Novel roles for intra- and extra-follicular factors during ovulation and oocyte growth

Stephany El-Hayek
Department of Biology
McGill University
Montreal, QC, Canada
July 2015

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy

Table of Contents

Abstract

During folliculogenesis, the multiple processes that culminate in the ovulation of a mature, developmentally competent egg are governed by an intercellular cooperation regulated by a myriad of factors from within or outside the follicle. This thesis discusses two such factors, the extra-follicular follicle-stimulating hormone (FSH), which is secreted by the pituitary, and the intra-follicular growth differentiation factor 9 (GDF9), which is derived from the oocyte. FSH is a glycoprotein hormone whose receptors are located on granulosa cells. FSH drives the late stages of folliculogenesis. Moreover, by upregulating the expression of luteinizing hormone (LH) receptor, FSH indirectly enables the LH-regulated release of the epidermal growth factor receptor (EGFR) ligands, which mediate the ovulatory response. The results discussed in Manuscript I identify a new role by which FSH establishes the EGFR signalling axis. Results show that FSH drives an increase in EGFR expression during late folliculogenesis and provide evidence that this increase is essential to enable the ovulatory response to EGF. By coordinating the expression of EGFR and the release of its ligands, FSH allows full-grown follicles to activate EGFR signalling at ovulation. In Manuscript II, another role for FSH is established. We show that by increasing the expression of junctional components in the oocyte and granulosa cells and increasing the number of actin-rich transzonal projections (TZPs) that physically link these two compartments, FSH enhances contact and communication between the oocyte and the surrounding granulosa cells, essential for oocyte development. In Manuscript III, a new role for the oocyte-derived paracrine factor GDF9 is described. GDF9 is a member of the TGFß superfamily and has been shown to regulate several processes in its target granulosa cells. I provide evidence that GDF9 signalling promotes the elaboration of actin-rich TZPs by the granulosa cells, highlighting that the formation of these projections is an active process. GDF9 also increases the expression of mRNAs encoding proteins that play key roles on filopodial extension and might mediate the formation of actin-rich TZPs. By establishing new functions for FSH and GDF9, the experiments reported in this thesis emphasize their indispensable function during oocyte and follicle growth, and subsequently embryo health, and highlight the importance of the interplay between intra-and extra-follicular factors during oocyte development.

Résumé

Durant la folliculogénèse, des processus divers qui permettent l'ovulation d'une ovule mature et compétente, sont régies par une coopération intercellulaire médiée par une myriade de facteurs provenants de l'intérieur ou à l'extérieur de la follicule. Cette thèse discute deux facteurs, l'hormone folliculo-stimulante (FSH) qui est sécrétée par l'hypophyse et le facteur de différenciation de croissance 9 (GDF9) qui est dérivé de l'ovocyte. La FSH est une glycoprotéine dont les récepteurs sont situés sur les cellules de la granulosa. Elle est essentielle pour l'accomplissement de l'étape finale de la folliculogenèse. En régulant l'expression de LHCGR, la FSH permet indirectement la production des ligands de l'EGFR. Les résultats discutés dans 'Manuscript I' montrent que j'ai identifié un nouveau rôle par lequel la FSH établit l'axe de signalisation de l'EGFR. Les résultats montrent que la FSH cause une augmentation de l'expression de l'EGFR, qui est nécessaire pour les processus d'ovulation: l'expansion des cellules cumulus et la maturation méiotique de l'ovocyte. En coordonnant l'expression de l'EGFR et la production de ses ligands, la FSH permet les follicules grandes d'activer la signalization de l'EGFR lors de l'ovulation. Dans 'Manuscript II', un autre rôle pour la FSH est demontré. Je montre que, en augmentant l'expression des protéines composantes les jonctions entre les cellules de la granulosa et l'ovocyte, et en augmentant le prolongement des projections trans-zonales (TZPs) qui relient physiquement ces deux, la FSH favorise le contact et la communication entre l'ovocyte et les cellules de la granulosa entourantes, ce qui est essentiel pour le développement de l'ovocyte. Dans le 'Manuscript III', un nouveau rôle pour le facteur paracrine GDF9 qui est dérivé de l'ovocyte est décrit. Le GDF9 est un membre de la superfamille TGFß, qui régule plusieurs processus dans les cellules de la granulosa, comme la prolifération, la différenciation, le metabolisme et l'expansion. Mes résultats montrent que le GDF9 favorise l'extension des TZPs riches en actine par les cellules de la granulosa, soulignants que la formation de ces extensions est un processus actif. On a trouvé que le GDF9 améliore l'expression de facteurs d'assemblage de l'actine dans les cellules de la granulosa qui pourraient médier la formation des TZPs riches en actine.

La FSH et le GDF9 sont connus pour être nécessaire pour folliculogénèse. Les résultats présentés dans cette thèse soulignent l'importance des rôles joués par ces deux facteurs pendant l'ovulation et durant la croissance de l'ovocyte, et la necessité et mettent en évidence que l'interaction entre ces facteurs est nécessaire pour l'ovocyte, le follicule, et l'embryon.

Acknowledgements

The research I have been working on during my PhD studies has made me fall in love with science even more, and it was all possible because of the creative, nurturing, and supportive lab which I am so lucky to have been part of. And for that, I must first thank my supervisor Dr. Hugh Clarke. Your guidance, patience, and support during these past four years have been invaluable. Your mentorship has allowed me to grow as a scientist. I will always appreciate the flexibility and autonomy you offered and will be forever grateful for the opportunities you unlocked in my career. It has been a privilege to work with you and learn from you. Thank you for always having my best interest as a student, as a researcher, and as a person, at heart.

I would also like to thank my committee members, Dr. Francois Fagotto and Dr. Makoto Nagano for the advice and direction in my projects, for the very long discussions at every committee meeting and for the genuine interest in the science I shared with you.

My lab family, Karl-Frédéric Vieux, Laleh Abbassi, Omar Farah, Suhaib Khayyat, Maria-Teresa Eyzaguirre, Nicole Edwards, and Suha Arab, your friendship and support have made these four years a very enjoyable experience. Thank you for being there for me for the science and the non-science. I am very lucky to have been part of such a collaborative and friendly environment. I would especially like to thank Dr. Qin Yang for all her help with everything from day one, and for always taking care of all of us at the lab.

Completing this degree has come at the expense of being far from the best family ever. Wanting to make you proud has always been my drive and your continuous support has helped me remain focused. Farid Gharazeddine, for learning about FSH and TZPs for me and for being so proud of me, thank you. Bassem Khalil, Samer Hanna, and Mayssa Moubarak for the oral presentations practices, and for always being there during these past years, thank you.

This thesis is dedicated to my father, who instilled motivation, determination, and the love of knowledge in me.

Publications and author contributions

1- El-Hayek S, Demeestere I, Clarke HJ. Follicle-stimulating hormone regulates expression and activity of epidermal growth factor receptor in the murine ovarian follicle. Proc Natl Acad Sci U S A 2014; 111:16778-16783. doi: 10.1073/pnas.1414648111. PMID 25385589 (Manuscript I)

2- El-Hayek S, Clarke HJ. Follicle-stimulating hormone increases gap junctional communication between the somatic and germ-line follicular compartments during murine oogenesis. Biol Reprod 2015. pii: biolreprod.115.129569. PMID 26063870 (Manuscript II)

3- El-Hayek S, Clarke HJ. Somatic-germ cell interactions during oocyte growth and development. Res. Prob. Cell Diff. (invited chapter, accepted)

4- El-Hayek S, Yang Q, Clarke HJ. Oocyte-driven remodeling of the somatic microenvironment enables intra-follicular communication. (Manuscript III; in preparation)

Contribution of authors

1- El-Hayek S, Demeestere I, Clarke HJ (2014)

Contribution: The candidate, El-Hayek S, performed the experiments presented in this manuscript, except the quantitative PCR analysis in Figures 7A, B and 13, which were performed by Demeestere I. El-Hayek S performed the data analysis for all experiments (except the two listed above) and the figure preparation. El-Hayek S and Clarke HJ wrote the manuscript. El-Hayek S, Demeestere I, Clarke HJ provided the intellectual contribution.

2- El-Hayek S, Clarke HJ (2015)

Contribution: The candidate, El-Hayek S, performed the experiments presented in this manuscript. El-Hayek S performed the data analysis for all experiments and the figure preparation. El-Hayek S and Clarke HJ wrote the manuscript. El-Hayek S and Clarke HJ provided the intellectual contribution.

3- El-Hayek S, Clarke HJ (accepted)

Contribution: The candidate, El-Hayek S, performed the literature research and the primary writing of the review. El-Hayek S and Clarke HJ performed the figure preparation. El-Hayek S and Clarke HJ provided the intellectual contribution.

4- El-Hayek S, Yang Q, Clarke HJ (in preparation)

Contribution: The candidate, El-Hayek S, performed the experiments presented in this manuscript, except the culture experiment in Figure 37A which was performed by Yang Q, and the tissue processing for transmission electron microscopy which was performed by a histology facility at McGill University. El-Hayek S performed the data analysis for all experiments and the figure preparation. El-Hayek S and Clarke HJ wrote the manuscript. El-Hayek S and Clarke HJ provided the intellectual contribution.

List of figures

List of tables

List of abbreviations

ALDOA	aldolase A
ALK5	activin receptor-like kinase-5
AKT	protein kinase B
AMH	anti-Müllerian hormone
AREG	amphiregulin
ATP	adenosine triphosphate
BFGF	basic fibroblast growth factor
BMP	bone morphogenetic protein
BMPRII	BMP type-II receptor
BSA	bovine serum albumin
BTC	betacellulin
CAMP	cyclic adenosine monophosphate
CDC	cell division cycle
CDH1	cadherin 1
CDH2	cadherin-2
CDK1	cyclin-dependent kinase 1
CGMP	cyclic guanosine monophosphate
CNP	C-type natriuretic peptide
COC	cumulus-oocyte complex
CREB	cAMP response element-binding protein
CYP	cytochrome P450
DAAM1	dishevelled-associated activator of morphogenesis 1
ECG	equine chorionic gonadotropin
EGF	epidermal growth factor
EGFR	EGF receptor
EREG	epiregulin
EU	5'-ethynyl uridine
FBS	fetal bovine serum

FDPS	farnesyl diphosphate synthase
FLIP	fluorescence loss in photobleaching
FOXO	forkhead box O
FOXL2	forkhead box protein L2
FRAP	fluorescence recovery after photobleaching
FSH	follicle-stimulating hormone
FSHR	FSH receptor
GJA	gap junction protein, alpha
GnRH	gonadotropin-releasing hormone
GOC	granulosa-oocyte complex
GPCR	G-protein coupled receptor
GVBD	germinal vesicle breakdown
HA	hyaluronan
HAS2	hyaluronan synthase 2
HPG	hypothalamic–pituitary–gonadal
HSD	hydroxysteroid dehydrogenase
ICSI	intra-cytoplasmic sperm injection
IGF-1	insulin-like growth factor 1
IAI	inter-α-inhibitor
IMDPH	inosine-5′-monophosphate dehydrogenase
IP3	inositol trisphosphate
ITS	insulin transferrin selenium
IVF	in vitro fertilization
KGF	keratinocyte growth factor
LIF	leukemia inhibitory factor
LHX8	LIM homeobox gene 8
LH	luteinizing hormone
LHCGR	luteinizing hormone receptor
MAPK	mitogen-activated protein kinase
MEM	minimal essential medium
MPF	maturation-promoting factor
MTOC	microtubule-organizing center

MTORC	mechanistic target of rapamycin complex 1
MYO10	myosin 10
NFKB	nuclear factor kappa B
NGF	nerve growth factor
NOBOX	newborn ovary homeobox protein
NPPC	natriuretic peptide C
NPR2	natriuretic peptide receptor B
NR4A	nuclear receptor subfamily 4 group A
NSN	non surrounded nucleolus
ODPF	oocyte-derived paracrine factor
OOX	oocytectomy
PBS	phosphate-buffered saline
PBST	tween- PBS
PCR	polymerase chain reaction
PD	postnatal day
PDE	phosphodiesterase
PDGF	platelet-derived growth factor
PFKP	phosphofructokinase
PAI	pre-α-inhibitor
PKA	protein kinase A
PTEN	phosphatase and tensin homolog
PTGS2	prostaglandin-endoperoxide synthase 2
PTX3	pentraxin 3
ROI	region of interest
RT	room temperature
SLC7A6	solute carrier family 7 member 6
SLC38A3	sodium-coupled neutral amino acid transporter 3
SMAD	sma-and mad-related
SN	surrounded nucleolus
SOHLH	spermatogenesis and oogenesis specific basic helix-loop-helix
SP1	specificity protein 1
SPAM1	sperm adhesion molecule 1

STAR	steroidogenic acute regulatory protein
TBP	TATA box-binding protein
TGFB	transforming growth factor beta
TNFAIP6	tumor necrosis factor, alpha-induced protein 6
TSC1	tuberous sclerosis 1
TSH	thyroid-stimulating hormone
TSG-6	tumor necrosis factor-inducible gene 6
TZP	trans-zonal projection

Chapter 1

Folliculogenesis

The mammalian ovarian follicle houses the oocyte, the female germ cell. In the mouse, oocyte development begins before the formation of follicles, when female primordial germ cells give rise to oogonia that enter meiosis at embryonic day 13.5 (Borum 1961), but it can only proceed if folliculogenesis occurs (Pepling and Spradling 2001; Rajah, Glaser, and Hirshfield 1992). Folliculogenesis is thus essential for oocyte growth, allowing the production of a mature fertilizable gamete.

Folliculogenesis begins with the formation of primordial follicles, consisting of a primary oocyte measuring approximately 12μm in diameter, arrested at the diplotene stage of prophase I, surrounded by a single layer of squamous granulosa cells. Primordial follicles are dormant, and constitute the largest population of follicles in the mammalian ovary, termed the ovarian reserve pool. This population is gradually depleted by the cyclical recruitment of primordial follicles into the growth phase, when primordial follicles are activated and oocyte growth begins. The follicle transitions into the primary stage, where the granulosa cells undergo a cell shape change and become cuboidal. The oocyte increases in size, and when it reaches around 30μm in diameter (Griffin et al. 2006) it begins to secrete an extracellular glycoprotein coat whose deposition outside the oocyte plasma membrane physically separates the oocyte from the granulosa cell layer. This coat, termed the *zona pellucida*, eventually encircles the oocyte entirely. The granulosa cells continue to divide mitotically giving rise to a multilayered secondary follicle. Theca cells deposit around basement membrane and the follicle also undergoes angiogenesis acquiring its own vasculature. As the granulosa cells increase in number, a fluid filled cavity termed an antrum forms within the follicle, now in the early antral stage. As the antrum expands, it physically separates the granulosa cells into two populations, the mural granulosa cells which line the follicular walls and the cumulus granulosa cells, which constitute the layers surrounding the oocyte, and the follicle is termed an antral follicle. Around this stage, the oocyte reaches full

size and acquires meiotic and developmental competence, the ability to resume meiosis and to sustain embryo development, respectively, and thus is now ready to be ovulated (Figure 1).

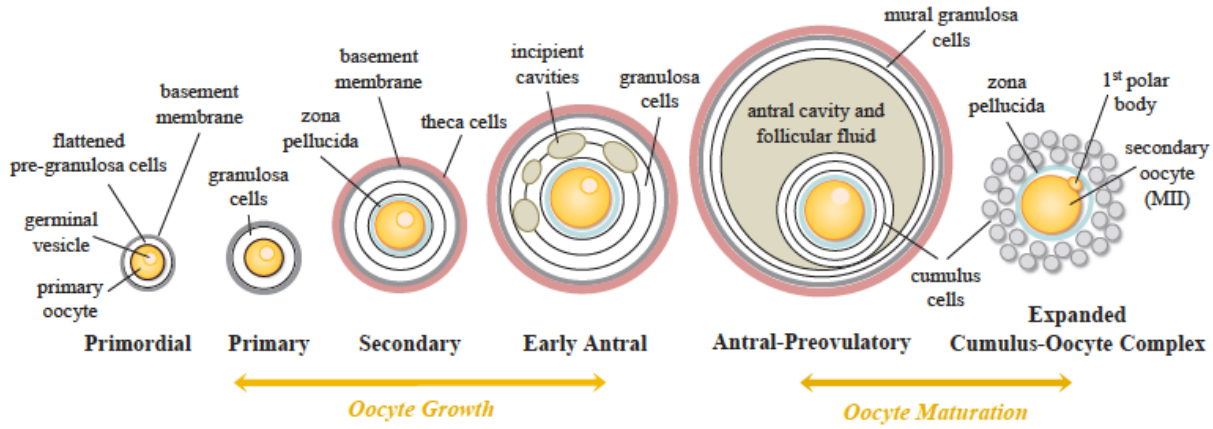


Figure 1: Folliculogenesis. Upon activation, primordial follicles, consisting of a primary oocyte surrounded by a single layer of squamous (flattened) granulosa cells, initiate their growth and form primary follicles, where the granulosa cells undergo a cell shape change and become cuboidal. The oocyte, which has grown in size, begins secreting the *zona pellucida*. The oocyte continues to grow and granulosa cells divide mitotically giving rise to a multilayered secondary follicle. Theca cells deposit around basement membrane. Fluid filled cavities form within the follicle, now in the early antral stage. As the antrum expands, it physically separates the granulosa cells into two populations, the mural and cumulus granulosa cells, and the follicle is termed a pre-ovulatory antral follicle. At ovulation, the cumulus expansion and meiotic maturation occur and the cumulus-oocyte complex is ovulated (from Collado-Fernandez, Picton, and Dumollard 2012).

Intra-follicular and extra-follicular factors mediate intercellular cooperation during folliculogenesis

The development of the follicle and the enclosed oocyte proceed synchronously. This synchronicity is governed by the interplay between intra- and extra-follicular factors. Moreover, within the follicle, various events occurring throughout folliculogenesis greatly manifest inter-cellular dependence and cooperation between germ cell (oocyte) and the somatic (granulosa and theca) compartments, mediated by such factors. This intra-follicular inter-dependence is partly mandated by the structural organization of the follicle and is not only advantageous and metabolically favorable, but also absolutely necessary for many major events of oocyte and follicle growth. The interplay between intra- and extra-follicular factors as well as the extent of this inter-cellular cooperation will thus directly affect the synchronicity and execution of these events, and in turn the competence of a follicle to outcompete other recruited follicles in the cycle and reach the ovulatory stage. The first sign of intercellular dependence is at the time of primordial follicle formation where the association of the primary oocytes with the somatic granulosa cells protects them from undergoing cell death (Felici et al. 1999; Pepling and Spradling 2001; Pesce, Di Carlo, and De Felici 1997; Rajah, Glaser, and Hirshfield 1992). This dependence remains as crucially important throughout all stages of folliculogenesis, until ovulation. Major events in pre-antral (I) and antral (II) folliculogenesis in which intra- and extra-follicular factors mediate inter-cellular cooperation are discussed below.

Ia) Primordial follicle activation

The activation of primordial follicles enables their entry into the growth phase. Throughout the female's reproductive life, a balance between signals that promote primordial follicle activation and those that inhibit it is established, ensuring a continuous recruitment of follicles while maintaining a resting reserve pool. Activation begins by the differentiation of granulosa cells (Braw-Tal 2002; Hirshfield 1991), manifested by a cell shape change from squamous to cuboidal, followed by the initiation of growth in the oocyte. The external signals (if they exist) that initiate this transition remain unknown; however, it is associated with an increase in the mitotic activity of the cells (Da Silva-Buttkus et al. 2008), which enables the granulosa cells to continue to fully cover the surface of the growing oocyte. Nonetheless, this transition is

apparently not indispensable for the initiation of oocyte growth (Lundy et al. 1999) and in mice lacking forkhead box protein L2 (*Foxl2)*, oocytes begin to grow even though the granulosa cells remain squamous (Schmidt et al. 2004; Uda et al. 2004). These oocytes remain very small, however, clearly demonstrating an essential role for FOXL2 to sustain normal oocyte growth.

In the oocyte, phosphoinositol 3-kinase (PI3K) and downstream protein kinase B (Akt) promote follicle activation both *in vivo* (Reddy et al. 2005) and *in vitro* (Li et al. 2010). Consistently, phosphatase and tensin homolog (PTEN) and its downstream forkhead box O3 (FOXO3) (John et al. 2008; Vlahos et al. 1994), which block PI3K/Akt signalling (Cantley and Neel 1999), inhibit the initiation of follicle growth (Castrillon et al. 2003; John et al. 2007; Li et al. 2010; Liu et al. 2006). Deletion of *Foxo3* or deletion of *Pten,* leading to nuclear export of *Foxo3*, causes a depletion of primordial follicle population due to a global activation (Castrillon et al. 2003; Hosaka et al. 2004; John et al. 2008). In contrast, oocyte tuberous sclerosis 1 (TSC1), through its inhibition of mechanistic target of rapamycin complex 1 (mTORC1), maintains follicle arrest and global follicle activation occurs in its absence (Adhikari and Liu 2010) (Figure 2). Oocyte transcription factors, spermatogenesis and oogenesis specific basic helix-loop-helix (SOHLH)1/2, LIM homeobox gene 8 (LHX8), and newborn ovary homeobox protein (NOBOX) have been shown to promote the transition from primordial to primary follicle. Deletion of *Sohlh1* causes a reduction in expression of *Lhx8*, and *Nobox* (Choi et al. 2008; Pangas et al. 2006). *Nobox* expression is also reduced in $Lhx8^{-/-}$ ovaries (Choi et al. 2008; Pangas et al. 2006). In mutants of these oocyte-specific transcription factors, the transition of primordial follicles into primary follicles fails to occur (Pangas et al. 2006; Pangas et al. 2004; Suzumori et al. 2002). NOBOX promotes the expression of genes including growth differentiation factor 9 (*Gdf9)* and *Bmp* (Bone morphogenetic protein) *15*, which are necessary for oocyte growth and development (Choi et al. 2010; Choi and Rajkovic 2006; Rajkovic et al. 2004), and deletion of *Nobox* causes a reduction in these transcripts (Rajkovic et al. 2004). Consistently, these transcripts are also reduced in *Sohlh1* and *Lhx8* mutants. These results suggest that *Sohlh1*, through *Lhx8* and *Nobox*, promotes follicle activation, at least in part by enhancing expression of necessary oocyte transcripts. On the other hand, some factors, such as nerve growth factor (NGF) may function in both the oocyte and the granulosa cells to promote follicle activation (Dissen, Garcia-Rudaz, and Ojeda 2009; Dissen et al. 1996; Dissen et al. 2001). This activation may also be stimulated by

extra-follicular factors such as stroma-derived growth factors, including BMP7, BMP4, and Keratinocyte growth factor (KGF), *in vitro* (Nilsson and Skinner 2003) and *in vivo* (Nilsson and Skinner 2003; Kezele, Nilsson, and Skinner 2005; Lee et al. 2001).

Early studies had suggested that follicle cells initiate the growth of the enclosed oocyte (Lintern-Moore and Moore 1979). Since then, a number of studies have elucidated the mechanism behind this, where the activation of granulosa cells not only precedes, but also induces the initiation of growth in the follicle. Kit ligand (KitL) is expressed by developing granulosa cells, and binds its receptor Kit on the oocyte, causing its phosphorylation (Manova et al. 1993). KitL-Kit signalling has been shown to be necessary for follicle activation both *in vivo* and in ovaries cultured *in vitro* (Packer et al. 1994; Parrott and Skinner 1999; Thomas et al. 2008; Yoshida et al. 1997). Specifically, KitL was then shown to induce the initiation of oocyte growth, through PI3K/Akt signalling in the oocyte (Liu et al. 2006; Reddy et al. 2008; Reddy et al. 2005), which as discussed above, promotes oocyte growth. A recent finding further shows that mTORC1 signaling in granulosa cells increases expression of KitL, placing PI3k in oocytes downstream of mTORC1-KitL in granulosa cells (Zhang et al. 2014). The signal that activates mTORC1 signaling, leading to the increase in KitL and subsequent initiation of oocyte growth, remains to be determined (Figure 2). Signaling by the granulosa cells is thus required for ending the quiescent phase of oocyte growth, and initiation of follicular growth.

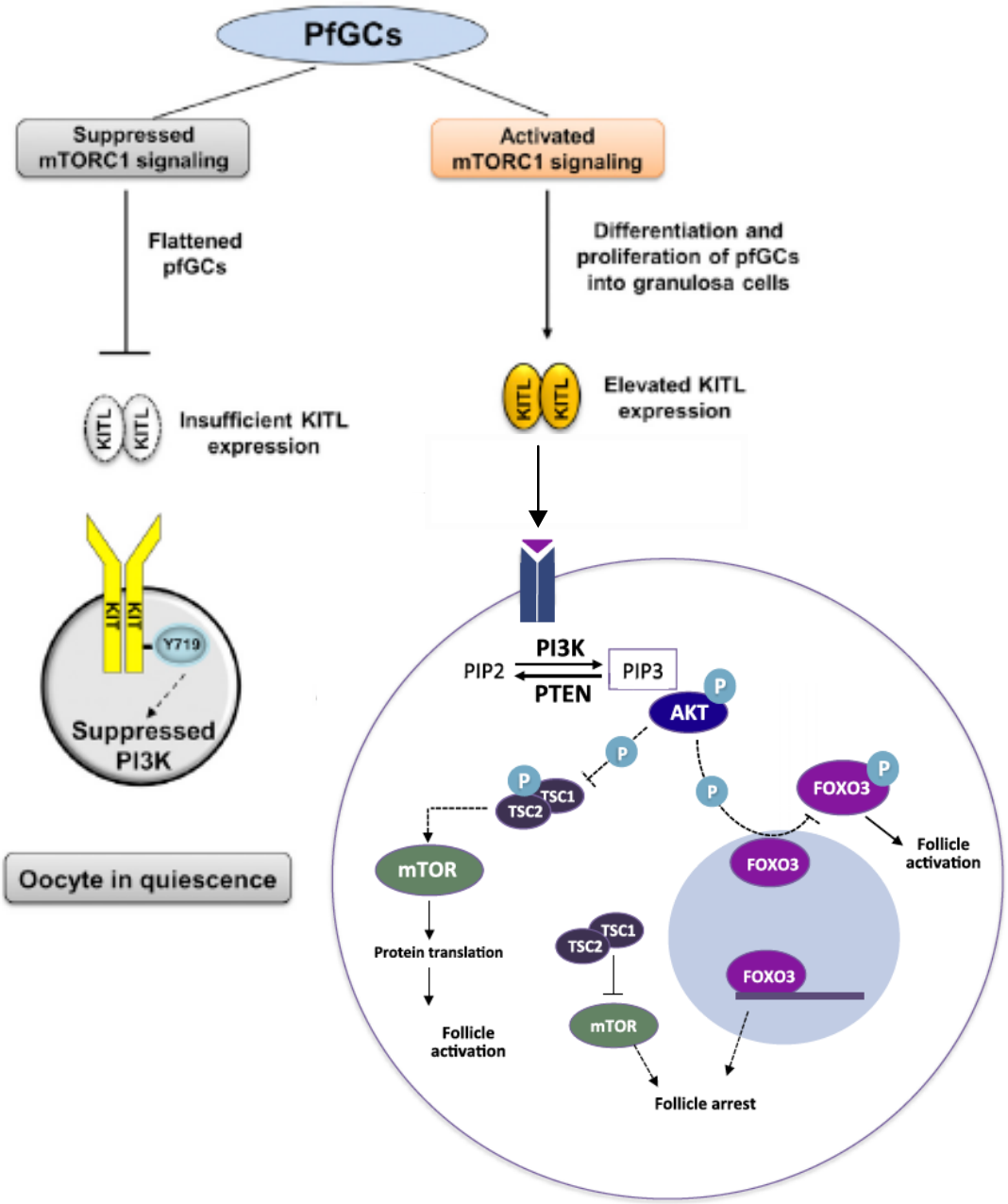


Figure 2: Primordial follicle activation. By an unknown mechanism, mTORC1 signalling in granulosa cells is activated, which causes an increase in expression of KitL. The binding of KitL to its receptor Kit on the oocyte activates PI3K/Akt signalling in the oocyte. This leads to the inactivation of FOXO3 and its translocation out of the nucleus, as well as the activation of mTORC1, enabling the initiation of follicle growth (from Sanchez and Smitz 2012; Zhang et al. 2014).

Ib) Metabolic coupling

It has long been established that one of the roles of granulosa cells is metabolic support (Brower and Schultz 1982; Gilula, Reeves, and Steinbach 1972; Zamboni 1970). Glycolysis, amino acid uptake, as well as cholesterol biosynthesis in the oocyte entail cellular cooperation. This outsourcing is favourable because it allows the oocyte to minimize its own energy expenditure, relying instead on the somatic cells.

Folliculogenesis is associated with an increased consumption of glucose and oxygen consumption, both *in vivo* (Harris et al. 2009) and *in vitro* (Boland et al. 1994; Harris et al. 2007). Many studies however, have shown that oocytes of several mammalian species, including human (Tsutsumi et al. 1990), mouse (Biggers, Whittingham, and Donahue 1967; Downs and Mastropolo 1994; Eppig 1976; Fagbohun and Downs 1992; Harris et al. 2009; Johnson et al. 2007; Sugiura, Pendola, and Eppig 2005) bovine (Rushmer and Brinster 1973; Zuelke and Brackett 1992), rat (Tsutsumi et al. 1992) and rhesus monkey (Brinster 1971), lack the ability to metabolize glucose but can utilize pyruvate directly to produce ATP. Consequently, granulosa cells metabolize glucose and transfer pyruvate to the oocyte (Brinster 1971; Cetica et al. 2002; Donahue and Stern 1968; Herubel et al. 2002; Leese and Barton 1985; Rieger and Loskutoff 1994; Sugiura, Pendola, and Eppig 2005; Tsutsumi et al. 1992; Tsutsumi et al. 1990; Zuelke and Brackett 1992).

This inability to use glucose is also a characteristic of oocytes of several non-mammalian species (Dworkin and Dworkin-Rastl 1991; Eppig and Steckman 1976; Ito et al. 2003; Monaco, Villecco, and Sanchez 2007; Rushmer and Brinster 1973; Waksmonski and Woodruff 2002). Nonetheless, oocytes may have a limited capacity to utilize glucose directly (Brinster 1968, 1971; Downs and Utecht 1999; O'Brien et al. 1996; Rieger and Loskutoff 1994; Saito, Hiroi, and Kato 1994; Urner and Sakkas 1999). One study traced the uptake of glucose by granulosa cells via glucose transporters and its transfer into the oocyte through gap junctions, suggesting that oocytes might indeed use this sugar (Wang et al. 2012). Nevertheless, oocytes have been shown to be deficient in expression of glycolytic enzymes including aldolase A (*Aldoa)* and phosphofructokinase (*Pfkp)* (Sugiura, Pendola, and Eppig 2005), while these enzymes are highly expressed by surrounding granulosa cells (Sugiura, Pendola, and Eppig 2005; Sugiura et al.

2007). Hence, even though oocytes might receive glucose molecules from surrounding granulosa cells, pyruvate, metabolized from glucose by the granulosa cells, rather than glucose itself seems to be the main source of energy for the mammalian oocyte. Alternatively, glucose transferred to the oocyte may be metabolized by the pentose phosphate or the hexosamine biosynthesis pathways (Collado-Fernandez, Picton, and Dumollard 2012). Hence the oocyte relies on the somatic compartment to obtain its source of ATP. As reduced ATP content in the oocyte has been linked to defects of the meiotic spindle (Zhang et al. 2006) as well as diminished developmental competence (Igarashi et al. 2005; Van Blerkom, Davis, and Lee 1995), this cooperation between the oocyte and granulosa cells is strictly essential for normal embryo development and fertility.

The high transcriptional activity of the oocyte and its accumulation of organelles are sustained by a large supply of amino acids. The oocyte, however, lacks the ability to uptake certain amino acids such as alanine, glycine, and proline *in vitro* (Colonna and Mangia 1983; Haghighat and Van Winkle 1990). Uptake significantly improves in the presence of surrounding granulosa cells, which transfer the amino acids through gap junctions (Colonna and Mangia 1983; Haghighat and Van Winkle 1990). Indeed, certain transporters including sodium-coupled neutral amino acid transporter 3 (SLC38A3), whose substrates include histidine and alanine (Eppig et al. 2005) and solute carrier family 7 member 6 (SLC7A6), a cationic amino acid transporter (Corbett et al. 2014), are present only on granulosa cells and not on oocytes. The oocyte's dependence is selective however, as certain amino acid transport systems are active in denuded oocytes (Colonna and Mangia 1983; Haghighat and Van Winkle 1990; Pelland, Corbett, and Baltz 2009). Nonetheless, they are functionally improved by the presence of surrounding granulosa cells (Pelland, Corbett, and Baltz 2009). Protein and nucleotide synthesis, methylation, pH and osmolarity regulation, as well as production of signalling molecules and glycoproteins all depend on the availability of amino acids. By supplying the oocyte with the amino acids for which it lacks transporters, granulosa cells thus ensure that these processes, which affect growth and development of the oocyte and embryo, occur optimally.

Yet another metabolic inter-cellular dependence involves cholesterol synthesis. Enzymes needed for the synthesis of cholesterol pathway, such as farnesyl diphosphate synthase (*Fdps)* and

cytochrome P450 (*Cyp)-51* are expressed at low levels in oocytes. Consistently, the conversion of acetate to cholesterol by granulosa-free oocytes is impaired (Su et al. 2008). Oocytes also lack the ability to transport cholesterol as they do not express receptors for high-density lipoprotein-cholesterol and low-density lipoprotein-cholesterol (Sato et al. 2003; Trigatti et al. 1999). Oocytes are thus deficient in both de-novo cholesterol synthesis and uptake of extracellular cholesterol. Instead, the surrounding granulosa cells, which express cholesterol pathway enzymes, synthesize cholesterol and transfer it to the oocyte (Su et al. 2008). Accumulation of cholesterol in oocytes is essential for normal embryo development (Comiskey and Warner 2007), as early preimplantation embryos are unable to synthesize cholesterol (Pratt 1982; Pratt, Keith, and Chakraborty 1980). The metabolic cooperation between the granulosa cells and the oocyte, which ensures the deposition of cholesterol in the oocyte, is thus critical for early embryo development.

The oocyte's own deficiencies force it to be strictly dependent in several metabolic aspects on the granulosa cells. However, the oocyte does not merely receive these metabolites from the granulosa cells. Rather, it instructs the somatic cells to synthesize and/or supply metabolic nutrients in order to meet its own metabolic requirements. In fact, the expression of several enzymes of the glycolytic pathway (Emori et al. 2013; Sugiura, Pendola, and Eppig 2005; Sugiura et al. 2007) and several amino acid transporters (Emori et al. 2013; Eppig et al. 2005) is enriched in the oocyte-adjacent cumulus cells compared to mural granulosa. Consistently, their expression, as well as the expression of several enzymes in the cholesterol synthesis pathway, is enhanced by oocytes or oocyte-derived paracrine factors (ODPFs) such as GDF9 and BMP15 (Emori et al. 2013; Eppig et al. 2005; Nakamura et al. 2015; Su et al. 2008; Sugiura, Pendola, and Eppig 2005; Sugiura et al. 2007; Zuelke and Brackett 1992). Hence, the metabolic cooperation between the oocyte and surrounding granulosa cells necessitates a bi-directional regulation by the two follicular compartments.

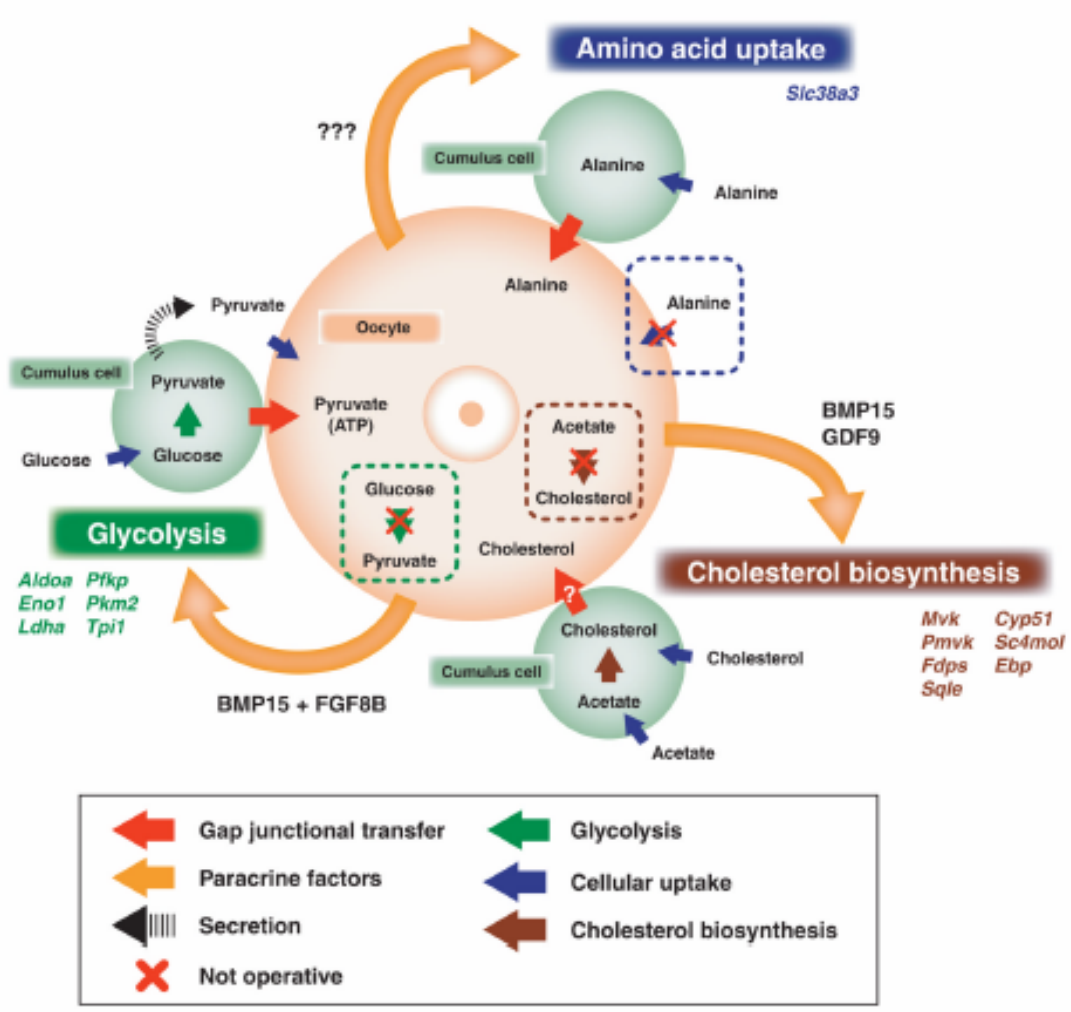


Figure 3: Metabolic coupling. The oocyte is unable to efficiently metabolize glucose, uptake certain amino acids, and synthesize cholesterol. By means of oocyte derived paracrine factors, the oocyte promotes the expression of metabolic enzymes and transporters in the adjacent granulosa cells. In turn, these granulosa cells supply the oocyte with pyruvate, amino acids, and cholesterol (from Su, Sugiura, and Eppig 2009).

Ic) Estradiol synthesis

Steroidogenesis occurs in the adrenal gland, ovary, testis, and placenta. In the ovary, this process has long been considered as an example of inter-cellular dependence. In fact, a model named the 'two-cell, two-gonadotropin' model, describing a cooperation between the granulosa and theca cells, was suggested in the 1970s (Fortune and Armstrong 1977). Steroidogenesis involves the uptake and use of cholesterol by several steroidogenic enzymes, and is driven by the pituitary gonadotropin hormones follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Conley et al. 1994; Parker and Schimmer 1995; Richards 1994; Richards et al. 2002; Tian, Berndtson, and Fortune 1995). Because theca cells do not express FSH receptor (Camp, Rahal, and Mayo 1991; Zeleznik, Midgley, and Reichert 1974), the theca-granulosa cooperation is necessary for estrogen synthesis. Steroidogenesis begins with the import of cholesterol imported by steroidogenic acute regulatory protein (StAR) (Arakane et al. 1996; Clark et al. 1994; Stocco 2001). Cholesterol is then cleaved by the mitochondrial enzyme, CYP-11A, producing pregnenolone. CYP-17, 3-β-hydroxysteroid dehydrogenase (HSD) and 17β-HSD then metabolize pregnenolone to produce androgens. These are then converted by aromatase (CYP-19) complex to estradiol (Miller 1988; Peter and Dubuis 2000; Simpson et al. 1994).

LH, whose receptor is on theca as well as granulosa cells (Camp, Rahal, and Mayo 1991; Erickson, Wang, and Hsueh 1979; Piquette et al. 1991; Segaloff, Wang, and Richards 1990; Zeleznik, Midgley, and Reichert 1974) drives androgen synthesis (Fortune and Armstrong 1977; Richards et al. 1987; Smyth et al. 1994), while FSH stimulates aromatase activity in granulosa cells (Dorrington, Moon, and Armstrong 1975; Erickson, Wang, and Hsueh 1979; Fitzpatrick and Richards 1991; Hickey et al. 1988; Whitelaw et al. 1992), enabling the production of estrogen from the theca-derived androgens. In combination with other follicular factors such as Insulin-like growth factor 1 (IGF-1), LH (Devoto et al. 1999; McGee et al. 1996; Schoppee, Garmey, and Veldhuis 2002; Sekar, Garmey, and Veldhuis 2000; Simone, Chorich, and Mahesh 1993; Willis et al. 1996; Zhang, Garmey, and Veldhuis 2000) and FSH (Adashi 1994; Balasubramanian et al. 1997; Glister et al. 2001; Hsu and Hammond 1987; Khalid, Haresign, and Luck 2000; Monniaux and Pisselet 1992; Pescador et al. 1997; Silva and Price 2002; Spicer, Chamberlain, and Maciel 2002; Yoshimura 1998) have been shown to increase expression of these steroidogenic enzymes in the theca and the granulosa cells, thus promoting androgen and

estrogen synthesis respectively, both *in vivo* and *in vitro*. Hence, the combined actions of theca cells and granulosa cells enable steroidogenesis in the developing ovarian follicle.

Some findings, however, suggest that steroidogenesis is in fact regulated by three, rather than two follicular cell types. The removal of an oocyte from a granulosa-oocyte complex *in vitro* by oocytectomy leads to reduction in estradiol levels, suggesting that the oocyte promotes its production (Vanderhyden, Cohen, and Morley 1993). Oocytes have been shown to enhance estradiol production in non-mammalian species as well (Sretarugsa and Wallace 1997; Yoshimura, Tischkau, and Bahr 1994). However, multiple studies have shown that oocyte signalling decreases the expression of steroidogenic enzymes (Diaz, Wigglesworth, and Eppig 2007b; Pangas et al. 2006; Wigglesworth et al. 2015) and suppresses FSH-stimulated estradiol synthesis (Glister et al. 2001; Vitt, McGee, et al. 2000). These seemingly contradicting results would suggest that the oocyte signalling promotes estradiol synthesis while also inhibiting the premature luteinization of the granulosa cells (Coskun et al. 1995; el-Fouly et al. 1970; Nekola and Nalbandov 1971), through independent pathways (Vanderhyden, Cohen, and Morley 1993; Vanderhyden and Macdonald 1998; Vanderhyden and Tonary 1995). The mechanism of how the oocyte achieves such a balanced effect remains to be elucidated. Theca, granulosa, and oocyte all cooperate to allow estradiol synthesis by granulosa.

The follicle itself is a target of the estradiol it produces (Berisha, Pfaffl, and Schams 2002; Britt and Findlay 2003; Juengel et al. 2006; LaVoie et al. 2002; Rosenfeld et al. 2001). Estradiol drives folliculogenesis (Couse et al. 2005; Emmen et al. 2005; Krege et al. 1998) and prevents follicular atresia (Britt et al. 2000; Dupont et al. 2000; Richards 1980; Robker and Richards 1998; Rosenfeld et al. 2001). It promotes the survival of granulosa cells as well as the oocyte by inhibiting the expression of pro-apoptotic genes (Billig, Furuta, and Hsueh 1993; Britt et al. 2000; Lund et al. 1999; Murdoch 1998; Quirk, Cowan, and Harman 2006; Toda et al. 2001). It also increases expression of IGF-1 (Hsu and Hammond 1987; Khalid, Haresign, and Luck 2000). Moreover, estradiol has also been shown to enhance the expression of natriuretic peptide receptor B (NPR2, see below), thus enabling the maintenance of meiotic arrest (Zhang et al. 2011). Hence, the cooperation between the various follicular compartments, resulting in the production of estradiol, is crucial for survival and development of the follicle as a whole.

