining. To examine the protein expression of YAP and pYAP T119 at GV, MI and MII stages of normal oocytes,
denuded oocytes from mature COC were either collected right away (GV oocytes) or were cultured for 7h in MEM-α medium before harvesting (MI oocytes). The in-vivo ovulated COC were placed in MEM-α medium with 1mg/ml hyaluronidase (Sigma) for 30s to help efficiently denude the MII oocytes before harvesting and subjected to immunoblotting. To test the effect of the PP2A inhibitor, okadaic acid (OA), on the phosphorylation of YAP1, denuded oocytes from mature COC were treated with 1µg/ml OA (Sigma) for 2h before harvesting and subjected to immunoblotting. To examine if the phosphorylation of YAP1 is also regulated by CDK1 activity, in-vivo ovulated COC were treated with 5µM CDK1 inhibitor RO3306 [330] (Enzo LifeScience) for 3h before denuding and harvesting the oocytes for immunoblotting assay later. To examine how F-actin modulators regulate oocyte maturation and possibly Hippo signaling activity, denuded oocytes from mature COC were cultured for 21 hours with or without 5µg/ml cytochalasin B (MP Biomedicals), an inhibitor for actin polymerization. Alternatively, cells were treated with 5 µM jasplakinolide (JASP, Calbiochem), an enhancer for actin polymerization for 2h and washed in control medium and continued to be cultured in control medium till the end of 21 h. Some oocytes from this experiment were subjected to immunoblotting, some were subjected to immunofluorescent staining. In all cases, 45~50 denuded oocytes were cultured in 25 µl drops and covered with mineral oil in 35mm petri dishes at 37°C in an incubator infused with a gas mixture of 5% CO₂, 20% O₂.

**Immunofluorescence**

Immunofluorescent staining of oocytes was conducted as described previously [331]. Briefly, oocytes were fixed in 4% PFA for 1 hour, except for oocytes that were stained for spindles, where oocytes were fixed with microtubule stabilizing buffer. After
fixing, oocytes were permeabilized with PBS (with 0.05% Tween and 0.2% Triton-X) for 15 min at room temperature followed by washing (3X) in PBST (1% BSA) and then blocked in goat serum blocking buffer (PBS, 2% goat serum, 1% BSA, 0.1% Triton-X and 0.05% Tween 20) for 1 hour followed immediately by incubation with YAP1 (1:200, Cell Signaling Technology) or pYAP 1 T119 (kind gift from Jixin Dong’s laboratory, University of Nebraska Medical Center, Omaha, Nebraska) or α,β-tubulin (1:200, Sigma) for spindle staining for 1 hour. After washing in PBST (1% BSA), oocytes were incubated with goat anti-rabbit IgG Alexa Fluor 594 (A11072) for 1 hour or phalloidin (Invitrogen A12379, 1:40) for 30min, followed by washing with PBST (1% BSA) and was mounted in prolonged anti-fade gold with DAPI (Life Technologies, P36941) on glass slides with etched rings to prevent cell rupture by the cover slip. Slides were imaged with Olympus Fluoview FV 1000 (Microscopy and Cytometry Facility at Penn State University) for YAP1, and Zeiss Axioskop2 Plus Microscopy and Axiovision for phalloidin imaging.

**Immunoblotting**

Oocytes were denatured by boiling for 5 min in Laemmli sample buffer (with 5% 2-Mercaptoethanol), followed by quenching on ice and immunoblotting preparation as previously described [80]. Proteins were separated on a Bolt 4~12% Bis-Tris Plus gel (ThermoFisher Scientific) and transferred to PVDF membrane (0.2 µm). The membranes were blocked in TBST+5% BSA for 1 hour with shaking at room temperature, followed by incubation with 1:1000 diluted pYAP1 T119 (kind gift from Jixin Dong’s laboratory, University of Nebraska Medical Center, Omaha, Nebraska), YAP1 (Cell Signaling Technology, D8H1X), and 1:5000 β-actin (Abcam, ab6276) antibodies with agitation at
4°C overnight. Following incubation, blots were washed 3~4 times, 10 min each with 1 X TBST, and incubated with HRP-labeled Goat anti-Rabbit (1:50,000) or Goat anti-Mouse secondary antibody for 1 hour at room temperature in the dark. Blots were washed and Pierce ECL Plus substrate (Life Technologies, 80197) was added for 5 min before detecting signal in a phosphorimager STORM 860.

**Statistical analyses**

Gene expression results, the results of CDK1 inhibitor and PP2A inhibitor experiments and the effect of VP on the spindle dimensions were analyzed by students’ t-test (Figure 6-1, 6-3C-E, 6-5B, 6-5D). The protein expression of YAP1 and pYAP1 during oocyte maturation was analyzed by one-way ANOVA followed by Tukey’s post-hoc test (Figures 6-4B, C). The effect of VP, Cytochalasin B and JASP on oocyte maturation was analyzed by Pearson’s Chi-Square test (Figure 6-2, 6-6). Minitab 17.1 software and Microsoft excel were used for all analyses. A p-value <0.05 was considered statistically significant.

**Results**

**Hippo transcripts are differentially expressed between oocytes and cumulus cells**

The relative expression level of Hippo transcripts in cumulus cells were compared to that in oocytes. Using Rpl19 as the normalizer, results showed that the relative abundance of Hippo transcripts differs between oocytes and cumulus cells (Figure 6-1). Oocytes expressed more abundant transcripts of Merlin, Kibra, Mob, Sav, upstream kinase Mst2 and transcriptional coactivator Yap1 than cumulus cells (P<0.05, Figure 6-1). In contrast, oocytes expressed lower level of transcripts of upstream kinase LATS1 and the other transcriptional coactivator WWTR1 than cumulus cells (P<0.05, Figure 6-1). However,
the transcripts of upstream kinases *Lats2* and *Mst1* are not significantly different between oocytes and cumulus cells. To localize YAP1 protein during meiosis, oocytes were stained for total YAP1. YAP1 protein localized prominently to the meiotic spindle in normal MII oocytes (Figure 6-2A).

**VP disrupts normal oocyte maturation and spindle morphology**

To investigate whether the Hippo pathway regulates oocyte maturation, the percentage of oocytes reaching normal MII were recorded. Denuded germinal vesicle (GV)-stage oocytes were treated with VP (200 nM or 1 µM) for 16 hours to undergo spontaneous meiotic resumption. VP treatment at 200 nM and 1 µM concentrations both significantly attenuated spontaneous maturation of denuded oocytes (P<0.05, Figure 6-2B-C). Most of the abnormal oocytes were arrested at metaphase I (Figure 6-2B). To further examine the cause of arrested MI oocytes treated by VP, oocytes were cultured for 5 hours with or without VP (1µM). Oocytes from control group were at MI stage with normal spindle morphology, whereas VP treated oocytes exhibited a smaller than usual spindle morphology (Figure 6-3A, B). All the MI oocytes from control and VP group were measured for the length, width and length/width ratio. For both length and width measurements, VP treated oocytes had significantly smaller size than control (n>40, P<0.05) (Figure 6-3C, D). However, the length/width ratio only showed a borderline significance between control and VP group (n>40, 0.05<P<0.1) (Figure 6-3E).

**The protein expression of YAP1 and phosphorylation at T119 site are upregulated during oocyte maturation.**

To quantify the protein expression of YAP1 at different stages of meiotic maturation, GV, MI and MII oocytes were lysed and subjected to immunoblotting (Figure
Oocytes before GVBD contain significantly lower YAP1 than oocytes undergoing meiotic resumption (P<0.05, N=3). A novel phosphorylation site at T119 was recently discovered to be phosphorylated by CDK1 during mitosis [185]. Levels of pYAP1 T119 were also examined during oocyte maturation. Similar to the total YAP1 expression, pYAP1 T119 was significantly increased in oocytes undergoing meiotic maturation, compared to that in GV oocytes (P<0.05, N=3) (Figure 6-4A, C). For both proteins examined, there wasn’t a significant difference between MI and MII oocytes.

**Regulation of YAP1 at T119**

During oocyte meiotic resumption, the cell cycle kinase CDK1 is activated and promotes the phosphorylation of meiotic phosphoproteins. A recent study in somatic cells revealed that YAP1 is phosphorylated and positively regulated by the CDK1 at 3 novel loci T119, S289 and S367 during mitosis and this regulation is critical for the oncogenic activity of YAP1 [185]. Therefore, to determine if T119 is also the potential target of CDK1 during oocyte maturation the CDK1 inhibitor, RO3306, was used to treat MII oocytes for 3h, and the expression of pYAP1 T119 was examined. Although lower, there wasn’t a significant difference between control and RO3306 groups (P>0.05, N=3) (Figure 6-5C, D).

**The phosphatase PP2A targets YAP1 at T119 site at the meiotic onset**

During oocyte maturation, the phosphorylation status of meiotic phosphoproteins is regulated by the balance between the kinase CDK1 and the phosphatase PP2A. PP2A remains at relatively high level before oocytes undergo meiotic resumption and its activity decreases as soon as oocytes resume meiosis and remains low [332]. To examine if PP2A potentially controls the Hippo activity by regulating the phosphorylation status
of pYAP1 at T119 in oocytes, GV oocytes were treated with PP2A inhibitor Okadaic Acid for 2h and the protein expression of pYAP1 T119 was examined. PP2A inhibitor OA significantly increased the expression of phosphorylated YAP1 at T119 site, compared to control (P<0.05, N=3) (Figure 6-5 A,B).

**Actin polymerization inhibitor cytochalasin B (CB) and enhancer JASP both arrest oocytes at MI stage**

While mechanical cues have been recognized as major inputs for Hippo signaling pathway in somatic cells, whether the actin cytoskeleton interacts with Hippo signaling to regulate oocyte function is not known. To investigate the roles that filamentous actin plays during oocyte maturation and whether or not that regulation is mediated through YAP1 activation, GV oocytes were treated with either negative (CB for 21 h) or positive (JASP for 2h, then washout and culture for 19 h) regulators of actin polymerization. Oocytes were then subjected to immunofluorescent staining for pYAP1 T119, actin and chromosomes. Surprisingly, CB and JASP treatments both arrested the oocytes at MI stage with percentages as high as 99.2% and 99%, respectively, and 0% of the oocytes treated by CB or JASP reached MII, whereas 82.7% of the oocytes in control group successfully reached MII (Figure 6-6). The localization of pYAP1 T119 was imaged using confocal microscopy. Interestingly, phosphorylated YAP1 at T119 was localized prominently to the meiotic spindle in not only normal MII oocytes where a polar body was extruded, but also MI oocytes that were results of either CB or JASP treatment.