Id) Maintenance of meiotic arrest

From the time it reaches prophase I of meiosis (at around E13.5) (Borum 1961), until the time of ovulation, the oocyte is in meiotic arrest. The maintenance of this prolonged arrest requires the inhibition of the maturation-promoting factor (MPF). MPF, consisting of cyclin-dependent kinase 1 (CDK1, encoded by *Cdc2*) and cyclin B1 (encoded by *Ccnb1*) (Brunet and Maro 2005), is partly inhibited through the continuous degradation of cyclin B1 by anaphase-promoting complex (APC)-CDH1 (encoded by *Cdc20*), preventing its accumulation and subsequent activation of MPF (Holt, Weaver, and Jones 2010; Marangos and Carroll 2004; Reis et al. 2006). In addition, MPF is activated by the phosphatase CDC25 and inhibited by the phosphatase WEE1 (Lew and Kornbluth 1996; Lincoln et al. 2002). The activity of WEE1 and CDC25 is in turn regulated by cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) in oocytes (Han et al. 2005; Oh, Han, and Conti 2010; Pirino, Wescott, and Donovan 2009; Zhang et al. 2008). PKA phosphorylates Wee1, a nuclear oocyte-specific member of WEE1/MYT family, activating it to inhibit CDK1 (Han et al. 2005; Oh, Han, and Conti 2010), and CDC25B, causing its cytoplasmic retention (Pirino, Wescott, and Donovan 2009; Solc et al. 2008; Zhang et al. 2008). Whether PKA also inhibits the activity of CDC25A, which is nuclear in prophase I oocytes, and thus isolated from the cytoplasmic cyclin B1 and CDK1, remains to be determined (Li, Yin, et al. 2008; Solc et al. 2008). Hence cAMP-dependent PKA activity is essential for inhibiting MPF activity and maintaining prophase I arrest during oocyte growth (Figure 4).

Consistently, early experiments showed that meiotic arrest can be maintained using cAMP (Cho, Stern, and Biggers 1974; Dekel and Beers 1978), or inhibitors of phosphodiesterase (PDE) (Bornslaeger, Wilde, and Schultz 1984; Dekel and Beers 1978, 1980; Vivarelli et al. 1983), enzymes that degrade cAMP, one of which, PDE3A, is expressed in oocytes of various species (Bornslaeger, Wilde, and Schultz 1984; Dekel and Beers 1978, 1980; Freudzon et al. 2005; Jensen et al. 2002; Masciarelli et al. 2004; Nogueira et al. 2006; Richard, Tsafriri, and Conti 2001; Sasseville et al. 2007; Shitsukawa et al. 2001; Tsafriri et al. 1996; Vivarelli et al. 1983). cAMP is produced by the oocyte (Olsiewski and Beers 1983; Urner et al. 1983) through the activity of a Gs-linked receptor (Hinckley et al. 2005; Ledent et al. 2005; Mehlmann et al. 2004; Norris et al. 2007; Mehlmann, Jones, and Jaffe 2002) and has been shown to regulate meiotic

arrest in several species (Deng et al. 2008; DiLuigi et al. 2008) by inhibiting MPF through PKA (Bornslaeger, Mattei, and Schultz 1986; Bornslaeger, Mattei, and Schultz 1988; Horner et al. 2003; Maller and Krebs 1977, 1980).

It has long been established that there is a 'Meiosis inhibitor' factor within the mammalian follicle, as fully-grown oocytes removed from the follicle undergo spontaneous maturation (Edwards 1965; Pincus and Enzmann 1935; Sato and Koide 1984; Tsafriri, Pomerantz, and Channing 1976), and blocking gap junctional communication with the oocyte causes meiotic resumption (Sela-Abramovich et al. 2006). The pathway involved, however, has only recently been fully elucidated. We now know that Natriuretic peptide C (NPPC), a precursor of C-type natriuretic peptide (CNP) synthesized by granulosa cells (Franciosi et al. 2014; Kawamura et al. 2011; Zhang et al. 2011; Zhang et al. 2010), activates the guanylyl cyclase NPR2 triggering cyclic guanosine monophosphate (cGMP) synthesis. cGMP enters the oocyte by gap junctions (Norris et al. 2009; Zhang et al. 2010) and blocks PDE, ensuring high cAMP-dependent PKA activity which prevents meiotic resumption (Vaccari et al. 2009) (Figure 4). This thus confirms earlier studies which suggested that cGMP plays a role in meiotic arrest (Tornell, Brannstrom, and Hillensjo 1984; Zhang, Tao, Xia, et al. 2005; Zhang, Tao, Zhou, et al. 2005).

Both FSH and estradiol have been shown to increase expression of *Nppc* and *Npr2* mRNA (Kawamura et al. 2011; Lee et al. 2013; Zhang et al. 2011; Zhang et al. 2010). In addition, the oocyte itself through factors including GDF9 and BMP15, promotes the expression of this ligand and receptor (Lee et al. 2013; Wigglesworth et al. 2015; Zhang et al. 2011; Zhang et al. 2010). Oocyte signaling enhances the expression of inosine-5′-monophosphate dehydrogenase (IMPDH), an enzyme required for the production of cGMP (Wigglesworth et al. 2013). The oocyte's meiotic arrest is thus maintained by the granulosa cells as well as by the oocyte itself. This cooperation between the two compartments prevents precocious meiotic maturation, ensuring that meiotic maturation only occurs following a protracted period of growth, when the oocyte becomes competent for fertilization.

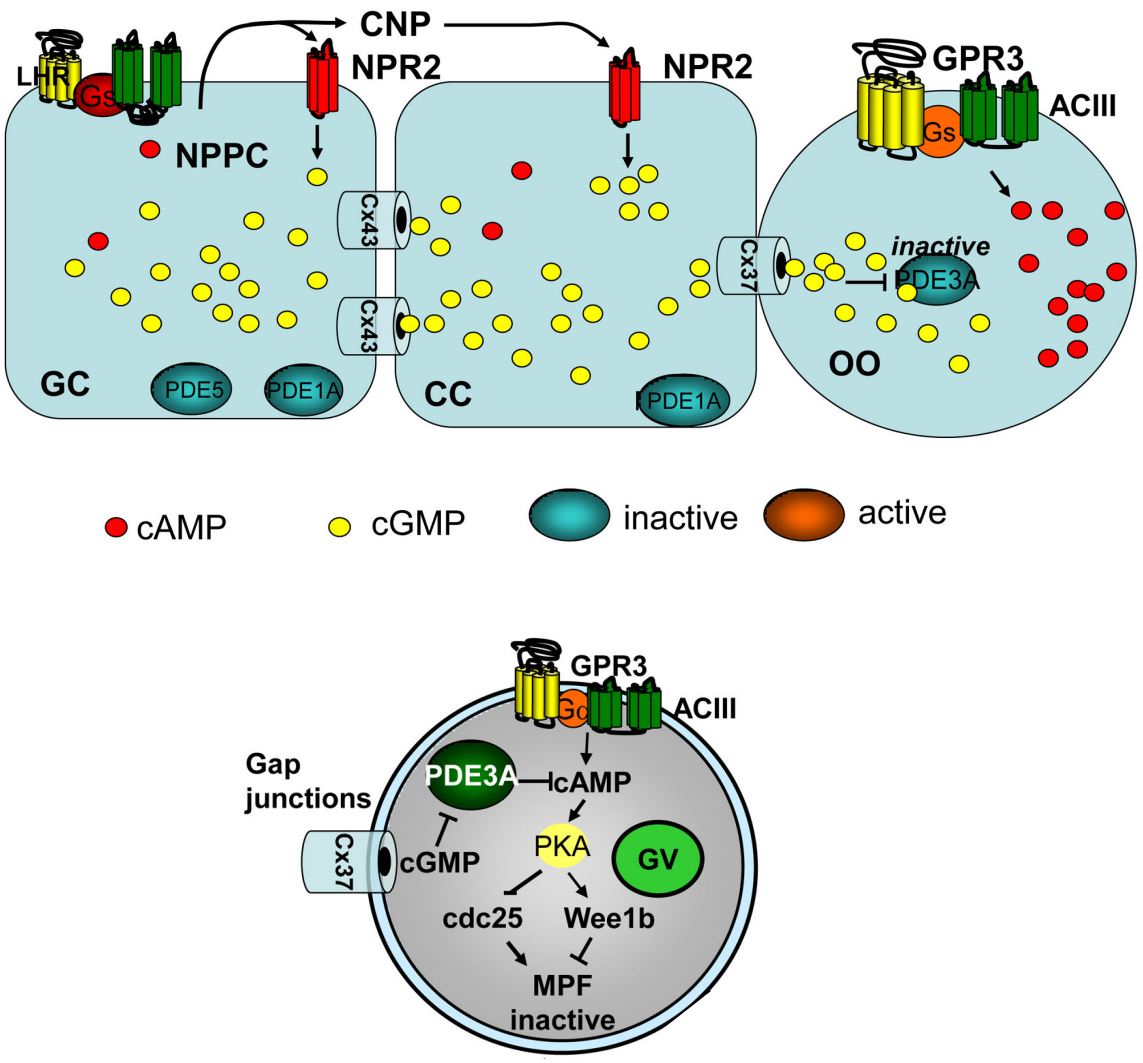


Figure 4: Maintenance of meiotic arrest. In the granulosa cells, NPPC activates the guanylyl cyclase NPR2, triggering cGMP synthesis. cGMP is transferred into the oocyte through gap junctions, where it blocks the activity of PDE3A, inhibiting it from degrading cAMP. This ensures a high cAMP-dependent PKA activity which prevents the activation of the MPF by inhibiting and activating CDC25 and Wee1, respectively, thus maintaining meiotic arrest (from Conti et al. 2012).

IIa) Chromatin remodeling and developmental competence

The initiation of oocyte growth in early folliculogenesis is accompanied by the initiation of high transcriptional activity. In preparation for embryogenesis, growing oocytes accumulate large quantities of RNA and protein (Bachvarova 1985; Cowden 1962; McGaughey, Montgomery, and Richter 1979; Moore et al. 1974). In the mouse, it has been estimated that an oocyte's RNA and protein contents increase by 300 and 38 fold, respectively (Schultz, Letourneau, and Wassarman 1979; Sternlicht and Schultz 1981). However, having reached full size, the oocyte ceases transcription (Bouniol-Baly et al. 1999; Schultz and Wassarman 1977; Wassarman and Letourneau 1976), which resumes at the 1 cell (rRNA transcription) or 2 cell stage (global transcription) (Braude et al. 1979; Johnson 1981; Lin et al. 2014). Coincidentally, chromatin progressively undergoes a conformational change from a decondensed chromatin configuration, termed non-surrounded nucleolus (NSN), to a partially condensed configuration, termed surrounded nucleolus (SN) (Debey et al. 1993; Mattson and Albertini 1990; Wickramasinghe, Ebert, and Albertini 1991). This change occurs in the oocytes of many mammalian species including human, pig, monkey, sheep, cow, and horse (Albertini 1987; Comizzoli, Pukazhenthi, and Wildt 2011; Fuhrer et al. 1989; Hinrichs et al. 1993; Jin et al. 2006; Lefevre et al. 1989; McGaughey, Montgomery, and Richter 1979; Parfenov et al. 1989; Russo et al. 2007; Schramm et al. 1993; Sui et al. 2005; Sun et al. 2009).

The change in chromatin configuration is also associated with a change in nuclear concentration of several transcription factors such as specificity protein 1 (SP1) and the TATA box-binding protein (TBP), a rearrangement of cytoplasmic components (appearance of microtubule-organizing centers-MTOCs) (Lodde et al. 2008; Mattson and Albertini 1990; Wickramasinghe, Ebert, and Albertini 1991) and a progressive repression of transcriptional activity, such that global transcriptional silencing occurs during both *in vivo* and *in vitro* growth (Bouniol-Baly et al. 1999; Christians et al. 1999; Worrad, Ram, and Schultz 1994). Although the events of chromatin remodelling and transcriptional silencing correlate temporally, they are not strictly dependent on each other (Abe et al. 2010; Andreu-Vieyra et al. 2010; De La Fuente 2006). Consistently, the dissociation of RNA polymerase II from DNA, an indication of transcriptional silencing, is not dependent on the NSN or SN conformation (Abe et al. 2010). *In vitro*, SN configuration is positively correlated with meiotic and developmental competence; human,

mouse, and bovine oocytes that have undergone the NSN to SN transition are more meiotically and developmentally competent than NSN oocytes (Lodde et al. 2007; Wickramasinghe, Ebert, and Albertini 1991; Zuccotti et al. 1998; Zuccotti et al. 2002), suggesting that the competence to undergo chromatin remodeling is associated with the acquiring of meiotic and developmental competence.

Several studies have shown that the oocyte's ability to undergo the transition into the SN configuration, as well as its meiotic and developmental competence, are affected by and dependent on the somatic granulosa cells. Gap junctional communication between both bovine and murine oocytes and their surrounding granulosa cells has been shown to promote chromatin condensation at the end of growth (Carabatsos et al. 2000; De La Fuente 2006; Lodde et al. 2007; Luciano et al. 2011). Oocytes cultured *in vitro* without surrounding granulosa cells, or oocyte-granulosa cell complexes exhibiting reduced gap junctional communication both fail to undergo the NSN-SN configuration (Carabatsos et al. 2000; Lodde et al. 2007). Other studies have also shown that developmental competence of the oocyte requires interaction with somatic cells (Chang et al. 2005; Luciano et al. 2005; Schramm and Bavister 1995). Consistently, models that exhibit reduced or impaired gap junctional communication between the oocyte and the granulosa cells also describe reduced developmental competence (Li and Mather 1997; Ratchford, Esguerra, and Moley 2008; Simon et al. 1997).

The precise mechanisms by which granulosa cells regulate and promote these events, however are not well understood. Granulosa cells are not necessary for the accumulation of proteins, such as cyclin B and CDH1 (Chesnel and Eppig 1995a) by oocytes, but they have been shown to regulate post-translational modifications on proteins, including those needed for meiotic maturation, in both murine and bovine oocytes, suggesting one possible mechanism (Cecconi et al. 1991; Colonna et al. 1989; Motlik et al. 1996). Moreover, a more recent study demonstrated that epidermal growth factor receptor (EGFR) signalling in granulosa cells is required for proper translation of specific maternal transcripts encoding key proteins for maturation, an effect mediated by the mTORC pathway in the oocyte (Chen et al. 2013). A more direct contribution by the granulosa cells, however, has been suggested by a study in the bovine model, describing the transfer of mRNAs and long noncoding RNA from the somatic cells into the oocyte

(Macaulay et al. 2014), a characteristic of non-mammalian oocytes. In *Drosophila*, there is a selective transfer of mRNA and protein from nurse cells to the oocyte during oogenesis (Ambrosio and Schedl 1985; St Johnston et al. 1989). In *C. elegans*, oocytes take advantage of RNA provided by germ cells transiently acting as nurse cells (Gibert, Starck, and Beguet 1984). Whether or not granulosa cells promote mammalian oocyte competence and its NSN-SN transition by supplying it with transcripts and/or proteins that regulate its development requires further studies. Regardless of the underlying mechanism, the role of the granulosa cells in mediating these events marking the final stages of the oocyte's growth and development is indispensable.

IIb) Cumulus expansion

Upon the mid-cycle LH surge, the large antral follicle ruptures and the enclosed oocyte is ovulated (Ball et al. 1982; Eppig 1979b, 1980; Espey 1980; Meinecke and Meinecke-Tillmann 1979; Parr 1974, 1975). The oocyte, however, is not extruded alone, but rather along with a mucified mass of expanded cumulus granulosa cells. Cumulus expansion facilitates transport of the complex through the oviduct (Chen, Russell, and Larsen 1993; Lam et al. 2000; Odor and Blandau 1973; Talbot, Shur, and Myles 2003). It is also essential for the process of fertilization, as it facilitates sperm binding and allows the sperm to pass through the cumulus layers to reach the oocyte (Salustri et al. 2004; Van Soom et al. 2002; Eisenbach 1999). In fact, the extent of cumulus expansion during *in vitro* maturation has been shown to affect the oocyte's developmental competence, fertilization, and embryo development (Ball et al. 1983; Foote 1987; Ng, Chang, and Wu 1999).

Cumulus expansion is characterized by the physical dispersal of the cumulus granulosa cells, which prior to the LH surge, are tightly layered surrounding the oocyte. This dispersal necessitates extensive cytoskeletal rearrangement, mediated by changes in filamentous (F)-actin composition (Sutovsky et al. 1993; Sutovsky, Flechon, and Pavlok 1995; Wert and Larsen 1989). In addition, cumulus expansion also involves the secretion of an extracellular matrix by the cumulus granulosa cells, which leads to the mucification of the ovulated mass. This matrix is produced by the cumulus granulosa cells and consists mostly of the glycosaminoglycan

hyaluronan (Eppig 1981; Fulop, Salustri, and Hascall 1997; Salustri et al. 1999; Salustri, Yanagishita, and Hascall 1989). Hyaluronan (HA) is made by the enzyme Hyaluronan synthase 2, encoded by the *Has2* gene (Fulop et al. 1997). Accordingly, a sperm membrane protein, PH-20 (encoded by Sperm adhesion molecule 1-*Spam1*), has been shown to exhibit hyaluronidase activity, allowing the sperm to penetrate through this mucified mass (Gmachl et al. 1993; Lin et al. 1994). The matrix also contains HA binding proteins (Chen et al. 1996; Fulop et al. 1997; Hess, Chen, and Larsen 1998; Sato et al. 2001; Yoshioka et al. 2000) as well as various proteoglycans (McArthur et al. 2000), which maintain the stability and structure of the matrix. The matrix is organized by the crosslinking of hyaluronan with the heavy chains of inter-α-inhibitor (IαI) and pre-α-inhibitor (PαI) (Sato et al. 2001; Scarchilli et al. 2007; Zhuo et al. 2001), both of which only enter the follicle upon the LH surge, when the blood–follicle barrier breaks down (Hess, Chen, and Larsen 1998; Irving-Rodgers et al. 2002; McClure et al. 1994). This interaction, allowing the heavy chains of IαI and PαI to form covalent links with HA, is essential for cumulus expansion (Fulop et al. 2003; Sato et al. 2001; Zhuo et al. 2001) and is mediated by proteins including pentraxin 3 (PTX3) and tumor necrosis factor-inducible gene 6 (TSG-6), encoded by *Ptx3* and *Tnfaip6*, respectively. (Figure 5) (Baranova et al. 2014; Fulop et al. 2003; Ievoli et al. 2011; Rugg et al. 2005; Salustri et al. 2004; Sanggaard et al. 2008; Scarchilli et al. 2007).

In vitro, cumulus expansion may be induced by several factors, including versican (Dunning et al. 2015), EGF (Dekel and Sherizly 1985; Downs 1989), cAMP analogs (Dekel and Kraicer 1978; Eppig 1989), and FSH (Dekel and Sherizly 1985; Eppig 1979b). *In vivo*, cumulus expansion is triggered by LH, whose receptors are on theca as well as mural granulosa cells (Amsterdam et al. 1975; Erickson, Wang, and Hsueh 1979; Peng et al. 1991). The LH signal is transduced to the cumulus granulosa cells by means of the EGFR pathway (Ashkenazi et al. 2005; Hsieh et al. 2007; Park et al. 2004), where LH triggers release of EGF-like peptides, betacellulin (BTC), amphiregulin (AREG) and epiregulin (EREG) (Ashkenazi et al. 2005; Park et al. 2004; Sekiguchi et al. 2004), which have been suggested to have redundant functions (Hsieh et al. 2007; Kim et al. 2011; Lee et al. 2004; Luetteke et al. 1999). These peptides in turn bind to EGFR located on both the mural and cumulus granulosa cells (Chabot et al. 1986; el-Danasouri, Frances, and Westphal 1993; Gall et al. 2004; Garnett, Wang, and Roy 2002; Goritz,

Jewgenow, and Meyer 1996; Hill et al. 1999; Qu et al. 2000; Singh, Rutledge, and Armstrong 1995).

Downstream of LH, EGFR signals through various downstream effectors including PI3K/Akt, mitogen-activated protein kinase (MAPK)14, and MAPK3/1, whose activation is necessary for cumulus expansion (Sela-Abramovich et al. 2005; Shimada et al. 2006; Su et al. 2003; Su et al. 2002; Hsieh et al. 2007; Keel et al. 1995; Panigone et al. 2008; Cameron et al. 1996; Carvalho et al. 2003; Downs and Chen 2008; Downs and Hunzicker-Dunn 1995; Li, Liang, et al. 2008; Tajima et al. 2003). Through these various effectors, as well as through PKA (independent of EGFR), LH induces the rapid upregulation of several transcription factors, including nuclear receptor subfamily 4 group A (NR4A) 1 and 2, Early growth response protein (EGR) 1 and 2, B-cell translocation gene (BTG) 1 and 2, by such altering gene expression in the ovulatory follicle (Carletti and Christenson 2009; Russell et al. 2003; Sirois and Richards 1993). Amongst its many target genes, are transcripts that are needed for cumulus expansion including *Ptgs2* (Prostaglandin-endoperoxide synthase 2) (Davis et al. 1999; Ochsner, Russell, et al. 2003; Sirois, Simmons, and Richards 1992), *Has2* (Sugiura, Su, and Eppig 2009) and *Tnfaip6* (Fulop et al. 1997; Fulop et al. 2003; Ochsner, Day, et al. 2003; Sugiura, Su, and Eppig 2009). *In vitro*, the upregulation in expression of these expansion-related transcripts has been shown to be mediated by PKA, MAPK14, MAPK3/1, or Sma-and Mad-related (SMAD) proteins (discussed below) (Diaz, Wigglesworth, and Eppig 2007b; Downs and Hunzicker-Dunn 1995; Dragovic et al. 2007; Gui and Joyce 2005; Panigone et al. 2008; Park et al. 2004; Shimada et al. 2006; Su et al. 2003; Su et al. 2002; Yamashita, Hishinuma, and Shimada 2009). This suggests that the actions of all these effectors may converge, downstream of LH, promoting the expression of expansion-related genes.

Hence, in the ovulatory follicle, the LH stimulus is transduced from the thecal and mural granulosa cells to the cumulus cells through the EGFR pathway, (and possibly through other mediators as well), enabling the assembly of an extracellular matrix around the cumulus cells that causes their expansion away from the oocyte. This process, however, also requires a signalling input by the oocyte itself, which by this stage, is meiotically and developmentally competent, as well as transcriptionally silent. Despite that, the oocyte signals to the cumulus

granulosa cells, such that, *in vitro*, these cells cannot undergo expansion in the absence of an oocyte. They can however, expand if they are co-cultured with oocytes or oocyte- conditioned medium (Buccione et al. 1990; Salustri et al. 1990; Vanderhyden et al. 1990). Many studies have demonstrated that in the mouse, signalling by oocyte-derived factors and their downstream effectors, SMADs, is needed for the upregulation of expansion related transcripts and for cumulus expansion (Diaz et al. 2006; Dragovic et al. 2005; Dragovic et al. 2007; Joyce et al. 2001; Mazerbourg et al. 2004; Moore, Otsuka, and Shimasaki 2003; Sasseville et al. 2010; Su et al. 2003; Su et al. 2010; Vanderhyden et al. 2003).

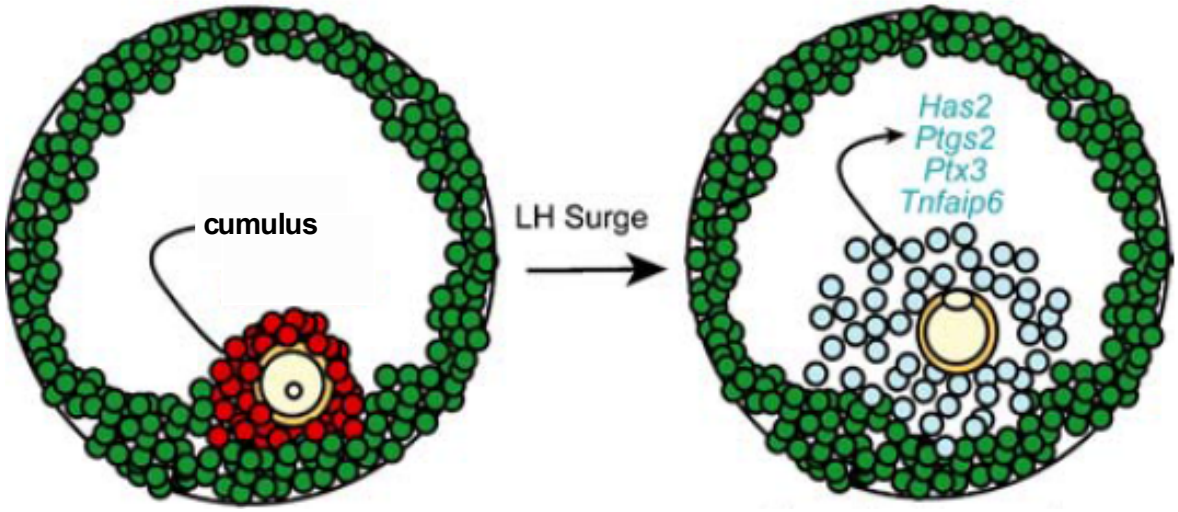


Figure 5: Cumulus expansion. Upon the LH surge, the cumulus granulosa cells upregulate the expression of expansion transcripts including *Has2*, *Ptgs2*, *Ptx3*, and *Tnfaip6* which enable the assembly of an extracellular matrix. This leads to the expansion and mucification of the cumulus-oocyte complex, which then gets extruded from the follicle in the process of ovulation (modified from Diaz, Wigglesworth, and Eppig 2007a).

Other studies have suggested that oocyte-derived factors are needed for activation of MAPK (Su et al. 2003), and for maintaining expression of EGFR (Su et al. 2010). The oocyte thus enables cumulus expansion, indirectly by promoting EGFR activity and/or directly by increasing the expression of expansion-related transcripts. Although the oocyte's ability to induce cumulus expansion *in vitro* is also a feature of several non-murine species (Prochazka et al. 1998; Prochazka et al. 2000; Vanderhyden 1993), in the cow, sheep, and pig, oocyte signalling is not required to induce cumulus expansion (Nagyova, Prochazka, and Vanderhyden 1999; Prochazka et al. 1991; Ralph, Telfer, and Wilmut 1995; Varnosfaderani Sh et al. 2013). Interestingly, oocytes of these species are capable of inducing the expansion of mouse cumulus granulosa cells (Vanderhyden 1993). Whether signalling from the oocyte is required during cumulus expansion in humans remains to be determined. In conclusion, in the mouse at least, in response to the LH stimulus, theca cells, mural and cumulus granulosa cells, as well as the oocyte, all cooperate together to allow cumulus cells to expand and mucify, a pre-requisite for fertilization.

IIc) Meiotic maturation

In response to the LH surge, and shortly before (in rodents) cumulus expansion (Dekel, Hillensjo, and Kraicer 1979; Eppig 1980), another ovulatory process, meiotic maturation, begins, manifested by the breakdown of the nuclear envelope. This is followed by the assembly and migration of the meiosis I spindle (Brunet and Maro 2007), allowing the extrusion of a small polar body, formation of the meiosis II spindle and then metaphase II arrest. This meiotic maturation marks the end of the long mitotic G2-like prophase I arrest. As it grows, the oocyte gradually acquires meiotic competence (Eppig et al. 1994; Sorensen and Wassarman 1976; Szybek 1972) and resumption only occurs when the oocyte has accumulated key cell cycle proteins (Chesnel and Eppig 1995b; de Vant'ery et al. 1996; Kanatsu-Shinohara, Schultz, and Kopf 2000; Mitra and Schultz 1996). As discussed, meiotic arrest is maintained by the inhibition of oocyte cAMP degradation and subsequent MPF activation by granulosa cell-derived cGMP. It thus follows that meiotic resumption, which occurs *in vivo* downstream of LH (Lei et al. 2001; Neal and Baker 1975), would involve relieving this inhibition by cGMP. Indeed, studies have demonstrated that meiotic resumption is associated with a decrease of cGMP transfer from

granulosa cells into the oocyte (Norris et al. 2008; Norris et al. 2009; Vaccari et al. 2009). This reduction is the consequence of a decrease in guanylyl cyclase activity of NPR2 (Vaccari et al. 2009; Egbert et al. 2014; Norris et al. 2009) and a decrease in expression of NPPC (Kawamura et al. 2011; Lee et al. 2013; Robinson et al. 2012), leading to decreased cGMP in the follicle (Norris et al. 2008; Norris et al. 2009; Vaccari et al. 2009) and in turn its subsequent diffusion out of the oocyte (Shuhaibar et al. 2015). This is followed by the closure of gap junctions (Andric, Thomas, and Ascoli 2010; Norris et al. 2008; Norris et al. 2010; Hsieh, Thao, and Conti 2011).

The fall in cGMP levels in the oocyte allows an increase in PDE3A activity, which then degrades cAMP (Richard, Tsafriri, and Conti 2001), causing a decrease in cAMP levels (Schultz, Montgomery, and Belanoff 1983; Vivarelli et al. 1983). This in turn decreases the activity of PKA causing dephosphorylation of Wee1 and CDC25B, which are translocated out of and into the nucleus, respectively (Oh, Han, and Conti 2010; Solc et al. 2008), allowing CDK1 activation. On the other hand, CDH1 becomes degraded allowing cyclin B1 accumulation (Mitra and Schultz 1996; Reis et al. 2007) and its nuclear translocation (Marangos and Carroll 2004; Reis et al. 2006). As in cumulus expansion, these LH-induced events leading to meiotic maturation are mediated by EGFR and MAPK (Ben-Ami et al. 2011; Fan et al. 2009; Kawamura et al. 2011; Lee et al. 2013; Norris et al. 2008; Norris et al. 2009; Peluffo et al. 2012; Sela-Abramovich et al. 2005). The maturation process also involves cytoplasmic redistribution of organelles such as endoplasmic reticulum (Mehlmann et al. 1995; Payne and Schatten 2003; Shiraishi et al. 1995), cortical granules (Liu et al. 2003; Nicosia, Wolf, and Inoue 1977; Szollosi 1967), mitochondria (Dalton and Carroll 2013; Van Blerkom, Davis, and Alexander 2003), and others (Hyttel, Greve, and Callesen 1989; Moreno, Schatten, and Ramalho-Santos 2002), as well as cortex reorganization (Chaigne et al. 2013; Larson et al. 2010). Moreover, during meiotic maturation, the translation of certain transcripts increases in the oocyte (Chen et al. 2011; Paynton and Bachvarova 1994; Su et al. 2007). Amongst these, cell cycle transcripts such as *Ccnb1* get translated, and this activity may be promoted by signalling from somatic cells (Chen et al. 2011).

Oocyte meiosis is unique in being lengthy, discontinuous, acentrosomic (Hertig and Adams 1967; Szollosi, Calarco, and Donahue 1972), and asymmetrical. Following a prolonged arrest,

meiotic maturation is indispensable for the formation of a haploid fertilizable egg. The timing of this maturation is also critical as oocytes that fail to resume meiosis in response to LH stimulation, often remain arrested and are unable to get fertilized either by *in vitro* fertilization (IVF) or by intra-cytoplasmic sperm injection (ICSI) (Calafell et al. 1991; Hartshorne, Montgomery, and Klentzeris 1999; Levran et al. 2002; Rudak et al. 1990; Strassburger et al. 2004). The intricate regulation of the processes governing meiotic resumption starting with signalling in the granulosa cells following the LH surge is thus critical for allowing the formation of a mature fertilizable egg.

Extra- and intra-follicular factors during folliculogenesis

From the onset of folliculogenesis, where pre-granulosa cells surround primary oocytes to form primordial follicles, and up until the ovulation of a mature fertilizable egg from the antral follicle, intra- and extra-follicular factors drive follicle and oocyte growth by regulating the essential inter-cellular cooperation within the ovarian follicle. Hormones, growth factors, nucleotides, and signalling molecules emanating from within or outside the growing follicle, to enable the development of the follicle and that of the enclosed oocyte to occur synchronously. Two of these many factors, FSH (extra-follicular) and GDF9 (intra-follicular) are discussed below, and the novel roles that they play during folliculogenesis constitute the original findings of my thesis project.

FSH, an extra-follicular factor that drives late folliculogenesis

FSH is a glycoprotein hormone secreted by the pituitary. It consists of two non-covalently linked subunits, the alpha subunit (chorionic gonadotropin alpha), which it shares with LH and thyroid-stimulating hormone (TSH), and the specific beta subunit (Papkoff and Ekblad 1970). The subunits are assembled then secreted by the gonadotrope cells of the anterior pituitary (Pierce and Parsons 1981). FSH is a component of the hypothalamic–pituitary–gonadal (HPG) axis, and is regulated by other factors along this axis including gonadotropin-releasing hormone (GnRH) (Cattanach et al. 1977; Mason et al. 1986; Pierce and Parsons 1981; Kumar et al. 2003; Lee et al. 2007; Pernasetti et al. 2001), activins, inhibins, follistatin (Baratta et al. 2001; Bilezikjian et al. 2004; Campen and Vale 1988; Corrigan et al. 1991; Kumar et al. 2003; Lee et al. 2007;

Pernasetti et al. 2001), BMPs (Faure et al. 2005; Ho and Bernard 2009; Lee et al. 2007; Otsuka and Shimasaki 2002; Young et al. 2008), androgens (Haisenleder et al. 2005; Paul et al. 1990), as well as estradiol (Gieske et al. 2008; Lindzey et al. 2006; Miller et al. 1983; Phillips et al. 1988).

FSH signals through its receptor, FSHR, a G-protein coupled receptor (GPCR). In addition to the activation of the classical Gαs/cAMP/PKA pathway (Dattatreyamurty, Figgs, and Reichert 1987; Maizels et al. 1998; Means and Huckins 1974; Yu et al. 2005), the binding of FSH to this GPCR leads to the activation of several signalling pathways, including Akt, Inositol trisphosphate (IP3), PI3K/mTORC and EGFR (Alam et al. 2004; Alam et al. 2009; Cottom et al. 2003; Dupont et al. 2010; Escamilla-Hernandez et al. 2008; Fan et al. 2010; Fan et al. 2008; Gonzalez-Robayna et al. 2000; Musnier et al. 2009; Nechamen et al. 2004; Park et al. 2005; Quintana et al. 1994; Zeleznik, Saxena, and Little-Ihrig 2003).

In the ovary, FSH targets the somatic granulosa cells, which express FSHR (Haisenleder et al. 2005; O'Shaughnessy, Dudley, and Rajapaksha 1996; Oktay, Briggs, and Gosden 1997; Simoni, Gromoll, and Nieschlag 1997), where it has been shown to regulate the expression of many target genes promoting survival, proliferation, and steroidogenesis (Grieshaber et al. 2003; Sasson et al. 2003). Target genes include transcription factors, GPCRs, signalling molecules, steroidogenic enzymes, cell cycle regulators, cytoskeletal elements, and growth factors (Alam et al. 2004; Alliston et al. 1997; Falender et al. 2003; Park et al. 2003; Piontkewitz, Sundfeldt, and Hedin 1997; Ratoosh et al. 1987; Russell et al. 2003; Salvador et al. 2004; Zeleznik, Midgley, and Reichert 1974; Sekiguchi et al. 2004; Sicinski et al. 1996; Turner et al. 1989; Woodruff et al. 1987; Yazawa et al. 2003).These FSH effects are mediated by transcription factors such as cAMP response element-binding protein (CREB), AP1, FOXO1, FOXO3, and nuclear factor kappa B (NFkB) (Cameron et al. 1996; Cottom et al. 2003; Fan et al. 2010; Fan et al. 2008; Gonzalez-Robayna et al. 2000; Nechamen et al. 2004; Park et al. 2005; Wang, Chan, and Tsang 2002), as well as by histone H3 phosphorylation and acetylation (Salvador et al. 2001).

Consistent with its role in promoting proliferation and differentiation of granulosa cells, studies of hypophysectomized mice, as well as mice lacking expression of FSH or its receptor, demonstrated that FSH is essential for the late stages of folliculogenesis (Yang et al. 2003; Burns

et al. 2001; Kumar et al. 1997). Follicular growth beyond the early-antral stage strictly requires FSH. Earlier stages though, are not dependent on it, although as the granulosa cells of the primary follicle acquire FSHR, the follicle becomes responsive to FSH (O'Shaughnessy, Dudley, and Rajapaksha 1996; Oktay, Briggs, and Gosden 1997). Thus *in vivo*, FSH drives late folliculogenesis. Consistently, women with mutations in *FSHB* or *FSHR* have impaired folliculogenesis and suffer from infertility (Aittomaki et al. 1995; Layman et al. 1997; Matthews et al. 1993; Tao and Segaloff 2009).

The role of FSH in follicle growth *in vitro* however, is controversial. Some studies show that follicular growth can be maintained in the absence of FSH. In fact, oocyte size and its meiotic competence, as well as overall follicle morphology *in vitro*, were not affected by FSH (Eppig et al. 1998; Kreeger et al. 2005). Conversely, others have demonstrated a necessity for FSH in order to sustain follicular growth and granulosa cell proliferation (Romero and Smitz 2010). As FSH is routinely administered to women undergoing assisted reproduction, and is extensively used in animal breeding (Lunenfeld 2004; Macklon et al. 2006), its role in follicle growth and oocyte development both *in vivo* and *in vitro* have to be reassessed.

GDF9, a paracrine intra-follicular factor

The concept of oocyte-derived factors regulating certain processes was brought forward in 1970 (el-Fouly et al. 1970), based on the observation that oocytes inhibit luteinization of granulosa cells (el-Fouly et al. 1970; Nekola and Nalbandov 1971). Almost two decades later, GDF9 was identified as an oocyte-specific factor (McGrath, Esquela, and Lee 1995), and later shown to be essential for fertility (Dong et al. 1996). GDF9 is a member of the TGFß superfamily, expressed in oocytes of several mammalian species, including humans and mice, as of the primary stage (Aaltonen et al. 1999; Bodensteiner et al. 1999; Duffy 2003; McGrath, Esquela, and Lee 1995; Prochazka et al. 2004). GDF9 exhibits effects on the three follicular compartments, oocyte, granulosa, and theca (Carabatsos et al. 1998; Dong et al. 1996; Elvin, Yan, et al. 1999).GDF9 signalling in the oocyte itself, however, is not required (Li et al. 2012). GDF9 is synthesized as a pre-protein which undergoes modifications to become the mature form of the protein (Shimasaki et al. 2004). As with other members of the transforming growth factor beta superfamily, it

signals through type-I and type-II receptors, namely, activin receptor-like kinase-5 (ALK5) type-I receptor and BMP type-II receptor (BMPRII). Upon GDF9 binding, the receptor complex activates the SMAD signalling pathway, specifically, SMAD 2 and 3, and the co-receptor, SMAD 4. The SMAD complex then translocates into the nucleus where it alters gene expression (Mazerbourg et al. 2004; Vitt et al. 2002).

The role of GDF9 in female fertility was elucidated by identifying mutations in women as well as by knockout mice. Women with mutations in *Gdf9* suffer from premature ovarian failure (Dixit et al. 2006; Kovanci et al. 2007; Laissue et al. 2006; Zhao et al. 2007); while others showed polyovulatory phenotype (Montgomery et al. 2004; Palmer et al. 2006). On the other hand, *Gdf9*$^{-/-}$ female mice are infertile, owing to a block in folliculogenesis at the primary stage (Dong et al. 1996). In addition, their oocytes show accelerated growth in size and acquire meiotic competence (Carabatsos et al. 1998) but lack developmental competence (Elvin, Clark, et al. 1999). These oocytes also had ultrastructural abnormalities and a reduced number of trans-zonal projections (Carabatsos et al. 1998). The phenotype of *Gdf9*$^{-/-}$ females has been proposed to be due to the upregulation of inhibin alpha expression in these mutants (Wu et al. 2004).

GDF9 exhibits several paracrine effects on granulosa cells. It has been shown to stimulate their proliferation (Hayashi et al. 1999; Hreinsson et al. 2002; Huang et al. 2009; Vitt, Hayashi, et al. 2000), promote their survival (Hussein et al. 2005; Orisaka et al. 2006), enable their differentiation into cumulus and mural cells (Diaz, Wigglesworth, and Eppig 2007b; Glister, Groome, and Knight 2003), as well as inhibit their luteinization (Diaz, Wigglesworth, and Eppig 2007b; Li et al. 2000). GDF9 also promotes metabolic coupling between the granulosa cells and the oocyte by regulating expression of metabolic enzymes and amino acid transport systems (Eppig et al. 2005; Su et al. 2008; Sugiura, Pendola, and Eppig 2005). At least some of these effects of GDF9 have been suggested to be mediated by its regulation of KitL levels (Elvin, Clark, et al. 1999; Joyce et al. 1999). Moreover, GDF9 can stimulate cumulus expansion *in vitro* (Diaz, Wigglesworth, and Eppig 2007a; Sugiura et al. 2010), and is required for expression of expansion-related transcripts (Su et al. 2004; Yan et al. 2001) and for MAPK activation downstream of LH-EGFR (Su et al. 2003). GDF9 also plays a role in maintaining meiotic arrest

by promoting expression of *Nppc* and *Npr2* (Lee et al. 2013; Zhang et al. 2011; Zhang et al. 2010).

In vivo, GDF9 is thought to function as a heterodimer with its homologue the oocyte-specific BMP15 (Peng et al. 2013). BMP15 also plays important roles in folliculogenesis, and has been shown to have synergistic effects with GDF9 (Dube et al. 1998; Emori et al. 2013; Galloway et al. 2000; Otsuka et al. 2000; Su et al. 2004; Yan et al. 2001).

New roles for FSH and GDF9

My thesis work focuses on the extra-follicular factor, FSH, and the intra-follicular factor GDF9. In Chapters 2 and 3, new roles for FSH are described. I found that FSH is necessary for the upregulation of EGFR, and the subsequent activation of EGFR signalling, which mediates the ovulatory events, cumulus expansion and meiotic maturation, downstream of LH. This constitutes a new mechanism by which FSH enables the ovulatory response. Moreover, my results show that by increasing the expression of cadherins and connexins in the oocyte and granulosa cells, and by enhancing the physical attachments between these two compartments, FSH promotes gap junctional communication within the follicle. By coupling the follicle-enclosed oocyte with the surrounding granulosa cells, FSH accelerates the growth and development of the oocyte. In Chapter 4, I identified GDF9 as an oocyte-secreted factor that promotes the extension of actin-rich trans-zonal projections (TZPs) by the granulosa cells. This role highlights a new mechanism by which GDF9 could promote the oocyte's developmental competence, as these projections enable communication with the granulosa cells, which is essential for the oocyte.

Chapter 2

Manuscript I
Follicle-stimulating hormone regulates expression and activity of epidermal growth factor receptor in the murine ovarian follicle

Stephany El-Hayek[1,2,4], Isabelle Demeestere[1,4,5] and Hugh J. Clarke[1,2,3,4]

Departments of Obstetrics and Gynecology[1], Biology[2], and Medicine[3], McGill University; Research Institute – McGill University Health Centre[4]; Montréal, Québec, Canada; Research Laboratory on Human Reproduction Fertility Clinic, Erasme-ULB 808 Route de Lennik, 1070 Brussels, Belgium[5]

Proc Natl Acad Sci U S A 2014; 111:16778-16783. doi: 10.1073/pnas.1414648111.

Correspondence: Hugh Clarke, Ph.D.
F3.50, Royal Victoria Hospital
687 av. des Pins O, Montréal, Québec, Canada H3A 1A1
hugh.clarke@mcgill.ca
Tel : 514-934-1934 x34748

Running title (50 char including spaces): FSH is required for EGFR signalling at ovulation

Keywords: ovary, follicle, FSH, EGFR, granulosa

Preface

FSH plays an essential role in the EGFR-mediated ovulatory process, where it induces the expression of LHCGR, allowing the follicle to respond to the LH stimulus (Erickson, Wang, and Hsueh 1979). *Fshb*$^{-/-}$ granulosa cells have reduced levels of LHCGR (Burns et al. 2001). Previous work from our lab showed that when oocytes from *Fshb*$^{-/-}$ mice were allowed to undergo meiotic maturation *in vitro*, thus bypassing the need for LH-dependent maturation and ovulation, fertilization was unexpectedly impaired. This result suggested that FSH may play an additional role in the LH-EGFR ovulatory pathway. To test this, the following objectives were designed:

Ia) Test the ability of *Fshb*$^{-/-}$ cumulus-oocyte complexes to undergo the ovulation-associated events of cumulus expansion and oocyte meiotic maturation, which are pre-requisites for fertilization.

Ib) Identify the underlying cause for the impairment of these ovulatory events in *Fshb*$^{-/-}$ females.

This work is discussed in Chapter 2 (Manuscript I). Chapter 2 contains slight modifications (Materials and Methods and Supplemental Data) from the published work.

Significance statement

Ovulation in mammals strictly requires activation of EGFR signalling within the ovarian follicle, but mechanisms responsible for implementing the EGFR network during follicular growth remain incompletely understood. The final phase of growth is driven by FSH. Here we show that during this phase EGFR expression increases sharply in follicular granulosa cells and that this increase requires FSH, and we provide evidence that the FSH-dependent increase is essential for EGFR signalling. FSH is also known to induce expression of LHCGR in the granulosa, permitting them to release EGFR-ligands in response to preovulatory LH. By coordinating receptor expression and ligand release, FSH endows full-grown follicles with the capacity to activate EGFR signalling at ovulation.