**Discussion**

In the previous chapter, we uncovered a novel role for the Hippo signaling pathway in mediating the peri-ovulatory transition in cumulus oocyte complexes (COC). In this
chapter, we aimed to examine potential roles of Hippo-YAP signaling in oocytes, particularly during oocyte maturation. Growing oocytes remain meiotically quiescent till ovulation, where ovulatory signals induce several cellular events in oocytes such as acquisition of SN configuration, reduction of cGMP and cAMP levels, cortical spindle positioning, thickening of cortical actin, and precise regulation of meiotic phosphoproteins that are regulated by the cell cycle kinase CDK1 and phosphatases such as PP2A. We first examined the relative abundance of Hippo transcripts in the COC and found that FGOs and cumulus cells differ in their expression of Hippo components at the mRNA level. Particularly, the expression of Yap1 transcript in oocytes is significantly more abundant than that in cumulus cells, which is different from its paralogue Wwtr1 and upstream kinase Lats1 which are significantly more abundant in cumulus cells than in FGOs (Figure 6-1). To investigate the potential functions of YAP1, we blocked YAP/TEAD binding and examined its effect on meiotic maturation starting from GV oocyte. Surprisingly, even at low concentration (200 nM), VP significantly blocked the completion of meiosis, causing nearly half of the oocytes to arrest before reaching metaphase II. This shows a novel role for YAP1 during oocyte maturation, which was unexpected given that the oocyte neither proliferates nor differentiates in the classic sense. Subsequent immunostaining revealed that YAP1 protein localized to the meiotic spindle (Figure 6-2A), but how YAP1 acts during meiosis is still unclear.

A very recent study on somatic mitosis revealed that YAP1 can be phosphorylated and positively regulated by the cell cycle kinase CDK1 at 3 novel loci: T119, S289 and S367 which are required for proper progression through the G2 to M transition during mitosis [333]. More importantly, although Hippo pathway activation mainly leads to the
phosphorylation of YAP1 at S127, the positive regulation of the novel phosphorylation loci is critical for oncogenic activity of YAP1 [185]. The same group then found that the mitotic phosphorylation of YAP1 controls the spindle checkpoint, through upregulating BubR1, a critical component of spindle checkpoint in mitosis [334]. In a very recent study, BubR1 was found essential for spindle checkpoint activity as well as the establishment of kinetochore-microtubule attachment in meiosis I of mouse oocytes [335]. It has also been known that CDK1 is an integral part of the MPF (maturation promoting factor) which is responsible for meiotic progression from GVBD to MI as well as the establishment of MII arrest. Meiosis and mitosis both largely rely on the kinetics of CDK1 activation [336], which is an essential cyclin-dependent kinase in both events [337, 338]. Meanwhile, the phosphorylation status of phosphoproteins by CDK1 also requires the concurrent inhibition of phosphatase protein 2A (PP2A) that is acted on by MASTL (microtubule-associated serine/threonine kinase-like) [339]. Therefore, it is tempting to speculate that MPF/CDK1-mediated phosphorylation of YAP1 and PP2A mediated dephosphorylation of YAP1 are critical events during meiotic progression. In this study, we found that as spontaneous meiosis progresses, YAP1 becomes increasingly phosphorylated at T119 site in MI and MII compared to GV oocytes. The CDK1 activity has been known to increase gradually during meiosis I, drop sharply during anaphase I and remain at high level till MII stage [338]. The activity of the phosphatase PP2A has been known to decrease gradually during meiosis I and remain low till MII stage [332]. Our hypothesized model therefore stated that CDK1 phosphorylates YAP1 at T119 whereas PP2A dephosphorylates the same site and both act coordinately with each other to tightly regulate the meiotic progression. By treating MII oocytes with a CDK1 kinase
inhibitor RO3306 for 3 hours, however, we didn’t observe a noticeable difference on pYAP1 T119 expression (Figure 6-5C, D), suggesting that CDK1 might not be the major kinase that contributes to the phosphorylation of YAP1 at T119 site. However, pYAP1 at T119 is already maximal in MII oocytes and perhaps does not require sustained MPF activity to maintain phosphorylation status. By contrast, when we treated the oocytes with a PP2A inhibitor OA at the onset of spontaneous meiosis for as short as 2 hours, we observed a significant increase of YAP1 phosphorylation at T119 site (Figure 6-5A, B). This is strong evidence that PP2A is indeed a major regulator of the phosphorylation status of YAP at T119.

It is important to notice that although the regulatory mechanisms underlying meiosis and mitosis share similarities, they are also quite different. In contrast to the prometaphase during mitosis, the prolonged prometaphase of meiosis I allows slow CDK1 activation, a bipolar spindle that’s slowly assembled by multiple microtubule-organizing centers (MTOC) and kinetochore-microtubule attachment which is inherently error-prone [340]. The activity of CDK1 reaches the peak level when the kinetochore-microtubule attachments are stabilized. However, reduced CDK1 activity led to more frequent meiotic errors that are presumably caused by overhasty kinetochore-microtubule interactions [340]. Among the various substrates that CDK1 phosphorylates, many are kinetochore-associated proteins which participate in kinetochore-microtubule interactions. Concomitantly, PP2A is not suppressed during meiosis I as CDK1 becomes slowly activated, as supported by data of normal progression of meiosis I from Mastl null oocytes [336]. However, resembling mitosis, inhibition of PP2A by MASTL is required in meiosis II, where a rapid activation of CDK1 is indispensable for oocytes to progress.
to MII [341]. Also interestingly, PP2A and PP1 phosphotase are known to dephosphorylate YAP1 and therefore positively regulate the Hippo target genes in other mammalian cells [342-344].

In summary, our current study uncovered that YAP1 which is primarily localized to the spindles, plays an essential role in oocyte maturation. The novel phosphorylation site of YAP1 at T119 didn’t seem to be under the regulation of CDK1 in MII oocytes but is the target of PP2A phosphatase at the onset of meiosis resumption. This study discovered previously unknown links between YAP1/Hippo and cell cycle regulators in oocytes. It still requires further investigation on how phosphorylation of YAP1, cell cycle regulators and actin cytoskeleton work together to support the cellular events that take place during oocyte maturation, and more importantly, whether that regulation directly or indirectly determines the spindle checkpoint (Figure 6-8).
Figure 6-1. Expression of *Lats1*, *Lats2*, *Wwtr1*, *Mst1*, *Mst2*, *Yap1*, *Merlin*, *Mob*, *Sav* and *Kibra* mRNAs in fully grown oocytes from 20 COC or cumulus cells from 10 COC, normalized to housekeeping mRNA *Rpl19*. Values are mean±SEM, N=3. *Indicates significant differences by Student’s t-test for each transcript, P<0.05.
Figure 6-2. A. Representative confocal images for YAP1 in normal MII oocytes (red=YAP1, blue=DAPI, scale = 50 µm). B. Representative confocal images of denuded oocytes treated with 1 µM verteporfin for 16 hours (Green=actin, blue=DAPI, scale = 50 µm). C. The effect of verteporfin (200 nM and 1µM) on oocyte maturation was quantified as percentage of normal MII oocytes. *Indicates significant differences from control by Pearson Chi-Square test, P<0.05, n>100 oocytes per treatment.
**Figure 6-3.** A. Representative images for YAP1 in normal MI oocytes (red=YAP1, blue=DAPI, green=spindle, scale = 50 µm). B. Representative images of YAP1 in oocytes treated with 1 µM verteporfin for 5 hours (red=YAP1, blue=DAPI, green=spindle, scale = 50 µm). C, D, E The effect of verteporfin (1µM) on the length, width and length/width ratio of the spindles were measured. *Indicates significant differences from control by students’ t test, P<0.05, # indicates borderline significant difference from control, 0.05<P<0.1, n>40 oocytes per treatment.
Figure 6-4. Immunoblotting for YAP1 (A, B) and pYAP1 T119 (A, C) of 50 oocytes at GV, MI and MII stages. The band density of each sample was normalized to β-actin, N=3~6. Values are mean±SEM. A, B indicate significant differences by ANOVA followed by Tukey’s post-hoc test, P<0.05.
Figure 6-5. A, B. Immunoblotting for pYAP1 T119 of 50 GV oocytes treated with control medium or medium with 1µg/ml okadaic acid (PP2A inhibitor) for 2h. C, D. Immunoblotting for pYAP1 T119 of 50 MII oocytes treated with control medium or 5µM RO3306 (CDK1 inhibitor) for 3h. The band density of each sample was normalized to β-actin, N=3. Values are mean±SEM. A,B indicate significant differences by students’ t test, P<0.05.
Figure 6-6. Representative confocal images for pYAP T119 in denuded fully grown oocytes cultured for 21h in control medium (A), with 5µg/ml cytochalasin B (B), or with 5 µM jasplakinolide (C) (red=pYAP T119, blue=DAPI, scale = 50 μm). Panel D is negative control immunostained with IgG. E and F, the effects of CB and JASP on oocyte maturation were shown by MI and MII oocytes percentages respectively. *Indicates significant differences from control by Pearson Chi-Square test, P<0.05, n>100 oocytes per treatment.
Figure 6-7. Schematic figure shows the change patterns of PP2A, CDK1 activity and our proposed change pattern of phosphorylated YAP1 at T119 site throughout oocyte meiotic progress, with pictures of GV, MI and MII oocytes indicating different stages of meiosis below.
Figure 6-8. Schematic figure shows the possible relationship between YAP1, pYAP1T119, PP2A, CDK1 and actin cytoskeleton.
Table 6-1. Primer Sequences used for qPCR.

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Chapter 7
Integrated Discussion

The Hippo signaling pathway, first discovered to control organ size in *Drosophila*, has now been recognized as a fundamental and functionally conserved regulatory pathway that plays many indispensable roles in a number of physiological processes in diverse species. With more inherent complexity in mammals, Hippo signaling pathway regulates cell proliferation, cell differentiation, stem cell maintenance [345], tumorigenesis and energy homeostasis in a variety of tissues [346]. However, the roles of the Hippo signaling in ovarian physiology are poorly understood.