Abstract

Fertility depends on the precise coordination of multiple events within the ovarian follicle to ensure ovulation of a fertilizable egg. FSH promotes late follicular development, including expression of LH receptor by the granulosa cells. This permits the subsequent LH surge to trigger the release of ligands that activate EGFR on the granulosa, thereby initiating the ovulatory events. Here we identify a new role for FSH in this signalling cascade. We show that follicles of *Fshb*$^{-/-}$ mice, which cannot produce FSH, have a severely impaired ability to support two essential EGFR-regulated events: expansion of the cumulus granulosa cell layer that encloses the oocyte and meiotic maturation of the oocyte. These defects are not due to an inability of *Fshb*$^{-/-}$ oocytes to produce oocyte-secreted factors or of *Fshb*$^{-/-}$ cumulus cells to respond. In contrast, whereas expression of both *Egfr* and EGFR increase during late folliculogenesis in *Fshb*$^{+/-}$ females, these increases fail to occur in *Fshb*$^{-/-}$ females. Remarkably, supplying a single dose of exogenous FSH activity to *Fshb*$^{-/-}$ females is sufficient to increase *Egfr* and EGFR expression and to restore EGFR-dependent cumulus expansion and oocyte maturation. These studies reveal that FSH induces an increase in EGFR expression during late folliculogenesis and imply that the FSH-dependent increase is necessary for EGFR physiological function. Our results uncover a novel role for FSH in establishing the signalling axis that coordinates ovulatory events and may contribute to the diagnosis and treatment of some types of human infertility.

Introduction

Fertility in mammals depends on the coordinated execution of multiple events within the fully grown ovarian follicle at the time of ovulation (Richards 2005; Richards et al. 2002). The oocyte undergoes meiotic maturation, during which it progresses to metaphase II of meiosis and acquires the ability to begin embryonic development (Channing, Hillensjo, and Schaerf 1978). Concomitantly, the layer of granulosa cells that immediately surrounds the oocyte, termed the cumulus, undergoes a process termed expansion, required for sperm to penetrate this layer and reach the oocyte (Eppig 1979b; Thibault, Gerard, and Menezo 1975; Fulop, Salustri, and Hascall 1997; Salustri et al. 1999). At the perimeter of the follicle, an inflammatory response associated with rupture of the follicular wall permits the cumulus-oocyte complex (COC) to escape from the follicle and enter the oviduct where fertilization will occur. These events are triggered by the preovulatory release of LH, which acts on LHCGR on the mural granulosa cells that line the interior wall of the fully grown follicle (Erickson, Wang, and Hsueh 1979).

Recent studies have identified a key downstream effector of LH activity at ovulation. Binding of LH to LHCGR triggers the release of the EGF-related peptides, AREG, BTC, and EREG (Ashkenazi et al. 2005; Park et al. 2004; Sekiguchi et al. 2004). These bind to EGFR located on both the mural and cumulus granulosa cells (Gall et al. 2004; Garnett, Wang, and Roy 2002; Hill et al. 1999; Chabot et al. 1986; Singh, Rutledge, and Armstrong 1995; Goritz, Jewgenow, and Meyer 1996; Qu et al. 2000; el-Danasouri, Frances, and Westphal 1993) and activate MAPK3/1 as well as other signalling networks (Downs and Chen 2008; Fan et al. 2009; Hsieh et al. 2007; Hsieh, Thao, and Conti 2011; Keel et al. 1995; Panigone et al. 2008; Prochazka, Blaha, and Nemcova 2012; Shimada et al. 2006; Su et al. 2002). Considerable evidence supports the view that the EGFR signalling mediates many or most ovulatory events. First, the release of the EGFR ligands follows the LH surge, but precedes the LH-dependent responses (Ashkenazi et al. 2005; Park et al. 2004; Sekiguchi et al. 2004). Second, EGF and the EGFR ligands can induce cumulus expansion and oocyte maturation *in vitro,* independently of LH (Downs 1989; Downs and Chen 2008; Ashkenazi et al. 2005; Park et al. 2004). Third, these events are impaired in mice bearing a hypomorphic *Egfr* allele that reduces EGFR activity by about one-half, as well as in mice in

which *Egfr* has been selectively inactivated in granulosa cells through a targeted mutation (Hsieh, Thao, and Conti 2011; Hsieh et al. 2007). Thus, activation of EGFR signalling in granulosa cells of mature follicles appears to be a major effector of the ovulatory response to LH.

FSH binds to receptors located on granulosa cells, and induces the expression of numerous genes including *Lhcgr* (Erickson, Wang, and Hsueh 1979; Law et al. 2013). *Lhcgr* expression is substantially impaired in mice that lack either FSH, owing to targeted mutation of the *Fshb* gene that encodes its β-subunit, or the FSH receptor, as well as in humans bearing spontaneous mutations, and these individuals fail to ovulate (Burns et al. 2001; Dierich et al. 1998; Tao and Segaloff 2009; Themmen and Huhtaniemi 2000). Thus, the ovulatory response to LH strictly depends on the prior FSH-dependent expression of *Lhcgr*, and in this manner FSH indirectly controls the LHCGR-regulated release of the EGFR ligands. We report here that FSH also drives an increase in EGFR expression during late folliculogenesis and provide evidence that this increase is essential to enable the ovulatory response to EGF. By coordinating the expression of EGFR and release of its ligands, FSH endows full-grown follicles with the capacity to activate EGFR signalling at ovulation.

Materials and Methods

Mice

All experiments were approved by the Animal Care Committee of the Royal Victoria Hospital and followed the regulations of the Canadian Council on Animal Care. Mice carrying a deletion in the gene encoding the β-subunit of FSH, *Fshb*, (Kumar et al. 1997) were obtained from Jackson Laboratories. $Fshb^{+/-}$ and $Fshb^{-/-}$ mice were generated by mating $Fshb^{+/-}$ females with $Fshb^{-/-}$ males. Genotyping of the offspring was done using the EZ Tissue/Tail DNA Isolation Plus PCR kit (EZ Bioresearch, St. Louis, MO). After DNA extraction from tail snips, PCR was used to detect the wild-type *Fshb* gene and the replacement gene *Hprt* using the following primers: *Fshb* primers TTCAGCTTTCCCCAGAAGAG and CTGCTGACAAAGAGTCTATG; *Hprt* primers CTTGCGCTCATCTTAGGCTT and GGACCTCTCGAAGTGTTGGAT. The following PCR program was used: 94°C for 60 sec, 55°C for 60 sec, and 72°C for 90 sec.

Following amplification, PCR products were detected and visualized on 1.5% agarose gels stained with ethidium bromide.

For some experiments, *Fshb*$^{-/-}$ females at postnatal day (PD) 16 or PD 21-23 received an intraperitoneal injection of 5 IU of equine chorionic gonadotropin (eCG; Sigma, Windsor, ON) 48 hr prior to sample collection.

Collection of follicles, COCs, granulosa cells and oocytes

Ovaries were collected from *Fshb*$^{+/-}$ and *Fshb*$^{-/-}$ females at different ages (according to experiment) in Hepes-buffered minimal essential medium (Hepes-MEM; Life Technologies, Burlington, ON) supplemented with sodium pyruvate (0.28 mM; Sigma), penicillin G (63 μ g/ml; Sigma), streptomycin (50 μ g/ml; Sigma), and bovine serum albumin (BSA; 3 mg/ml; Sigma) at 37°C. To obtain intact follicles, ovaries were carefully dissected using fine needles. To obtain COCs, follicles protruding from the ovarian surface were punctured to allow the release of COCs. Purified granulosa cells and oocytes were obtained from follicles using mechanical dissection and enzymatic digestion using Collagenase (10 μ g/ml; Cedarlane, Burlington, ON) and DNase I (10 μ g/ml; Sigma). To examine cumulus expansion, COCs were incubated for 16 hr in serum-free bicarbonate-buffered MEM (MEM-$NaHCO_3$, Life Technologies) in the presence of 10 ng/ml EGF (Becton Dickinson; Mississauga, ON) at 37^{o}C in a humidified atmosphere of 5% CO_2 in air. For the *in vitro* culture experiment, follicles were isolated from PD 16 *Fshb*$^{-/-}$ females, then cultured for 48 hrs on collagen membrane inserts (Becton Dickinson) in 750 µl of MEM-$NaHCO_3$ supplemented with ITS (10 µg/mL insulin, 5.5 µg/mL transferrin and 5 ng/mL selenium; Sigma) ±10 mIU/ml of FSH (EMD Serono, Mississauga, ON). To assess response to EGF, follicles (freshly isolated or cultured) or COCs were incubated as above for 10 minutes, then processed. To assess oocyte maturation, follicles were incubated with EGF for 16 hr, after which the oocytes were removed from the follicles and examined using light microscopy and the fraction that had undergone germinal vesicle breakdown (GVBD) was recorded.

Oocytectomy

Oocytectomy (OOX) was performed using fine glass needles. OOX complexes were co-cultured with oocytes (5 oocytes per complex) in 25-µl drops of MEM-$NaHCO_3$ under paraffin oil under

the conditions described above. After 16 hr, the expansion of the COCs was qualitatively assessed.

RNA purification and quantitative PCR

Total RNA was purified from cells (oocytes, granulosa cells, COCs or intact follicles) using a Picopure RNA isolation kit (Life technologies), according to manufacturer's instructions. An additional DNase I (Qiagen, Toronto, ON) treatment was used to ensure purity. RNA was eluted in 10 µl of the provided elution buffer. To obtain cDNA SuperScript II Reverse Transcription kit (Life technologies) was used, beginning with the addition of 1 µl of deoxyribonucleotides (10 mM each) and 200 ng of random primers to the 10 µl of RNA. After a 2 min incubation at 25°C, followed by the addition of 1 µl of SuperScript II, mixture was then successively incubated for 10, 50, and 15 min, at 25°C, 42°C, and 70°C respectively. Quantitative PCR analysis was performed using a Corbett Rotorgene 6000 (Montréal Biotech, Montréal, QC, Canada). Each reaction contained 4 µl of EvaGreen Mix (Montréal Biotech), 13 µl of UltraPure DNase/RNase-free distilled water (Life technologies), 1 µl of 10 µM primers and 2 µl of cDNA (diluted by 1:20 from original stock). Primers were designed on Primer-BLAST (National Institutes of Health, Bethesda, MD) and ordered from Sigma. Primer sequences are given in Table 1. For each primer pair, a standard curve was generated using serial dilutions of cDNA prepared from ovarian RNA and used to determine the efficiency of amplification. Melt-curve analysis and electrophoresis of amplified products ensures that only a single product of the expected size was generated. Data were analyzed using software provided by the manufacturer. Relative quantities of amplified product were calculated according to the $2^{-\Delta\Delta CT}$ method, using *Actb* (actin) for normalization.

Gene/transcript	Primer Sequence
Actb	F: 5'-GGCTGTATTCCCCTCCATCG-3' R: 5'-CCAGTTGGTAACAATGCCATGT-3'
Areg	F: 5'- CCTTCTGGCAGTGAACTCTCCAC-3' R: 5'-GGTCCTTGTCATCCTCGCTGTGA-3'
Bax	F: 5'-CGAGCTGATCAGAACCATCA-3' R: 5'-GAAAAATGCCTTTCCCCTTC-3'

Bcl2	F: 5'-TAAGCTGTCACAGAGGGGCT-3' R: 5'-TGAAGAGTTCCTCCACCACC-3'
Bmp15	F: 5'-GAGCGAAAATGGTGAGGCTG-3' R: 5'-GGCGAAGAACACTCCGTCC-3'
Btc	F: 5'-CGGGTAGCAGTGTCAGCTC-3' R: 5'-CGATGTTTCCGAAGAGGATG-3'
Cyp19a1	F: 5'-ACACGTCTGGTCTCCTGCTAGAGT-3' R: 5'-GATCCACCGTAAGCAACTGGGTTT-3'
Egfr	F: 5'-GTGGAGGGACATCGTCCAAA-3' R: 5'-ATTGGGACAGCTTGGATCACAT-3'
Ereg	F: 5'-AACTCAGGAACAATTTACGTCTCTG-3' R: 5'-GCTTTGGTTCTCAGTATAGAGAGAGA-3'
Fas	F: 5'-GAGAATTGCTGAAGACATGACAATCC-3' R: 5'-GTAGTTTTCACTCCAGACATTGTCC-3'
Fasl	F: 5'-TTAGCTTCTCTGGAGCAGTCAGCGTC-3' R: 5'-CCTTCTTCTTTAGAGGGGTCAGTGGC-3'
Fshr	F: 5'-CAGGTCAACATACCGCTTGA-3' R: 5'-GATCCCCAGGCTGAGTCATA-3'
Gdf9	F: 5'-GCTCTATAAGACGTATGCTACC-3' R: 5'-CAGAGTGTATAGCAAGACCGAT-3'
Has2	F: 5'-AAGACCCTATGGTTGGAGGTGTT-3' R: 5'-CATTCCCAGAGGACCGCTTAT-3'
Il6	F: 5'-AGAGGATACCACTCCCAACAGA-3' R: 5'-ATCTCTCTGAAGGACTCTGGCT-3'
Il6r	F: 5'-CACTCCTTGGATAGCAGAGCC-3' R: 5'-GACACAGAGAAGCAACCCAAAC-3'
Lhcgr	F: 5'-CGGACCCTCCCAGATGTTTCG-3' R: 3'-GTGGCGATGAGCGTCTGAAGT-3'
Ptgs2	F: 5'-CCTTCCTCCCGTAGCAGATG-3' R: 5'-ATGAACTCTCTCCGTAGAAGAACCTT-3'

p53	F: 5'-GGAGTATTTGGACGACCG-3' R: 5'-TCAGTCTGAGTCAGGCCC-3'
Tnfaip6	F: 5'-GATGGTCGTCCTCCTTTGCTT-3' R: 5'- TATCTGCCAGCCCGAGCTT-3'

Table 1: Primer sequences used for quantitative PCR analysis

Immunoblotting

Cells (granulosa cells, COCs or intact follicles) were lysed in 20 µl of 2× Laemmli buffer (Bio Rad, Saint-Laurent, QC) to allow protein denaturation. Proteins were separated on a 10% polyacrylamide gel and transferred onto a polyvinylidene fluoride membrane (Amersham, Oakville, ON) under 100V. Blocking was done with 5% skimmed milk, in 0.1% Tween-phosphate-buffered saline (PBST). Following blocking, membranes were put in primary antibody solutions (diluted in 3% BSA in PBST) overnight at 4°C with gentle shaking. After 3 PBST washes, membranes were then incubated with 1:5000 dilution of corresponding secondary antibodies conjugated to horseradish peroxidase (Promega, Madison, WI) in fresh blocking buffer for 1 hr at room temperature. Following 3 washes with PBST, bound antibody signals were visualized using bound antibody was revealed by ECL plus (Amersham) using a Storm 860 phosphorimager (GE Healthcare; Baie d'Urfé, QC). For EGFR and the pSMADs, each sample was loaded into one lane of a gel and used to analyze both the protein of interest and tubulin. For MAPK3/1 analysis, each sample was suspended in loading buffer, heat-denatured, and then divided into two aliquots, one of which was used to detect total MAPK3/1 and the other to detect pMAPK3/1. Antibodies: EGFR (Santa Cruz Biotechnology, sc-03,1:200); tubulin (Sigma T8203, 1:2000); pSMAD2 (Ser465/467, Cell Signaling Technology, Whitby, ON, 3108, 1:1000); pSMAD1/5 (Ser463/465, Cell Signaling 9516, 1:1000); MAPK3/1 (Santa Cruz Biotechnology sc-94, 1:1000); pMAPK3/1 (Cell Signaling 9106, 1:1000). Signals were quantified using Image J software (National Institutes of Health) and normalized to the respective control (EGFR and pSMADs to tubulin; pMAPK3/1 to total MAPK3/1).

Histological sections

After overnight fixation in 4% paraformaldehyde at 4°C with gentle shaking, ovaries were dehydrated, paraffin-embedded, cut at 5 μm, mounted on slides, deparaffinized with xylene, and rehydrated with a decreasing ethanol gradient, PBS, and water. Sections were then stained with hematoxylin and eosin (Sigma). Sections were scanned visually to identify large follicles and photographed using a Leica DM6000 microscope (Leica Microsystems, Richmond Hill, ON).

Granulosa cell monolayer

Granulosa cells were isolated from ovaries of *Fshb*$^{+/-}$ and *Fshb*$^{-/-}$ females, as described above. Cells were then seeded onto glass chamber culture slides (Becton Dickinson) and cultured in MEM-$NaHCO_3$ supplemented with 5% FBS (fetal bovine serum; Life Technologies) at 37^{o}C in an atmosphere of 5% CO_2 in air, for a 2-3 days to reach confluency. Bright-field images of the monolayers were taken with a LSM 510 confocal microscope (Zeiss, Toronto, ON).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.0 (San Diego, CA). Single-sample *t*-test, two-sample *t*-test, or one-way ANOVA followed by Tukey HSD test were used, depending on the experiment. $P<0.05$ was considered significant. All values represent the mean ± standard error of the mean of three or more independent experiments.

Results

COCs of *Fshb*$^{-/-}$ females do not expand or upregulate expansion-related transcripts in response to EGF

To examine whether FSH was required for activation of follicular EGFR signalling, we analyzed *Fshb*$^{-/-}$ mice, which cannot produce FSH (Kumar et al. 1997). Follicular development in *Fshb*$^{-/-}$ females is overtly normal until the early antral stage, but then becomes arrested. These females fail to ovulate and hence are infertile (Kumar et al. 1997; Demeestere et al. 2012). Nonetheless, there is little or no effect on the expression of GC genes not regulated by FSH (Burns et al. 2001) and the follicles retain the ability to support the development of fully grown meiotically competent oocytes and to maintain them in prophase arrest (Demeestere et al. 2012). Although

the oocytes can mature *in vitro*, they develop poorly after fertilization, however, indicating that some aspect of their development is abnormal (Demeestere et al. 2012; Burns et al. 2001). *Fshb*$^{+/-}$ females are fertile (Burns et al. 2001; Demeestere et al. 2012) and served as controls in our experiments. Because the absence of FSH eventually leads to follicular atresia (Chun et al. 1996), we used prepuberal *Fshb*$^{-/-}$ females. This enabled us to study the large cohort of follicles that initiates growth shortly after birth in the mouse, reaching full-size after about three weeks. By using prepuberal *Fshb*$^{+/-}$ and *Fshb*$^{-/-}$ animals of the same age, we were able to compare follicles that had been growing for the same period of time, in the presence or absence of FSH, before follicular atresia occurs in *Fshb*$^{-/-}$ females.

We first assessed cumulus expansion, which occurs downstream of EGFR signalling (Ashkenazi et al. 2005; Park et al. 2004). When we obtained COCs of *Fshb*$^{+/-}$ females at post-natal days (PD) 21-23 and incubated them in serum-free medium in the presence of EGF for 16 hr, they expanded as expected (Figure 6A). Although growing follicles of *Fshb*$^{-/-}$ females do not form large antra (Demeestere et al. 2012; Kumar et al. 1997), we were able to puncture the largest follicles and recover oocytes enclosed by granulosa cells, which we provisionally term COCs. In contrast to the COCs of *Fshb*$^{+/-}$ females, *Fshb*$^{-/-}$ COCs of the same age did not expand in response to EGF (Figure 6B). Hence, this EGFR-dependent event failed to occur in COCs from follicles that had grown in the absence of FSH. We note that COCs from hypogonadal mice, which also lack FSH, can undergo expansion *in vitro* using medium that contains serum (Diaz, Wigglesworth, and Eppig 2007a).

Cumulus expansion requires EGFR-dependent up-regulation of a subset of genes, including *Has2, Ptgs2*, and *Tnfaip6* (Ochsner, Day, et al. 2003; Fulop et al. 2003; Ochsner, Russell, et al. 2003; Fulop et al. 1997; Fulop, Salustri, and Hascall 1997; Sugiura, Su, and Eppig 2009). We therefore isolated COCs from PD 21-23 *Fshb*$^{+/-}$ and *Fshb*$^{-/-}$ females and examined basal and EGF-stimulated expression of these genes. Prior to EGF treatment, *Has2, Ptgs2,* and *Tnfaip6* mRNA levels were lower in *Fshb*$^{-/-}$ COCs than in heterozygotes (Figure 13). Upon EGF stimulation, the quantity of all three transcripts increased significantly more in COCs of *Fshb*$^{+/-}$ females than in those of *Fshb*$^{-/-}$ females (Figure 6C). Several lines of evidence indicate that the impaired *Fshb*$^{-/-}$ response is unlikely to reflect incipient atresia of the follicles (Chun et al. 1996).

First, histological sections of *Fshb*$^{-/-}$ ovaries at PD 24 showed no evidence of increased pycnotic nuclei in *Fshb*$^{-/-}$ as compared to *Fshb*$^{+/-}$ ovaries (Figure 14A). Second, granulosa cells isolated from *Fshb*$^{+/-}$ and *Fshb*$^{-/-}$ follicles at PD 21-23 formed indistinguishable monolayers in tissue-culture (Figure 14B). Third, there was no difference between the two genotypes in the follicular expression of genes associated with atresia or apoptosis (Lefevre 2011; Matsuda et al. 2012) (Figure 14C). We conclude that COCs of *Fshb*$^{-/-}$ females express lower constitutive levels of the three EGF-regulated transcripts and show an impaired functional and transcriptional response to EGF stimulation.

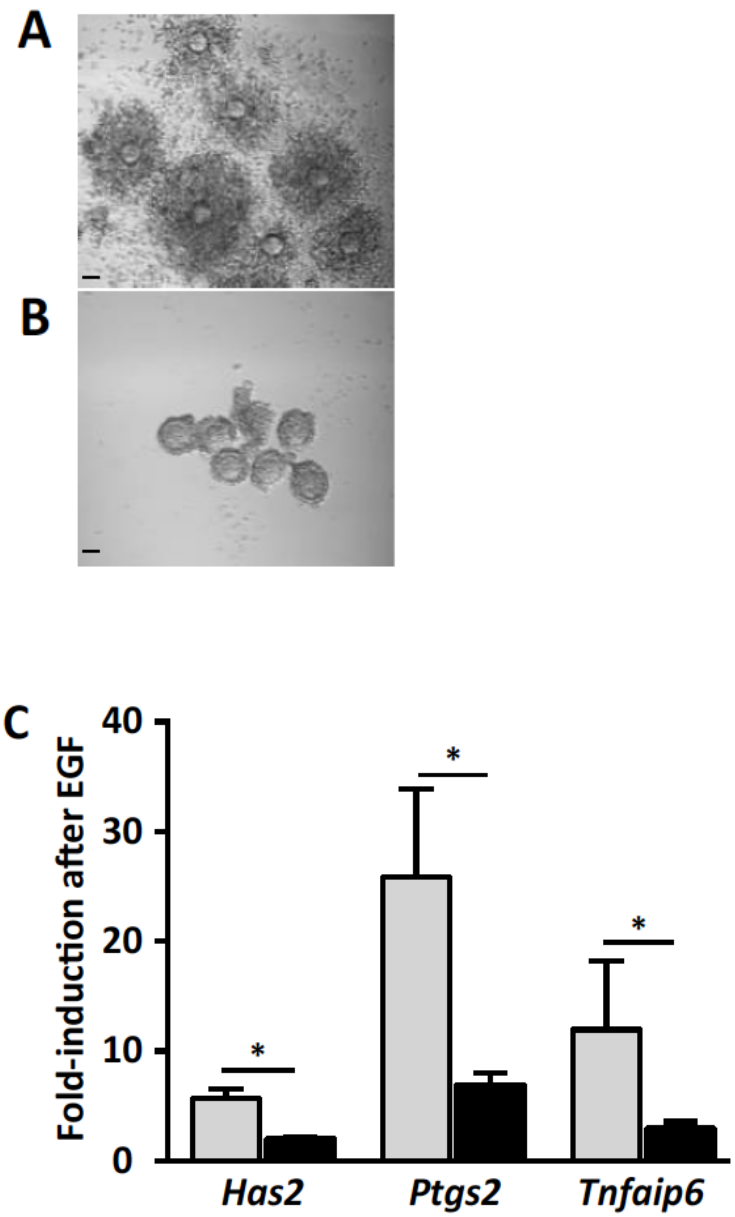


Figure 6: COCs of *Fshb*$^{-/-}$ females do not undergo cumulus expansion or up-regulate expansion-related mRNAs in response to EGF. (A, B) COCs isolated from follicles of *Fshb*$^{+/-}$ (A) or *Fshb*$^{-/-}$ (B) females, respectively, at PD 21-23 and incubated for 16 hr in the presence of EGF. Micrographs are representative of three independent experiments. Scale bar = 50 μm. (C) Fold-increase in *Has2*, *Ptgs2*, and *Tnfaip6* mRNAs in COCs of *Fshb*$^{+/-}$ (gray bars) and *Fshb*$^{-/-}$ (black bars) females following EGF stimulation relative to non-stimulated follicles of the same genotype. Data was analyzed using a two-sample *t*-test. Asterisks indicate $p<0.05$.

ODPFs required for EGF responses are functional in COCs of *Fshb*$^{-/-}$ females

ODPFs, including GDF9 and BMP15 are essential for EGF-induced *Has2*, *Ptgs2*, and *Tnfaip6* up-regulation and cumulus expansion (Buccione et al. 1990; Diaz et al. 2006; Dragovic et al. 2007; Nagyova et al. 2011; Sasseville et al. 2010; Su et al. 2010). Their effects in granulosa cells are transduced by activation of the SMAD pathway. We therefore tested whether the failure of *Fshb*$^{-/-}$ COCs to expand in response to EGF could be attributed to insufficient production of ODPFs or their inability to activate SMAD signalling.

We first quantified *Gdf9* and *Bmp15* mRNAs, and found no difference in transcript levels between *Fshb*$^{+/-}$ and *Fshb*$^{-/-}$ females in either growing (PD 12) or fully grown (PD 21-23) oocytes (Figure 7A, B). To directly test whether the oocytes produced ODPFs, we then performed a 'cross-fostering' experiment. We removed the oocyte from COCs of *Fshb*$^{+/-}$ and *Fshb*$^{-/-}$ females at PD 21-23 and incubated the OOX complexes with oocytes derived from the other genotype. As expected, neither *Fshb*$^{+/-}$ nor and *Fshb*$^{-/-}$ OOXs expanded in response to EGF when cultured in the absence of oocytes (Figure 7C, D, left panels). Heterozygous OOX underwent expansion in response to EGF when incubated with oocytes of *Fshb*$^{+/-}$ females (Figure 7C, middle), or with oocytes of *Fshb*$^{-/-}$ females (Figure 7C, right). Thus, the oocytes of *Fshb*$^{-/-}$ females produce sufficient ODPFs to permit cumulus expansion in response to EGF. In contrast, *Fshb*$^{-/-}$ OOX failed to expand in response to EGF when incubated with oocytes of either *Fshb*$^{+/-}$ (Figure 7D, middle) or *Fshb*$^{-/-}$ females (Figure 7D, right). The levels of phosphorylated (p) SMAD2/3 (GDF9 effector) and pSMAD1/5 (BMP15 effector) in COCs were similar, however, in PD 21-23 *Fshb*$^{+/-}$ and *Fshb*$^{-/-}$ females (Figure 7E). This suggests that the *Fshb*$^{-/-}$ complexes activated SMAD signalling in response to ODPFs. Together, these results indicate that the inability of the *Fshb*$^{-/-}$ complexes to expand in response to EGF is not due to a failure of the oocytes to produce ODPFs or of granulosa cells to respond.

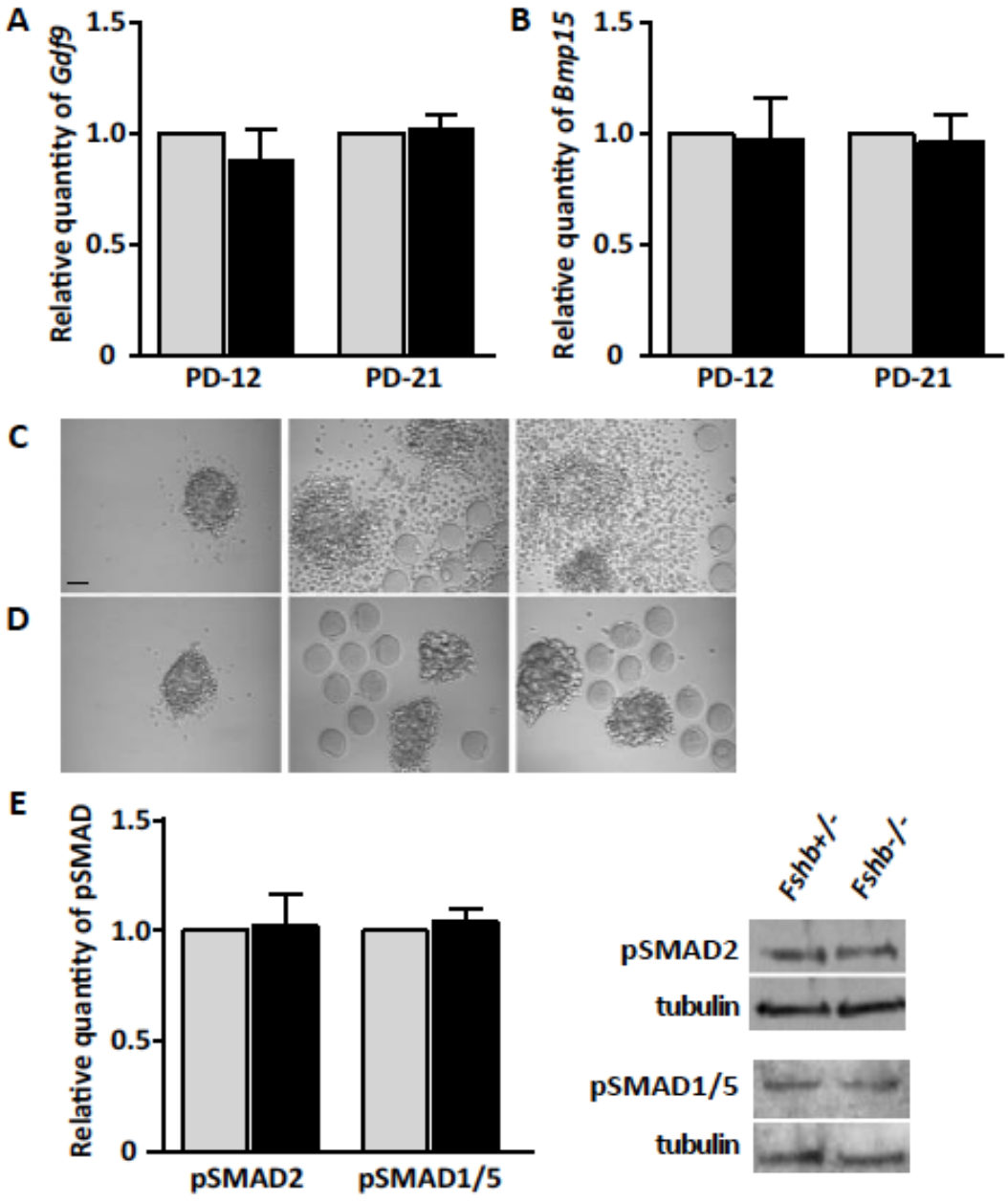


Figure 7: *Fshb*$^{-/-}$ follicles produce ODPFs required for response to EGF. (A, B) Relative quantity of *Gdf9* (A) and *Bmp15* (B) in growing (PD 12) and fully-grown (PD 21-23) oocytes of *Fshb*$^{+/-}$ (gray bars, value set to 1 for each mRNA) and *Fshb*$^{-/-}$ (black bars) females. (C, D) *Fshb*$^{+/-}$ (C) or *Fshb*$^{-/-}$ (D) OOX following incubation for 16 h in the presence of EGF with no oocytes (left), or oocytes of *Fshb*$^{+/-}$ (middle) or *Fshb*$^{-/-}$ (right) females. Micrographs are representative of three independent experiments. Scale bar = 50 µm. (E) Basal levels of phosphorylated (p)SMAD2 and pSMAD1/5 in freshly collected COCs of *Fshb*$^{+/-}$ (gray bars) and *Fshb*$^{-/-}$ (black bars) females at PD 21-23. Representative immunoblots are shown. Data in (A, B, E) was analyzed using a single-sample *t*-test.

***Egfr* and EGFR expression and activity are reduced in the granulosa cells of *Fshb*$^{-/-}$ females**

In mice that bear the hypomorphic *Egfr*$^{wa/wa}$ allele, EGFR activity in granulosa cells is reduced by about one-half, and oocyte maturation and ovulation are severely impaired (Hsieh et al. 2007). This demonstrates that a relatively modest reduction in EGFR activity can have a significant physiological effect and prompted us to examine whether the absence of cumulus expansion in response to EGF in the *Fshb*$^{-/-}$ females was associated with a reduced expression of *Egfr*. At PD 12, when the population of growing follicles is at the secondary pre-antral stage, we observed no difference in the quantity of *Egfr* mRNA in granulosa cells of *Fshb*$^{+/-}$ and *Fshb*$^{-/-}$ females (Figure 8A). By PD 21-23, however, *Egfr* had significantly increased in granulosa cells of early antral follicles of *Fshb*$^{+/-}$ females, whereas no increase was apparent in granulosa cells of follicles of *Fshb*$^{-/-}$ females of the same age (Figure 8A). Moreover, the increase in *Egfr* in granulosa cells of growing follicles of *Fshb*$^{+/-}$ females between PD 12 and PD 21-23 was accompanied by a quantitatively similar increase in EGFR protein (Figure 8B). However, consistent with the reduced quantity of *Egfr*, granulosa cells of follicles of *Fshb*$^{-/-}$ females at PD 21/23 contained only about one-half as much EGFR than those of the *Fshb*$^{+/-}$ females (Figure 8C). Thus, *Egfr* mRNA and protein both accumulate during late follicular growth, but fail to accumulate normally in follicles that grow in the absence of FSH. In contrast, the amounts of its ligand-encoding mRNAs, *Areg*, *Btc*, and *Ereg*, did not significantly differ between granulosa cells of PD 21-23 *Fshb*$^{+/-}$ and *Fshb*$^{-/-}$ females (Figure 15).

Because granulosa cells of *Fshb*$^{-/-}$ follicles contained detectable EGFR, albeit reduced compared to the heterozygotes, we tested whether EGFR signalling activity was impaired. MAPK3/1 are the principal effectors of EGFR signalling and their activation by phosphorylation is necessary for cumulus expansion (Fan et al. 2009; Ochsner, Day, et al. 2003; Sasseville et al. 2010; Su et al. 2002). Upon addition of EGF, phosphorylation of MAPK3/1 increased significantly in *Fshb*$^{+/-}$ follicles, but did not detectably change in *Fshb*$^{-/-}$ follicles (Figure 8D). Thus, the reduced expression of EGFR was associated with impaired EGFR signalling in *Fshb*$^{-/-}$ follicles.

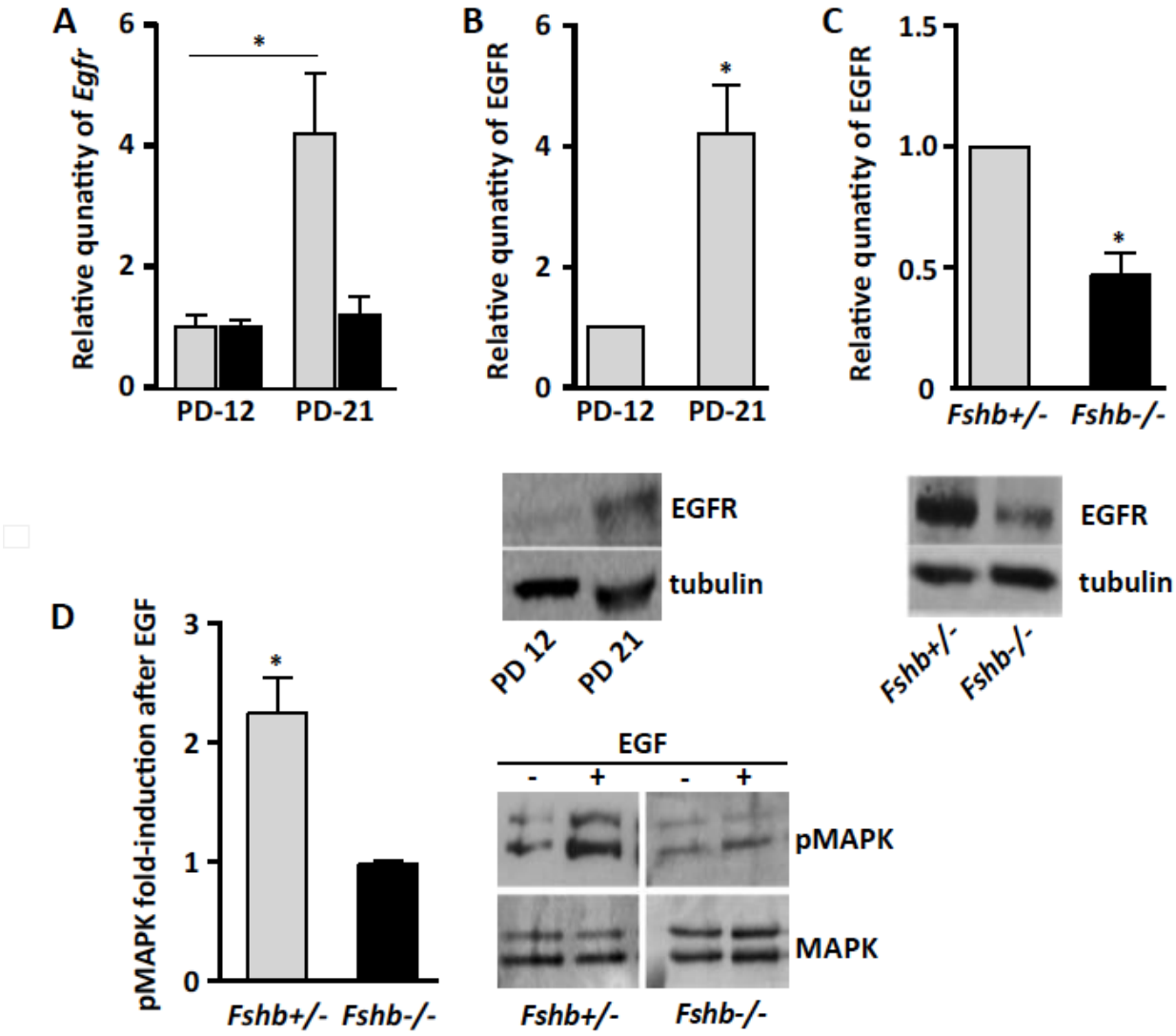


Figure 8: FSH increases expression and activity of EGFR in granulosa cells. (A) Relative quantity of *Egfr* in granulosa cells of *Fshb*$^{+/-}$ (gray bars) and *Fshb*$^{-/-}$ (black bars) females at PD 12 and PD 21-23. (B) Relative quantity of EGFR in granulosa cells of *Fshb*$^{+/-}$ females at PD 12 and PD 21-23. (C) Relative quantity of EGFR in granulosa cells of *Fshb*$^{+/-}$ (gray bars) and *Fshb*$^{-/-}$ (black bars) females at PD 21-23. (D) Fold-increase in phosphorylated MAPK3/1 in follicles of *Fshb*$^{+/-}$ (gray bars) and *Fshb*$^{-/-}$ (black bars) females at PD 21-23 following stimulation with EGF compared to non-stimulated controls of the same genotype. Data was analysed using single-sample (B, C, D) or two-sample *t*-test (A). Asterisks indicate $p<0.05$. Asterisk in D denotes a significant difference between EGF-stimulated and non-stimulated follicles of the same genotype. Representative immunoblots are shown (note lanes shown in C are from the same blot but were not adjacent as shown here).

Exogenous FSH activity rescues EGFR expression, signalling and activity in granulosa cells of *Fshb*$^{-/-}$ females

To further clarify the link between FSH and EGFR expression in granulosa cells, we then tested whether providing a brief pulse of FSH activity could restore EGFR expression and signalling in follicles that had grown in the absence of FSH. We injected equine chorionic gonadotropin (eCG), which exhibits FSH activity and is commonly used *in vivo* due to its long half-life (Murphy and Martinuk 1991), into *Fshb*$^{-/-}$ females at PD 16, After 48 hr, we measured *Egfr* and EGFR expression in follicles of these females as well as in those of non-injected PD 18 *Fshb*$^{-/-}$ and *Fshb*$^{+/-}$ females. Both *Egfr* (Figure 9A) and EGFR (Figure 9B) were significantly higher in eCG-injected *Fshb*$^{-/-}$ females than in non-injected *Fshb*$^{-/-}$ females, and were close to the amounts in *Fshb*$^{+/-}$ females. Two known FSH targets, *Cyp19a1* and *Lhcgr*, were reduced in *Fshb*$^{-/-}$ females as previously reported (Burns et al. 2001), and up-regulated after eCG-priming (Figure 16). In contrast with previous results (Burns et al. 2001), we found no change *in Fshr* in granulosa cells of *Fshb*$^{-/-}$ females, although it increased following eCG-priming.

Coupled to the eCG-induced increase in *Egfr* expression was an increase in the phosphorylation of MAPK3/1 in response to EGF (Figure 9C). Moreover, EGF stimulation of follicles of eCG-primed *Fshb*$^{-/-}$ females caused an increase in the quantity of the three expansion-related mRNAs to levels similar to those observed in heterozygous females (Figure 10A) and induced cumulus expansion in primed *Fshb*$^{-/-}$ females (Figure 10B). Further confirming the link between FSH and *Egfr* expression, priming PD 16 *Fshb*$^{+/-}$ females with eCG similarly caused a significant increase in *Egfr* mRNA compared to non-primed *Fshb*$^{+/-}$ females (Figure 17A). The upregulation of expansion-related mRNAs in response to EGF was not augmented in the eCG-primed *Fshb*$^{+/-}$ females (Figure 17B), which may indicate that endogenous EGFR is sufficient to maximally activate its signalling pathway. Hence, providing exogenous FSH activity to *Fshb*$^{-/-}$ females increased follicular EGFR expression and signalling activity and restored the physiological responses to EGF.

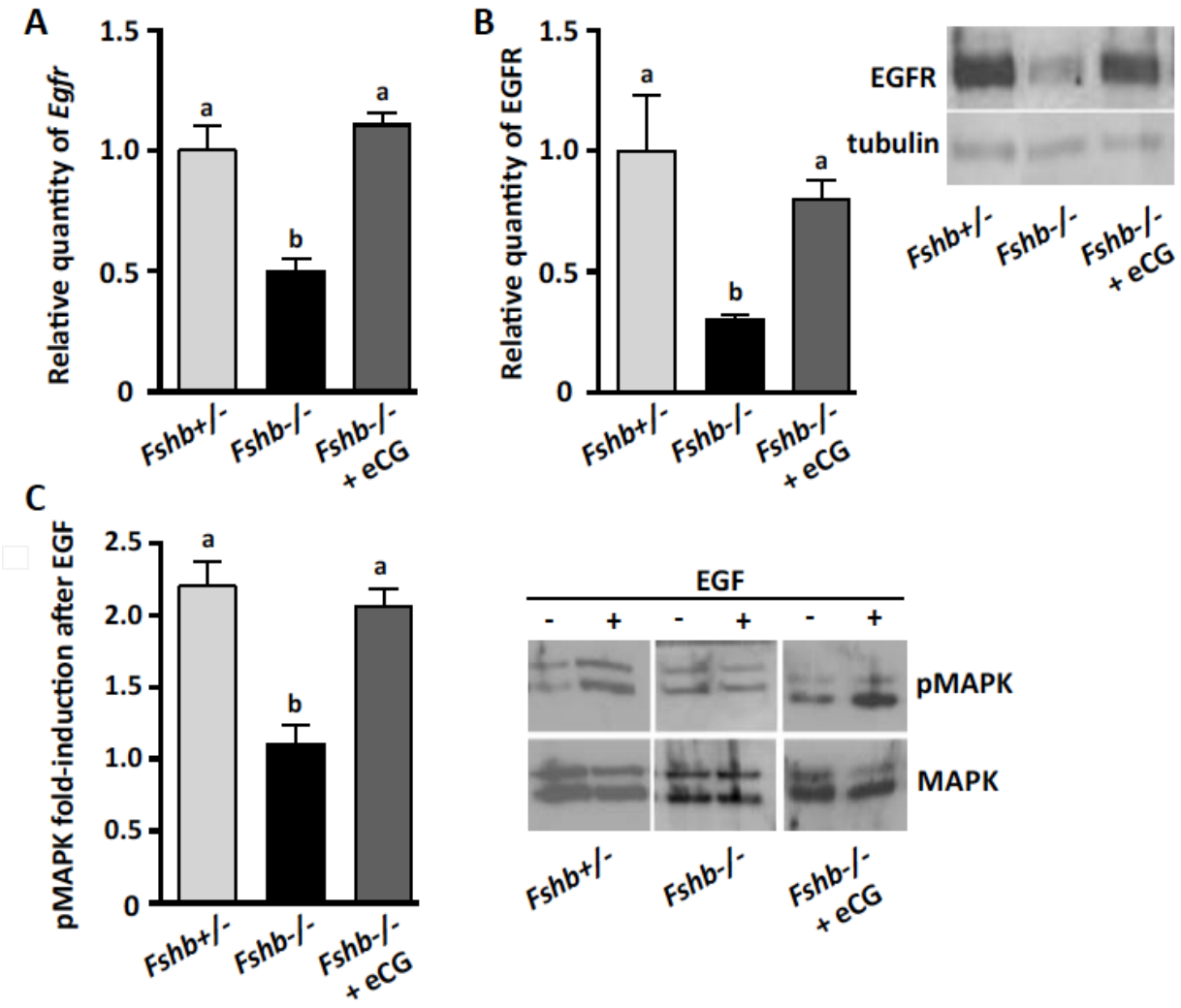


Figure 9: Supplying FSH activity to follicles of *Fshb*$^{-/-}$ females *in vivo* restores EGFR expression and activity. All histograms show follicles of PD 18 *Fshb*$^{+/-}$ females (gray bars), PD 18 *Fshb*$^{-/-}$ females (black bars), and PD 18 *Fshb*$^{-/-}$ females injected 48 h previously with eCG (dark gray bars). (A) Relative quantity of *Egfr*. (B) Relative quantity of EGFR. (C) pMAPK3/1 following EGF stimulation relative to non-stimulated controls. Data was analysed using one-way ANOVA and Tukey HSD. Different letters above bars indicate $p<0.05$. Representative immunoblots are shown.