Ablation of the *Lats1* gene was reported to be associated with fewer ovarian follicles, no corpora lutea and development of ovarian stromal tumors in adult mice from a study in 1999 using a global *Lats1* knockout model [176]. Later, LATS1 was reported to phosphorylate FOXL2 in ovarian follicles and a mutation of *Foxl2* was found in some patients with premature ovarian failure (POF) [347]. The phosphorylation of FOXL2 by LATS1 represses the expression of the StAR gene which indicates granulosa cell differentiation [347]. Few studies since then have further characterized the roles of LATS1 or any other components of Hippo pathway during ovarian follicular development. In Chapter 3, with the same *Lats1* knockout strain used in the early study, we further characterized the ovarian phenotypes following *Lats1* ablation and showed increased germ cell apoptosis at birth, loss of primordial follicles and development of abnormal ovarian cysts during early postnatal development. These results suggested that LATS1 is essential for prenatal germ cell survival, disruption of which could lead to the
extensive follicle depletion observed later after birth. However in 11d cultured $Lats1^{-/-}$ ovaries where cyst breakdown was supposed to be complete, few to no morphologically normal granulosa cells surrounded the growing oocytes. This demonstrates that LATS1 is required for normal cyst breakdown and deletion of $Lats1$ gene led to uncoupling of growth of oocytes and granulosa cells. Canonical Hippo signaling states that LATS1 acts to phosphorylate and inhibit the nuclear localization and the growth promoting activity of the transcriptional coactivators YAP1 and WWTR1. Thus our original prediction was that deletion of $Lats1$ would promote the proliferation of granulosa cells during follicle activation. However, the observed lack of granulosa cell over-proliferation in $Lats1^{-/-}$ ovaries indicated that there might be other mechanisms that control the activity of YAP1 and WWTR1 when LATS1 is absent. For example, LATS2 may compensate for LATS1 ablation. Importantly, based on the finding that LATS1 represses the expression of the granulosa cell differentiation marker StAR in early follicles [347], ablation of LATS1 might lead to premature differentiation in these abnormal follicles in our study. But further studies are needed to test these hypotheses. On the other hand, the fast growth of cystic structures in $Lats1^{-/-}$ mutant ovaries could be due to the decreased inhibitory effect of LATS1 on YAP1 and WWTR1. Though not clear if these cysts were the “precursors” of the stromal tumors reported previously, the appearance of epithelium-like cells lining the inside of the cyst suggests that these structures might be derived from a different origin. As a matter of fact, YAP1 has been recognized as an oncogenic marker in several ovarian carcinomas, which are derived from epithelium of the ovarian surface or lining the oviduct [179, 219, 257]. WWTR1, the other downstream effector of LATS1, was reported to facilitate the proliferation and migration of ovarian epithelial cancer cells
Therefore, the abnormal cystic structure observed in \textit{Lats1}^-^- ovaries was likely to develop into an epithelium-derived cell type due to the upregulation of YAP1, although further characterization is needed to draw a valid conclusion. In addition, our other data in Chapter 3 also revealed that in normal ovaries, Hippo transcripts are decreased as follicles progress from primordial to antral follicles and their proteins are widely expressed in follicles at different stages, which were supported by similar findings by others [258, 305].

As important transcriptional coactivators in the Hippo pathway that are phosphorylated by the kinases LATS1/2, YAP1 and WWTR1 have been extensively studied in the past decade. A few studies in mice suggested that YAP1/WWTR1 in progenitor cells promotes cell proliferation in a variety of organs [217, 218]. Their nuclear (active) versus cytoplasmic (inactive) localization is directly associated with tissue homeostasis. For example, upon tissue damage, YAP1/WWTR1 is diverted to the nucleus to stimulate proliferation and tissue regeneration [228]. YAP1 and WWTR1 share structural and functional similarities but also have divergent roles in a context-dependent way, shown in studies on liver, pancreas, salivary glands, kidney, lung, heart, skin and nervous system [228]. In mammalian ovaries, YAP1 was first reported to be expressed at a high level in ovarian serous cystadenocarcinoma compared to normal ovarian tissues [179]. A later study in 2011 revealed that YAP1 de-repression leads to ovarian clear cell carcinoma [219]. These studies along with a few others suggested a strong correlation between nuclear YAP1 localization and poor prognosis in ovarian cancer patients, thus making YAP1 an attractive therapeutic target. Meanwhile, genome-wide association studies revealed \textit{Yap} as a susceptibility gene for PCOS [349]. However,
we have limited information on how YAP1 plays its role during normal ovarian follicular
development which involves germ cell growth and somatic cell proliferation and
differentiation. In Chapter 4, we reported the roles of YAP1 during early follicular
development by ablating YAP1 with either a small molecule inhibitor verteporfin (VP) or
an inducible transgenic mice model. Our findings show that in cultured ovaries, YAP1 is
important for normal follicle activation and for maintaining germ cell pool during cyst
breakdown. These were the first direct evidence showing that YAP1 participates in
follicular development as early as during cyst breakdown. But how YAP1 plays its role in
germ cells and somatic cells for these processes is not quite clear. The p53 signaling is
one of the ubiquitous mechanisms that regulate programmed cell death in various cell
types [350]. Tumor suppressor LATS2 was shown to activate p53 [351]. In the event of
oncogenic stress, LATS2 phosphorylates ASPP1 (apoptosis-stimulating protein of p53-1),
activates its nuclear translocation and further upregulates p53 activity to promote the
apoptosis [188]. However, the oncoprotein YAP1 was reported to disrupt LATS2-ASPP1
binding through competitively binding with LATS2, which in turn counteracts with the
pro-apoptotic activity of p53 in a human diploid cell line [188]. The germ cell apoptosis
during cyst breakdown can be stimulated by TNF-α signaling [22, 23] and is regulated by
the balance between anti-apoptotic Bcl-x and pro-apoptotic Bax [22, 24]. Given that p53
responds to TNF signaling [352] and is also a well-known regulator of Bcl-x and Bax in
various mammalian cells [353], it would be reasonable to speculate that YAP1
contributes to germ cell survival in early development by impinging on p53 activity.
Without proper function of YAP1, for instance by blocking it with inhibitor VP, the
antagonizing effect on p53 is presumably diminished; therefore germ cells would be more
prone to undergo apoptosis. The same mechanism, if proved correct, could also explain possible functional changes in somatic cells during follicle activation. For example, impeded follicle activation by VP may be partly due to the increased apoptosis in granulosa cells which, without VP, are supposed to be activated and become cuboidal. Additionally, during follicle activation, granulosa cells produce LIF (Leukaemia inhibitory factor) to induce KL expression [53] which binds to its cognate receptor c-kit and regulates oocyte growth and function such as the expression of $Bmp15$. It was reported that the tyrosine kinase, Yes, phosphorylates YAP1 to mediate the YAP1-TEAD2 dependent transcription induced by LIF through an unknown mechanism in embryonic stem cells [186]. Then several recent studies in breast cancer cell lines revealed that LIF acts to facilitate the membrane accumulation of Scribble (a scaffolding protein), which sequesters YAP1 in the cytoplasm so YAP1 activity is suppressed [354, 355]. Together, it is expected that in ovarian follicles, blunted YAP1-TEAD binding would hinder YAP1-TEAD2 dependent transcription induced by LIF and therefore any downstream effects would be potentially attenuated, for example $Bmp15$ expression, which is critical for oocyte growth and granulosa cell activation. On a different note, the stimulated follicle activation in Yap1 F/Cre ovaries could be attributed to the inherent (one Yap1 allele already deleted) and later induced lower YAP1 expression. If sequestering YAP1 expression or activity to a certain point is actually required for LIF-induced KL expression in follicular cells during follicle activation, then it may not be surprising that the Yap1 inducible knockout ovaries exhibited stimulated follicle activation, as opposed to impeded follicle activation in VP treated ovaries with normal
genetic background. However, the results with the inducible knock out ovaries should be interpreted with caution because the extent of gene deletion was not known.

Without a doubt, YAP1 is essential for early follicular development. Very recently, a novel role of YAP1 acting downstream of filamentous actin following ovarian physical disruption was uncovered [221, 258]. These studies clearly demonstrated a positive relationship between fragmentation-triggered actin polymerization, increased nuclear YAP1 accumulation and follicular development [221, 258]. This was the first published study showing that YAP1 plays an essential role in follicular development in a “malignancy-free” context. Even more significant was the clinical application of those discoveries, which showed that stimulating actin polymerization with potential therapeutic drugs JASP and S1P [221], along with Akt stimulator [258] can efficiently stimulate follicular growth. In fact, physically disruptive procedures have already been applied clinically in treating POI and PCOS patients.

Starting from follicle activation, oocytes increase in size while potently stimulating surrounding granulosa cells to proliferate. Oocytes also promote granulosa cells to structurally and metabolically support oocyte growth, maintain the oocyte in meiotic arrest and induce transcriptional silencing in fully grown oocytes. In large antral follicles before ovulation, granulosa cells are highly proliferative under the stimulation of both oocyte-secreted factors and FSH. At ovulation, the ovulatory LH signal propagates throughout the entire follicle by stimulating production of EGF-like peptides. As a result of ovulatory signals, both cumulus and mural granulosa cells cease proliferation, and start differentiating as indicated by increase in progesterone production and cumulus expansion. Ovulatory signals also cause the oocyte to resume meiosis and arrest at
metaphase II until fertilization. Due to the dynamic changes of granulosa cell function and oocyte meiotic maturation, COC during the ovulatory transition is a very unique model to study the function and regulation of Hippo signaling. A better understanding of the ovulatory process would improve our currently limited understanding of the mechanisms responsible for this tightly coordinated process and the changes occurring in oocytes and cumulus cells. In Chapter 5 and 6, we uncovered previously unknown regulatory mechanisms that involve the Hippo signaling pathway in the control of granulosa cells proliferation and differentiation and oocyte maturation.

In Chapter 5, we found that before ovulation, oocytes stimulate granulosa cell proliferation and inhibit premature differentiation by suppressing Hippo activity and inducing the growth-promoting activity of YAP1. This finding is the first to clearly show which oocyte-stimulated pathway promotes granulosa cell proliferation. During the ovulatory transition, ovulatory signal initiates a cascade of events leading granulosa cells to undergo terminal differentiation, by stimulating Hippo signaling and inhibiting YAP1 nuclear activity. The action of oocytes on modulating Hippo activity in granulosa cells is believed to be partly through SMAD2/3 pathway, since a pSMAD2 specific inhibitor reduced inhibitory impact of oocyte co-culture on expression of Hippo transcripts. Coincidently, a recent study demonstrated that two of OSFs, GDF-9 and BMP-15, form heterodimers and mediate SMAD2/3 phosphorylation through a unique receptor complex in granulosa cells [73]. More importantly, this new heterodimer form was found to possess more bioactivity than the homodimer forms of GDF-9 or BMP15 that were known for years [73], which supports the idea that SMAD2/3 is one of the major mechanisms through which oocytes regulate granulosa cell function. Nevertheless, our
observation that the pSMAD2 inhibitor did not restore Hippo activity to the same level as oocytectomy did suggests that SMAD2/3 pathway is required but not necessarily sufficient in mediating oocytes’ action in granulosa cells. Other pathways that mediate the action of OSFs require future investigation, for example, SMAD1/5/8 signaling. Also, the impact of oocytes on Hippo signaling before ovulation was currently demonstrated on the transcriptional level, leaving the question unanswered of how Hippo components are modulated at post-transcriptional, translational level and post-translational levels. For example, genes encoding transcriptional coactivators YAP1 and WWTR1 were not affected by whether or not oocytes were present, which was different from genes encoding most of the upstream regulators such as STK4 and LATS2. After all, the post-translational phosphorylation and the nucleocytoplasmic shuttling of YAP and WWTR are the key determinant of their function and therefore the main target of the Hippo pathway [356]. Canonical Hippo signaling states that the phosphorylation of YAP1 at S127 site creates 14-3-3 binding consensus binding site which sequesters YAP1 in the cytoplasm. But some results suggest that the phosphorylation of YAP1 at S127 does not necessarily result in its cytoplasmic localization in some cases [357, 358], which apparently added another level of complexity in dissecting the regulation of Hippo signaling in our model. On the other hand, while pSMAD2/3 was shown to act upstream of Hippo transcripts in our present study, SMADs were reported to interact with YAP1 and WWTR1 at the protein level in other cell contexts [253, 254, 307, 308], which could also be a potential mechanism which mediates oocyte regulation of granulosa functions.