EGF-induced oocyte meiotic maturation is impaired in *Fshb*$^{-/-}$ follicles

To test whether follicular growth in the presence of FSH was more broadly required for EGFR-dependent signalling at ovulation, we examined meiotic maturation of the oocyte. EGFR ligands do not appear to initiate maturation by acting directly on the oocyte, but instead reduce the transmission of inhibitory molecules to the oocyte from the EGFR-expressing granulosa cells (Ashkenazi et al. 2005; Hsieh, Thao, and Conti 2011; Jamnongjit, Gill, and Hammes 2005; Park et al. 2004). We isolated follicles from *Fshb*$^{+/-}$ and *Fshb*$^{-/-}$ females at PD 21-23, when developing oocytes of both genotypes have acquired meiotic competence (Demeestere et al. 2012). After incubating the follicles overnight in the presence or absence of EGF, we removed the oocytes and recorded the fraction that had undergone GVBD, indicative of maturation initiation (Schuetz 1974). In the absence of EGF, only a small fraction of the oocytes in both *Fshb*$^{-/-}$ and *Fshb*$^{+/-}$ follicles underwent GVBD (Figure 11). This demonstrates that the follicles of PD 21-23 *Fshb*$^{-/-}$ females retained the ability to hold oocytes in meiotic arrest. Addition of EGF triggered maturation of most oocytes within *Fshb*$^{+/-}$ follicles, but this response was significantly attenuated in *Fshb*$^{-/-}$ follicles (Figure 11). When *Fshb*$^{-/-}$ females were injected with eCG 48 hr prior to follicle isolation, however, the fraction of oocytes that underwent GVBD in response to EGF was restored to that of the *Fshb*$^{+/-}$ females. Hence, FSH signalling promotes EGFR-dependent ovulation-associated events in the germ-line as well as in the somatic compartment of the follicle.

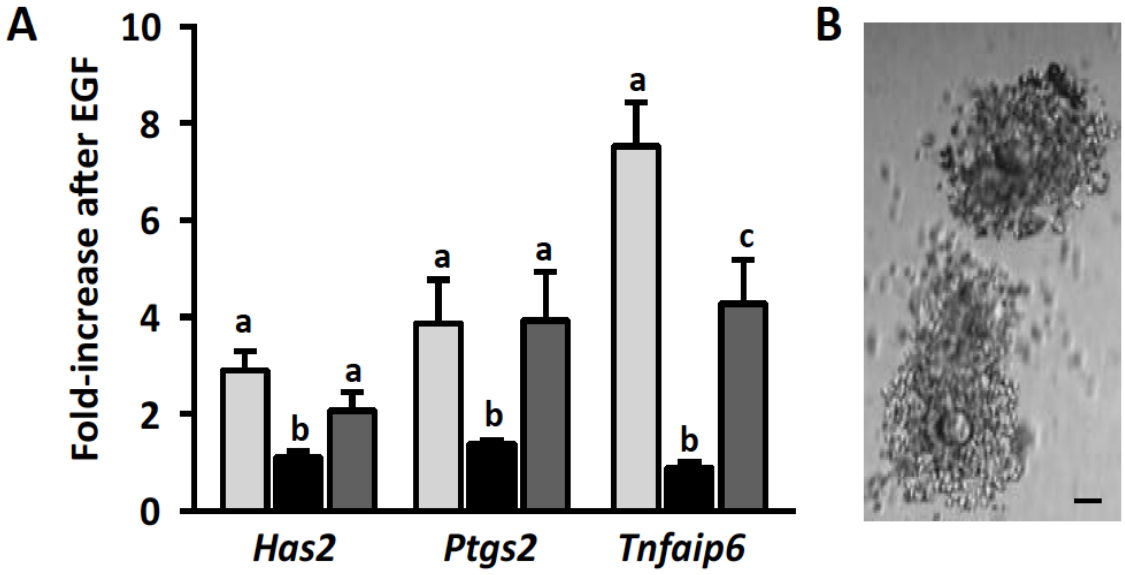


Figure 10: Supplying FSH activity to *Fshb*$^{-/-}$ females restores the ability to undergo cumulus expansion *in vitro*. (A) Relative quantity of *Has2*, *Ptgs2*, and *Tnfaip6* in follicles of PD 18 *Fshb*$^{+/-}$ females (gray bars), *Fshb*$^{-/-}$ females (black bars), and *Fshb*$^{-/-}$ females injected 48 h previously with eCG (dark gray bars), after stimulation with EGF as compared to non-stimulated follicles of the same genotype. (B) Cumulus expansion in COCs of eCG-injected *Fshb*$^{-/-}$ females following EGF stimulation.Scale bar = 50 µm. Data was analysed using one-way ANOVA and Tukey HSD. Different letters above bars indicate $p<0.05$.

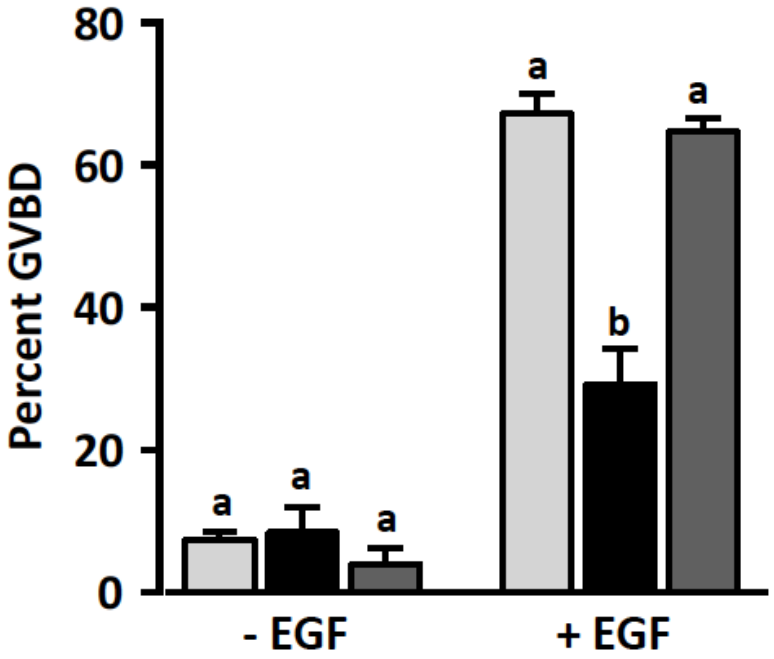


Figure 11: Prior exposure to FSH promotes EGF-stimulated oocyte meiotic maturation. Follicles of *Fshb*$^{+/-}$ (gray bars), *Fshb*$^{-/-}$ (black bars) or eCG-injected *Fshb*$^{-/-}$ (dark gray bars) females at PD 21-23 were incubated for 16 hrs in the presence or absence of EGF and the percentage of oocytes that underwent GVBD was recorded. Data was analysed using one-way ANOVA and Tukey HSD. Different letters above bars indicate $p<0.05$.

Discussion

The EGFR signalling axis is a major effector of LH-dependent ovulatory events (Downs and Chen 2008; Hsieh et al. 2007; Panigone et al. 2008; Park et al. 2004; Ashkenazi et al. 2005). We demonstrate here that follicles of *Fshb*$^{-/-}$ females suffer a severely impaired ability to undergo two key EGFR-regulated events, expansion of the cumulus cells and oocyte meiotic maturation, in response to EGF. In other respects, however, *Fshb*$^{-/-}$ follicles resemble wild-type follicles, at least during the time-period studied here. For example, they show little change in the expression of numerous genes, apart from those known to be FSH-regulated (Burns et al. 2001), are able to support growth and the acquisition of meiotic competence, and are able to hold competent oocytes in meiotic arrest (Demeestere et al. 2012), which requires the production of cGMP by granulosa cells and its transfer to the oocyte (Norris et al. 2009; Vaccari et al. 2009). Our results also imply that granulosa cells of *Fshb*$^{-/-}$ females can activate SMAD signalling in response to ODPFs. In addition, transcript levels of EGF-like peptides were normal. Thus, the inability of the *Fshb*$^{-/-}$ follicles to initiate cumulus expansion or oocyte maturation in response to EGF is unlikely to reflect a non-specific loss of follicular function in the absence of FSH, but rather indicates that FSH promotes specific events that enable the ovulatory response to EGF.

Several lines of evidence suggest that FSH promotes EGF responsiveness by increasing the expression of EGFR. First, expression of both *Egfr* and EGFR increased during late folliculogenesis in *Fshb*$^{+/-}$ but not *Fshb*$^{-/-}$ females. Second, a ~50% decrease in EGFR activity in granulosa cells, similar to what we observed in *Fshb*$^{-/-}$ females, is sufficient to severely impair the ovulatory response (Hsieh et al. 2007). Third, eCG injection into *Fshb*$^{-/-}$ females increased expression of *Egfr* as well as that of known FSH targets. It also restored both cumulus expansion and oocyte maturation, which are independently regulated downstream of EGFR activation. We propose that FSH stimulates an increase in EGFR expression and activity in granulosa cells and that this increase is essential to enable its ligands to trigger cumulus expansion, oocyte maturation and perhaps other events of ovulation. Notwithstanding the link between the increase in EGFR expression and response to EGF, other FSH-dependent events may also prepare granulosa cells to respond to EGFR ligands.

Egfr expression increases during antral folliculogenesis in the hamster and this increase is abolished when FSH is depleted by hypophysectomy (Garnett, Wang, and Roy 2002). *Egfr* is also expressed in antral follicles of humans (el-Danasouri, Frances, and Westphal 1993; Guzman et al. 2013). In the goat, stimulation with exogenous FSH increases the expression of *Egfr* in the cumulus granulosa cells (Almeida et al. 2011). These results are consistent with ours and suggest that FSH may play a key conserved role in regulating follicular EGFR expression in mammals. Although mutations in *Fshb* or the FSH receptor are relatively rare in humans (Tao and Segaloff 2009; Themmen and Huhtaniemi 2000; Casarini, Pignatti, and Simoni 2011), women bearing these mutations would be candidates for assisted reproduction including *in vitro* maturation owing to the probable absence of LHCGR on granulosa cells. EGF is increasingly used during *in vitro* maturation, where it has been shown to increase its efficiency (Zamah et al. 2010; Peluffo et al. 2012; Ben-Ami et al. 2011). Our results suggest that women lacking FSH activity might require a modified therapeutic intervention.

The mechanism by which FSH regulates expression of *Egfr* remains to be established. Some effects of FSH are mediated through estradiol (Adashi and Hsueh 1982). However, although cumulus expansion was impaired in some follicles of mice lacking estrogen receptor-β, it was normal in others (Couse et al. 2005). These ovaries also showed normal induction of *Has2* in response to LH, but reduced basal levels of *Ptgs2* and *Tnfaip6* (Couse et al. 2005; Binder et al. 2013), implying that EGFR signalling was partially functional in the absence of estrogen receptor-β. Alternatively, β-catenin and the transcription factor SP1, both of which are implicated in FSH-regulated gene expression (Alliston et al. 1997; Parakh et al. 2006; Law et al. 2013), are important regulators of *Egfr* expression in other cell types (Brandt et al. 2006; Kageyama, Merlino, and Pastan 1988; Guturi et al. 2012).

Our results do not exclude a role for other factors in regulating *Egfr* expression, and previous work has shown that ODPFs promote the expression of *Egfr* in the cumulus granulosa cells (Diaz et al. 2006; Dragovic et al. 2007; Nagyova et al. 2011; Pangas and Matzuk 2005; Sasseville et al. 2010; Su et al. 2010). The impaired *Egfr* expression we describe here is not likely due to an absence of ODPFs, however, as oocytes of *Fshb*$^{-/-}$ females express *Bmp15* and *Gdf9*, and induce expansion of oocytectomized *Fshb*$^{+/-}$ complexes, indicating that they produce biologically active

ODPFs. It is also worth noting that, as ODPFs typically generate differences between the cumulus and mural granulosa cells (Gilchrist, Lane, and Thompson 2008), they might not be expected to regulate *Egfr* expression in the mural cells. FSH and ODPFs may each contribute to establishing physiological levels of EGFR expression and activity.

FSH has long been known to play an indispensable role in preparing the mature follicle to respond to the pre-ovulatory LH surge (Erickson, Wang, and Hsueh 1979), through stimulating the expression of LHCGR by the mural granulosa cells. Recent work has shown that the LH induces the mural granulosa cells to release ligands that bind to EGFR on the mural and cumulus granulosa cells and are the proximate trigger of LH-regulated ovulatory events (Ashkenazi et al. 2005; Hsieh et al. 2007; Panigone et al. 2008; Park et al. 2004; Shimada et al. 2006). Our results reveal that prior exposure to FSH is required for follicles to respond to EGF and that this is associated with an FSH-dependent increase in EGFR expression and activity. Thus, FSH appears to play a larger role than previously thought in the ovulatory cascade (Figure 12) – it not only promotes the expression of LHCGR on the mural granulosa cells, enabling them to release EGFR ligands in response to LH, but also promotes the expression of EGFR itself in these cells. The FSH-dependent remodeling of the late-follicular environment may ensure an efficient and coordinated response to ovulatory signals.

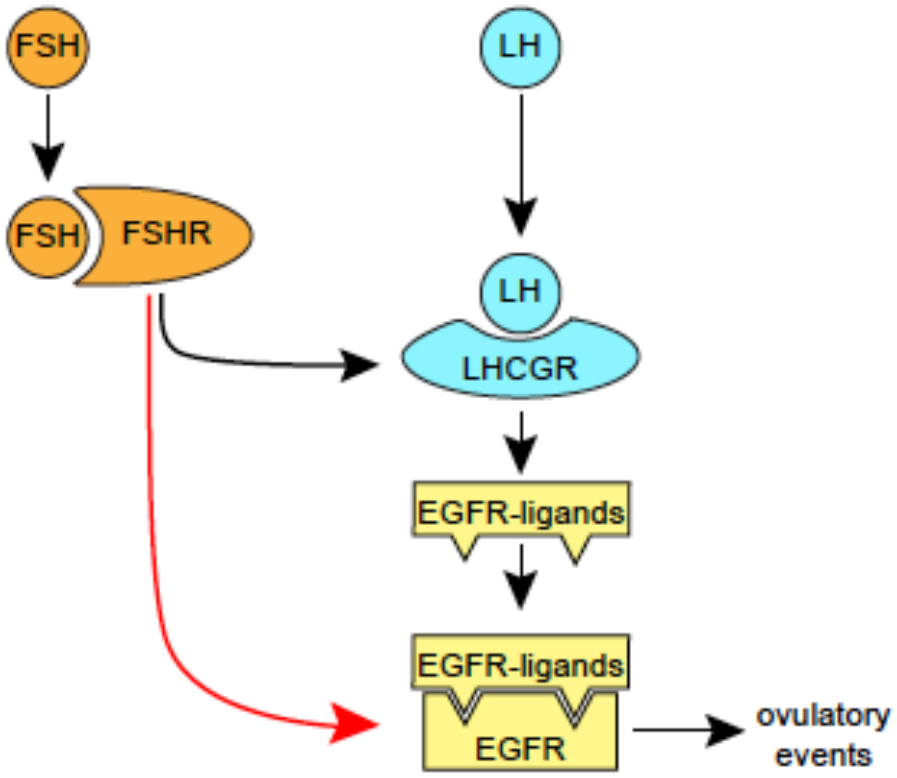


Figure 12: Dual role of FSH in establishing the EGFR signalling axis in the ovarian follicle. FSH induces granulosa cells to express both LHCGR, which enables them to release EGFR ligands in response to the pre-ovulatory LH surge, and EGFR, which enables them to respond to these ligands. The two-step FSH-driven remodeling of the late-follicular environment may ensure an efficient and coordinated response to ovulatory signals.

Acknowledgements

Supported by the Canadian Institutes of Health Research (H.J.C.) and by the Royal Victoria Hospital Foundation. S.E.H. received support from the Research Institute of the McGill University Health Centre and the CIHR Training Program in Reproduction, Early Development, and the Impact on Health. I.D. was supported by les Fonds de la Recherche Scientifique (Belgium).

Supplemental data

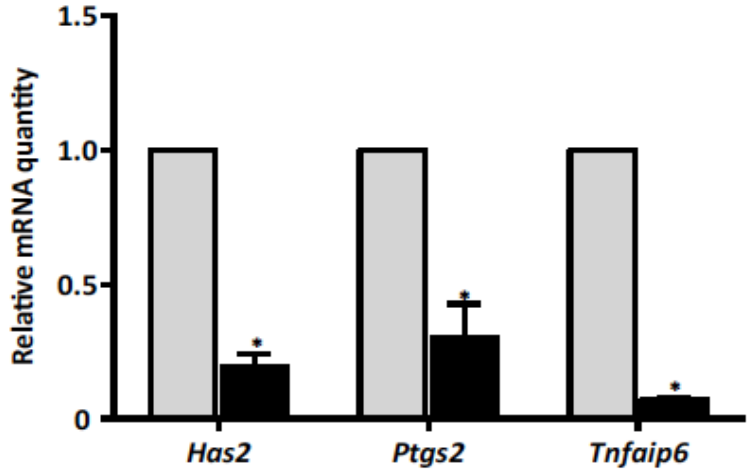
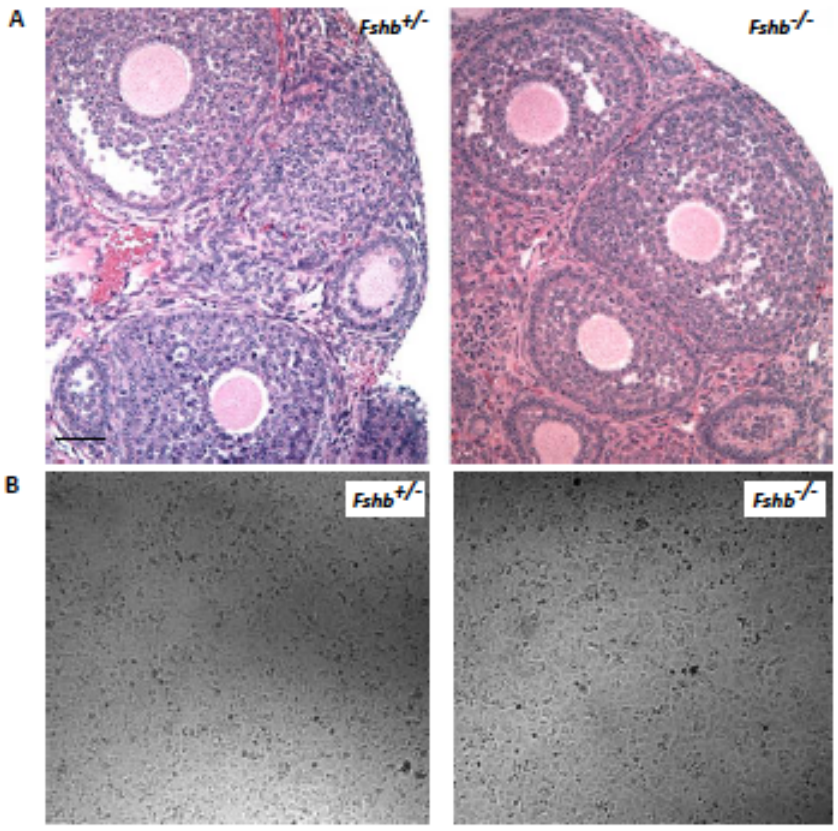


Figure 13: Quantities of *Has2*, *Ptgs2*, and *Tnfaip6* are higher in COCs of *Fshb*$^{+/-}$ females (gray bars) than *Fshb*$^{-/-}$ females (black bars) at PD 21-23. Data was analyzed using single-sample *t*-test. P-values <0.05 were considered statistically significant, as indicated by asterisks.



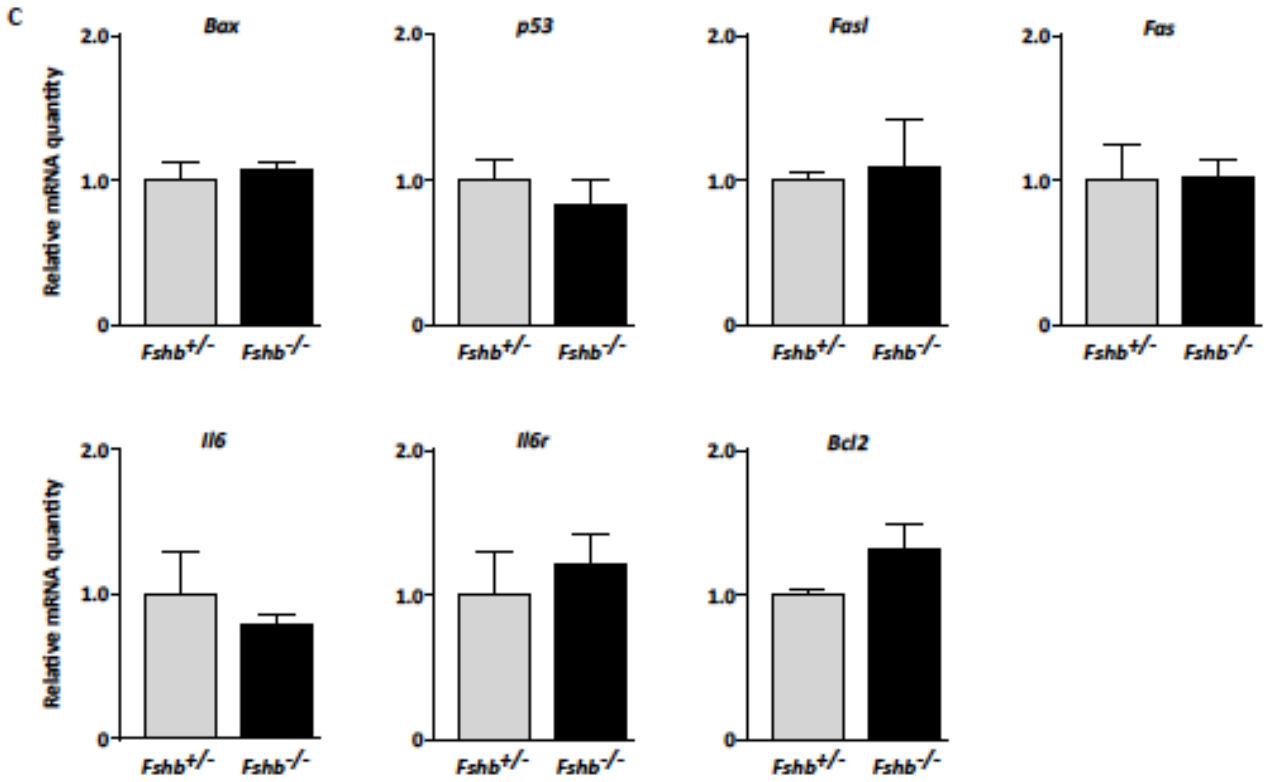


Figure 14: (A) Ovarian follicles of prepuberal *Fshb*$^{-/-}$ females do not contain an increased number of pycnotic or otherwise abnormal nuclei. Histological sections of ovaries of *Fshb*$^{+/-}$ and *Fshb*$^{-/-}$ females at PD 24. (B) Granulosa cells of *Fshb*$^{+/-}$ and *Fshb*$^{-/-}$ females form morphologically indistinguishable monolayers *in vitro*. Brightfield images of monolayers formed by granulosa cells of *Fshb*$^{+/-}$ and *Fshb*$^{-/-}$ females collected at PD 21 and cultured for three days. c. Quantities of mRNAs encoding pro-apoptotic and anti-apoptotic factors are similar in follicles of *Fshb*$^{+/-}$ and *Fshb*$^{-/-}$ females. qPCR analysis of the pro-apoptotic factors *Bax*, *p53*, *Fasl*, and *Fas* and the anti-apoptotic factors *Il6*, *Il6r*, and *Bcl2* in follicles of *Fshb*$^{+/-}$ (gray bars) and *Fshb*$^{-/-}$ (black bars) females at PD 18. Data was analyzed using single-sample *t*-test. P-values <0.05 were considered statistically significant.

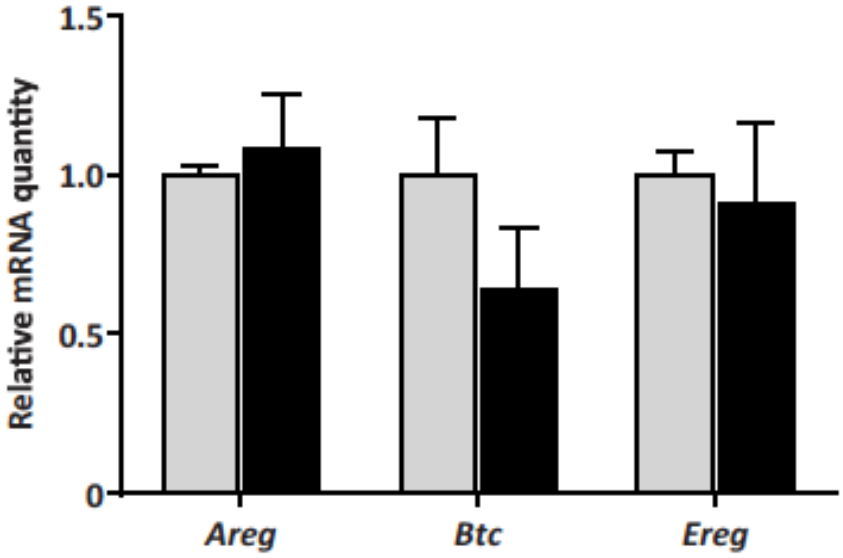


Figure 15: Quantities of *Areg*, *Ereg*, and *Btc* are not significantly different between GCs of *Fshb*$^{+/-}$ females (gray bars) and *Fshb*$^{-/-}$ females (black bars) at PD 21-23. Data was analyzed using two-sample *t*-test. P-values <0.05 were considered statistically significant.

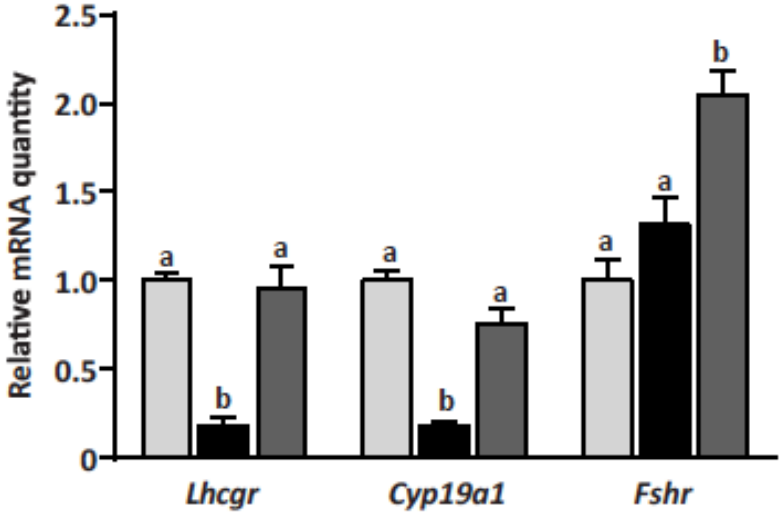


Figure 16: Quantities of *Lhcgr, Cyp19a1,* and *Fshr* in follicles of *Fshb*$^{+/-}$ (gray bars), *Fshb*$^{-/-}$ (black bars) or eCG-injected *Fshb*$^{-/-}$ (dark gray bars) PD 18 females. Data was analysed using one-way ANOVA and Tukey HSD. Different letters above bars indicate $p<0.05$.

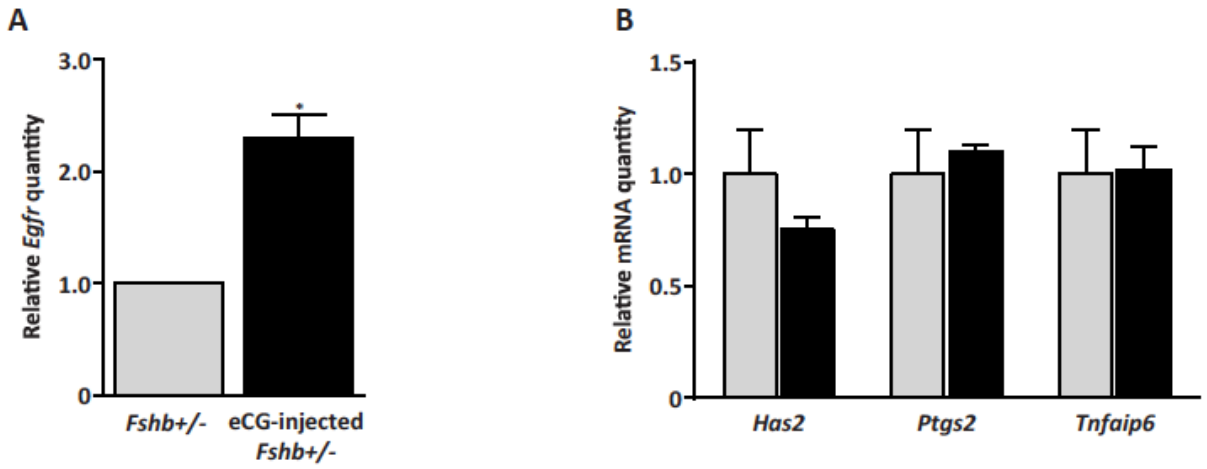


Figure 17: Quantity of *Egfr* in follicles of *Fshb*$^{+/-}$ and eCG-injected *Fshb*$^{+/-}$ PD 18 females. Data was analyzed using single-sample *t*-test. P-value <0.05 was considered statistically significant, as indicted by asterisk. b. Quantity of *Has2*, *Ptgs2*, and *Tnfaip6* after EGF stimulation of follicles of *Fshb*$^{+/-}$ (gray bars) and eCG-injected *Fshb*$^{+/-}$ (black bars) PD 18 females. Data was analyzed using two-sample *t*-test. P-value <0.05 was considered statistically significant.

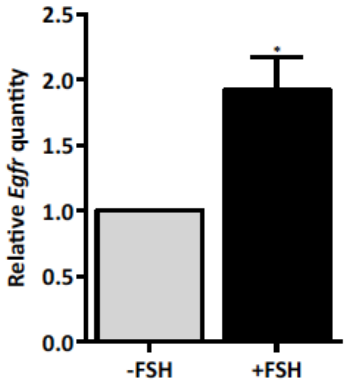


Figure 18: Quantity of *Egfr* in granulosa oocyte complexes isolated from of PD 16 *Fshb*$^{-/-}$ females and cultured for 48 hrs ± FSH. Data was analyzed using single sample *t*-test. P-value <0.05 was considered statistically significant, as indicated by asterisk.

Chapter 3

Manuscript II
Follicle-stimulating hormone increases gap junctional communication between the somatic and germ-line follicular compartments during murine oogenesis

Stephany El-Hayek[1,2,4] and Hugh J. Clarke[1,2,3,4]
Departments of Obstetrics and Gynecology[1], Biology[2], and Experimental Medicine[3]
McGill University Research Institute of the McGill University Health Centre[4]

Biol Reprod 2015. pii: biolreprod.115.129569.

Running title: Germ cell-somatic communication

Key words: FSH, oocyte, granulosa, gap junctions

Significance statement: FSH increases gap junctional communication between the growing oocyte and surrounding somatic follicular granulosa cells

Author contributions: S.E.H. and H.C. designed the experiments. S.E.H. performed the experiments. S.E.H. and H.C. wrote the manuscript.

Grant support: Supported by the Canadian Institutes for Health Research (H.J.C.). S.E.H. was supported by the CIHR Training Program in Reproduction, Early Development, and the Impact on Health, the Réseau Québécois en Reproduction, and by the Research Institute of the McGill University Health Centre.

Competing interests: The authors declare no competing interests

Correspondence: Hugh Clarke
MUHC Research Institute 1001 Decarie Blvd, Block E-M0.2218, Montreal, QC CANADA H4A 3J1
hugh.clarke@mcgill.ca

Preface

In a study previously published by our lab, it was shown that, the oocytes of *Fshb*$^{-/-}$ mice grow normally in size and diameter. However, these oocytes have reduced developmental competence (Demeestere et al. 2012). The results discussed in Chapter 2 provide a mechanism for how FSH affects the ovulatory response, yet they do not demonstrate how it promotes the oocyte's development. Hence, in this part of my studies, I sought to determine how FSH affects the oocyte's development. Since the oocyte is not a direct target of FSH as it does not express the receptor for FSH, the mechanism by which FSH affects the oocyte's development has to be indirect. Several studies have shown that FSH increases the expression of cellular junctional components in granulosa cells. We thus hypothesized that FSH may also increase the expression of these junctional components at the interface between the oocyte and the granulosa cells. As such FSH would enhance the communication between the two cell types, thus improving the oocyte's developmental competence. To test this, the following objectives were designed:

IIa) Determine whether FSH increases expression of connexin37 and E-cadherin, components of cellular junctions between the oocyte and the granulosa cells

IIb) Determine whether FSH increases oocyte - granulosa cell gap junctional communication

This work is discussed in Chapter 3 (Manuscript II). Chapter 3 contains some modifications from the published work, in the 'Materials and Methods' section, as well as some additional experiments shown in the 'Supplemental data' section.

Abstract

Germ cells develop in intimate contact and communication with somatic cells of the gonad. In female mammals, oocyte development depends crucially on gap junctions that couple it to the surrounding somatic granulosa cells of the follicle. Yet the mechanisms that regulate this essential intercellular communication remain incompletely understood. FSH drives the terminal stage of follicular development. We found that FSH increases the steady-state levels of mRNAs encoding the principal connexins that constitute gap junctions and cadherins that mediate cell attachment. This increase occurs both in granulosa cells, which express the FSH-receptor, and in oocytes, which do not. FSH also increased the number of transzonal projections that provide the sites of granulosa cell-oocyte contact. Consistent with increased connexin expression, FSH increased gap junctional communication between granulosa cells and between the oocyte and granulosa cells, and it accelerated oocyte development. These results demonstrate that FSH regulates communication between the female germ cell and its somatic microenvironment. We propose that FSH-regulated gap junctional communication ensures that differentiation processes occurring in distinct cellular compartments within the follicle are precisely coordinated to ensure production of a fertilizable egg.

Introduction

Germ cells develop in continuous communication with somatic cells of the gonad, which generate a microenvironment that both supports homeostatic functions of the germ cells and regulates their differentiation (Starich, Hall, and Greenstein 2014; Brower and Schultz 1982; De La Fuente and Eppig 2001; Oatley and Brinster 2012; Eppig 1979a). When this communication is disrupted, germ cells do not develop normally and mature gametes, capable of participating in fertilization, are not produced (Simon et al. 1997; Li and Mather 1997; Juneja et al. 1999; Ackert et al. 2001; Eppig 1979a; Dong et al. 1996). Gap junctions couple the germ-line and somatic compartments (Anderson and Albertini 1976) and, together with secreted and membrane-bound factors, play an indispensable role in mediating this intercellular communication (Eppig 1991). Identifying the mechanisms that regulate gap junctional communication between the germ-line and soma is central to understanding how the somatic microenvironment supports germ cell development.

In mammalian females, the oocyte becomes enclosed by somatic granulosa cells shortly after the germ cell enters meiosis, when the two cell types assemble to generate a primordial follicle. During reproductive life, cohorts of primordial follicles regularly enter a prolonged growth phase, during which the oocyte volume increases more than 100-fold (Sorensen and Wassarman 1976; Griffin et al. 2006) and it acquires the ability to develop as an embryo following fertilization (Eppig and Schroeder 1989). As the oocyte grows, the granulosa cells proliferate so that they continue to fully enclose the expanding oocyte. Although the growing oocyte generates a thick extracellular matrix, termed the *zona pellucida*, which physically separates it from the surrounding granulosa cells, narrow cytoplasmic extensions of the granulosa cells, termed trans-zonal projections (TZPs), traverse the zona to reach the oocyte plasma membrane. These TZPs enable the growing oocyte and granulosa cells to remain in physical contact. Importantly, gap junctions become assembled at the tips of the TZPs, where they contact the oocyte plasma membrane (Motta et al. 1994; Anderson and Albertini 1976; Albertini and Rider 1994). These enable the oocyte to remain coupled to the surrounding granulosa cells throughout its growth. During the final stage of oocyte development, termed meiotic maturation, the TZPs retract from

the oocyte surface and gap junctional communication with granulosa cells is lost (Combelles et al. 2004).

The function and importance of oocyte-granulosa gap junctional communication has been well defined. This coupling permits granulosa cells to provide the growing oocyte with nucleotides, amino acids, and energy substrates that it is unable to obtain itself (Wang et al. 2012; Corbett et al. 2014; Biggers, Whittingham, and Donahue 1967; Sugiura, Pendola, and Eppig 2005; Eppig et al. 2005). Communication with granulosa cells is also necessary for the growing oocyte to maintain a stable intracellular pH *in vitro* (FitzHarris and Baltz 2009) and promotes chromatin remodeling and the acquisition of meiotic competence (Lodde et al. 2013). Near the end of oocyte growth, cyclic nucleotides that prevent precocious initiation of meiotic maturation are transmitted via gap junctions from granulosa cells to the oocyte (Vaccari et al. 2009; Norris et al. 2009; Richard and Baltz 2014; Carabatsos et al. 2000). Direct evidence of the indispensable role of the gap junctions in oocyte development is provided by experiments where the gene encoding the principal gap junction protein present in the oocyte (*Gja4*, encoding connexin-37) was deleted. These oocytes fail to establish gap junctional communication with granulosa cells, exhibit severely impaired development, and cannot be fertilized (Simon et al. 1997; Gittens and Kidder 2005). Deletion of the gene encoding the principal gap junction protein present in granulosa cells (*Gja1*, encoding connexin-43) also prevents oocyte development, although oocyte-granulosa gap junctional communication remains intact (Juneja et al. 1999; Ackert et al. 2001; Gittens and Kidder 2005). These experiments establish the indispensability and function of communication with granulosa cells during oocyte development. In contrast, mechanisms that regulate this communication, and thereby might control the timing or efficacy of oocyte development, have been little explored.

FSH is a pituitary glycoprotein whose receptors are located on granulosa cells (Erickson, Wang, and Hsueh 1979; Zeleznik, Midgley, and Reichert 1974). The early stages of oocyte and follicular growth can proceed in the absence of FSH, but more advanced stages become responsive to and subsequently dependent on it (Kumar et al. 1997). Oocyte development is retarded *in vivo* in mice lacking FSH and embryos derived from these oocytes develop poorly, indicating a key role for FSH in oocyte development *in vivo* (Demeestere et al. 2012). The FSH

receptor is expressed by the granulosa cells of the follicle, not the oocyte, however, indicating that the influence of FSH on oocyte development must be exerted via granulosa cells (Demeestere et al. 2012).

Previous studies have shown that FSH promotes gap junctional communication between granulosa cells. The quantity of connexin-43 encoded by gap junction protein, alpha 1 *(Gja1)* in the granulosa increases during FSH-dependent follicle growth, and exogenously supplied FSH activity increases its abundance (Granot and Dekel 2002, 1997). FSH also triggers the translocation of connexin-43 from the cytoplasm to the plasma membrane *in vivo* and *in vitro*, accompanied by increased connexin-43 phosphorylation (Granot and Dekel 1997; Wang et al. 2013; Burghardt and Matheson 1982; Yogo et al. 2002). In addition, FSH increased gap junctional communication in an immortalized cell line derived from granulosa cells (Sommersberg et al. 2000). Given the essential role in oocyte development of gap junctional communication with granulosa cells and the ability of FSH to increase communication between granulosa cells, we speculated that FSH might increase communication between granulosa cells and the oocyte. We report that FSH increases the expression of junctional components in oocyte and granulosa cells, increases the density of of actin-rich TZPs physically linking these two compartments, and increases granulosa-granulosa and granulosa-oocyte gap junctional communication. These results identify FSH as an extra-follicular regulator of gap junctional communication which, by increasing communication between the germ cell and somatic compartments, promotes the growth and development of the oocyte within its follicular microenvironment.

Materials and Methods

Mice

All experiments were performed in compliance with the regulations and policies of the Canadian Council on Animal Care and were approved by the Animal Care Committee of the Royal Victoria Hospital. C57BL/6 mice carrying a deletion in the *Fshb* gene (Kumar et al. 1997) were obtained from Jackson Laboratories and a colony was established at McGill. The mice were housed and bred in a temperature- and light-controlled room and provided food and water *ad*

libitum. *Fshb*$^{-/-}$ females were generated by mating *Fshb*$^{+/-}$ females with *Fshb*$^{-/-}$ males. *Fshb* $^{+/-}$ females of the same litter were used as controls. Genotyping of the offspring was done using the EZ Tissue/Tail DNA Isolation Plus PCR kit (EZ Bioresearch, St. Louis, MO). After DNA extraction from tail snips, PCR was used to detect the wild-type *Fshb* gene and the replacement targeting vector using the following primers: *Fshb*: TTCAGCTTTCCCCAGAAGAG, CTGCTGACAAAGAGTCTATG; targeting vector: CTTGCGCTCATCTTAGGCTT, GGACCTCTCGAAGTGTTGGAT. The following PCR program was used: 94°C for 60 sec, 55°C for 60 sec, and 72°C for 90 sec. PCR products were visualized on 1.5% agarose gels stained with ethidium bromide. For some experiments, *Fshb*$^{-/-}$ females at PD 16 were given an intraperitoneal injection of 5 IU of eCG (Sigma, Windsor, ON), and used at PD 18. CD-1 mice were obtained from Charles River (St Constant, QC).

Collection and culture of cells

Ovaries were removed from *Fshb*$^{+/-}$ and *Fshb*$^{-/-}$ females, transferred to MEM (Life Technologies, Burlington, ON), supplemented with sodium pyruvate (0.28 mM; Sigma), penicillin G (63 μg/ml; Sigma), streptomycin (50 μg/ml; Sigma), and BSA (3 mg/ml; Sigma) at 37°C in air, and dissected using 30G1/2 needles. For all experiments, cells were collected and manipulated in MEM buffered using Hepes (MEM-H; pH 7.2), and were incubated in MEM buffered using $NaHCO_3$ in an atmosphere of 5% CO_2 in air. Taking advantage of the synchronous follicular growth that occurs during the first three weeks of post-natal life in mice, secondary follicles containing mid-growth oocytes (50-60 μm diameter) and early antral follicles containing late-growth oocytes (60-75 μm diameter) were obtained from females at PD 10-12 and PD 18-21, respectively. To obtain granulosa-oocyte complexes (GOCs) containing mid-growth oocytes, ovaries from PD 10-12 females were dissected into several fragments and incubated in MEM-H supplemented with collagenase (10 μg/ml; Cedarlane, Burlington, ON) and DNase I (10 μg/ml; Sigma) at 37^{o}C in air. At 2- to 3 minute intervals, the fragments were gently pipetted to disrupt them. Individual GOCs were collected using a mouth-controlled micropipette and transferred to fresh medium (Demeestere et al. 2012). To obtain complexes containing late-growth oocytes, follicles on the surface of ovaries obtained from PD 18-21 females were punctured using 30G1/2 needles and the released complexes were collected using a mouth-controlled micropipette and

transferred to a fresh dish of medium. Purified granulosa cells and oocytes were obtained by further manipulating complexes using a fine-bore mouth-controlled micropipette.