YAP1 has also been shown to block differentiation of tissue specific progenitor cells in a variety of tissues such as pancreatic acinar cells [313], myoblasts [312], and neuronal
cells [314-316]. In pre-ovulatory follicles, granulosa cells undergo terminal differentiation after ovulatory signals are received. Here we show that blocking YAP1 activity induces premature granulosa cell differentiation even though there were no ovulatory signals. This finding was not surprising given the role of YAP1 in blocking cell differentiation reported by other studies. However, the cumulus cell differentiation markers were not induced by YAP1 inhibitor with the same degree as observed during normal cumulus expansion [224], which suggests that there might be other mechanisms that also contribute to guarding the proliferation/differentiation switch, for example, the WWTR1, which in this case was not hindered and may prevent the granulosa cells to reach full differentiation potential. Additionally, as mentioned earlier, phosphorylation of FOXL2 by LATS1 represses granulosa cell differentiation, as indicated by blunted StAR expression [347]. Given the phosphorylation of YAP1 by LATS1 sequesters YAP1 activity, it is thus plausible to hypothesize in future studies that YAP1 and FOXL2 may act through two different LATS1-regulated mechanisms that determine the cell fate of granulosa cells.

During ovulatory transition, our hypothesized model stated that ovulatory signals induce Hippo signaling activity and abolish YAP1 function to allow cumulus differentiation and block proliferation. Indeed, our data suggested that in vivo ovulatory signals stimulated by hCG lead to an increase in Hippo pathway activity. Consistently, the in vitro ovulatory signal EGF, when delivered acutely, induced the expression of most Hippo transcripts and also high pYAP1 S127 protein level which is direct evidence that Hippo activity is upregulated during ovulation in vivo and maturation of the COC in vitro. Prolonged EGF treatment, on the other hand, caused a decrease in total YAP1
protein while it did not affect Yap1 mRNA at the transcription level. First, these different observations implied that post-translational mechanisms may play an indispensable role in modulating YAP1 at the protein level. Among many post-translational mechanisms, β-TRCP-dependent proteosomal degradation could be a promising mechanism to investigate in the future, as it was shown to be responsible for the turnover of both YAP1 and WWTR1 in other tissues [182, 317]. More importantly, the time-sensitive modulation of Hippo signaling by ovulatory signal is an interesting and important issue in that the differentiation process of ovulated COC in vivo has a limited time frame before they are functionally ready to be fertilized. Ultimately in a physiological context, COC are actually not exposed to the ovulatory signals for a very long time. This is probably the main reason why acute EGF treatment induced Hippo activity that recapitulated in vivo induction of Hippo pathway induced by hCG. In both cases the nuclear activity of YAP1 is suppressed which then removes a block to cell differentiation.

Finally, the various aspects of our studies on follicular cell functions are almost all geared towards the ultimate question: how to make a developmentally competent oocyte? Our understanding of oocyte growth and maturation has been extensively studied in the past. The oocyte maturation process involves chromosome modification, cytoplasmic rearrangement, storage and regulation of maternal RNA, all of which are crucial for the achievement of oocyte developmental competence [359, 360]. In Chapter 6, we were interested in examining the role of Hippo components in oocytes during maturation, especially given that almost no studies reported on determining its functions in reproductive germ cells. First, the relatively abundant expression of Yap1 transcript but not Wwtr1 or others in oocytes compared to cumulus cells made YAP1 an attractive
candidate for follow-up studies. Then we demonstrated the necessity of YAP1 for achieving meiotic competence by showing that blocking YAP1 with VP greatly reduced the proportion of oocytes reaching the MII stage. Here we also reported that YAP1 localizes to the meiotic spindles. More interestingly, most of the VP treated oocytes that failed to reach MII were arrested at MI stage but with a mildly intense actin staining at the cortex which was a hint that YAP1 may be indispensable for the meiotic progression from MI to MII. Therefore next, with a shorter culture time, oocytes treated with or without VP were only allowed to reach MI stage before comparison. Intriguing enough, the spindles of VP treated MI oocytes exerted what appeared to be a smaller spindle structure that was also co-localized with YAP1. This observation strongly suggested that YAP1 participates in spindle behavior at MI stage. During the long prometaphase of meiosis I, the spindle is slowly assembled by multiple microtubule-organizing centers (MTOC) and kinetochore-microtubule attachment that is inherently error-prone [340]. The eccentric positioning of meiotic spindle does not rely on astral microtubules and centrosomes [321]. Instead, the F-actin cytoplasmic meshwork plays key roles in determining the positions of the spindles and migration of chromosomes. Fortunately with advanced techniques, actin meshwork during oocyte meiosis has been very well visualized [322, 323]. Particularly, the formation of actin filaments is dependent on actin-nucleating proteins Formin2 (FMN2), Spire 1/2 and ARP2/3, which are also required for the positioning of spindles [320, 322-325]. A model of two-phase migration mechanism was introduced: First, activated FMN2 induced F-actin polymerization drives the spindle to move randomly within short range away from the center of oocyte. Then, chromosomes were moved faster to the cortex with more fixed trajectory, driven by a
continuous cytoplasmic flow that generate a pushing force [325]. Another model of spindle migration proposed in some studies stated that the motor protein myosin II, located at the spindle poles acts to anchor the leading spindle at the cortex area and pull on it by an actomyosin-based contractile mechanism [323, 327] and this process is under the regulation of Mos-MAPK [329]. In our current study, though blocking YAP1 at MI allowed spindles to localize to the cortex (some even presented what appears to be “over-eccentric” positioning), the length and width of spindles were greatly reduced and spindles were not properly structured. Given the primary roles of F-actin meshwork in spindle migration, it is possible that YAP1 interacts with FMN2, Spire 1/2 and ARP2/3 during this process and the lack of YAP1 leads to the disruption of actin meshwork that supports the normal dynamics of spindles at MI. It is also possible that ablating YAP1 disrupts the normal actomyosin contraction by the motor protein myosin II at the spindle poles, especially since the spindles seemed not properly “pulled” when YAP1 was blocked. Therefore further investigations should also focus on the crosstalk between Mos-MAPK and YAP1. In addition to the spindle dynamics, F-actin meshwork also modulates the stiffness of the cortex throughout maturation which is essential for cytokinesis during dividing [328, 329]. The cortex becomes less stiff while the actin cap becomes more thickened near MI spindle which is regulated by Mos-MAPK and its downstream ARP2/3 [328, 329]. Various findings on Hippo pathway identified YAP1 as a sensor and mediator of mechanical cues that are instructed by the cell niche (as reviewed in [212]), it is therefore also very necessary to study the function of YAP1 in mediating the changes of cortex stiffness during oocyte cytokinesis.
However, the dynamic behaviors of chromosomes, spindles and actin cytoskeleton cannot be discussed without acknowledging the cell cycle kinase CDK1 which has been recognized as an important integral part of the MPF (maturation promoting factor). It is well known that the activity of CDK1 and its antagonist PP2A phosphatase, act in opposition throughout oocyte meiosis [341, 361, 362], also shown in Figure 6-7. Normally when kinetochore-microtubule attachments are stabilized the activity of CDK1 reaches plateau. A suboptimal level of CDK1 would lead to unstable kinetochore-microtubule interactions that was believed to cause more frequent meiotic errors [340]. CDK1 phosphorylates various kinetochore-associated proteins that are also substrates of phosphatase PP2A [336, 363]. During MI to MII transition, a rapid CDK1 activation and a concurrent inhibition of PP2A by MASTL is required, which is also what happens in mitosis [341]. Intriguingly, a very recent study in somatic mitosis revealed 3 novel sites on YAP1 (T119, S289 and S367) that are phosphorylated and positively regulated by the CDK1 and this regulation is required for G2 to M progression during mitosis [333]. Plus, PP2A is already known to dephosphorylate YAP1 and positively regulate the Hippo target genes in other mammalian somatic cells [342-344]. Taken together, it is highly appealing to investigate if any of these novel phosphorylation sites of YAP1 are under the direct control of CDK1 and PP2A in oocyte meiosis. We found that the phosphorylation status of YAP1 at T119 was not altered much by inhibiting CDK1 for 3h in MII oocytes but it was clear that pYAP1 at T119 was a target of PP2A at the onset of meiotic resumption. Nevertheless, when we examined the phosphorylation of YAP1 at T119 along the progression of oocyte meiosis, we indeed found an increase in pYAP1 expression, which potentially parallels with shifts in CDK1 activity. Future studies are
necessary to draw a solid conclusion on the relation between pYAP1 and CDK1/PP2A at different stages of meiosis, particularly during the abrupt changes of CDK1 activity in MI to MII transition.