For *in vitro* growth experiments, intact follicles or GOCs were transferred to type I collagen 3.0 micron inserts (Becton-Dickinson, Mississauga, ON) in 24-well plates containing 750 µl of pre-equilibrated serum-free MEM supplemented with ITS (10 µg/mL insulin, 5.5 µg/mL transferrin and 5 ng/mL selenium; Sigma) and cilostamide (10 µM, Sigma) and incubated in the presence or absence of 10 mIU/ml FSH (EMD Serono, Mississauga, ON), a dose previously shown to sustain follicular growth *in vitro* (Adriaens, Cortvrindt, and Smitz 2004). For culture of GOCs, two-thirds of the medium was replaced every third day. To measure the diameter of oocytes within the complexes, pseudo-brightfield images were recorded using a Zeiss confocal laser scanning microscope (CLSM) 510 (Zeiss, Toronto, ON) and analyzed using the software provided by the manufacturer. To permit meiotic maturation, complexes were transferred to cilostamide-free MEM and incubated overnight. The following day, the maturation state of the oocytes was assessed visually by assessing for GVBD.

Immunohistochemistry

Ovaries of PD 21 CD-1 mice were fixed overnight at 4^{o}C in freshly prepared 4% *para*-formaldehyde in PBS (pH 7.5) with continuous agitation, and then embedded in paraffin. Sections were cut at 5 µm, deparaffinized and rehydrated, then boiled for 40 min in Tris-EDTA (pH 9.0). After cooling to room temperature (RT), slides were blocked with 1.35% goat serum in PBST for 30 min at RT in a humidified chamber. Following PBST washes, slides were incubated at 4^{o}C overnight with one of the following antibodies diluted in blocking buffer: N-cadherin (1:100, Ab18203, Abcam, Toronto, ON), connexin-43 (1:400, C6219, Sigma), E-cadherin (1:100, Ab15148, Abcam), connexin-37 (1:250, 40-4300, Life Technologies). Slides were washed in PBST and incubated for 1 hr at RT with anti-rabbit Alexa 488 (1:100, Life Technologies) and DRAQ5 (1:1000, New England Biolabs, Whitby, ON) as a DNA stain. Slides were then washed and mounted in Mowiol (Sigma), and examined using the CLSM 510 microscope (Zeiss).

RNA extraction and quantitative real-time PCR

Thirty mid-growth oocytes and their associated granulosa cells or 20 late-growth oocytes and their associated granulosa cells were used for total RNA extraction using a Picopure RNA isolation kit (Life Technologies), according to the manufacturer's instructions. RNA was eluted in 10 µl of the provided elution buffer. SuperScript II Reverse Transcription kit (Life Technologies) was used to generate cDNA. One µl of deoxyribonucleotides (10 mM each) and 200 ng of random primers were added to the 10 µl of RNA. The mixture was then incubated for 5 min at 65°C, followed by the addition of 4 µl of 5x first strand synthesis buffer, 2 µl of 0.1 M dithiothreitol and 1 µl RNaseOUT. After a 2 min incubation at 25°C, followed by the addition of 1 µl of SuperScript II, the mixture was then successively incubated for 10, 50, and 15 min, at 25°C, 42°C, and 70°C respectively. PCR amplification was performed using a Corbett Rotorgene 6000 (Montréal Biotech, Montreal, QC). Each reaction contained 4 µl of EvaGreen Mix (Montréal Biotech), 13 µl of UltraPure DNase/RNase-free distilled water (Life Technologies), 1 µl of 10 µM primers and 2 µl of cDNA (diluted by 1:20 from original stock). Primers were designed using Primer-BLAST (National Institutes of Health, Bethesda, MD) and obtained from Sigma. Primer sequences are given in Table 1. For each primer pair, a standard curve was generated using serial dilutions of cDNA prepared from ovarian RNA and used to determine the efficiency of amplification. Melt-curve analysis and electrophoresis of amplified products confirmed that only a single product of the expected size was generated. Data was analyzed using software provided by the manufacturer. Relative quantities of amplified product were calculated according to the $2^{-\Delta\Delta CT}$ method, using *Actb* (actin) for normalization.

Gene/transcript	Primer sequence
Actb	F: 5′-GGCTGTATTCCCCTCCATCG-3′
	R: 5′-CCAGTTGGTAACAATGCCATGT-3′
Ccnb1	F: 5′-AAGGTGCCTGTGTGTGAACC-3′
	R: 5′-GTCAGCCCCATCATCTGCG-3′
Cdh1	F: 5′-AACGCTCCCATCCCAGAACCTC-3′
	R: 5′-TGCCACGATTCCCGCCTTCAT-3′
Cdh2	F: 5′-CCCAAGTCCAACATTTCCATCCTGC-3′
	R: 5′-TCGTCTAGCCGTCTGATTCCCACG-3′
Gja1	F: 5′-CGCCCAGCCGTTTGATTTCCCT-3′
	R: 5′-CCCCTTTCCCTACTTTTGCCGCC-3′
Gja4	F: 5′-AGCTCTGCATCCAAGAAGCAGTA -3′
	R: 5′-AGTTGTCTCTGAGGTGCCTTTGA-3′
Mos	F: 5′-TGGCTGGTTTTGAGAATCAAGG-3′
	R: 5′-GTCACATGAGACACTAGGGAGA -3′
Slbp	F: 5′-ATGGCCTGCAGACCTAGAAG-3′
	R: 5′-CTGGCCCAGTCAGAACATCT-3′

Table 2: Primer sequences for quantitative PCR analysis

Immunoblotting

Granulosa cells from 75 GOCs containing mid-growth oocytes or 50 complexes containing late-growth oocytes were transferred to a microfuge tube in MEM, briefly centrifuged to pellet them and resuspended in 20 µl of 2× Laemmli buffer (Bio-Rad, Saint-Laurent, QC). After heating to 95^{o}C for 5 min, the samples were subjected to Tris-glycine electrophoresis using 12% polyacrylamide gels as described (Yang et al. 2010). Proteins were transferred onto a polyvinylidene fluoride membrane (Amersham GE Healthcare, Mississauga, ON) under constant voltage (100V) for 1.5 hr. The membrane was subsequently blocked in 5% non-fat milk, in PBST. The membrane was then incubated overnight at 4°C with primary antibody diluted in PBST containing 3% BSA: connexin-43 (1:8000, C6219, Sigma), N-cadherin (1:1000, Ab18203, Abcam), tubulin (1:2000, T8203, Sigma). After washing, the membrane was incubated in secondary antibody conjugated to horseradish peroxidase (Promega Fisher, Ottawa, ON) diluted 1:5000 in PBST containing 5% non-fat milk for 1 hr at RT. After washing, fluorescence was revealed using ECL+ (Amersham GE Healthcare), imaged using a Storm phosphorimager (Amersham GE Healthcare), and quantified using Image J (National Institutes of Health). N-cadherin and connexin-43 signals were normalized to tubulin in the same sample.

Detection of RNA synthesis

RNA synthesis was detected using Click-it technology (Life Technologies), which utilizes the modified nucleotide, 5'-ethynyl uridine (EU). Late-growth oocytes obtained from PD 18-20 females were incubated in MEM supplemented with 100 μM EU for 2 hr. They were then fixed for 15 min at RT in freshly prepared 2% *para*-formaldehyde in PBS (pH 7.5). EU detection was carried out following the manufacturer's instructions. Oocytes were mounted on glass slides and fluorescence was recorded using the confocal microscope and quantified using Image J.

Detection and quantification of Cy3-phalloidin fluorescence

Oocytes were fixed for 15 min at RT in freshly prepared 2% *para*-formaldehyde in PBS, then washed with PBST. To stain actin, cells were incubated for 1 hr at RT in Cy3-conjugated phalloidin (P1951, Sigma) diluted 1:100 in PBST. To assess chromatin configuration (Bouniol-Baly et al. 1999; Bellone et al. 2009), cells were incubated for 1 hr at RT in DRAQ5 diluted 1:1000 in PBST. To mount the cells, a 9 mm x 0.12 mm spacer (GBL654008, Sigma) was attached to a glass microscope slide. A 2-μl drop of PBS was placed in the centre of the spacer and covered with 20 μl of mineral oil. Cells were then transferred into the drop of PBS and a cover slip was placed on top. Using the 63x objective of the confocal microscope, the equatorial plane of the oocyte or complex was identified visually. Two concentric circles, one at the oocyte plasma membrane and the second at the granulosa cell plasma membranes apposed to the *zona pellucida*, were traced using the image-analysis software supplied with the confocal system, taking care to exclude the Cy3-stained plasma membranes of the oocyte and granulosa cell bodies. Cy3 fluorescence between the two circles, which corresponds to the TZPs within the *zona pellucida*, was recorded, quantified using Image J, and expressed per unit area.

Measurement of granulosa cell communication using fluorescence recovery after photobleaching (FRAP)

A protocol for FRAP was adapted from a previous study that utilized it to monitor gap junctional communication between cumulus granulosa cells within cumulus-oocyte complexes (Santiquet et al. 2012).Complexes isolated from ovaries of PD 18-21 $Fshb^{+/-}$ and $Fshb^{-/-}$ females were incubated for 15 min in MEM containing calcein-AM (Life Technologies, 1 μM, diluted from 5

mM stock) and then transferred into calcein-free medium. FRAP was performed using the ROI (region of interest)-Bleach function of the CLSM 510 Meta software. Using the 63× objective, an individual granulosa cell was bleached using a single series of 50 iterations spanning 2 sec of the argon laser at 100% transmission strength. Using the BP500-550 filter, the fluorescence within the cell was recorded 24 sec before bleaching, at the time of bleaching, and at 16 sec intervals after bleaching for 4 min. Two granulosa cells on diametrically opposite sides of the enclosed oocyte were bleached in each complex, and 10 complexes per experimental group were analyzed. In some cases, the gap junction blocker, carbenoxolone (Sigma, 100μM), was added to the medium 30 min before and during incubation with calcein-AM.

Measurement of oocyte-granulosa cell communication using fluorescence loss in photobleaching (FLIP)

A protocol for FLIP was adapted from a previous study (Maeda et al. 2012). Complexes isolated from ovaries of PD 10-12 or PD 18-21 females were incubated for 15 min in MEM supplemented with calcein-AM as above and then for 60 min in calcein-free MEM to allow transfer of calcein from the granulosa cells into the oocyte. A circular ROI of fixed diameter (10 μm, to ensure the bleaching did not extend to the surrounding granulosa cells) was set in the center of the enclosed oocyte and was subjected to 60 repetitive bleaches of 50 iterations (1 min total duration) using the argon laser at 50% transmission strength. The intensity of fluorescence in the oocyte and the layer of granulosa cells immediately surrounding it was recorded before bleaching and after the 10^{th}, 20^{th}, 30^{th}, 40^{th}, 50^{th}, and 60^{th} bleach. Loss of fluorescence was calculated as intensity of fluorescence in the granulosa cell layer at each time-point divided by the pre-bleach intensity of the layer. As oocytes of all experimental groups were subjected to the same bleaching procedure within the fixed ROI, the loss in fluorescence was further divided by initial pre-bleach intensity of the oocyte normalized to intensity of fluorescence in the oocyte at the given time-point, in order to correct for differences in bleaching effect in oocytes of different sizes. To verify that loss of fluorescence depended on gap junctional communication, some complexes were first incubated with calcein-AM and wash medium to allow dye entry into the oocyte and then incubated in the presence of carbenoxolone for 30 min, after which FLIP was performed as described.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.0. Single-sample *t*-test, two-sample *t*-test, Chi-square test, or one-way ANOVA followed by Tukey HSD test was used, depending on the experiment. $p<0.05$ was considered significant. For single-sample *t*-test, two-sample *t*-test, and one-way ANOVA, reported values represent the mean ± standard error of the mean (s.e.m.) of three or more independent experiments. For the Chi-square test, reported values represent the mean of three independent experiments.

Results

FSH increases the expression of connexins and cadherins in granulosa cells and oocytes

Growth of the oocyte and follicle is conventionally divided into morphologically defined stages. Primordial follicles consist of a non-growing oocyte enclosed by a small number of squamous granulosa cells. Upon entry into the growth phase, the follicles are termed primary and are characterized by a growing oocyte surrounded by a single layer of cuboidal granulosa cells. As the granulosa cells proliferate, they generate multiple layers around the growing oocyte, and the follicles are termed secondary. Subsequently, a fluid-filled cavity termed the antrum develops and the oocyte, upon reaching full size, stops growing. Such follicles are termed antral, and large antral follicles ovulate in response to gonadotropins. Because FSH drives the transition from secondary to antral follicle (Demeestere et al. 2012; Kumar et al. 1997), we focused on these two stages. We examined the expression of genes encoding the principal gap junction proteins and the cadherins that mediate attachment of the granulosa cells to the oocyte. As shown in Figure 19, granulosa cells mainly express connexin-43 (encoded by *Gja1*) and N-cadherin (*Cdh2;* Cadherin-2), whereas oocytes mainly express connexin-37 (*Gja4*) and E-cadherin (*Cdh1;* Cadherin-1) (Mora et al. 2012; Valdimarsson, De Sousa, and Kidder 1993; Simon et al. 1997; Machell and Farookhi 2003; Wright et al. 2001; Veitch et al. 2004; Teilmann 2005). Using $Fshb^{+/-}$ and $Fshb^{-/-}$ females, we isolated complexes consisting of an oocyte and surrounding granulosa cells from secondary and early antral follicles, mechanically separated the two cell types, and analyzed gene expression in each.

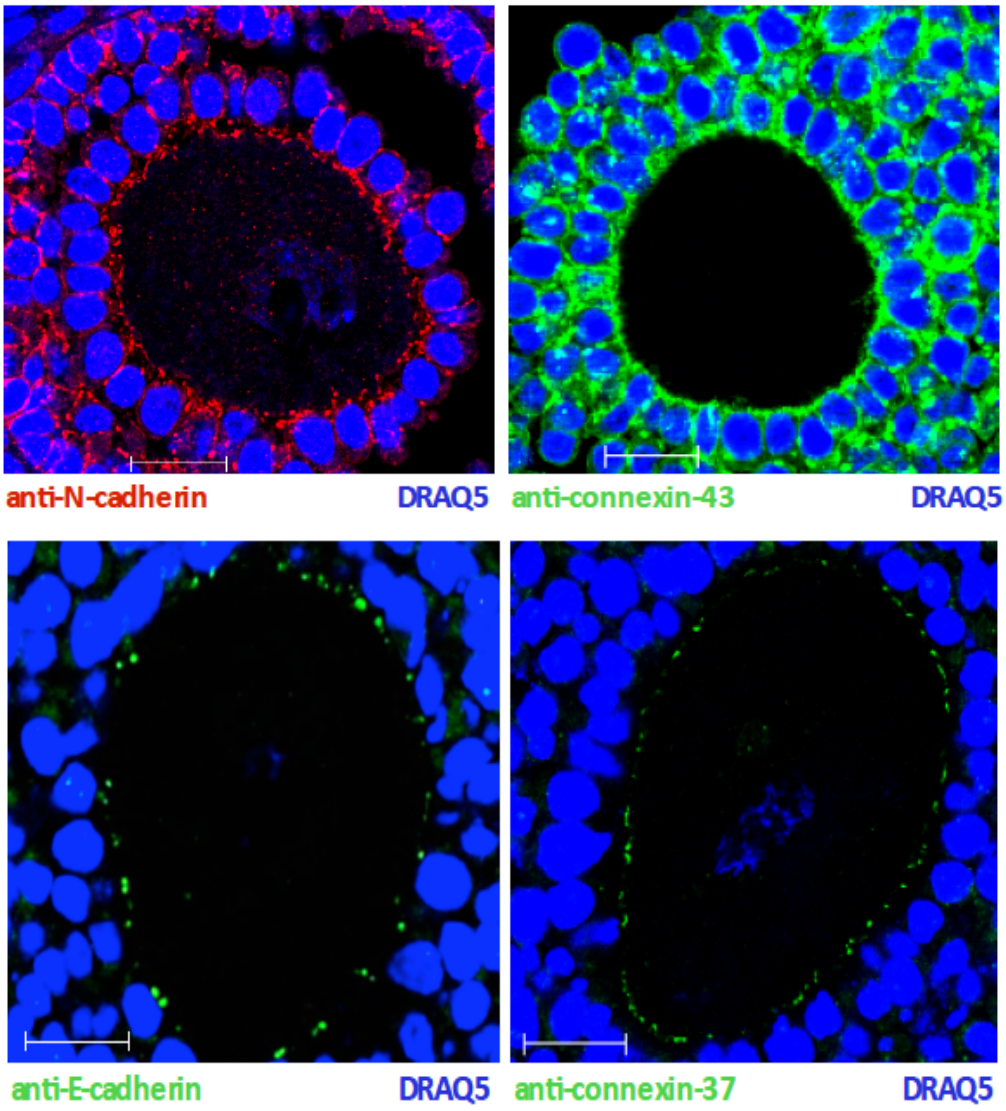


Figure 19. Expression of connexins and cadherins in oocytes and neighboring granulosa cells. Ovarian sections of wild-type CD-1 mice were stained using the indicated antibodies. Images shown are of early-antral follicles. N-cadherin and connexin-43 are detectable in granulosa cells, and E-cadherin and connexin-37 in oocytes. DNA stained using DRAQ5 is shown in blue. Scale bar = 20 μm

In secondary follicles, granulosa cells of *Fshb*$^{+/-}$ females already contained significantly more *Gja1* and *Cdh2* than those of *Fshb*$^{-/-}$ females (Figure 20A), and a similar difference was observed in the amount of encoded protein (Figure 20B). During progression to the early antral stage, the quantity of both *Gja1* and *Cdh2* mRNAs increased substantially in granulosa of *Fshb*$^{+/-}$ follicles. In contrast, a much smaller increase was observed during the same period in granulosa of *Fshb*$^{-/-}$ follicles (Figure 20C, D). These results showed that the steady-state levels of *Gja1* and *Cdh2* in granulosa cells increased during progression from the secondary to early antral stage, and that this increase was partially dependent on FSH.

We observed similar results when we examined the expression of *Gja4* and *Cdh1* in the oocyte. Both mRNAs were more abundant in oocytes of secondary follicles of *Fshb*$^{+/-}$ females than in those of *Fshb*$^{-/-}$ females (Figure 20E). We were unable to detect the encoded proteins by immunoblotting using available antibodies. Both mRNAs increased significantly in oocytes of *Fshb*$^{+/-}$ females during progression from the secondary to early antral stage, but this increase failed to occur in oocytes of *Fshb*$^{-/-}$ females (Figure 20F, G). We observed no difference, however, between oocytes of *Fshb*$^{+/-}$ and *Fshb*$^{-/-}$ females in the expression of other developmentally important genes (Figure 20H) or global transcriptional activity as measured by intensity of EU labelling (Figure 20I), implying that transcription was not non-specifically reduced in oocytes of *Fshb*$^{-/-}$ females. Similarly, the absence of FSH does not non-specifically reduce transcription in granulosa cells (El-Hayek, Demeestere, and Clarke 2014; Burns et al. 2001). Thus, although the oocyte does not express the FSH receptor and thus cannot be a direct target of the hormone, the steady-state levels of the mRNAs encoding the major oocyte connexin and cadherin were relatively low in females that lack FSH.

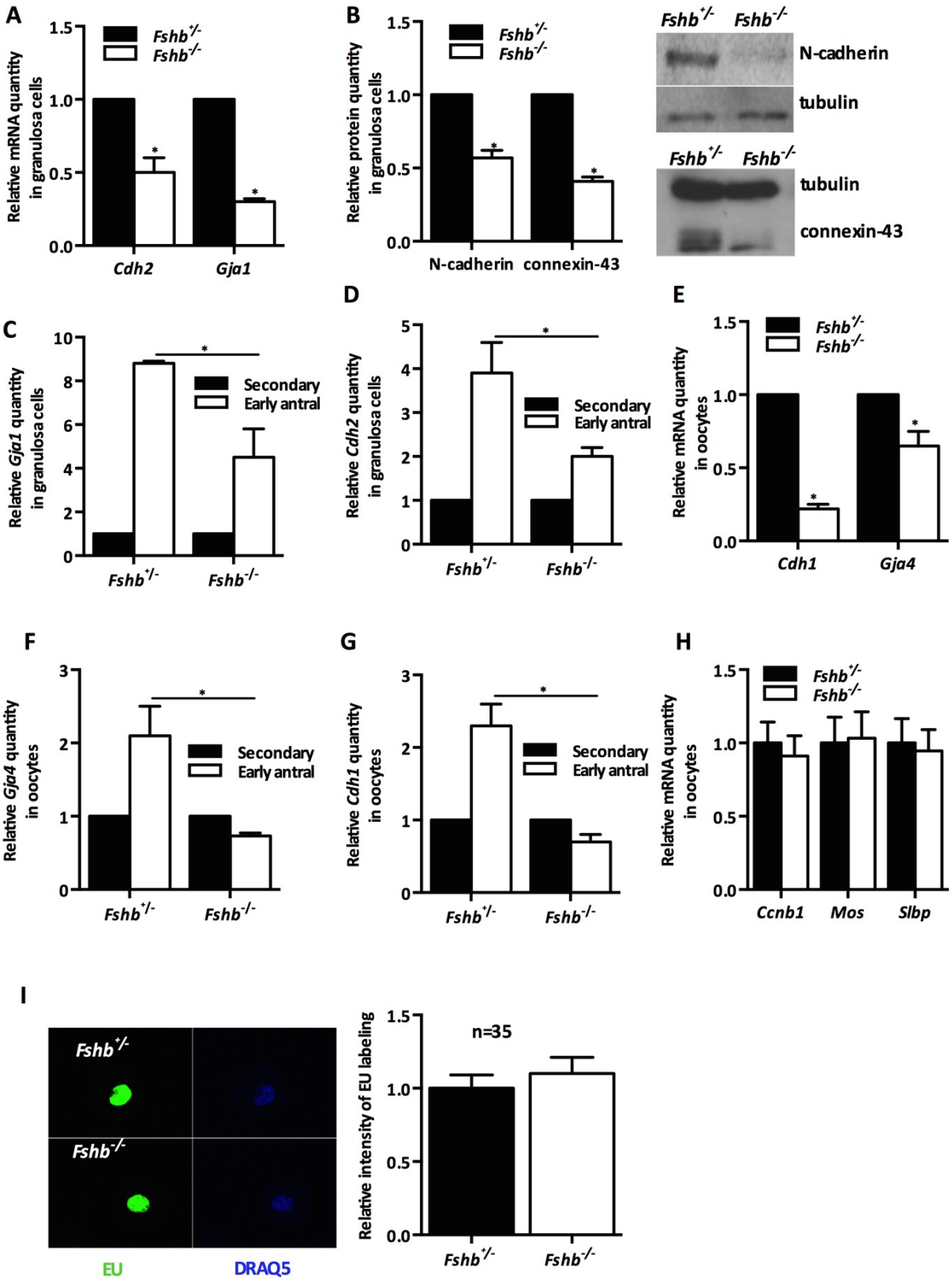
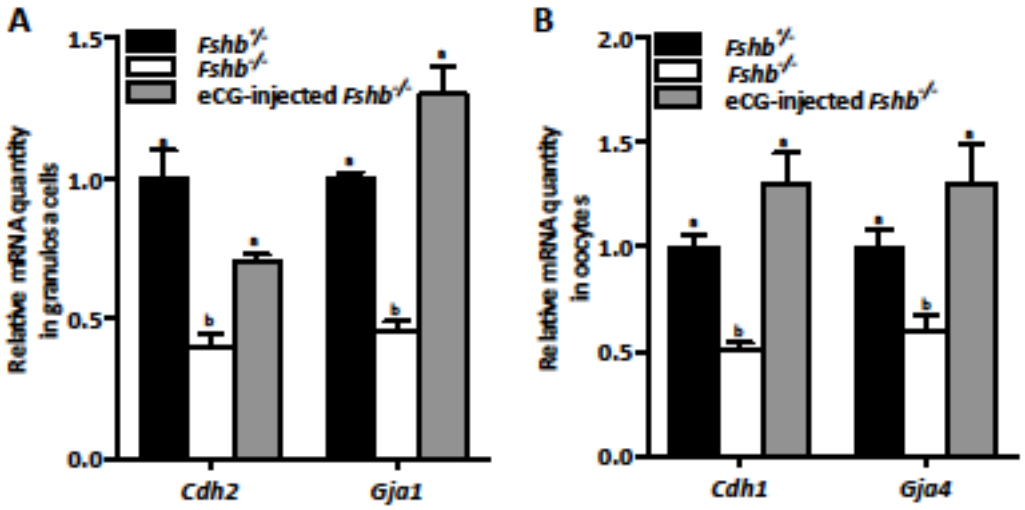

A
Relative mRNA quantity in granulosa cells
Fshb+/-
Fshb-/-
Cdh2
Gja1
B
Relative protein quantity in granulosa cells
N-cadherin
connexin-43
Fshb+/- Fshb-/-
N-cadherin
tubulin
tubulin
connexin-43
C
Relative Gja1 quantity in granulosa cells
Secondary
Early antral
D
Relative Cdh2 quantity in granulosa cells
E
Relative mRNA quantity in oocytes
Cdh1
Gja4
F
Relative Gja4 quantity in oocytes
G
Relative Cdh1 quantity in oocytes
H
Relative mRNA quantity in oocytes
Ccnb1
Mos
Slbp
I
EU
DRAQ5
Relative intensity of EU labeling
n=35

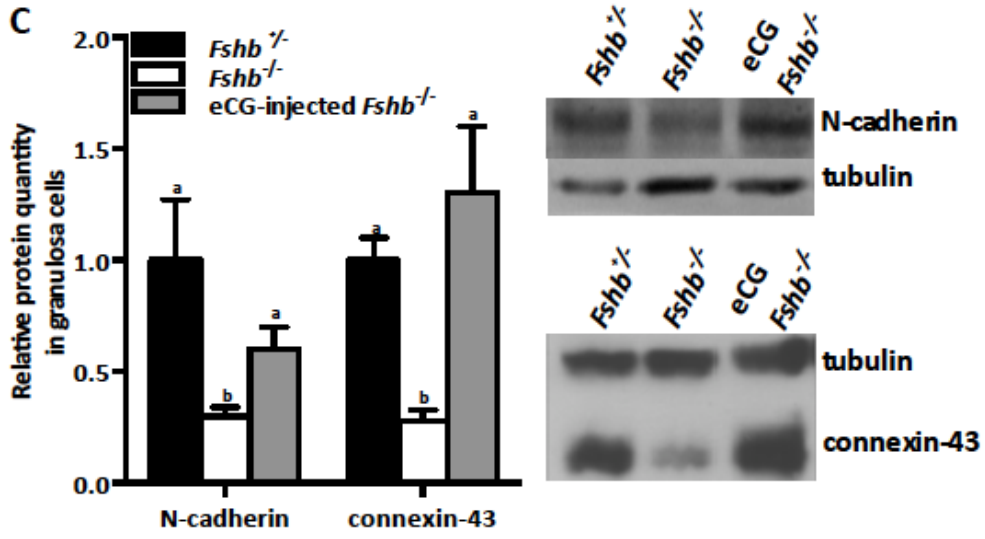
Figure 20: Expression of mRNAs encoding cadherins and connexins is reduced in granulosa cells and oocytes of *Fshb*$^{-/-}$ females. (A) Granulosa cells were isolated from granulosa-oocyte complexes of secondary follicles of *Fshb*$^{+/-}$ and *Fshb*$^{-/-}$ females and the quantities of *Gja1* and *Cdh2* were assayed using qPCR. (B) As in (A) except that the quantity of N-cadherin and connexin-43 was measured using immunoblotting and normalized to tubulin in the same sample. Representative immunoblots are shown. (C, D) Granulosa cells were isolated from complexes of secondary and early antral follicles of *Fshb*$^{+/-}$ and *Fshb*$^{-/-}$ females. *Gja1* (C) and *Cdh2* (D) were quantified. (E) As in (A), except that *Gja4* and *Cdh1* were quantified in oocytes. (F, G) As in (C) except that *Gja4* (F) and *Cdh1* (G) were quantified in oocytes. (H) As in (D), except that *Ccnb1*, *Mos* and *Slbp* were quantified in oocytes of early antral follicles. (I) Representative images of EU detection assay in oocytes of early antral *Fshb*$^{+/-}$ (upper panels) and *Fshb*$^{-/-}$ (lower panels) follicles, with EU labeling in green and DRAQ5 in blue. Histogram shows quantitation of EU intensity in oocytes of *Fshb*$^{+/-}$ and *Fshb*$^{-/-}$ females. Number of oocytes examined per group is indicated. In (A) to (H), histograms show the mean and s.e.m of three or more independent replicates. (I) shows the mean and s.e.m. of 35 oocytes of each genotype. Data was analysed using single-sample (A, B, E) or two-sample *t*-test (C, D, F, G, H, I). Asterisks denote $p<0.05$.

To test whether the reduced gene expression was due to the absence of FSH, we injected eCG, which exhibits FSH activity (Murphy and Martinuk 1991), into *Fshb*$^{-/-}$ females and 48h later collected GOCs from them, as well as from age-matched non-injected *Fshb*$^{-/-}$ and *Fshb*$^{+/-}$ females, and separated the granulosa cells from the oocytes for analysis. eCG-injected *Fshb*$^{-/-}$ females contained significantly more *Gja1* and *Cdh2* in granulosa cells (Figure 21A) and *Gja4* and *Cdh1* in oocytes (Figure 21B) than non-injected *Fshb*$^{-/-}$ females. Notably, the quantity of each mRNA did not differ significantly from that of *Fshb*$^{+/-}$ females. Similar increases in the quantities of connexin-43 and N-cadherin protein were also observed (Figure 21C). These results indicated that a 2-day exposure to FSH activity *in vivo* was sufficient to increase the steady-state quantities of these mRNAs.

As a second test of the role of FSH in regulating expression of these genes, we collected secondary follicles from *Fshb*$^{-/-}$ females and cultured them for two days in the presence or absence of FSH. Follicles cultured in the presence of FSH contained significantly more *Gja1* and *Cdh2* in granulosa cells and *Gja4* and *Cdh1* in the oocyte than those cultured in its absence (Figure 21D, E). FSH also increased the steady-state level of *Gja4* and *Cdh1* in oocytes of wild-type secondary follicles of CD-1 females that were cultured *in vitro* (Figure 21F), indicating that its effect was not limited to the *Fshb*$^{-/-}$ background. These results indicate that FSH increases the

steady-state level of mRNAs encoding the major connexins and cadherins of both granulosa cells and oocytes.





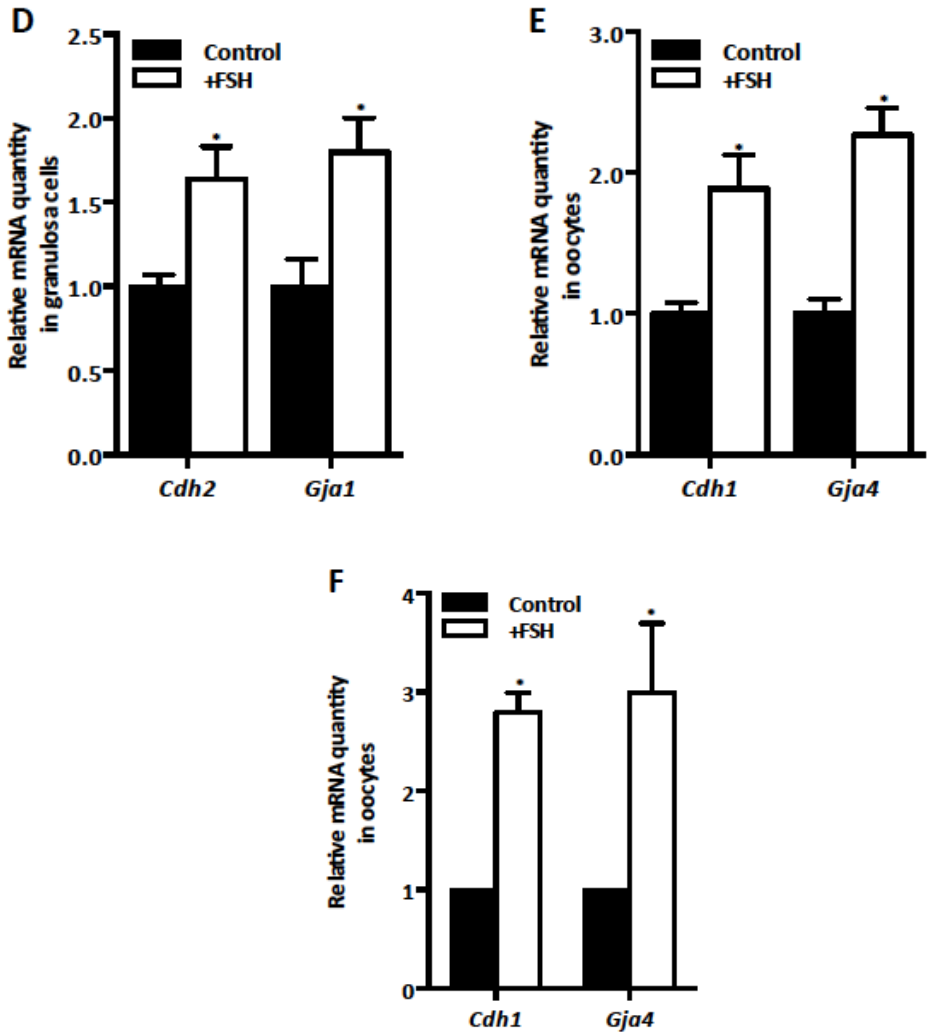


Figure 21 Supplying FSH activity *in vivo* or *in vitro* increases the expression of cadherins and connexins. (A) Granulosa cells were isolated from granulosa-oocyte complexes of *Fshb*$^{+/-}$ females, *Fshb*$^{-/-}$ females, and *Fshb*$^{-/-}$ females injected 48 h previously with eCG. *Gja1* and *Cdh2* were quantified using qPCR. (B) *Gja4* and *Cdh1* were quantified in oocytes of the same complexes as in (A). (C) As in (A), except that the quantity of proteins encoded by *Gja1* and *Cdh2* was measured using immunoblotting. Representative immunoblots are also shown. (D) *Fshb*$^{-/-}$ follicles were cultured for 2 days in the absence or presence of FSH. Granulosa cells were isolated and *Cdh2* and *Gja1* were quantified. (E) *Cdh1* and *Gja4* were quantified in oocytes of the same follicles as in (D). (F) Secondary follicles of wild-type CD-1females were cultured for 5 days in the presence or absence of FSH, then processed as in (E). Histograms show the mean and s.e.m of three or more independent replicates. Data was analysed using one-way ANOVA followed by Tukey HSD (A, B, C), two-sample *t*-test (D, E) or single-sample *t*-test (F). Different letters or asterisk above bars denote $p<0.05$.

FSH increases contact between the oocyte and its surrounding granulosa cells

The gap junctions that connect granulosa cells and the oocyte are located where the actin-rich TZPs contact the oocyte plasma membrane (Motta et al. 1994; Albertini and Rider 1994; Anderson and Albertini 1976). We reasoned that, if FSH increased gap junctional communication between the two cell types, this might be associated with an increase in the number of actin-rich TZPs. We isolated complexes from early antral follicles, which are responsive to FSH, mechanically removed the bodies of granulosa cells, and stained the oocytes using Cy3-conjugated phalloidin, which binds to F-actin. Using confocal microscopy, we measured the fluorescence of an equatorial optical section of the *zona pellucida* and normalized this to the area that was sampled to generate a value corresponding to the density of F-actin. We observed significantly greater phalloidin fluorescence in the *zona pellucida* of oocytes of $Fshb^{+/-}$ females than in those of $Fshb^{-/-}$ females (Figure 22A, B).

We then examined oocytes obtained from $Fshb^{-/-}$ females that had been injected 48 h previously with eCG as above. The phalloidin fluorescence within the *zona pellucida* of these oocytes was indistinguishable from oocytes of $Fshb^{+/-}$ females (Figure 22A, B), indicating that supplying FSH activity *in vivo* could increase the density of actin-rich TZPs. We also isolated wild-type GOCs from secondary follicles of CD-1 females, cultured them in the presence or absence of FSH, and then stained the oocytes using Cy3-phalloidin. Oocytes of GOCs that had been cultured in the presence of FSH showed increased density of phalloidin fluorescence as compared to those cultured in the absence of FSH (Figure 22C). Although we cannot exclude that FSH increased the actin content of the TZPs without changing their number, these results imply that FSH increases the density of actin-rich TZPs within the *zona pellucida*.

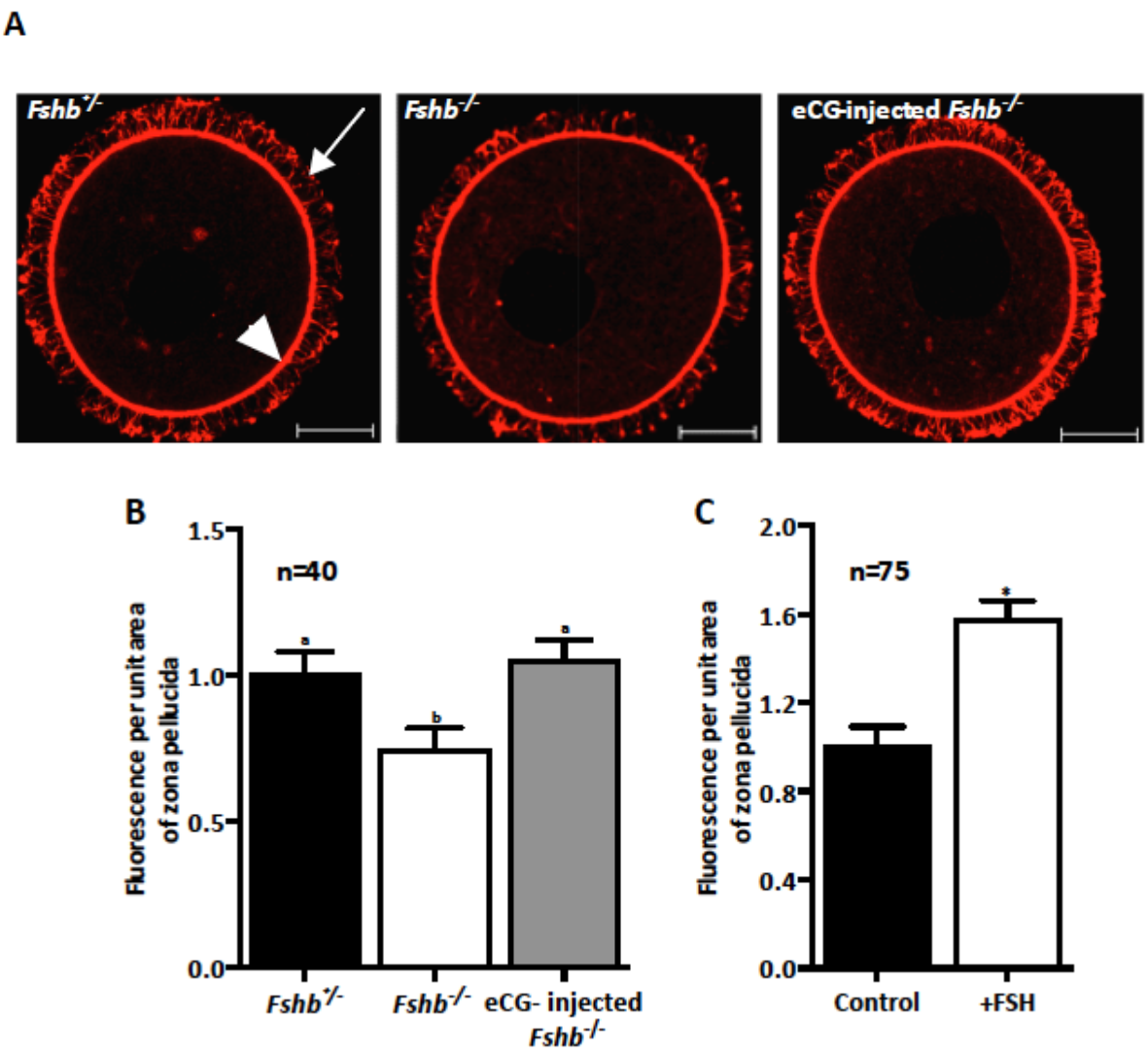


Figure 22. FSH increases the density of actin-rich TZPs i*n vivo* and *in vitro*. (A) Actin-rich TZPs (arrow) in oocytes from early antral follicles of *Fshb*$^{+/-}$, *Fshb*$^{-/-}$, and eCG-injected *Fshb*$^{-/-}$ PD 21 females, stained using Cy3-phalloidin. Granulosa cell bodies have been removed to make the TZPs easier to see. Arrowhead indicates cortical actin in oocyte. Scale bar = 20 µm. (B) Cy3-phalloidin fluorescence in the *zona pellucida* of an equatorial optical section of early antral oocytes of *Fshb*$^{+/-}$, *Fshb*$^{-/-}$, and eCG-injected *Fshb*$^{-/-}$ females was quantified using confocal microscopy. Results are expressed per unit area of the *zona*. Forty oocytes of each group were analyzed. (C) As in (B), using oocytes obtained from complexes cultured for 5 days in the absence or presence of FSH. Seventy-five oocytes of each group were analyzed. Data was evaluated using one-way ANOVA followed by Tukey HSD (B) or two-sample *t*-test (C). Different letters or asterisk above bars denote $p<0.05$.

FSH increases gap junctional communication within the follicle

Because FSH increased the steady-state levels of mRNAs encoding the major gap-junctional components of granulosa cells and oocytes and increased the density of the TZPs that harbor oocyte-granulosa gap junctions, we examined whether it increased gap junctional communication within the follicle. We first examined communication between granulosa cells, using FRAP, which has previously been used for this purpose (Santiquet et al. 2012; Johnson et al. 2002). Cells to be analyzed using FRAP are briefly incubated in the presence of calcein-AM, a dye that upon entry into cells becomes fluorescent and cell-impermeable but can be transmitted between cells via gap junctions. Using the laser of a confocal microscope, the fluorescence in a target cell is bleached and the recovery of fluorescence in the bleached cell is recorded, reflecting transfer of fluorescent calcein from neighbouring cells via gap junctions (Wade, Trosko, and Schindler 1986; Ishikawa-Ankerhold, Ankerhold, and Drummen 2012).

Granulosa-oocyte complexes were isolated from ovaries of PD 18-21 *Fshb*$^{+/-}$ and *Fshb*$^{-/-}$ females, incubated in the presence of calcein-AM, then transferred to calcein-free medium and analyzed using FRAP. We observed a slower recovery of fluorescence, indicating decreased gap junctional activity, in complexes of *Fshb*$^{-/-}$ females compared to complexes of *Fshb*$^{+/-}$ females (Figure 23B). Injection of eCG into *Fshb*$^{-/-}$ females 2 days prior to collection of the complexes, however, increased the rate of fluorescence recovery, although it remained below that observed using complexes of *Fshb*$^{+/-}$ females (Figure 23B). No fluorescence recovery was observed when FRAP was performed in the presence of a gap junction blocker, carbenoxolone (Figure 23B), confirming that recovery depended on gap junctional activity (Davidson and Baumgarten 1988)

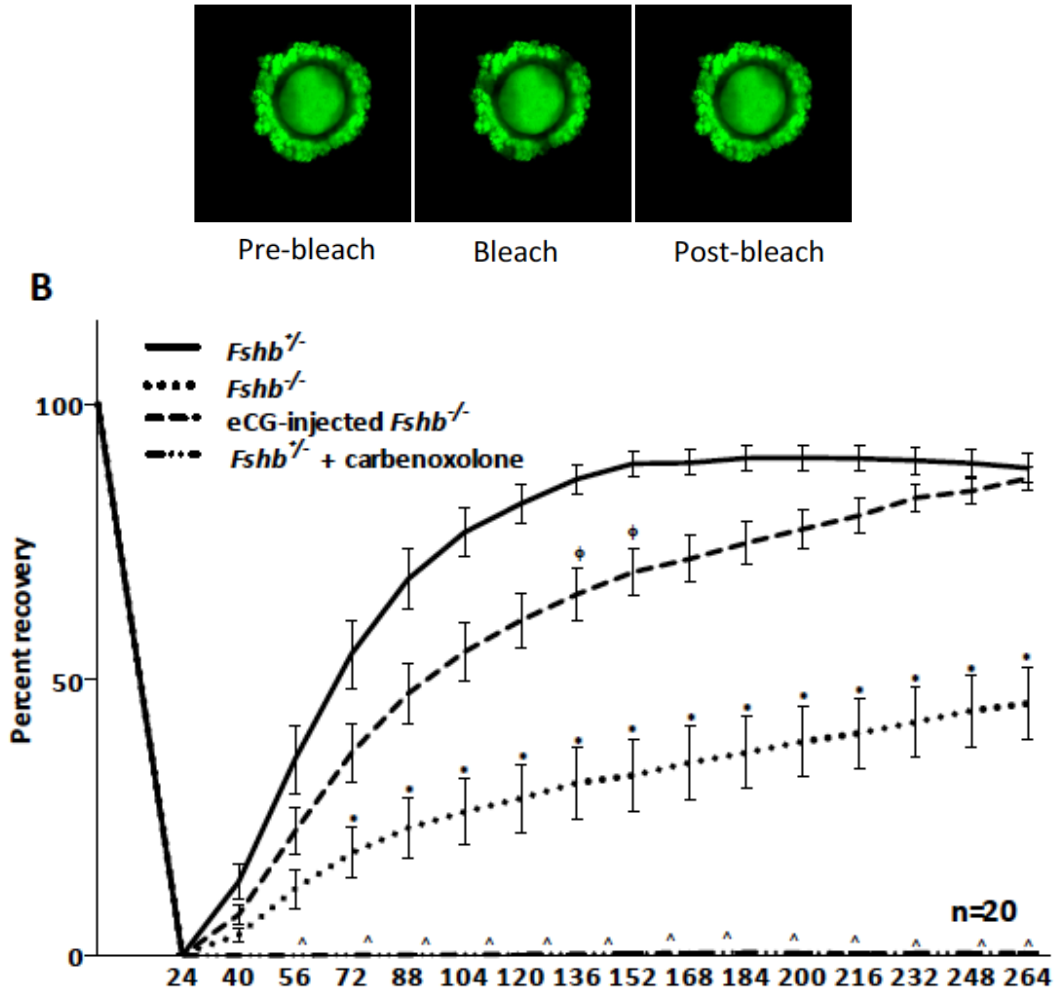
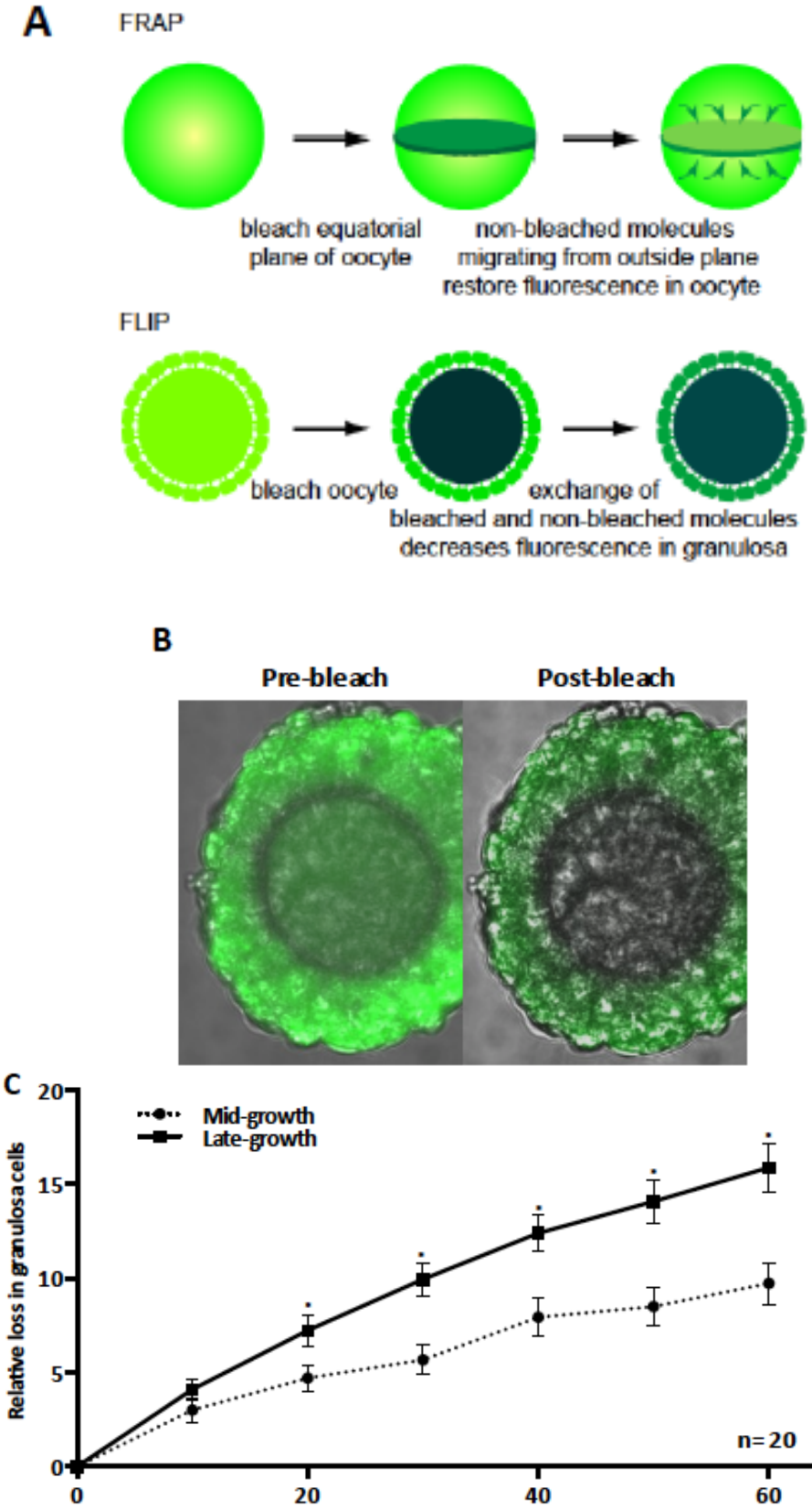


Figure 23: FSH increases gap junctional communication between granulosa cells. (A) Fluorescent images showing a GOC pre-bleach, at the time of bleching, and post-bleach. (B) FRAP was performed on granulosa-oocyte complexes isolated from *Fshb*$^{+/-}$, *Fshb*$^{-/-}$, and eCG-primed *Fshb*$^{-/-}$ females. In a fourth group *Fshb*$^{+/-}$ GOCs were pre-treated with carbenoxolone. Number of cells analyzed in each experimental group is shown. X-axis shows time in seconds. Data was analysed using one-way ANOVA followed by the Tukey HSD. For clarity, the symbols indicate where the value differs from the value for the *Fshb*$^{+/-}$ group at the same time-point.

We then examined gap junctional communication between granulosa cells and the oocyte. FRAP analysis using the oocyte is unsuitable, however, as migration of non-bleached calcein from the oocyte cytoplasm that was outside the plane of bleaching, as well as from coupled granulosa cells, could contribute to the fluorescence recovery after bleaching (Figure 24A, upper). We therefore adapted a method termed FLIP, which is commonly employed to monitor intracellular

trafficking (Ishikawa-Ankerhold, Ankerhold, and Drummen 2012). GOCs were loaded with calcein-AM and incubated to allow its transfer to the oocyte. We then photo-bleached the oocyte and recorded the loss of fluorescence in the layer of granulosa cells immediately surrounding the oocyte, which reflects migration of non-bleached calcein from granulosa cells into the oocyte (Figure 24A, lower). All oocytes were subjected to the same laser treatment conditions and bright-field images of the complexes before and after bleaching were recorded to confirm that the oocyte remained intact after bleaching (Figure 24B). We observed a more rapid loss of fluorescence in granulosa cells of early antral follicles than in those of secondary follicles (Figure 24C). This indicates that there is more extensive gap junctional communication between granulosa cells and the oocyte in early antral follicles than in secondary follicles.

To assess whether FSH contributed to this increased oocyte-granulosa communication, we performed FLIP on complexes isolated from early antral follicles of *Fshb*$^{+/-}$ and *Fshb*$^{-/-}$ females. We observed a more rapid loss of granulosa fluorescence in complexes from *Fshb*$^{+/-}$ females than in those from *Fshb*$^{-/-}$ females (Figure 24D). eCG-priming of *Fshb*$^{-/-}$ females 48 h prior to collection of the complexes, however, increased the rate of fluorescence loss to nearly that of *Fshb*$^{+/-}$ females, indicating that it increased gap junctional communication between the oocyte and granulosa cells. Very little loss of granulosa cell fluorescence occurred in the presence of carbenoxolone, confirming that it depended on gap junction activity (Figure 24D). As a second test of the link between FSH and oocyte-granulosa cell communication, we isolated GOCs from secondary follicles of wild-type CD-1females and cultured them for 5 days in the presence or absence of FSH and then assessed communication. We observed a more rapid loss of granulosa cell fluorescence in complexes that were cultured in the presence of FSH as compared to in its absence (Figure 24E). These results indicate that, both *in vivo* and *in vitro*, FSH increases gap junctional communication between the oocyte and its surrounding granulosa cells.


A
FRAP
bleach equatorial plane of oocyte
non-bleached molecules migrating from outside plane restore fluorescence in oocyte
FLIP
bleach oocyte
exchange of bleached and non-bleached molecules decreases fluorescence in granulosa
B
Pre-bleach
Post-bleach
C
Mid-growth
Late-growth
Relative loss in granulosa cells
n=20
0
5
10
15
20
0
20
40
60

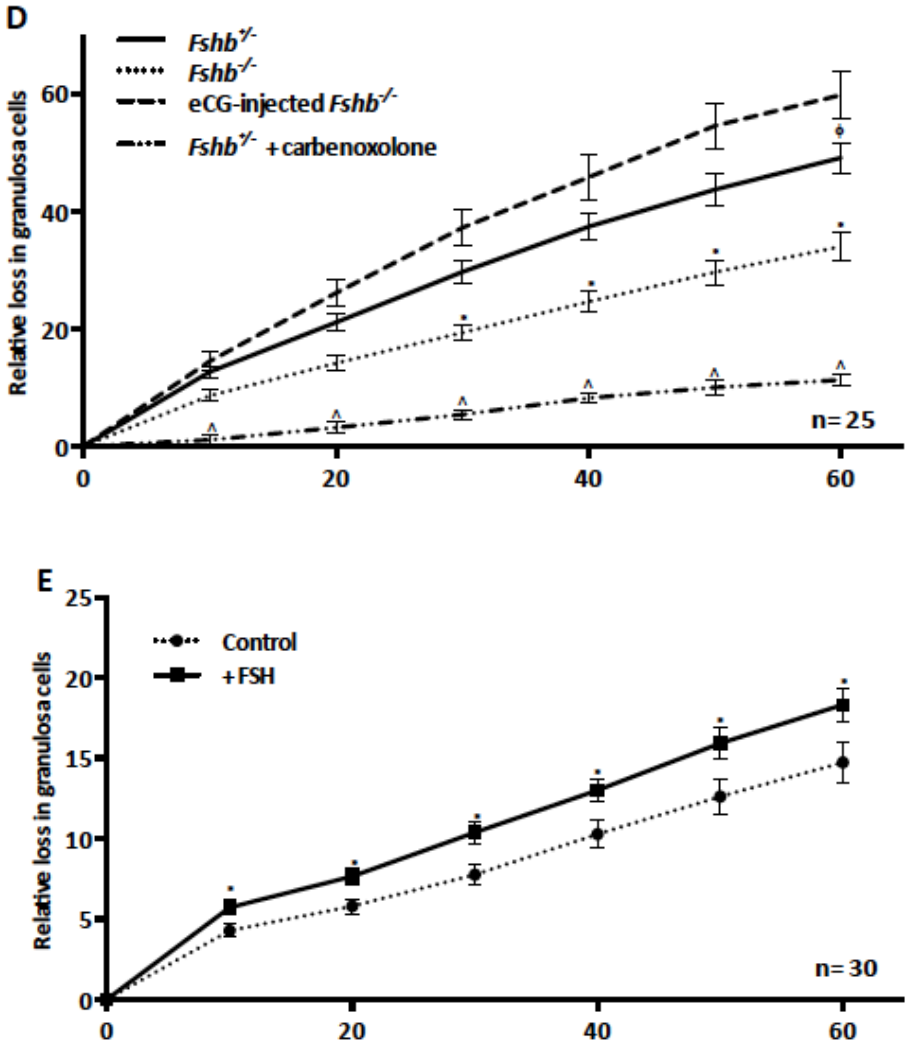


Figure 24: FSH increases gap junctional between granulosa cells and the oocyte *in vivo* and *in vitro*. (A) Upper: FRAP is not suitable to measure recovery of fluorescence in bleached oocytes, because unbleached molecules from oocyte cytoplasm above and below the plane of bleaching may contribute to fluorescence recovery. Hence, the rate of recovery does not solely reflect gap junctional communication with the neighbouring granulosa cells. Oocyte is shown in equatorial view, with granulosa not shown for clarity. Lower: Schematic diagram illustrating use of FLIP to assess oocyte-granulosa cell communication. After the oocyte is bleached, exchange of bleached and unbleached molecules through gap junctions reduces fluorescence in the layer of granulosa cells that surrounds the oocyte. Oocyte is shown in polar view. (B) Merge of brightfield and fluorescent images of GOC before and after photobleaching for FLIP. (C) FLIP was performed using granulosa-oocyte complexes obtained from secondary or early antral follicles of CD-1 females. The loss of fluorescence in the layer of granulosa cells surrounding the oocyte is plotted as a function of time. (D) FLIP was performed as in (C), using complexes of *Fshb*$^{+/-}$, *Fshb*$^{-/-}$, and eCG-primed *Fshb*$^{-/-}$ females. In a fourth group *Fshb*$^{+/-}$ GOCs were treated with carbenoxolone before FLIP. (E) FLIP was performed using GOCs of wild-type CD-1 females that had been cultured for 5 days in the presence or absence of FSH. Number of cells

analyzed per experimental group is indicated. X-axes show time in seconds. Data was analysed using one-way ANOVA followed by the Tukey HSD (D) or the two-sample *t*-test (C, E). Different letters or asterisks denote $p<0.05$. In (D), for clarity, the symbols indicate where the value differs from the value for the *Fshb*$^{+/-}$ group at the same time-point.

FSH accelerates oocyte development *in vitro.*

We then tested whether the increased gap junctional communication in the presence of FSH was associated with an acceleration of oocyte development. As FSH is known to accelerate oocyte development *in vivo* (Demeestere et al. 2012), we examined its effect *in vitro*, focusing on three well-characterized parameters – (i) the increase in diameter of the growing oocyte; (ii) the change in chromatin configuration known as the NSN to SN transition (Bellone et al. 2009; Bouniol-Baly et al. 1999), which occurs at the end of growth and is correlated with the ability of the oocyte to develop as an embryo; and (iii) the ability to undergo meiotic maturation, termed meiotic competence. We isolated GOCs from secondary follicles of wild-type CD-1 females and cultured them for 5 days in the presence or absence of FSH. At the end of the culture period, the diameter of the enclosed oocyte was measured by bright-field imaging and its chromatin configuration was assessed using a fluorescent DNA stain. To assess meiotic competence, we extended the culture period to 8 days, after which the oocytes were allowed to undergo meiotic maturation. Addition of FSH to the culture medium was associated with significant increases in oocyte diameter, the percentage of oocytes showing the SN configuration, and the percentage oocytes that could undergo germinal vesicle breakdown (Figure 25). Thus, in addition to its activity to increase gap junctional communication between the oocyte and granulosa cells, FSH also accelerates oocyte development.

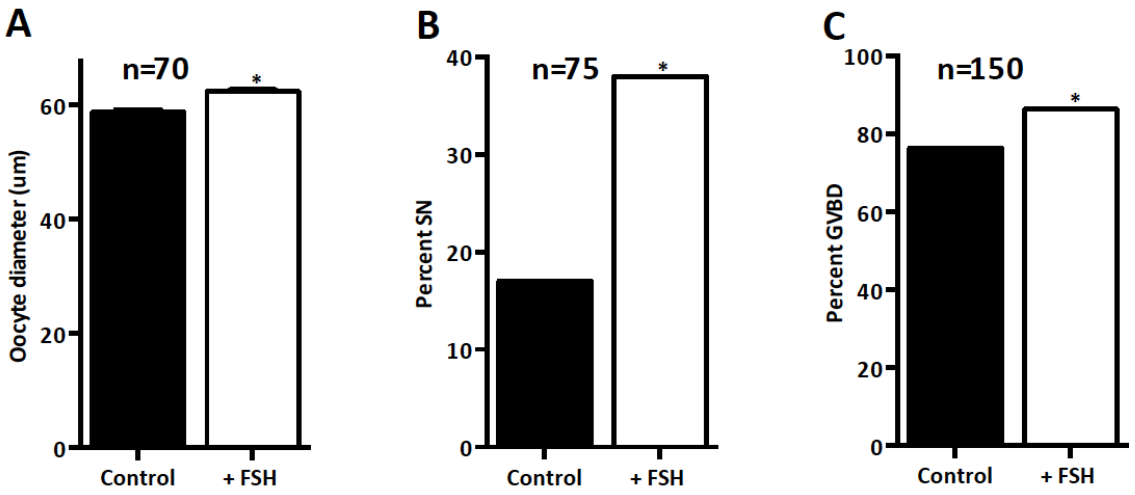


Figure 25: FSH accelerates oocyte growth and development *in vitro*. GOCs obtained from PD 10 wild-type CD-1 females were cultured for five (A, B) or eight (C) days in the presence or absence of FSH, after which the enclosed oocytes were isolated and examined. (A) Oocyte diameter. (B) Percentage of oocytes displaying the SN chromatin configuration. (C) Percentage of oocytes that were able to undergo GVBD. Number of oocytes examined per experimental group is shown. Data was analysed using two-sample *t*-test (A) or chi-square test (B, C). Asterisks denote $p<0.05$.

Discussion

The essential role of contact and communication with the somatic compartment of the gonad during germ cell development is well-established, but the factors and conditions that regulate this contact in mammals remain relatively poorly understood (Simon et al. 1997; Li and Mather 1997; Ratchford, Esguerra, and Moley 2008; Gittens and Kidder 2005). We show here that FSH increases the expression of genes encoding the principal connexins and cadherins in both granulosa cells and oocytes, increases the number of TZPs that harbor the sites of contact, and increases gap junctional communication between granulosa cells and between the oocyte and granulosa cells. FSH also accelerates oocyte development, a process known to require gap junctional communication with the granulosa. These results identify FSH as a factor that regulates germ line-somatic communication during late folliculogenesis in mammals.

Previous studies have shown that connexin-43 and N-cadherin protein and mRNA increase during folliculogenesis and that their expression is regulated by FSH (Wright et al. 2001; Wang et al. 2013; Sommersberg et al. 2000; Mora et al. 2012; Machell and Farookhi 2003; Granot and Dekel 1997; Farookhi et al. 1997). Our results confirm these earlier reports. FSH may increase the expression of connexins and cadherins through the estradiol pathway, as *Gja1* and *Cdh2* increase in the ovary upon estradiol treatment (Blaschuk and Farookhi 1989; Grummer, Traub, and Winterhager 1999; Wiesen and Midgley 1994). Also, in other cell types, *Gja1* expression is regulated by PKA activity (Yun et al. 2012). As PKA is a downstream effector of FSH, the stimulation of *Gja1* expression by FSH in granulosa cells may be PKA-dependent. Additionally, FSH has recently been shown to increase *Gja1* expression in granulosa cells through the β-catenin pathway (Wang et al. 2013). These results suggest that FSH most likely increases steady-state levels of these mRNAs by upregulating their transcription.

Although the link between FSH and increased expression, phosphorylation, and membrane localization of connexin-43 in granulosa cells is well established (see Introduction), surprisingly few studies have directly assessed the effect of FSH on gap junctional communication. FSH increased communication in a transformed cell line derived from granulosa cells of large antral follicles in the rat (Sommersberg et al. 2000). In contrast, FSH increased communication in primary cultures of granulosa cells obtained from small follicles, but not from mid-size or large

follicles, in the cow (Johnson et al. 2002). Moreover, BMP15, which is secreted by the oocyte, decreased expression of *Gja1* and connexin-43 and reduced gap junctional communication in an immortalized human granulosa cell line (Chang et al. 2014). This last observation highlights potential differences that may arise depending on the context in which communication is assayed. Our results demonstrate that FSH increases granulosa cell communication in the physiological context of the granulosa-oocyte complex. FSH also increases gap junctional communication in primary cultures of sertoli cells (Pluciennik, Joffre, and Deleze 1994), which in males are descended from the same embryonic precursor cells that in females give rise to granulosa cells. Our results taken together with the previous work suggests that FSH increases communication through both acute mechanisms including phosphorylation and translocation of connexin-43 and long-term effects on the steady-state level of the protein (Sommersberg et al. 2000; Johnson et al. 2002; Pluciennik, Joffre, and Deleze 1994).

FSH also increased the expression of *Gja4* and *Cdh1* in oocytes, even though these cells do not express the FSH receptor, and increased their gap junctional communication with the surrounding granulosa cell layer. The reduced expression of *Gja4* and *Cdh1* in oocytes of *Fshb*$^{-/-}$ females does not reflect an arrest of oocyte development in the absence of FSH, as expression of other genes tested was unaffected and previous studies have shown that these oocytes grow to full-size and acquire meiotic competence (Kumar et al. 1997; El-Hayek, Demeestere, and Clarke 2014; Demeestere et al. 2012; Burns et al. 2001). Rather, acting through granulosa cells that express its receptor, FSH increases expression of *Gja4* and *Cdh1* in the oocyte. A feedback mechanism shown to regulate connexin gene expression (Chakraborty et al. 2010) provides a potential mechanism for this indirect effect. Connexins exist in equilibrium between a cytoplasmic pool of free molecules and those assembled into gap junctions. When free connexins are assembled into new gap junctions, mRNA transcription increases to replenish the depleted cytoplasmic pool of protein. We suggest that the direct effect of FSH on *Gja1* expression in granulosa cells increases the amount of connexin-43, which is then assembled into new gap junctional plaques at the plasma membrane. These may recruit or stabilize connexin-37 in the oocyte plasma membrane as new gap junctions are assembled. The resulting depletion in free connexin-37 would in turn increase *Gja4* expression in the oocyte. It should be noted here that it is unclear whether it is connexin-37 or -43 in granulosa cells that establish gap junctions with the

oocyte, but gene-knockout studies have established that both are able to do so (Veitch et al. 2004; Gittens and Kidder 2005). A similar feedback mechanism could account for the FSH-driven increase in oocyte *Cdh1* expression during late folliculogenesis.

Using a novel assay based on photo-bleaching, we further identified FSH as a factor that increases gap junctional communication between the oocyte and granulosa cells. This increased communication is likely enabled at least in part by the increased expression of *Gja4*. The larger number of TZPs linking granulosa cells to the oocyte observed in the presence of FSH may play an important role also, as these would provide a greater granulosa cell surface area available for gap junction assembly. As discussed in the Introduction, gap junctions permit granulosa cells to supply the oocyte with a wide range of small molecules, including nucleotides and amino acids. We observed that FSH also accelerated oocyte development *in vitro*, albeit modestly, as assessed using three independent measures. Another study also reported that FSH accelerates oocyte growth *in vitro* (Thomas et al. 2005); moreover, oocytes of *Fshb*$^{-/-}$ females develop more slowly *in vivo* than those of *Fshb*$^{+/-}$ females (Kumar et al. 1997; El-Hayek, Demeestere, and Clarke 2014; Demeestere et al. 2012; Burns et al. 2001). We propose that the increased communication in the presence of FSH permits oocytes to progress more rapidly through the developmental programme of growth. Thus, by regulating gap junctional communication with granulosa cells, FSH indirectly controls the rate of oocyte development during folliculogenesis.

Although FSH increases gap junctional communication with granulosa cells and accelerates oocyte development, oocytes grown *in vitro* in the absence of FSH can be fertilized and will give rise to normal offspring (Eppig et al. 1998; Eppig et al. 2000). Thus, *in vitro*, oocytes are able to complete development normally in the absence of the FSH-driven increase in communication with granulosa cells. *In vivo*, however, oocytes of *Fshb*$^{-/-}$ females develop poorly after fertilization and very few reach the blastocyst stage (Kumar et al. 1997; El-Hayek, Demeestere, and Clarke 2014; Demeestere et al. 2012; Burns et al. 2001). The differing importance of FSH during oocyte growth *in vivo* and *in vitro* may reflect that oocyte growth *in vivo* occurs in the context of growth and differentiation of the follicle. Follicular development comprises a wide range of processes, including development of the cumulus granulosa cell layer, which undergoes a process termed expansion at the time of ovulation that is essential for the fertilizing sperm to

reach the egg; acquisition by the mural granulosa cells of the ability to undergo luteinization following ovulation; and the ovulatory process itself. We suggest that FSH, by increasing communication between the oocyte and granulosa cells, helps to coordinate development of the germ-line with that of the somatic compartments. More broadly, as FSH also increases communication between granulosa cells, it may play an essential role in ensuring that the diverse differentiation processes occurring in different cellular compartments within the growing follicle are precisely coordinated and synchronized to ensure ovulation of a fertilizable, developmentally competent egg.

Acknowledgements

We thank Nicolas Santiquet and François Richard (Université Laval) for discussions and advice regarding FRAP.

Supplemental data

Detection and quantification of Cy3-phalloidin fluorescence

Oocytes were collected from PD18 and PD 23 *Fshb*$^{+/-}$ and *Fshb*$^{-/-}$ females, fixed for 15 min at RT in freshly prepared 2% para-formaldehyde in PBS, and washed with PBST. To stain actin, cells were incubated with Cy3-conjugated 1:100 phalloidin (P1951, Sigma) in PBST, then mounted on glass slides as described in the 'Materials and Methods' section. Cells were imaged and the intensity of Cy3 fluorescence, corresponding to the TZPs within the zona pellucida, was recorded, quantified using Image J, and expressed per unit area, as described in the 'Methods' section.

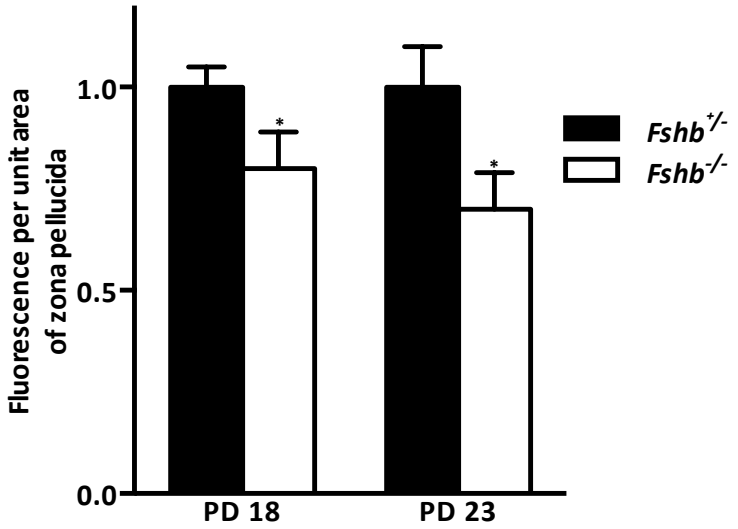


Figure 26: Cy3-phalloidin fluorescence in the *zona pellucida* of an equatorial optical section of early antral oocytes of $Fshb^{+/-}$ and $Fshb^{-/-}$ PD 18 and PD 23 females was quantified using confocal microscopy. Results are expressed per unit area of the *zona pellucids*. Fifty oocytes of each group were analyzed. Fifty oocytes of each group were analyzed. Data was evaluated using two-sample *t*-test. Asterisk above bars denotes $p<0.05$.

Detection and quantification of FM-143 fluorescence

Two distinct populations of TZPs link the granulosa cells and the oocyte: the numerous actin-rich TZPs and the less numerous tubulin-rich TZPs. Since we observed that the density of actin-rich TZPs is significantly reduced in $Fshb^{-/-}$ females, we next examined whether the total density of TZPs is affected as well. Oocytes were collected from PD 18-23 $Fshb^{+/-}$ and $Fshb^{-/-}$ females, washed with ice cold PBS and incubated in 5 μg/mL of FM-143 (Life Technologies) in ice-cold PBS for 15 min. Oocytes were then imaged, while still in the staining solution, using the 63X objective of the confocal microscope. For image analysis, the fluorescence intensity was expressed per unit area, as described in the 'Materials and Methods' section.

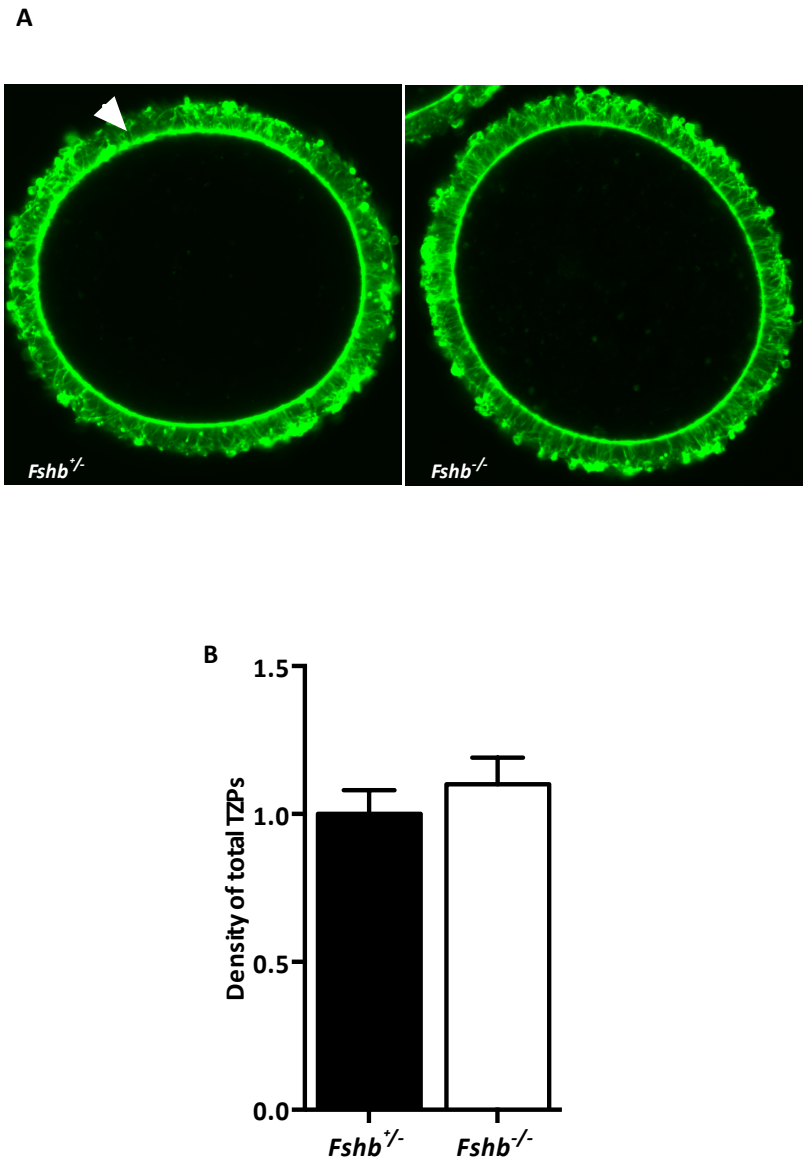


Figure 27: (A) Total TZPs (arrow) in oocytes from early antral follicles of *Fshb*$^{+/-}$ and *Fshb*$^{-/-}$ stained using FM-143. Granulosa cell bodies have been removed to make the TZPs easier to see. Scale bar = 20 µm. (B) FM-143 fluorescence in the *zona pellucida* of an equatorial optical section of early antral oocytes of *Fshb*$^{+/-}$ and *Fshb*$^{-/-}$ was quantified using confocal microscopy. Results are expressed per unit area of the *zona*. Fifty oocytes of each group were analyzed. Data was evaluated using two-sample *t*-test. Asterisk above bars denotes $p<0.05$.

Measurement of granulosa cell communication using FRAP in granulosa cell monolayers

Granulosa cells were obtained by enzymatic digestion of late-growth GOCs (of early-antral follicles) of PD 21 females, seeded onto cell-chamber slides (Becton Dickinson), and cultured for 2 days in MEM. For FRAP, cells were then incubated in MEM containing calcein-AM (Invitrogen, 1 μM, diluted 1:5000 from 5 mM stock), for 15 min, which was then replaced by calcein-free MEM. A granulosa cell fully surrounded by other granulosa cells was bleached by a single hit of 50 iterations of the Argon laser (100% strength) of a LSM 510 Meta confocal microscope. In each monolayer, 4 individual cells were bleached; 5 monolayers per experimental group were used for a total number of 20 bleached cells. Each cell was imaged 24 sec before bleaching, at the time of bleaching, and up to 2.4 min after bleaching, at 11 sec intervals. For blocking gap junctions during the FRAP assay, monolayers were incubated in MEM containing 100μM carbenoxolone (Sigma) for 30 min then loaded with calcein-AM in medium containing carbenoxolone.

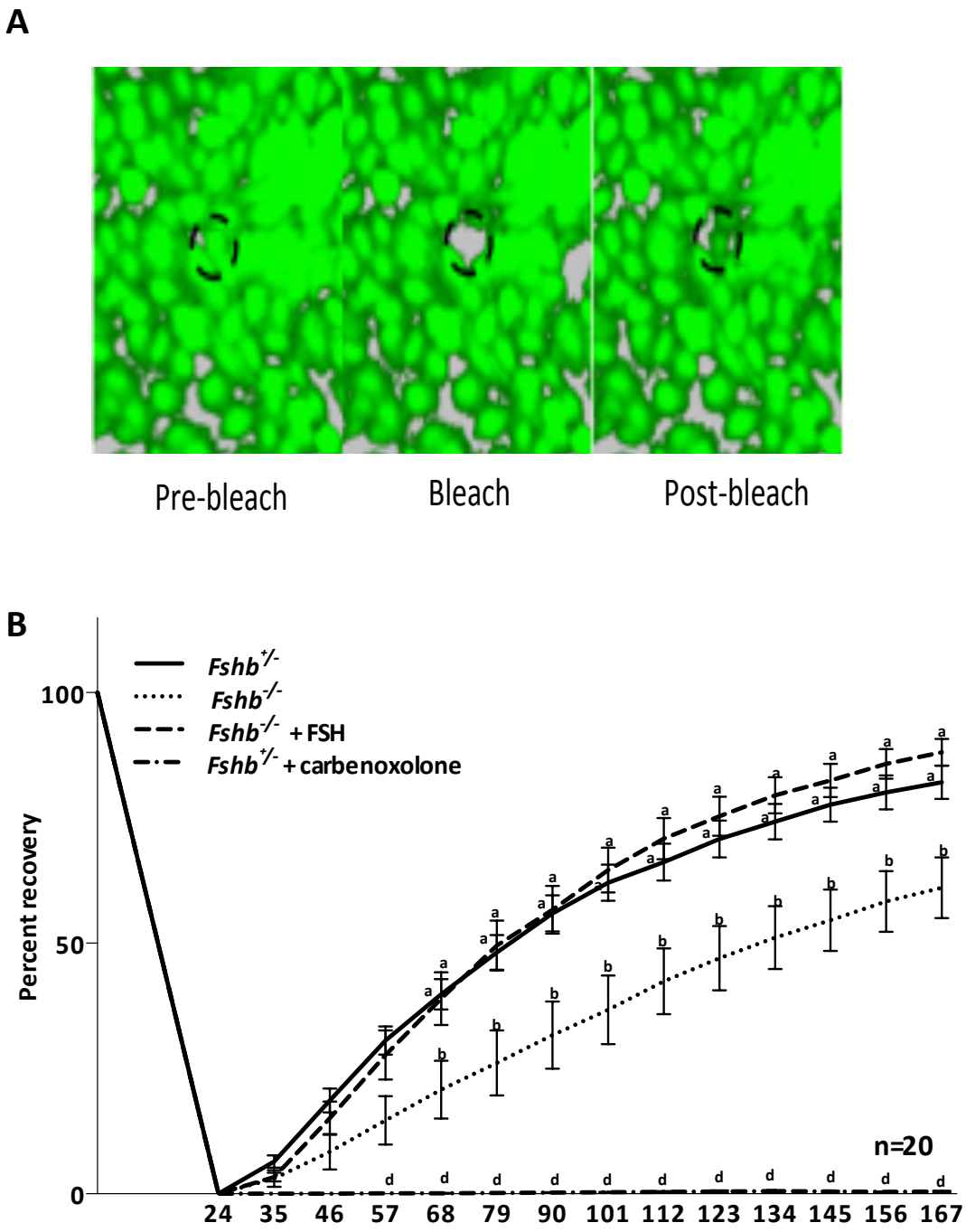


Figure 28: (A) Pre-bleach, bleach, and post-bleach fluorescent images, illustrating FRAP analysis on granulosa cell monolayer after calcein-AM loading. (B) FRAP analysis was performed on monolayers of *Fshb*$^{+/-}$ or *Fshb*$^{-/-}$ granulosa cells, as well as *Fshb*$^{-/-}$ granulosa cells cultured with 10 mIU/ml FSH, and *Fshb*$^{+/-}$ granulosa cells pre-treated with carbenoxolone. The relative amount of fluorescence in the photo-bleached cell is plotted as a function of time (seconds).

Immunoblotting for Ser-368 phosphorylated connexin-43

Ser368 is a site on connexin-43 whose phosphorylation is associated with increased junction permeability. We aimed to determine the levels of phospho-Ser368 connexin-43 (p-connexin-43) in *Fshb*$^{+/-}$ and *Fshb*$^{-/-}$ granulosa cells. For analysis of connexin-43 phosphorylation, and in order to obtain equal loading of total connexin-43 levels in *Fshb*$^{+/-}$ and *Fshb*$^{-/-}$ samples, 50 *Fshb*$^{+/-}$ and 100 *Fshb*$^{-/-}$ granulosa-oocyte complexes (based on results shown in Figure 20B) were collected from PD 18-21 females and their granulosa cells were mechanically isolated as described in 'Materials and Methods' section. Granulosa cells were suspended in loading buffer, heat-denatured, and then divided into two aliquots, one of which was used to detect total connexin-43 (C6219, Sigma, 1:8000) and tubulin (T8203, Sigma, 1:2000) and the other to detect Ser-368 phosphorylated connexin-43 (3511, Cell Signaling Technology, Whitby, ON 1:1000). p-connexin-43 was normalized to connexin-43 of the same sample run in a separate lane of the same gel, and connexin-43 was further normalized to tubulin of the same sample.

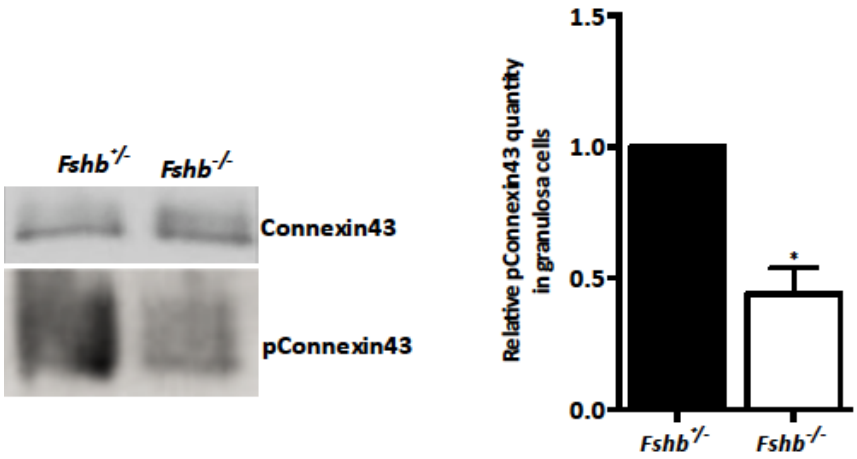


Figure 29: Quantity of p-connexin43 relative to that of total connexin-43 in granulosa cells obtained from early antral follicles of *Fshb*$^{+/-}$ and *Fshb*$^{-/-}$ females. Histogram represents mean and s.e.m of 3 independent experiments. Results were analyzed using a single-sample t-test. Asterisk above bar denotes $p<0.05$. Representative immunoblot is shown.

Measurement of calcein uptake into GOCs

To assess coupling of oocytes with granulosa cells in secondary and early antral follicles, complexes consisting of an oocyte and its surrounding granulosa cells were isolated from the follicles, and incubated in the presence of 1 μM calcein-AM for 15 min, followed by a 15 min incubation in calcein-free MEM to allow dye transfer from granulosa cells into oocyte. GOCs were then imaged using the confocal microscope. To block gap junctions in GOCs of early antral follicles, carbenoxolone (100μM) was added to the medium containing calcein-AM and the post-calcein wash. An equatorial section was visually identified and, using a long path 505 filter, the intensity of fluorescence in the area corresponding to the oocyte was recorded, then quantified and divided by the total area of the oocyte to obtain the fluorescence per unit area.

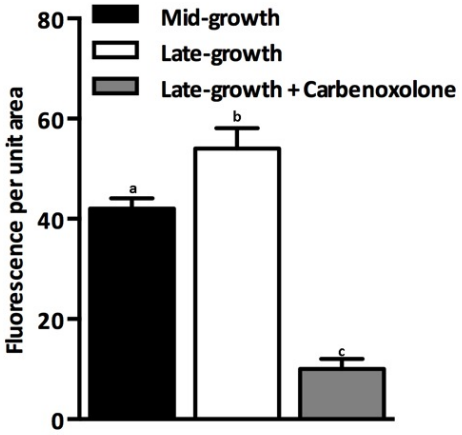


Figure 30: Fluorescence in an equatorial optical section of the oocyte normalized to the oocyte surface area of the section in early- and late-growth GOCs as well as late-growth GOCs that had been pre-treated with the gap junction blocker carbenoxolone. Twenty GOCs were used per experimental group. Data was analysed using one-way ANOVA followed by the Tukey HSD. Different letters denote $p<0.05$.

Chapter 4

Manuscript III
Oocyte-driven remodeling of the somatic microenvironment enables intra-follicular communication

El-Hayek S, Yang Q, and Clarke HJ

Preface

In the experiments described in manuscript II, we found that the density and distribution of TZPs were altered in *Fshb*$^{-/-}$ females. This suggested that these structures, which have been long assumed to be passively generated during oocyte growth (Chiquoine 1960), might be actively elaborated from the granulosa cells surrounding the oocyte. Intriguingly, TZPs are disorganized and reduced in number in oocytes of *Gdf9*$^{-/-}$ females (Carabatsos et al. 1998). Moreover, although GDF9 signalling is not required in the oocyte itself (Li et al. 2012), oocytes of young *Gdf9*$^{-/-}$ females lack developmental competence. This demonstrates that GDF9 induces effects on the granulosa cells, which in turn regulate oocyte competence. The mechanism underlying these effects, however, remains unknown. To further explore this, the following objectives were designed:

IIIa) Examine the dynamics of TZPs during growth

IIIb) Determine the role that GDF9 plays in the extension of TZPs

This work is discussed in Chapter 4 (Manuscript III).

Abstract

Germ cells develop in a microenvironment generated by the somatic compartment of the gonad. In female mammals, somatic granulosa cells enclose the developing oocyte. As the oocyte grows, it secretes an extracellular matrix termed the *zona pellucida* that separates it from the granulosa cells. The granulosa bypass this physical barrier by means of narrow cytoplasmic extensions, termed transzonal projections (TZPs), which traverse the *zona pellucida* and contact the oocyte. TZPs have been identified in all mammalian species studied to date, and exist in two populations: abundant actin-rich TZPs and rare tubulin-TZPs. As the sole means by which the granulosa cells establish physical contact with the oocyte, TZPs play a fundamental and indispensable role in oocyte development. However, despite their crucial role in fertility, TZPs have aroused little interest, likely because they have been assumed to form passively as the thickening *zona pellucida* pushes the granulosa cell bodies away from the oocyte. This model predicts that the number of TZPs should not increase during oocyte development. In contrast, we found that actin-rich TZPs increase both in number and density as the oocyte grows. This demonstrates that TZPs are newly generated as oocytes develop, notably after the *zona pellucida* has formed. Intriguingly, we found that the granulosa cells adjacent to the oocyte, as well as actin-rich TZPs, express Myosin (MYO)10, Dishevelled-associated activator of Morphogenesis (DAAM) 1, and fascin, which are actin assembly factors required for the formation of filopodia. Moreover, signalling from the oocyte itself, including the oocyte-derived paracrine factor growth differentiation factor 9(GDF9), promotes the expression of mRNAs encoding these factors and the generation of actin-rich TZPs by granulosa cells adjacent to the oocyte. We propose that actin-rich TZPs are specialized filopodia that are extended by granulosa cells and directed by oocyte-derived paracrine factors towards the oocyte, where they establish contact and communication required for normal oocyte development. Our data identifies an important parameter to monitor in future work aimed at growing oocytes *in vitro* to preserve human and animal fertility.

Introduction

Germ cells develop in a microenvironment, often termed a niche, generated by the somatic compartment of the gonad. Interaction with this somatic compartment is essential for the development of all germ cells, as somatic cells supply nutrients that sustain their basal metabolic activity and send signals that regulate their development (Buccione, Schroeder, and Eppig 1990; Dumesic et al. 2015; Matunis, Stine, and de Cuevas 2012; Oatley and Brinster 2012; Sanchez and Smitz 2012; Starich, Hall, and Greenstein 2014). In female mammals, the oocyte grows and develops within the ovarian follicle surrounded by somatic granulosa cells. The two cell types become associated when a small number of granulosa cells enclose an oocyte forming a primordial follicle. Primordial follicles then enter a prolonged growth phase in preparation for ovulation and fertilization, throughout which the oocyte maintains contact and communication with the granulosa cells (Eppig 1991; Gilchrist, Ritter, and Armstrong 2004; Kidder and Vanderhyden 2010). The growing oocyte increases more than 200-fold in volume (Griffin et al. 2006) as it accumulates an enormous quantity of mRNAs, proteins and organelles, establishes maternal genomic imprints, and undergoes chromatin and ultrastructural remodeling, in preparation for fertilization events and early embryonic development (Bachvarova 1985; De La Fuente and Eppig 2001; Lucifero et al. 2002; Mahrous, Yang, and Clarke 2012).