As noted before, actin polymerization has been hypothesized to be essential for spindle migration, as shown in experiments that disrupted FMN2 [325]. In order to link the actin polymerization with pYAP1 T119, which results from the balance of CDK1 and PP2A activity, we interfered with actin polymerization of fully grown GV oocytes with either cytochalasin B (inhibitor) or JASP (enhancer) and followed their meiotic progression. Consistent with early studies using cytochalasin B (CB), CB treated oocytes were almost all arrested at MI with no oocytes reaching MII phase in comparison to control where a high percentage of oocytes progressed to MII phase [364]. We also observed aggregation or patches of amorphous actin structures in the cortex area of the oocytes, as supported by a few early studies [365, 366]. Unexpectedly, JASP which is an actin polymerization enhancer, also arrested majority of the oocytes at MI phase which was not reported previously. Due to the fact that JASP competes with phalloidin for actin binding, we were not able to show the direct changes of actin that were caused by JASP treatment. However, it is worth noting that based on the potential toxicity of JASP in prolonged tissue culture indicated by others [221], oocytes were only exposed to JASP treatment in the first 2h of the culture in our study, after which JASP was washed out. The high percentage of oocytes bearing the arrested MI phenotype at the end of 21h culture strongly suggested that the effect of acute JASP treatment at the beginning persisted throughout the meiotic maturation. Lastly, we also reported that normal MII oocytes in control group as well as arrested MI oocytes treated by either CB or JASP all
presented phosphorylated YAP1 T119 that was localized at the spindles. Together, our investigation in roles of YAP1 and actin polymerization in oocyte meiotic maturation made a new connection between Hippo-YAP1, cell cycle regulators CDK1, PP2A and actin cytoskeleton, which has great potential for expanding our understanding on the dynamics of maturating oocytes, where to gain developmental competency is crucial.

In summary, the findings from our current studies lie in three areas. First, we characterized the possible roles of Hippo pathway, in particular, the kinase LATS1 and transcriptional coactivator YAP1 in germ cell survival and follicle activation during early follicular development. Secondly, we reported a few previously unknown mechanisms through which YAP1 acts to mediate the growth promoting effect of oocytes and ovulatory signals to modulate the balance between proliferation and differentiation in cumulus granulosa cells. Thirdly, we proposed a novel model that links YAP1, filamentous actin and cell cycle regulators CDK1 and PP2A during oocyte meiosis. Though many still need further investigations, all the above contributed to our understanding on oocyte and cumulus functions at different stages of follicular development. Meanwhile, our follicular paradigm also provided a unique platform to grow our knowledge on how Hippo signaling pathway regulates cell proliferation and cell differentiation.
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PUBLICATIONS

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