The intense metabolic activity required to sustain this complex program of differentiation is supported by nucleotides, amino acids, and energy substrates transferred to the growing oocyte by the granulosa cells via the communicating gap junctions (Eppig 1991; Eppig et al. 2005; Wigglesworth et al. 2013; Brower and Schultz 1982). The granulosa cells also synthesize lipids required by the growing oocyte, maintain its intracellular pH, and transfer small molecules that prevent precocious entry of the oocyte into the final phase of development, termed meiotic maturation (FitzHarris and Baltz 2009; Norris et al. 2009; Vaccari et al. 2009; Su et al. 2008). This inter-cellular communication between the germ cell and the somatic compartment is therefore essential for the growth, development, and survival of the oocyte, as well as its ability to undergo fertilization and develop into a healthy embryo. It is thus not surprising that when oocyte-granulosa cell communication is disrupted, such as by targeted deletion of gap junctional components (Eppig 1979a; Li and Mather 1997; Simon et al. 1997), oocyte development is severely abnormal and the individual is infertile.

Growing oocytes and granulosa cells also communicate via growth factors. Membrane-bound KitL on the granulosa cells stimulates oocyte growth *in vitro* (Thomas et al. 2008; Packer et al. 1994). Moreover, GDF9 and BMP15, members of the TGFβ superfamily secreted by the oocyte, are required for proliferation and differentiation of the granulosa cells (Peng et al. 2013; Dube et al. 1998; Dong et al. 1996). Strikingly, although genetic deletion of downstream effectors has shown that GDF9 signalling in the oocyte itself is not required (Li et al. 2012), oocytes of *Gdf9*$^{-/-}$ mice develop abnormally and cannot give rise to embryos (Carabatsos et al. 1998; Dong et al. 1996; Elvin, Clark, et al. 1999). This observation not only emphasizes the importance of bidirectional communication between the oocyte and granulosa cells, but also demonstrates that development of a healthy oocyte requires GDF9-dependent processes in the granulosa cells. The nature of these processes, however, remains unknown.

At the primary follicle stage, when the oocyte diameter is approximately 30 μm in the mouse (Griffin et al. 2006), it secretes glycoproteins that become assembled into an extracellular matrix, termed the *zona pellucida*, that gradually envelopes the oocyte entirely, thereby physically separating it from the adjacent granulosa cells (Chiquoine 1960). The *zona pellucida*, which prevents polyspermic fertilization and protects the embryo prior to implantation, progressively thickens to a final diameter of ~7 μm in mice and ~16 μm in humans, after which it persists throughout oocyte growth (Avella, Xiong, and Dean 2013; Green 1997; Dean 1992; Wassarman and Mortillo 1991; Wassarman 1988). To permit contact and communication between the oocyte and granulosa cells across this barrier, thin cytoplasmic projections, termed TZPs, extend from the granulosa cells adjacent to the oocyte through the *zona pellucida* and contact the oocyte plasma membrane. TZPs have been identified in all mammalian species studied to date, including rabbit, guniea pig, mouse, cat, sheep, tiger, cow, monkey, hamster, pig, and human (Adams and Hertig 1964; Anderson and Albertini 1976; Anderson and Beams 1960; Cran, Moor, and Hay 1980; De Lesegno et al. 2008; Fleming and Saacke 1972; Franchi 1960; Hertig and Adams 1967; Hope 1965; Odor 1960; Sotelo and Porter 1959; Szollosi and Hunter 1973; Tesoriero 1981; Yamada 1957; Zamboni 1970; Gjorret et al. 2002; Weakley 1966). Morphologically similar structures have been identified in non-mammalian species (Pascucci et al. 1996; Kessel et al. 1985; Schroeder 1981). At their tips, TZPs harbour adherens and gap

junctions that couple the granulosa cells to the oocyte (Anderson and Albertini 1976; Gilula, Epstein, and Beers 1978; Sotelo and Porter 1959; Zamboni 1970, 1974). Most TZPs are rich in actin filaments; in addition, a smaller population of tubulin-rich TZPs also exists (Albertini and Rider 1994; De Smedt and Szollosi 1991). The TZPs thus act as a bridge between the granulosa cells and the growing oocyte, enabling them to physically communicate.

As the sole means by which the granulosa cells establish contact-dependent communication with the oocyte, TZPs play a fundamental and indispensable role in oocyte development. Despite their crucial role in fertility, TZPs have aroused little interest, likely because they have been assumed to form passively as the thickening *zona* pushes the granulosa cell bodies away from the oocyte, gradually giving rise to slender elongated extensions. This model predicts that the number of TZPs should not increase substantially during oocyte growth, as granulosa cells that are born by cell division after the deposition of the *zona* would not exhibit these extensions. This passive generation of TZPs also implies that if these extensions are damaged or broken they may not be repaired or regenerated.

Because these intercellular bridges have received so little attention, despite their indispensable role, we re-examined their origin and function. We report that actin-rich TZPs increase both in number and density as the oocyte grows. This thus implies that new TZPs are actively generated as oocytes grow. Granulosa cells adjacent to the oocyte or the actin-rich TZPs themselves express proteins previously implicated in actin assembly in filopodia, including myosin 10 (MYO10), fascin, and dishevelled-associated activator of morphogenesis 1 (DAAM1). Intriguingly, GDF9 signalling maintains the number of actin-rich TZPs and increases the expression of mRNAs encoding these actin-assembly factors. Our data thus suggests that actin-rich TZPs are actively generated during oogenesis, under the instruction of the oocyte itself. The novel concept that these intercellular bridges, which are essential to produce a healthy oocyte, are dynamically regulated during growth sheds the light on potential directions for diagnosing and treating infertility, including the possibility that TZPs that become damaged during procedures such as cryopreservation may be repaired and thereby improve the quality of the enclosed oocyte.

Materials and Methods

Mice

All experiments were performed in compliance with the regulations and policies of the Canadian Council on Animal Care and were approved by the Animal Care Committee of the Royal Victoria Hospital. CD-1 mice were obtained from Charles River (St Constant, QC). For some experiments, females at PD 19 were given an intraperitoneal injection of 5 IU of eCG (Sigma, Windsor, ON), and used at PD 21.

Collection and culture of cells

Ovaries were removed from CD-1 females, transferred to MEM (Life Technologies, Burlington, ON), supplemented with sodium pyruvate (0.28 mM; Sigma), penicillin G (63 μg/ml; Sigma), streptomycin (50 μg/ml; Sigma), and BSA (3 mg/ml; Sigma) at 37°C in air, and dissected using 30G1/2 needles. For all experiments, cells were collected and manipulated in MEM buffered using Hepes (MEM-H, pH 7.2), and were incubated in MEM buffered using $NaHCO_3$ (MEM) in an atmosphere of 5% CO_2 in air. Taking advantage of the synchronous follicular growth that occurs during the first three weeks of post-natal life in mice, secondary follicles containing mid-growth oocytes (50-60 μm diameter) and early antral follicles containing late-growth oocytes (60-75 μm diameter) were obtained from females at PD 10-12 and PD 18-21, respectively. To obtain GOCs containing mid-growth oocytes, ovaries from PD 10-12 females were dissected into several fragments and incubated in MEM-H supplemented with collagenase (10 μg/ml; Cedarlane, Burlington, ON) and DNase I (10 μg/ml; Sigma) at 37^{o}C in air. At 2- to 3-minute intervals, the fragments were gently pipetted to disrupt them. Individual GOCs were collected using a mouth-controlled micropipette and transferred to fresh medium (Demeestere et al. 2012). To obtain COCs containing late-growth oocytes, follicles on the surface of ovaries obtained from PD 18-21 females were punctured using 30G1/2 needles and the released complexes were collected using a mouth-controlled micropipette and transferred to a fresh dish of medium. Granulosa-free oocytes were obtained by further manipulating GOCs or COCs using a fine-bore mouth-controlled micropipette.

For *in vitro* growth experiments, GOCs were transferred to type I collagen 3.0 micron inserts (Becton-Dickinson, Mississauga, ON) in 24-well plates containing 750 μl of pre-equilibrated

serum-free MEM supplemented with ITS (10 μg/mL insulin, 5.5 μg/mL transferrin and 5 ng/mL selenium; Sigma), cilostamide (10 μM, Sigma), estradiol (10 nM; Sigma) and FSH (10 mIU/ml; EMD Serono, Mississauga, ON). For cultures longer than 3 days, two-thirds of the medium was replaced every third day. In some experiments, GDF9 (100ng/mL, R&D systems, Minneapolis, MN) or SB431542 (10 μM, Sigma) was added to the medium. For oocytectomy, COCs were collected and oocytectomized using fine glass needles. Oocytectomized complexes (OOX) were collected fresh, cultured overnight, or cultured overnight with GDF9, then analyzed.

Immunofluorescence and image analysis

To visualize total TZPs, oocytes were pre-washed with ice cold PBS and then incubated in 5 μg/mL of FM-143 (Life Technologies) in ice-cold PBS for 15 minutes. Oocytes were then imaged, while still in the staining solution in Fluorodish cell culture dishes (World Precision Instruments, Sarasota, FL). To detect fascin, cells were fixed in methanol for 5 min at -20 ^{o}C, washed with PBST, then incubated overnight in anti-fascin (1:100, Ab78487, Abcam, Toronto, ON). The following day, after PBST washes, oocytes were incubated for 1 hr at RT in anti-mouse Alexa 647 secondary antibody (1:100; Life Technologies). For all other stainings, oocytes were fixed for 15 min at RT in freshly prepared 2% *para*-formaldehyde in PBS, then washed with PBST. To stain actin, oocytes were incubated for 1 hr at RT in Cy3-conjugated phalloidin (P1951, Sigma) diluted 1:100 in PBST. To stain tubulin, cells were incubated overnight with anti-tubulin (1:100; T8203, Sigma). The following day, after PBST washes, oocytes were incubated for 1 hr at RT in 1:100 anti-mouse Alexa 647 secondary antibody. To detect MYO10 and DAAM1 cells were incubated overnight with anti-MYO10 (1:100, HPA024223, Sigma; 1:200, Novus Biologicals, Oakville, ON) or anti-DAAM1,(1:100, sc-55929, Santa Cruz Biotechnology, Dallas, Tx), respectively. The following day, after PBST washes, oocytes were incubated for 1 hr at RT in anti-rabbit or anti-goat Alexa 488 secondary antibody (1:100, Life Technologies), respectively, with Cy3-conjugated phalloidin. To mount the oocytes, a 9 mm x 0.12 mm spacer (GBL654008, Sigma) was attached to a glass microscope slide. A 2 μl drop of PBS was placed in the centre of the spacer and covered with 20 μl of mineral oil. Oocytes were then transferred into the drop of PBS and a cover slip was placed on top. Images were taken using the 63x objective of the confocal microscope.

To measure the diameter of oocytes, pseudo-brightfield images were recorded using a CLSM 510 confocal microscope (Zeiss, Toronto, ON) and analyzed using the software provided by the manufacturer. To quantify the number of actin, tubulin, or total TZPs, images were analyzed using Image J (National Institutes of Health, Bethesda, MD). We counted the number of peaks of fluorescence above a threshold (defined as 20% of intensity in oocyte's cortex in a given image) in 4 arcs (90^o apart) around the oocyte's circumference, each of 10 μm length. The average number of peaks per arc was then calculated, thus generating a value corresponding to the density of the TZPs within each oocyte. We then converted this to an estimate of the total number of TZPs in the circumference of the *zona*, based on the diameter of the oocyte.

To determine the MYO10 foci count, the "Particle analysis" tool on ImageJ was used to determine the number of foci at the base of the granulosa cells, by screening for particles that have a size greater than 0.05 μm^2. To examine colocalization, an ImageJ plugin, "Coloc2", was used to determine Pearson's colocalization coefficient, measuring the overlay between red (phalloidin) staining and green (anti-DAAM1) staining within the *zona pellucida*.

Transmission electron microscopy

Preparation and tissue processing for transmission electron microscopy was performed by a histology core service at McGill University. Ovaries of PD 10 and 15 CD-1 mice were fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer overnight at 4^oC, then processed for sectioning. Sections were cut at a 90-100 nm thickness, and images were obtained using a FEI Tecnai 12 transmission electron microscope (TEM) equipped with an AMT XR80C 8 megapixel CCD camera.

Immunohistochemistry

Ovaries of PD 5, 10, 15, and 21 CD-1 mice were fixed overnight at 4^oC in freshly prepared 4% *para*-formaldehyde in phosphate-buffered saline (PBS, pH 7.5) with continuous agitation, and then embedded in paraffin. Sections were cut at 5 μm, deparaffinized and rehydrated, then boiled for 40 min in Tris-EDTA (pH 9.0). After cooling to RT, slides were blocked with 1.35% goat serum in PBST for 30 min at RT in a humidified chamber. Following PBST washes, slides were incubated at 4^oC overnight with anti-MYO10 diluted in blocking buffer (1:100). Slides were washed in PBST and incubated for 1 hr at RT with 1:100 anti-rabbit Alexa 488, anti-mouse

Alexa 647, or anti-goat Alexa 488. Slides were then washed and mounted in Mowiol (Sigma), and examined using the CLSM 510 microscope.

RNA extraction and quantitative real-time PCR

GOCs, COCs, or OOX were used for total RNA extraction using a Picopure RNA isolation kit (Life Technologies), according to the manufacturer's instructions. RNA was eluted in 10 µl of the provided elution buffer. SuperScript II Reverse Transcription kit (Life Technologies) was used to generate cDNA. One µl of deoxyribonucleotides (10 mM each) and 200 ng of random primers were added to the 10 µl of RNA. The mixture was then incubated for 5 minat 65°C, followed by the addition of 4 µl of 5x first strand synthesis buffer, 2 µl of 0.1 M dithiothreitol and 1 µl RNaseOUT. After a 2 min incubation at 25°C, followed by the addition of 1 µl of SuperScript II, the mixture was then successively incubated for 10, 50, and 15 min at 25°C, 42°C, and 70°C respectively. PCR amplification was performed using a Corbett Rotorgene 6000 (Montréal Biotech, Montreal, QC). Each reaction contained 4 µl of EvaGreen Mix (Montréal Biotech), 13 µl of UltraPure DNase/RNase-free distilled water (Life Technologies), 1 µl of 10 µM primers and 2 µl of cDNA (diluted by 1:20 from original stock). Primers were designed using Primer-BLAST (National Institutes of Health) and obtained from Sigma. Primer sequences are given in Table 3. For each primer pair, a standard curve was generated using serial dilutions of cDNA prepared from ovarian RNA and used to determine the efficiency of amplification. Melt-curve analysis and electrophoresis of amplified products confirmed that only a single product of the expected size was generated. Data was analyzed using software provided by the manufacturer. Relative quantities of amplified product were calculated according to $2^{-\Delta\Delta CT}$ method, using *Actb* (actin) for normalization.

Gene transcript	Primer Sequence
Actb	F: 5'-GGCTGTATTCCCCTCCATCG-3' R: 5'-CCAGTTGGTAACAATGCCATGT-3'
Daam1	F: 5'-GCGGCTGCTCAGAGTATAGAAA-3' R: 5'-AAACATGGCTTCCCTGTGTTTG -3'
Fscn1	F: 5'-AGAACGCCAGCTGCTACTTT-3' R: 5'-CGAGGAATCACTACCCACCG -3'
Myo10	F: 5'-TCCAGACAGACTATGGGCAGG-3' R: 5'-GGAAGCCATGTCGTCCACG -3'

Table 3: Primer sequences for quantitative PCR analysis.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.0. Single-sample *t*-test, two-sample *t*-test, Chi-square test, or one-way ANOVA followed by Tukey HSD test was used, depending on the experiment. $P<0.05$ was considered significant. For single-sample *t*-test, two-sample *t*-test, and one-way ANOVA, reported values represent the mean ± standard error of the mean (s.e.m.) of three or more independent experiments.

Results

Two distinct populations of TZPs link the granulosa cells with the oocytes and allow the formation of cell-cell junctions.

TZPs can be visualized by electron and fluorescence microscopy as elongated structures that emanate from granulosa cells and extend through the *zona pellucida*, eventually making contact with the oocyte plasma membrane (Figure 31A, B). Two populations of TZPs can be detected in growing as well as fully-grown oocytes: numerous actin-rich TZPs (Figure 31C) and scarce tubulin-rich TZPs cell (Figure 31D). Co-staining oocytes with both Cy3-conjugated phalloidin and tubulin antibody demonstrates that these two populations are distinct (Figure 31E). Notably, although they are extensions of the granulosa cells, TZPs can be still visualized in granulosa-free oocytes, as TZPs shafts remain embedded within the *zona pellucida* after removal of granulosa cell bodies surrounding the oocyte. As previously described (Anderson and Albertini 1976; Gilula, Epstein, and Beers 1978; Sotelo and Porter 1959; Zamboni 1970, 1974), at the contact sites that TZPs make with the oocyte plasma membrane, cell-cell junctions form (Figure 31F). Connexin-based gap juctions as well as cadherin-based adherens junctions assemble at these contact sites, thus linking the oocyte with its surrounding granulosa cells.

New actin-rich TZPs are generated during oocyte growth

To examine the dynamics of TZP formation during oocyte growth, oocytes of different diameters were collected from PD 8-21 females. Oocytes were then stained with FM-143, a membrane dye that allows visualization of all TZPs. Alternatively, oocytes were fixed and stained with Cy3-

conjugated phalloidin or with anti-tubulin antibody to visualize for actin-rich and tubulin-rich TZPs, respectively. After imaging, the number of TZPs around the circumference of the zona pellucida of each oocyte was plotted as a function of its diameter. As shown in Figure 32A, the number of total TZPs progressively increased as the oocyte diameter increased, such that the fully-grown oocytes (>75 μm) had the highest number of TZPs. The same relationship was observed when the number of actin-rich TZPs was plotted as a function of oocyte diameter, (Figure 32B). Notably, the number of total TZPs and actin-rich TZPs, as determined using FM-143 and phalloidin staining, respectively, were very similar. Interestingly, the density of actin-rich TZPs, which was determined as the number of those TZPs per 10 μm, also increases as the oocytes diameter increases (Figure 33A). In striking contrast, the number of tubulin-rich TZPs did not detectably change during oocyte growth (Figure 32C). It is also noteworthy that the number of tubulin-rich TZPs that could be detected in an oocyte was less than one-tenth that of actin-rich TZPs, confirming the relative scarcity of tubulin-rich TZPs.

We next examined whether this increase in actin-rich TZPs occurred during *in vitro* growth of oocytes. GOCs were isolated by enzymatic digestion of PD 10 ovaries and either fixed immediately or cultured for 5 days. Then the diameter of each oocyte in the non-cultured and cultured groups, as well as the number of TZPs, was recorded. We observed that in addition to a significant increase in diameter, the oocytes of cultured controls had a significantly higher density as well as total count of actin-TZPs in the circumference of their *zona pellucida* (Figure 33B). These results demonstrate that, during oocyte growth both *in vivo* and *in vitro*, the granulosa cells surrounding the oocyte make new actin-rich TZPs and extend them towards the oocyte. We next explored the potential molecular basis of TZP formation.

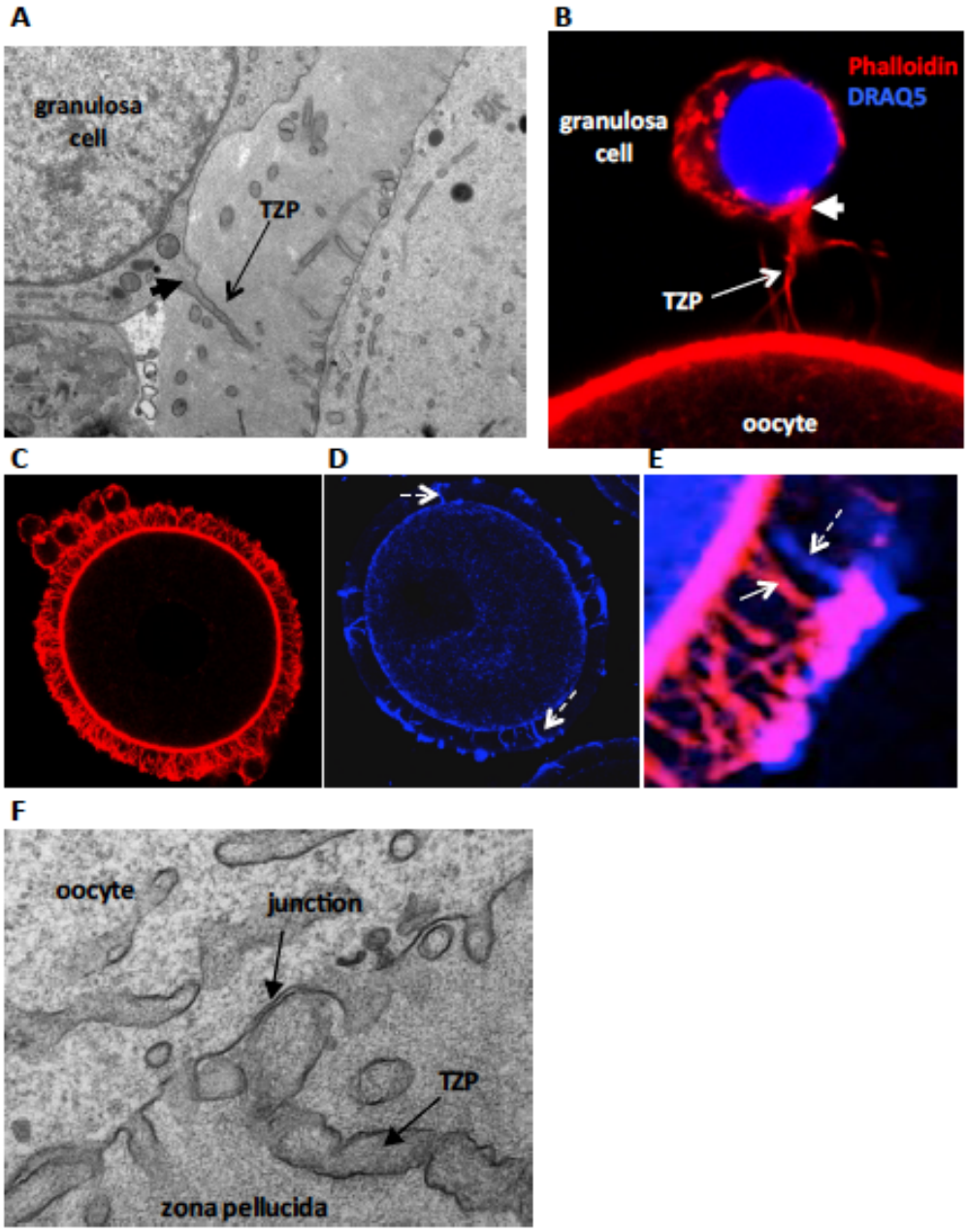


Figure 31: TZPs physically link granulosa cells with *zona*-enclosed oocyte, enabling inter-cellular communication. (A) Electron micrograph of an ovarian section showing a TZP emanating from the base (arrowhead) of a granulosa cell, directed towards the oocyte. (B) Fluorescent images of a GOC stained with Cy3-phalloidin, showing an actin-rich TZP extending from a granulosa cell towards the oocyte's surface. The DNA marker DRAQ5 shown in blue labels the cell nucleus. (C-D) Fluorescent images of oocytes stained with Cy3-phalloidin (C) and anti-tubulin antibody (D), showing actin- and tubulin-rich TZPs, respectively. (E) Fluorescent images of an oocyte co-stained with Cy3-phalloidin and anti-tubulin antibody to visualize showing actin (red) - and tubulin (blue)-TZPs respectively. (F) Electronmicrograph of an ovarian section showing the interface between a single TZP and the oocyte cell membrane, where a cell junction forms.

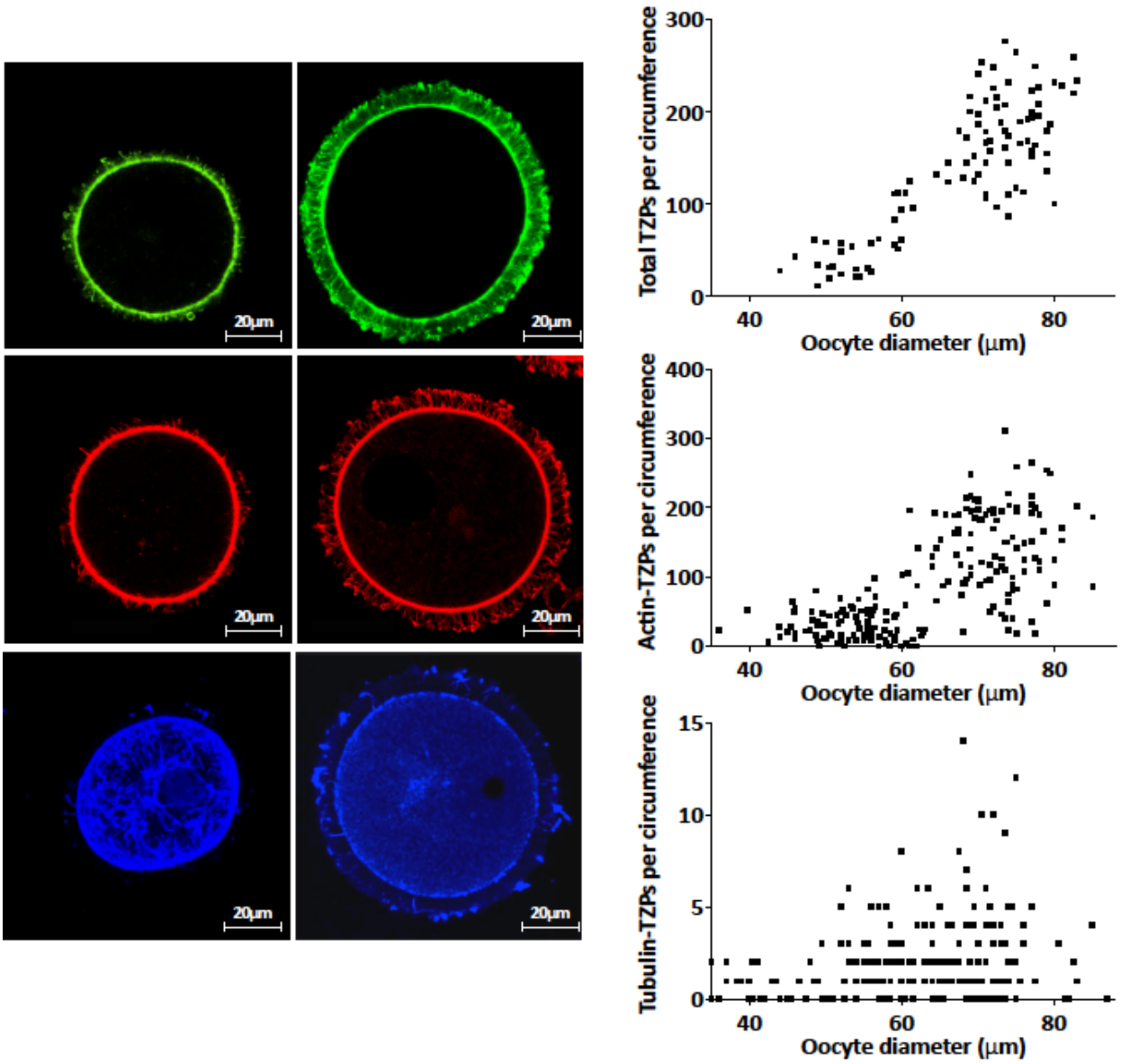


Figure 32: The number of actin-rich TZPs increases during growth. (A-B-C) Oocytes were collected from PD 8-21 females and stained with the membrane dye FM143 (A), Cy3-phalloidin (B) or anti-tubulin antibody (C) to visualize all TZPs, actin-rich TZPs, and tubulin-TZPs, respectively. Scatter plots represent the number of TZPs around the oocyte's circumference as a function of the oocyte's diameter. Scale bars represent 20 µm. 100 (A), 200 (B), and 230 (C) oocytes were examined.

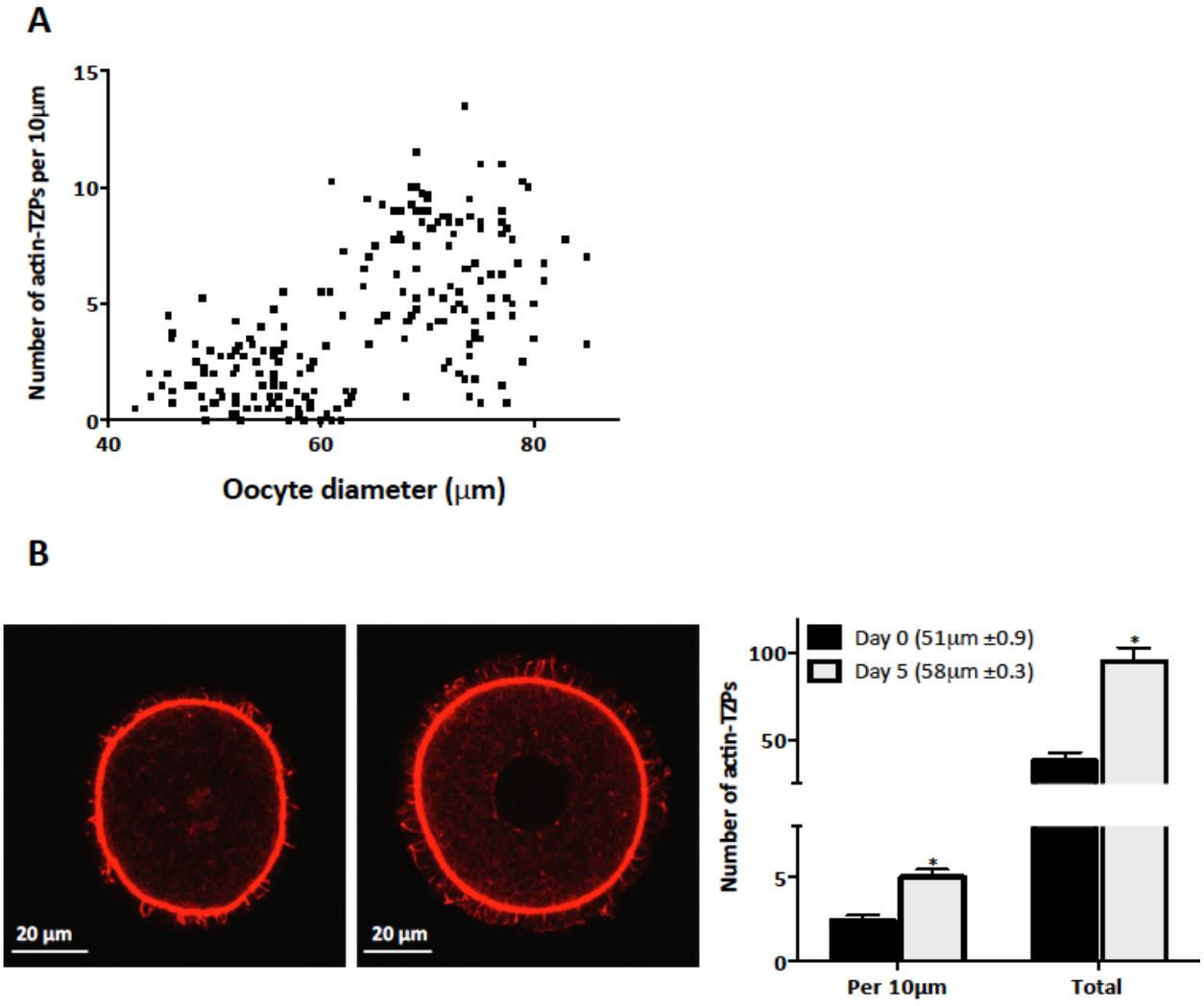


Figure 33: Growth, *in vivo* and *in vitro,* is correlated with an increased density of actin-rich TZPs. (A) Scatter plots showing the density of actin-rich TZPs (number of TZPs per 10µm arc of circumference) as a function of the oocyte's diameter. 200 oocytes were examined. (B) Representative fluorescent images of oocytes isolated from freshly collected (left) GOCs or GOCs cultured *in vitro* for 5 days (right), stained with Cy3-phalloidin to visualize actin-rich TZPs. Histogram shows the density and total count of actin-rich TZPs in the oocytes' circumference. Scale bars represent 20 µm. Data was analyzed using two-sample t-test (B). Asterisk above bars denotes $P < 0.05$. 30 oocytes per group were examined (B).

DAAM1 and fascin can be detected in TZPs and granulosa cells adjacent to the oocyte.

Actin-rich TZPs exhibit morphological similarities to filopodia, which are finger-like extensions of the plasma membrane that range from 0.1-0.4 μm in diameter and 1-200 μm in length and contain tight parallel bundles of F-actin that provide their backbone (Fairchild and Barna 2014; Kornberg and Roy 2014; Bornschlogl 2013). Interest in filopodia has surged recently as new and unexpected roles have begun to emerge in diverse biological processes, including early embryogenesis, neural growth cone guidance, metastatic invasion, viral infection, and even long-range delivery of morphogens to their targets (Fairchild and Barna 2014; Kornberg and Roy 2014; Sainath and Gallo 2015; Kelley et al. 2014; Fierro-Gonzalez et al. 2013; Nurnberg, Kitzing, and Grosse 2011). Filopodia grow by assembly of linear chains of F-actin. Assembly is promoted by members of the formin family of actin-assembly nucleators, such as DAAM 1 (Liu et al. 2010; Jaiswal et al. 2013). Fascin stabilizes the bundled actin filaments to generate stiff fibers; filopodia become buckled in its absence (Vignjevic et al. 2006). To determine whether actin-rich TZPs are regulated by mechanisms similar to those regulating filopodia, we began by examining the expression of some of these conserved filopodial assembly factors. GOCs containing growing oocytes were isolated from PD 10-15 mice and processed for immunofluorescence using antibodies directed against DAAM1, fascin and MYO10.

We observed robust anti-DAAM1 staining along a portion of the TZPs. Three patterns were observed. In some cases, DAAM1 was detectable along the entire length of the TZP (Figure 34A, left), in others it was not detectable on the TZP (Figure 34A, right), and in a third group it was detectable along a portion of the TZP. In this latter group, DAAM1 was always detected on the proximal portion of the TZP adjacent to the granulosa cell. We never observed DAAM1 localized only on the distal portion adjacent to the oocyte. To determine whether the pattern of DAAM1 staining on the TZP changed during oocyte growth, we determined the degree of co-localization between signal of anti-DAAM1 antibody and Cy3-conjugated phalloidin within the TZPs in oocytes whose diameter was less than or more than 60 μm. The co-localization coefficient was significantly higher in the larger oocytes (Figure 34B). This implies that the DAAM1 became increasingly abundant on the TZPs as oocytes progress through growth.

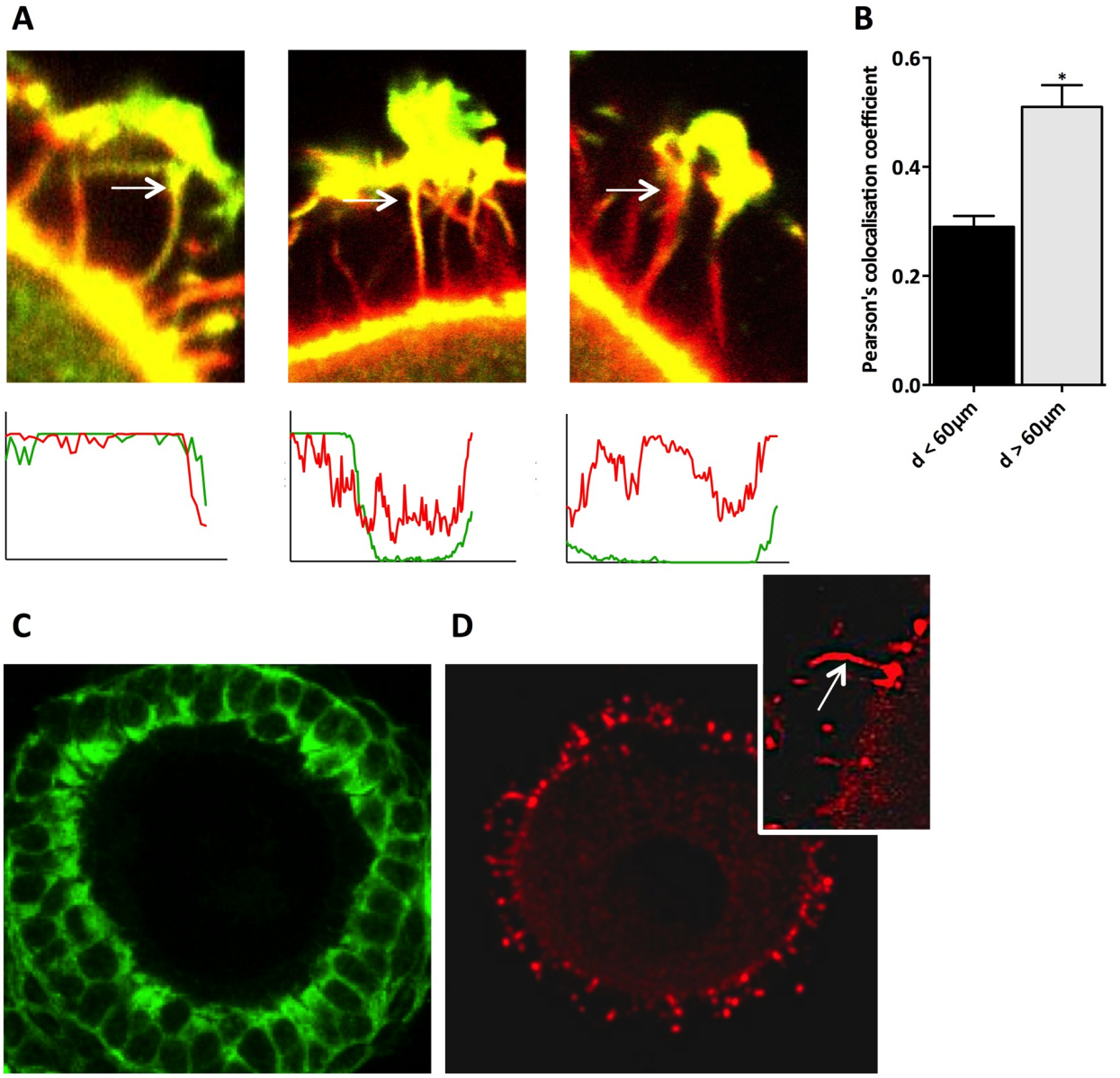


Figure 34: Fascin and DAAM1 can be detected in TZPs and granulosa cells adjacent to the oocyte. (A) Fluorescent images of oocytes co-stained with Cy3-phalloidin (red) and anti-DAAM1 antibody (green). Histograms below images show quantification of intensity of phalloidin and DAAM1 staining along the TZP indicated by the arrow. (B) Colocalization coefficient of DAAM1 intensity versus phalloidin intensity around the circumference in oocytes of different sizes. 20 oocytes were examined per group and data was analyzed using two-sample t-test (B). Asterisk above bars denotes $P < 0.05$. (C, D) Fluorescent images of GOC (C) and oocyte (D) stained with anti-fascin antibody. Arrow in inset shows a TZP positive for fascin.

We next examined the expression of fascin, an actin-bundling protein, which localizes along filopodial shafts (Jaiswal et al. 2013). In intact GOCs, fascin could be detected in the cytoplasm of the granulosa cells, and seemed to be particularly abundant in the apical cytoplasm adjacent to the oocyte (Figure 34C). To determine whether it was also present in TZPs, the granulosa cells were stripped from oocytes, which were then fixed and stained. The methanol fixation severely distorts the morphology of the oocyte. Nevertheless, it was possible to detect anti-fascin staining along the shafts of some TZPs (Figure 34D). These results show that DAAM1 and fascin, known to promote filopodial formation through interactions with actin filaments, are expressed by granulosa cells adjacent to the oocyte and localize along TZPs.

MYO10 expression pattern is consistent with a role in actin-rich TZP formation

MYO10 is an evolutionarily conserved unconventional myosin, (Mattila and Lappalainen 2008; Applewhite et al. 2007) that is localized in filopodia and has been implicated in their formation and growth (Liu et al. 2012; Kerber and Cheney 2011). To examine the localization of MYO10, ovarian histological sections were immunostained with an anti-MYO10 antibody. Beginning at the late primary stage of folliculogenesis, prominent foci were detected at the base of some of the granulosa cells that immediately surround the oocyte. These foci were not detectable in primordial or early primary follicles (Figure 35A). The same pattern of localization at the base of granulosa cells adjacent to the oocyte was detected when intact GOCs were imaged and when a second antibody raised against a different portion of MYO10 was used (Figure 35B, C). Notably, foci were never observed in granulosa cells that were not adjacent to the oocyte.

Because we observed that oocyte growth is coupled with an increase in actin-rich TZPs, we hypothesized that if MYO10 foci play a role in the initiation of TZP formation then the number of foci should increase during growth. We quantified the number and total area of MYO10 foci in GOCs at different stages of growth. As predicted, we observed that the number of MYO10 foci is higher in GOCs containing larger oocytes (Figure 35D). Consistent with this, the number of MYO10 foci also increased when GOCs were grown in vitro for five days (Figure 35E). Moreover, the number of actin-rich TZPs and the number of MYO10 foci showed a strong positive correlation (R^2= 0.75) (Figure 35F). These results show that during oocyte growth, there is an increase in MYO10 foci in granulosa cells adjacent to the oocyte, which accompanies the

increase in the number of actin-rich TZPs. This suggests that MYO10 might play a role in TZP generation, similar to the role it plays in initiation of filopodia formation.

GDF9 signalling promotes the expression of actin assembly factors in granulosa cells.

Because TZPs project exclusively from the granulosa cells adjacent to the oocyte, we wondered whether they might be induced by a factor provided by the oocyte itself. The oocyte regulates various processes in the granulosa cells by means of ODPFs. These bind to their receptors in granulosa cells and activate downstream effectors – often SMADs, which act as transcription factors. In young mice lacking the ODPF, GDF9, oocytes grow to full-size but cannot give rise to embryos. Intriguingly, the number of TZPs that project to the oocytes is dramatically reduced, and those that exist often do not project directly to the oocyte but are buckled (Carabatsos et al. 1998). We hypothesized that, through GDF9 signalling, the oocyte enhances the expression of actin assembly factors, thus promoting the extension of actin-rich TZPs. Although GDF9 likely acts as a heterodimer with BMP15 *in vivo*, GDF9 alone is equally effective *in vitro* (Peng et al. 2013). We thus cultured GOCs for 5 days in the presence and absence of GDF9, then measured mRNA levels of *Daam1*, *Fscn1*, and *Myo10*.

We found that GOCs growing in the presence of GDF9 had significantly higher levels of the transcripts encoding each actin assembly factor (Figure 36A). Moreover, GDF9 also increased the number of MYO10 foci in granulosa cells adjacent to the oocyte (Figure 36B). Consistent with these results, when GOCs were cultured for two days in the presence of SB431542, a drug that inhibits ALK5, the receptor for GDF9 in the granulosa cells, mRNA levels of *Daam1*, *Fscn1*, and *Myo10* significantly decreased (Figure 36C). SB431542 treatment also caused a significant decrease in the number of MYO10 foci (Figure 36D) as well as the in the degree of colocalization between anti-DAAM1 and phalloidin staining (Figure 36E).

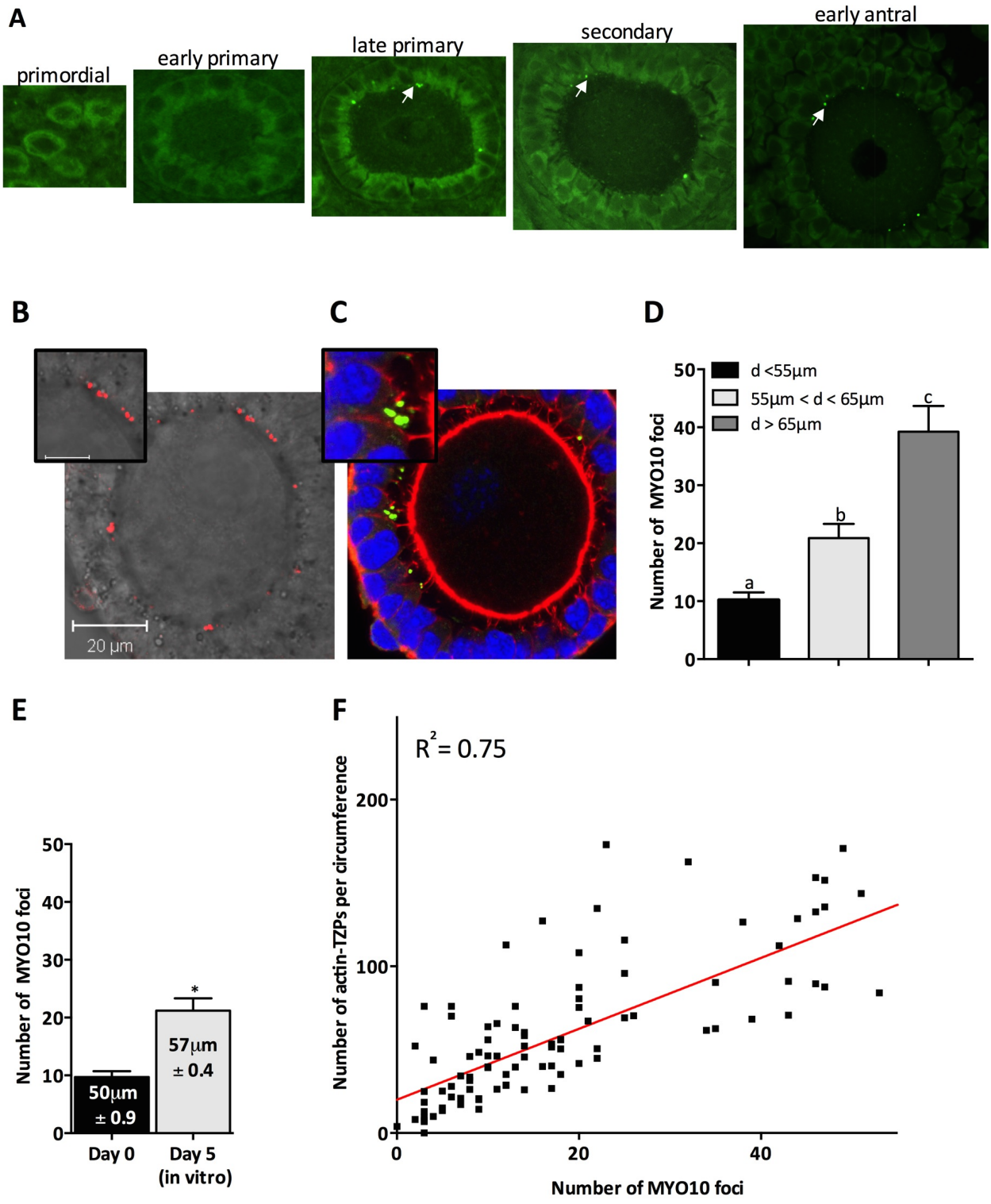


Figure 35: MYO10 expression in granulosa cells adjacent of growing oocytes. (A) Ovarian sections were stained using anti-MYO10 antibody. Images show primordial, early primary, late primary, secondary, and early antral follicles. MYO10 foci are detectable in granulosa cells adjacent to the oocyte, as of the late primary stage. (B, C) Fluorescent images of GOCs stained

with two different anti-MYO10 antibodies (red in B, green in C). In (C), DRAQ5 is in blue and Cy3- phalloidin is in red. (D) Quantification of the number of MYO10 foci located in granulosa cells adjacent to oocytes of different diameters. (E) Quantification of the number of MYO10 foci located in granulosa cells adjacent to the oocyte in freshly collected GOCs and GOCs cultured *in vitro* for 5 days. The average oocyte diameter in each group is indicated. 30 GOCs per group were examined (D, E). Data was analyzed using one-way ANOVA followed by Tukey HSD (D) or two-sample t-test (E). Different letters or asterisks above bars denote $P < 0.05$. (F) Scatter plot showing the number of actin-rich TZPs around the circumference as a function of the number of MYO10 foci in granulosa cells adjacent to the oocyte. The correlation coefficient (R^2) is indicated.

We then tested a role for ODPF using a different approach. COCs were oocytectomized (ie their oocytes were microsurgically removed), thus removing the source of the ODPF. Oocytectomy caused a significant reduction in the levels of *Daam1, Fscn1*, and *Myo10*. Strikingly, when oocytectomized complexes were incubated in the presence of exogenous GDF9, expression of these mRNAs was restored fully (*Daam1* and *Myo10*) or partially (*Fscn1*) (Figure 36F). These results indicate that GDF9 promotes the expression of actin assembly factors in granulosa cells.

Treatment with SB431542 causes a reduction in actin-rich TZP numbers.

Because we observed that GDF9 signalling promotes the expression of *Daam1*, *Fscn1*, and *Myo10*, we next examined whether it can also promote the generation of actin-rich TZPs. We isolated GOCs and cultured them in vitro for 2 days in the presence or absence of SB431542. We observed that treatment of GOCs with SB431542 caused a significant reduction in the number of actin-rich TZPs projecting to the oocytes (Figure 37). Importantly, the diameters of the oocytes within the GOCs growing in the presence or absence of SB431542 were not significantly different, indicating that the decrease in actin-rich TZPs was not due to an arrest in oocyte growth. These results suggest that, by promoting the expression of actin assembly factors, GDF9 signalling maintains the formation of actin-rich TZPs.

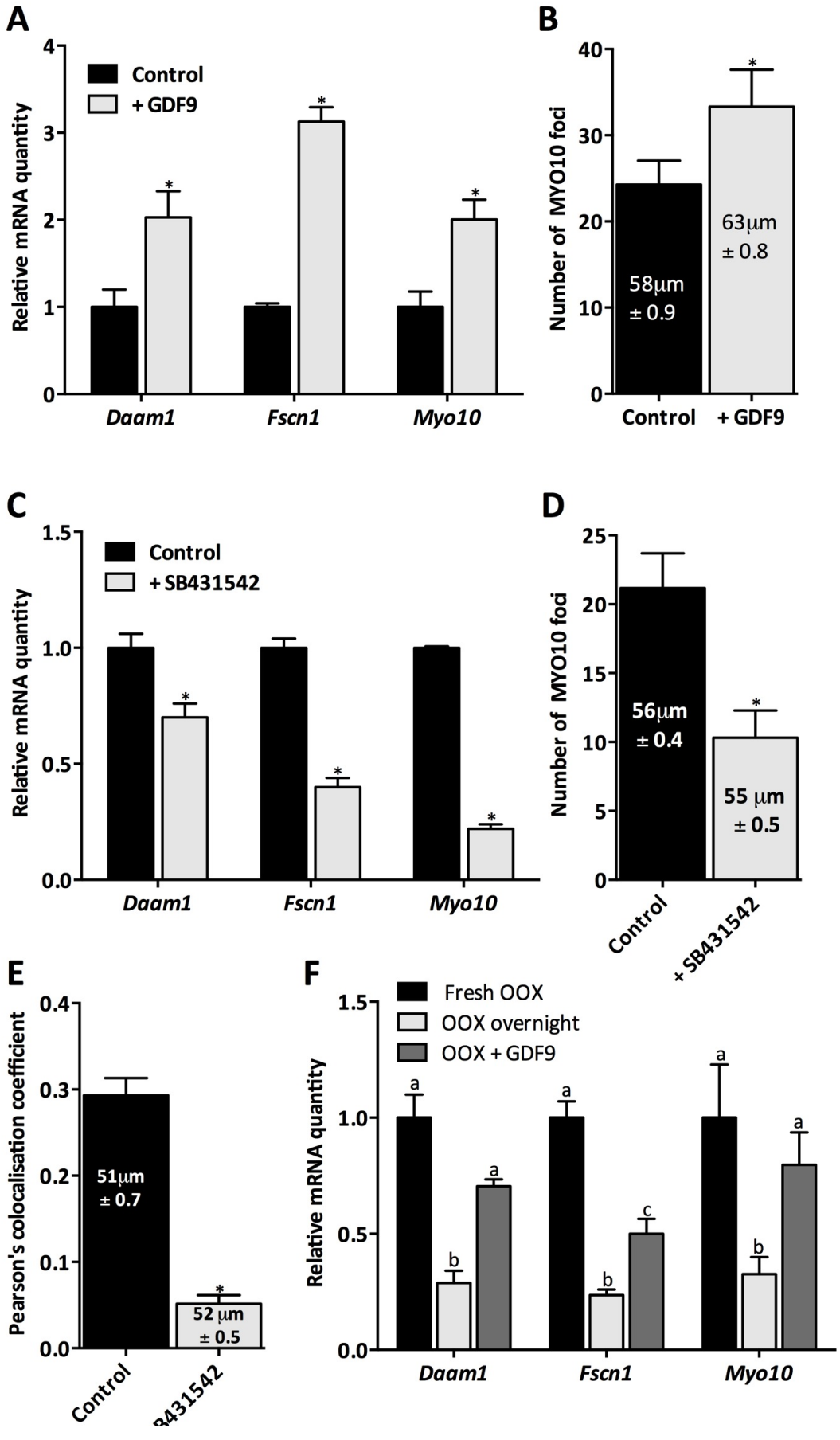

A
Relative mRNA quantity
Control
+ GDF9
Daam1
Fscn1
Myo10
B
Number of MYO10 foci
58µm
± 0.9
63µm
± 0.8
Control
+ GDF9
C
Relative mRNA quantity
Control
+ SB431542
Daam1
Fscn1
Myo10
D
Number of MYO10 foci
56µm
± 0.4
55 µm
± 0.5
Control
+ SB431542
E
Pearson's colocalisation coefficient
51µm
± 0.7
52 µm
± 0.5
Control
F
Relative mRNA quantity
Fresh OOX
OOX overnight
OOX + GDF9
Daam1
Fscn1
Myo10

Figure 36: GDF9 signalling enhances the expression of *Daam1*, *Fscn1*, and *Myo10 in vitro*. GOCs obtained from PD 10 females were cultured for 5 (A, B) or 2 (C, D, E) days in the presence or absence of GDF9 (A, B) or SB431542 (C, D, E), after which the levels of *Daam1*, *Fscn1*, and *Myo10* mRNA were quantified (A, C), the number of MYO10 foci in granulosa cells adjacent to the oocyte was quantified (B, D), or the colocalization coefficient between anti-DAAM1 and phalloidin staining was determined (E). The average oocyte diameter in each group is indicated. 25 GOCs per group were examined (B, D). Levels of *Daam1*, *Fscn1*, and *Myo10* mRNA in freshly collected OOX (black bars), OOX incubated overnight (light gray bars), and OOX incubated overnight in the presence of GDF9 (dark gray bars). Data was analyzed using two-sample t-test (A, B, C, D, E) or one-way ANOVA followed by Tukey HSD (F). Different letters or asterisks above bars denote $P < 0.05$.

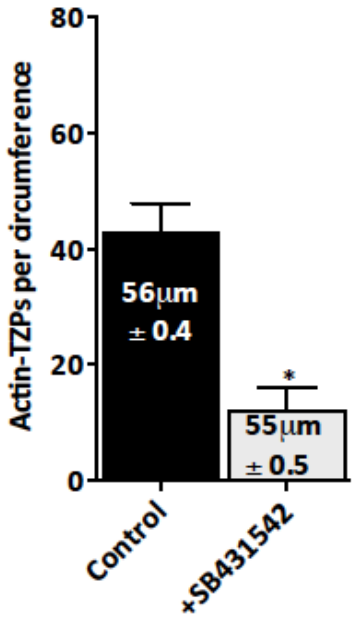


Figure 37: GDF9 signalling maintains the extension of actin-rich TZPs *in vitro*. GOCs obtained from PD 10 females were cultured for 2 days in the presence or absence of SB431542, after which the number of actin-rich TZPs around their oocytes' circumference was determined. The average oocyte diameter in each group is indicated. 20 GOCs per group were examined. Data was analyzed using two-sample t-test. Asterisks above bars denote $P < 0.05$.

Discussion

The importance of the physical association between the oocyte and the somatic granulosa cells is manifested by two simple observations. On one hand oocytes cannot grow *in vitro* without their surrounding granulosa cells (Klinger and De Felici 2002; Cecconi and Colonna 1996). On the other hand, when GOCs consisting solely of an oocyte and its surrounding granulosa are isolated from murine follicles at a very early stage of development and cultured in serum-free media, the oocyte can complete its development and give rise to a viable embryo (O'Brien, Pendola, and Eppig 2003). Thus, the granulosa are necessary and sufficient (at least *in vitro*) to generate the somatic microenvironment that developing oocytes need. Cellular extensions linking the somatic cells to the oocyte have been known for over one hundred years. The mechanism of generation of these extensions however, is still not defined. These structures, known as TZPs, are the sole means by which the granulosa cells are able to physically contact and communicate with the *zona*-enclosed oocyte.

By enabling communication with the somatic granulosa cells, which is crucial for the oocyte to grow and acquire developmental competence, TZPs play an essential role in female fertility. We here report that oocyte growth is associated with a significant increase in the number of TZPs extending from the granulosa cells towards the oocyte, as determined by staining oocytes with the membrane dye, FM-143. When the actin-rich TZPs were examined by phalloidin staining, their number showed a similar pattern. This increase in actin-rich TZPs occurred during both *in vivo* and *in vitro* growth. This indicates that, as the oocyte was growing within the follicle *in vivo* or within GOCs *in vitro*, the adjacent granulosa cells actively generated new actin-rich TZPs and extended them towards the oocyte. It is important to note that the quantification of TZP numbers was also determined in oocytes within intact GOCs, as well as granulosa-free oocytes. The numbers of TZPs around the oocyte circumference at different stages was similar in both cases, thus confirming that the reduced number of TZPs in smaller oocytes is not due to these TZPs being less resistant to the chemical or physical stress of the oocyte isolation process.

Since the increase in actin-rich TZP numbers was observed in oocytes in which the *zona pellucida* had already been deposited, this implies that active generation of TZPs must be taking place after the deposition of this layer. This observation thus contradicts a long-standing model

of TZP generation, where TZPs were thought to constitute elongated portions of the granulosa cell membrane that remained stuck to the oocyte as the two cells were progressively separated by the deposition of the *zona pellucida* (Chiquoine 1960). Actin-rich TZPs and total TZPs (FM-143 stained) showed very similar patterns across growth and very similar numbers were obtained when their numbers were determined. This confirms that the actin-rich TZPs are the major contributors to the total number of TZPs. This observation also demonstrates that it is the number of the TZP structures themselves, and not the actin content within existing TZPs, that increases during growth. In other words, it eliminates the possibility that TZPs are as abundant in early as in late stages of oocyte growth, but do not contain enough actin to be detected by phalloidin. Rather, our results demonstrate the granulosa cells are forming and extending new actin-rich TZPs even after the *zona pellucida* has been fully formed.

How TZPs physically cross the zona is not known. They may need to digest their way through the *zona pellucida* in order to reach the oocyte plasma membrane. Metalloproteinases are expressed by granulosa cells and play in role in the ovulatory process (Portela, Veiga, and Price 2009). It is possible that they are also important for the penetration of TZPs through the *zona pellucida*. Whether TZP penetration requires digestion of the *zona* and whether metalloproteinases or other enzymes localize to granulosa cells adjacent to the oocyte and to TZPs is yet to be determined.

In contrast to actin-rich TZPs, the number of tubulin-TZPs did not increase throughout different stages of growth. This observation suggests that the two sub-populations of TZPs are regulated by different mechanisms. The functional difference between tubulin- and actin-rich TZPs is not known. Although the actin-rich TZPs are typically represented as harbouring the gap junctions (Li and Albertini 2013), no experimental evidence to date supports (or refutes) this notion.

To determine the mechanism of generation of actin-rich TZPs, we examined whether they might be formed and regulated similarly to filopodia, which are also thin and actin-rich. We found that actin-rich TZPs and filopodia share the expression of several actin-assembly factors. Fascin and DAAM1 have been shown to co-localize along the filopodial shaft. Both proteins have been shown to be necessary for proper filopodia formation and we found that both were present in

TZPs. The similar pattern of localization of these two proteins along filopodia and along TZPs suggests that they could serve the same function. We were unable to examine whether fascin and DAAM1 co-localize along TZPs, as is the case in filopodia, because the antibodies used require different fixation techniques. However, consistent with a potential role in promoting the formation of TZPs, co-localization between DAAM1 and phalloidin staining was higher in larger oocytes, suggesting that DAAM1 expression increases along with the increase in actin-rich TZP extension during growth.

Foci of MYO10 were detected at the base of granulosa cells adjacent to the oocyte. The number of these foci increased along with growth, showing a strong correlation with the increase in actin-rich TZPs and suggesting that MYO10 could be involved in the formation and growth of actin-rich TZPs, as it does in filopodia (Kerber and Cheney 2011; Liu et al. 2012; Mattila and Lappalainen 2008). Moreover, MYO10 is absent from primordial and early primary follicles, in which the oocyte and granulosa cells are still in direct contact, and TZPs are absent (McLaren 1988).

That the oocyte regulates certain granulosa cell processes was first suggested by El Fouly et al (el-Fouly et al. 1970). We now report that the oocyte, through GDF9 signalling can enhance the expression of actin assembly factors and maintain the extension of actin-rich TZPs by the granulosa cells. This is consistent with previous results, showing that the TZPs projecting to the oocytes in *Gdf9*$^{-/-}$ mice are disorganized and dramatically reduced in number (Carabatsos et al. 1998), highlighting the importance of this ODPF in promoting actin-rich TZP extension. Intriguingly, in melanocytes, BMP6, another member the TGFß superfamily, has been shown to increase the expression of MYO10 and promote filopodia formation through the SMADs (Pi et al. 2007). Hence, GDF9 and downstream SMAD effectors could have a similar mechanism of action in granulosa cells.

However, since TZPs are present in oocytes of *Gdf9*$^{-/-}$ females, albeit in reduced numbers, other factors must be involved in the formation of actin-rich TZPs. The oocyte may regulate their extension by other ODPFs such as BMP15. Moreover, somatic factors are likely to be involved in the formation of these TZPs. We have shown that oocytes of *Fshb*$^{-/-}$ females have a reduced

number of actin-rich TZPs. Oocytes of *Fshb*$^{-/-}$ females however, have normal levels of *Gdf9* and *Bmp15* (El-Hayek, Demeestere, and Clarke 2014). These results highlight the concept that the extension of TZPs by the granulosa cells likely requires the cooperation of signals emanating from both the granulosa cells (such as FSH) as well as the oocyte (such as GDF9).

It is well established that communication with its somatic microenvironment is crucial for oocyte growth and development (Simon et al. 1997; Li and Mather 1997; Ratchford, Esguerra, and Moley 2008; Gittens and Kidder 2005). As the oocyte grows within the follicle, it prepares to support post-fertilization events. As such, its reliance on surrounding granulosa cells for metabolic requirements, signalling molecules, and transcripts is likely to increase. Consistent with this notion, we previously reported that follicular growth is associated with an increase in expression of junctional components and with an enhanced coupling between the enclosed oocyte and the surrounding granulosa cells. As gap junctions which enable this communication occur at the contact sites made by these TZPs, it follows that more contact sites and hence more TZPs would be required to enable the increase in communication during oocyte growth. As predicted, we found that along with oocyte growth there is an increase in the number of communication-enabling TZPs. Taken together, these results suggest that the enhanced extension of TZPs, and the increase in expression levels of E-cadherin and connexin-37 provide a platform for increased granulosa cell-oocyte junctional contact and communication during growth. We thus propose that the continuing generation of TZPs provides the basis for increasing gap junctional communication between the growing oocyte and surrounding granulosa cells, and that this increased communication helps the oocyte acquire the ability to develop as an embryo. In addition, the concept that TZPs are actively generated may have important clinical implications. Cryopreservation, an intervention critically needed for female patients undergoing certain treatments, has been shown to cause the disruption of TZPs (Brambillasca et al. 2013; Vandevoort et al. 2008). Understanding the exact mechanism for TZP generation would thus shed the light on possible ways to repair such damaged TZPs and preserve fertility.

Supplemental data

Effect of FSH on extension of actin- rich **TZPs**

In previous work, we showed that the density of Cy3-conjugated phalloidin fluorescence within the *zona pellucida* was significantly reduced in oocytes of *Fshb*$^{-/-}$ females compared to those of *Fshb*$^{+/-}$ females. Here we determined whether this reduction is due to a decrease in the number of TZPs. Fully-grown oocytes were isolated from PD 21 *Fshb*$^{+/-}$ and *Fshb*$^{-/-}$ females, stained with Cy3-conjugated phalloidin, and analysed with ImageJ to determine the number of actin-rich TZPs per 10μm arc as well as their total number in the circumference, as described in the 'Materials and Methods' section. To further explore the effect of FSH, PD 19 wildtype CD-1 females were injected with the FSH analogue eCG. Two days later, their oocytes were isolated, stained with Cy3-conjugated phalloidin, and analysed with ImageJ, in comparison with oocytes of age-matched non-injected controls. Consistent with our previous results, we found that the number of actin-rich TZPs is significantly lower in fully-grown oocytes of *Fshb*$^{-/-}$ females compared to those of *Fshb*$^{+/-}$ females. In addition, in wild-type females, after treatment with eCG (FSH analog), the oocytes had a significantly higher number of actin-rich TZPs compared to oocytes of non-treated age-matched controls. Taken together, these results show that FSH enhances the extension of actin-rich TZPs by granulosa cells.

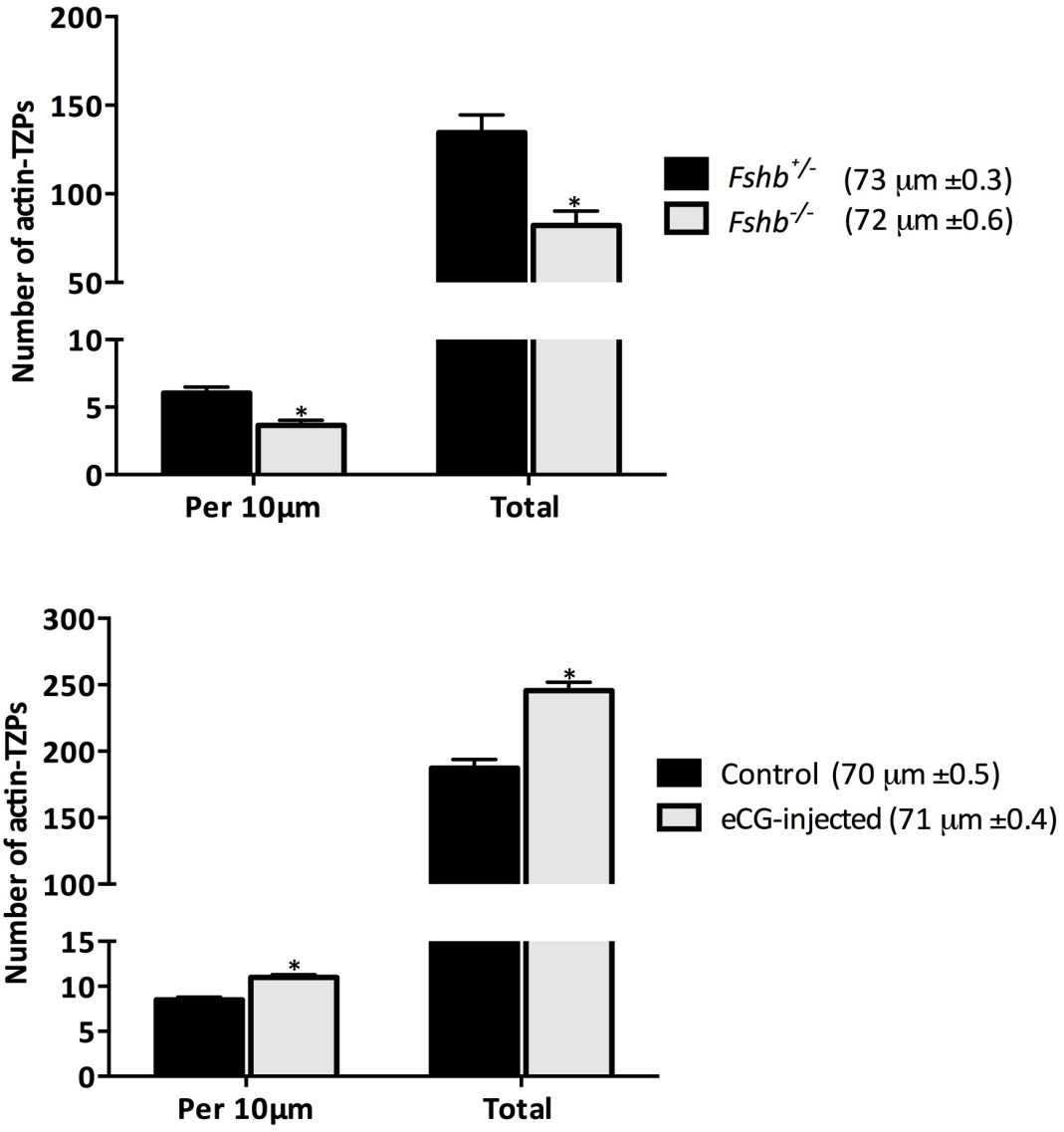


Figure 38: (A) Number per 10µm and total number around circumference of actin-rich TZPs in fully-grown oocytes of *Fshb*$^{+/-}$ (black bars) and *Fshb*$^{-/-}$ (light gray bars). (B) Number per 10µm and total number around circumference of actin-rich TZPs in fully-grown oocytes of controls (black bars) and eCG-injected females (light gray bars). The average oocyte diameter in each group is indicated (A, B). 50 oocytes were examined per group (A, B). Data was analyzed using two-sample t-test (A, B). Asterisks above bars denote $P < 0.05$.

Chapter 5

Discussion: synergistic effects of FSH and GDF9 during oocyte growth and ovulation

The ultimate goal of folliculogenesis is the ovulation of a mature, developmentally competent egg, able to undergo fertilization. Given the structure of the ovarian follicle, achieving this goal mandates intercellular cooperation and dependence. In every female reproductive cycle, a growing follicle is one out of many that have been recruited into the growth pool, competing for dominance. In fact, the follicle not only seeks dominance, but also survival, as follicles that do not ovulate eventually undergo atresia (Byskov 1974; Oakberg 1979). The processes discussed in the introduction as well as several other important events during folliculogenesis such as the transition from a primary to a secondary follicle (Braw-Tal 2002; Dong et al. 1996; Galloway et al. 2000; Parrott and Skinner 1999; Yoshida et al. 1997), control of granulosa cell lutenization and differentiation (Nekola and Nalbandov 1971; Vanderhyden, Cohen, and Morley 1993; Vanderhyden and Macdonald 1998; Vanderhyden and Tonary 1995; Li et al. 2000; Glister, Groome, and Knight 2003; Eppig et al. 1997), as well as theca cell recruitment (Spicer et al. 2008; Dong et al. 1996; Liu et al. 2015), critically require cellular cooperation between the follicular cell types, the oocyte, granulosa cells and theca cells. Optimal intercellular cooperation would thus culminate in the proper execution of all these events, favouring follicle survival and promoting its dominance. This intercellular cooperation is mediated by a myriad of extra- and intra- follicular factors, two of which, FSH and GDF9, are the focus of this thesis.

FSH is a pituitary glycoprotein whose receptors are located on granulosa cells (Erickson, Wang, and Hsueh 1979; Zeleznik, Midgley, and Reichert 1974). The early stages of oocyte and follicular growth can proceed in the absence of FSH, but more advanced stages become responsive to and subsequently dependent on it (Kumar et al. 1997). By upregulating the expression of *Lhcgr*, FSH indirectly enables the LHCGR-regulated release of the EGFR ligands, which mediate the ovulatory response. The results discussed in chapter 2 identify a new role by which FSH establishes the EGFR signalling axis. Our results show that FSH also drives an increase in EGFR expression during late folliculogenesis and provide evidence that this increase

is essential to enable the ovulatory response to EGF. By coordinating the expression of EGFR and the release of its ligands, FSH allows full-grown follicles to activate EGFR signalling at ovulation. In chapter 3, yet another role for FSH is highlighted. We show that by increasing the expression of junctional components in oocyte and granulosa cells and increasing the extension of actin-rich TZPs physically linking these two compartments, FSH enhances contact and communication between the oocyte and the surrounding granulosa cells, essential for the oocyte's development. Combined together, and with conserved role of FSH in driving the late stages of folliculogenesis by promoting the proliferation, differentiation, and survival of the granulosa cells, these results demonstrate that FSH coordinates multiple events in the follicle. By implementing the EGFR signalling axis, as well as coupling the oocyte with the granulosa cells, FSH ensures that when the LH surge occurs, the follicle is ready to respond and ovulate, and the enclosed oocyte is a competent gamete capable of getting fertilized and developing into an embryo.

The importance of FSH during *in vitro* culture has been controversial. Some studies show that follicular growth can be maintained in the absence of FSH (Eppig et al., 1998; Kreeger et al., 2005). Conversely, others have demonstrated a necessity for FSH in order to sustain follicular growth and granulosa cell proliferation (Romero and Smitz, 2010). My thesis work thus shows that despite not being necessarily essential for *in vitro* growth, FSH nonetheless enhances and accelerates the development of both the follicle and the enclosed oocyte, making it a beneficial component of *in vitro* culture medium.

FSH has been shown to regulate the expression of many target genes promoting survival, proliferation, and steroidogenesis (Sasson et al. 2003; Grieshaber et al. 2003). Target genes include transcription factors, GPCRs, signalling molecules, steroidogenic enzymes, cell cycle regulators, cytoskeletal elements, and growth factors (Alam et al. 2004; Alliston et al. 1997; Park et al. 2003; Piontkewitz, Sundfeldt, and Hedin 1997; Ratoosh et al. 1987; Russell et al. 2003; Salvador et al. 2004; Sekiguchi et al. 2004; Sicinski et al. 1996; Turner et al. 1989; Woodruff et al. 1987; Yazawa et al. 2003; Zeleznik, Midgley, and Reichert 1974). In manuscripts I and II, our results show new gene targets for FSH including *Egfr* and *Cdh2* in granulosa cells as well as *Cdh1* and *Gja4* in the oocyte. FSH regulates gene expression by transcription factors such as

CREB, AP1, FOXO1A, FOXO3A, LEF, and NFkB (Cameron et al. 1996; Cottom et al. 2003; Fan et al. 2010; Fan et al. 2008; Gonzalez-Robayna et al. 2000; Nechamen et al. 2004; Park et al. 2005; Wang, Chan, and Tsang 2002), as well as by histone H3 phosphorylation and acetylation (Salvador et al. 2001). The mechanism by which it affects the transcription levels of these new targets remains to be elucidated.

The ODPF GDF9 is a member of the TGFß superfamily, that has been shown to regulate several processes in its target granulosa cells, including proliferation, survival, differentiation, metabolism, luteinization, and expansion. It has also been shown to play a role in theca cell recruitment. The work discussed in manuscript III further adds a new role played by this ODPF, by which GDF9 signalling helps to maintain the extension of actin-rich TZPs by the granulosa cells. Experiments described in manuscript III demonstrate that GDF9 signalling enhances the expression of actin assembly factors, MYO10, DAAM1, and fascin, identifying by such new targets for the SMADs in granulosa cells. Whether other ODPFs, such as BMP15, play a similar role in the formation of actin-rich TZPs remains to be determined.

Results of manuscript III also demonstrate that the interface between granulosa cells and the oocyte is dynamically remodeled during growth, where actin-rich TZPs are actively emanating from granulosa cell bodies towards the oocyte. We also show that granulosa cells are capable of actively projecting actin-based cellular structures. These results have also highlighted differences between actin- and tubulin-rich TZPs. The distribution of the latter does not seem to change significantly during growth and further studies are needed to determine how they arise and whether they, like actin-rich TZPs, are affected by oocyte signalling. Importantly, future work should aim to examine whether TZPs may be regenerated after damage caused by cryopreservation for instance (Brambillasca et al. 2013; Vandevoort et al. 2008), possibly through GDF9 and/or FSH signalling.

The newly identified roles which FSH and GDF9 play constitute additional examples of intercellular cooperation within the follicle. Moreover, my work provides evidence for synergistic actions of FSH and GDF9, where (1) both FSH (manuscript I) and GDF9 (Diaz et al. 2006; Dragovic et al. 2007; Nagyova et al. 2011; Pangas and Matzuk 2005; Sasseville et al.

2010; Su et al. 2010) enhance the expression of *Egfr*, enabling the ovulatory response; and (2) both FSH (manuscript II, III) and GDF9 (manuscript III) plays a role in maintaining the extension of actin-rich TZPs by the granulosa cells, enabling contact and communication with the *zona*-enclosed oocyte. Even though these two factors probably work independently, as the levels and activity of GDF9 were intact in follicles of *Fshb*$^{-/-}$ females, whether FSH promotes actin-rich TZPs by a similar mechanism as GDF9 (by promoting expression of actin assembly factors) is to be determined.

Conclusion

The essence of female fertility is the production of a mature, developmentally competent gamete, able to undergo fertilization and produce a healthy embryo. It is thus not surprising that most if not all follicular processes reviewed in the introduction and discussed in the three manuscripts seem to, either directly or indirectly, serve the oocyte's own growth and development. The activation of follicular growth, immense increase in oocyte size, establishment of metabolic coupling, accumulation of maternal transcripts and organelles, meiotic resumption, cumulus expansion, and all underlying molecular processes that support or mediate these events, occur in preparation for fertilization and embryo development. The work described in the three manuscripts highlights how essential the interplay between intra- and extra-follicular factors is for the growth of the follicle as a whole, but especially for the development of the oocyte, and subsequently the embryo. This interplay governs the relationship between the different follicular compartments, providing an ideal niche for the development of the germ cell within its local somatic microenvironment. As the human population's needs for fertility treatments and interventions are at a constant rise, further exploring the roles played by different intra- and extra-follicular factors could potentially have great clinical significance.

References

Aaltonen, J., M. P. Laitinen, K. Vuojolainen, R. Jaatinen, N. Horelli-Kuitunen, L. Seppa, H. Louhio, T. Tuuri, J. Sjoberg, R. Butzow, O. Hovata, L. Dale, and O. Ritvos. 1999. 'Human growth differentiation factor 9 (GDF-9) and its novel homolog GDF-9B are expressed in oocytes during early folliculogenesis', *J Clin Endocrinol Metab*, 84: 2744-50.

Abe, K., A. Inoue, M. G. Suzuki, and F. Aoki. 2010. 'Global gene silencing is caused by the dissociation of RNA polymerase II from DNA in mouse oocytes', *J Reprod Dev*, 56: 502-7.

Ackert, C. L., J. E. Gittens, M. J. O'Brien, J. J. Eppig, and G. M. Kidder. 2001. 'Intercellular communication via connexin43 gap junctions is required for ovarian folliculogenesis in the mouse', *Dev Biol*, 233: 258-70.

Adams, E. C., and A. T. Hertig. 1964. 'Studies on Guinea Pig Oocytes. I. Electron Microscopic Observations on the Development of Cytoplasmic Organelles in Oocytes of Primordial and Primary Follicles', *J Cell Biol*, 21: 397-427.

Adashi, E. Y. 1994. 'Endocrinology of the ovary', *Hum Reprod*, 9: 815-27.

Adashi, E. Y., and A. J. Hsueh. 1982. 'Estrogens augment the stimulation of ovarian aromatase activity by follicle-stimulating hormone in cultured rat granulosa cells', *J Biol Chem*, 257: 6077-83.

Adhikari, D., and K. Liu. 2010. 'mTOR signaling in the control of activation of primordial follicles', *Cell Cycle*, 9: 1673-4.

Adriaens, I., R. Cortvrindt, and J. Smitz. 2004. 'Differential FSH exposure in preantral follicle culture has marked effects on folliculogenesis and oocyte developmental competence', *Hum Reprod*, 19: 398-408.

Aittomaki, K., J. L. Lucena, P. Pakarinen, P. Sistonen, J. Tapanainen, J. Gromoll, R. Kaskikari, E. M. Sankila, H. Lehvaslaiho, A. R. Engel, E. Nieschlag, I. Huhtaniemi, and A. de la Chapelle. 1995. 'Mutation in the follicle-stimulating hormone receptor gene causes hereditary hypergonadotropic ovarian failure', *Cell*, 82: 959-68.

Alam, H., E. T. Maizels, Y. Park, S. Ghaey, Z. J. Feiger, N. S. Chandel, and M. Hunzicker-Dunn. 2004. 'Follicle-stimulating hormone activation of hypoxia-inducible factor-1 by the phosphatidylinositol 3-kinase/AKT/Ras homolog enriched in brain (Rheb)/mammalian target of rapamycin (mTOR) pathway is necessary for induction of select protein markers of follicular differentiation', *J Biol Chem*, 279: 19431-40.

Alam, H., J. Weck, E. Maizels, Y. Park, E. J. Lee, M. Ashcroft, and M. Hunzicker-Dunn. 2009. 'Role of the phosphatidylinositol-3-kinase and extracellular regulated kinase pathways in the induction of hypoxia-inducible factor (HIF)-1 activity and the HIF-1 target vascular endothelial growth factor in ovarian granulosa cells in response to follicle-stimulating hormone', *Endocrinology*, 150: 915-28.

Albertini, D. F. 1987. 'Cytoplasmic reorganization during the resumption of meiosis in cultured preovulatory rat oocytes', *Dev Biol*, 120: 121-31.

Albertini, D. F., and V. Rider. 1994. 'Patterns of intercellular connectivity in the mammalian cumulus-oocyte complex', *Microsc Res Tech*, 27: 125-33.

Alliston, T. N., A. C. Maiyar, P. Buse, G. L. Firestone, and J. S. Richards. 1997. 'Follicle stimulating hormone-regulated expression of serum/glucocorticoid-inducible kinase in rat

ovarian granulosa cells: a functional role for the Sp1 family in promoter activity', *Mol Endocrinol*, 11: 1934-49.
Almeida, K. C., A. F. Pereira, A. S. Alcantara Neto, S. R. Avelar, L. R. Bertolini, M. Bertolini, V. J. Freitas, and L. M. Melo. 2011. 'Real-time qRT-PCR analysis of EGF receptor in cumulus-oocyte complexes recovered by laparoscopy in hormonally treated goats', *Zygote*, 19: 127-36.
Ambrosio, L., and P. Schedl. 1985. 'Two discrete modes of histone gene expression during oogenesis in Drosophila melanogaster', *Dev Biol*, 111: 220-31.
Amsterdam, A., Y. Koch, M. E. Lieberman, and H. R. Lindner. 1975. 'Distribution of binding sites for human chorionic gonadotropin in the preovulatory follicle of the rat', *J Cell Biol*, 67: 894-900.
Anderson, E., and D. F. Albertini. 1976. 'Gap junctions between the oocyte and companion follicle cells in the mammalian ovary', *J Cell Biol*, 71: 680-6.
Anderson, E., and H. W. Beams. 1960. 'Cytological observations on the fine structure of the guinea pig ovary with special reference to the oogonium, primary oocyte and associated follicle cells', *J Ultrastruct Res*, 3: 432-46.
Andreu-Vieyra, C. V., R. Chen, J. E. Agno, S. Glaser, K. Anastassiadis, A. F. Stewart, and M. M. Matzuk. 2010. 'MLL2 is required in oocytes for bulk histone 3 lysine 4 trimethylation and transcriptional silencing', *PLoS Biol*, 8.
Andric, N., M. Thomas, and M. Ascoli. 2010. 'Transactivation of the epidermal growth factor receptor is involved in the lutropin receptor-mediated down-regulation of ovarian aromatase expression in vivo', *Mol Endocrinol*, 24: 552-60.
Applewhite, D. A., M. Barzik, S. Kojima, T. M. Svitkina, F. B. Gertler, and G. G. Borisy. 2007. 'Ena/VASP proteins have an anti-capping independent function in filopodia formation', *Mol Biol Cell*, 18: 2579-91.
Arakane, F., T. Sugawara, H. Nishino, Z. Liu, J. A. Holt, D. Pain, D. M. Stocco, W. L. Miller, and J. F. Strauss, 3rd. 1996. 'Steroidogenic acute regulatory protein (StAR) retains activity in the absence of its mitochondrial import sequence: implications for the mechanism of StAR action', *Proc Natl Acad Sci U S A*, 93: 13731-6.
Ashkenazi, H., X. Cao, S. Motola, M. Popliker, M. Conti, and A. Tsafriri. 2005. 'Epidermal growth factor family members: endogenous mediators of the ovulatory response', *Endocrinology*, 146: 77-84.
Avella, M. A., B. Xiong, and J. Dean. 2013. 'The molecular basis of gamete recognition in mice and humans', *Mol Hum Reprod*, 19: 279-89.
Bachvarova, R. 1985. 'Gene expression during oogenesis and oocyte development in mammals', *Dev Biol (N Y 1985)*, 1: 453-524.
Balasubramanian, K., H. A. Lavoie, J. C. Garmey, D. M. Stocco, and J. D. Veldhuis. 1997. 'Regulation of porcine granulosa cell steroidogenic acute regulatory protein (StAR) by insulin-like growth factor I: synergism with follicle-stimulating hormone or protein kinase A agonist', *Endocrinology*, 138: 433-9.
Ball, G. D., M. E. Bellin, R. L. Ax, and N. L. First. 1982. 'Glycosaminoglycans in bovine cumulus-oocyte complexes: morphology and chemistry', *Mol Cell Endocrinol*, 28: 113-22.
Ball, G. D., M. L. Leibfried, R. W. Lenz, R. L. Ax, B. D. Bavister, and N. L. First. 1983. 'Factors affecting successful in vitro fertilization of bovine follicular oocytes', *Biol Reprod*, 28: 717-25.

Baranova, N. S., A. Inforzato, D. C. Briggs, V. Tilakaratna, J. J. Enghild, D. Thakar, C. M. Milner, A. J. Day, and R. P. Richter. 2014. 'Incorporation of pentraxin 3 into hyaluronan matrices is tightly regulated and promotes matrix cross-linking', *J Biol Chem*, 289: 30481-98.

Baratta, M., L. A. West, A. M. Turzillo, and T. M. Nett. 2001. 'Activin modulates differential effects of estradiol on synthesis and secretion of follicle-stimulating hormone in ovine pituitary cells', *Biol Reprod*, 64: 714-9.

Bellone, M., M. Zuccotti, C. A. Redi, and S. Garagna. 2009. 'The position of the germinal vesicle and the chromatin organization together provide a marker of the developmental competence of mouse antral oocytes', *Reproduction*, 138: 639-43.

Ben-Ami, I., A. Komsky, O. Bern, E. Kasterstein, D. Komarovsky, and R. Ron-El. 2011. 'In vitro maturation of human germinal vesicle-stage oocytes: role of epidermal growth factor-like growth factors in the culture medium', *Hum Reprod*, 26: 76-81.

Berisha, B., M. W. Pfaffl, and D. Schams. 2002. 'Expression of estrogen and progesterone receptors in the bovine ovary during estrous cycle and pregnancy', *Endocrine*, 17: 207-14.

Biggers, J. D., D. G. Whittingham, and R. P. Donahue. 1967. 'The pattern of energy metabolism in the mouse oocyte and zygote', *Proc Natl Acad Sci U S A*, 58: 560-7.

Bilezikjian, L. M., A. L. Blount, A. M. Leal, C. J. Donaldson, W. H. Fischer, and W. W. Vale. 2004. 'Autocrine/paracrine regulation of pituitary function by activin, inhibin and follistatin', *Mol Cell Endocrinol*, 225: 29-36.

Billig, H., I. Furuta, and A. J. Hsueh. 1993. 'Estrogens inhibit and androgens enhance ovarian granulosa cell apoptosis', *Endocrinology*, 133: 2204-12.

Binder, A. K., K. F. Rodriguez, K. J. Hamilton, P. S. Stockton, C. E. Reed, and K. S. Korach. 2013. 'The absence of ER-beta results in altered gene expression in ovarian granulosa cells isolated from in vivo preovulatory follicles', *Endocrinology*, 154: 2174-87.

Blaschuk, O. W., and R. Farookhi. 1989. 'Estradiol stimulates cadherin expression in rat granulosa cells', *Dev Biol*, 136: 564-7.

Bodensteiner, K. J., C. M. Clay, C. L. Moeller, and H. R. Sawyer. 1999. 'Molecular cloning of the ovine Growth/Differentiation factor-9 gene and expression of growth/differentiation factor-9 in ovine and bovine ovaries', *Biol Reprod*, 60: 381-6.

Boland, N. I., P. G. Humpherson, H. J. Leese, and R. G. Gosden. 1994. 'The effect of glucose metabolism on murine follicle development and steroidogenesis in vitro', *Hum Reprod*, 9: 617-23.

Bornschlogl, T. 2013. 'How filopodia pull: what we know about the mechanics and dynamics of filopodia', *Cytoskeleton (Hoboken)*, 70: 590-603.

Bornslaeger, E. A., P. M. Mattei, and R. M. Schultz. 1988. 'Protein phosphorylation in meiotically competent and incompetent mouse oocytes', *Mol Reprod Dev*, 1: 19-25.

Bornslaeger, E. A., P. Mattei, and R. M. Schultz. 1986. 'Involvement of cAMP-dependent protein kinase and protein phosphorylation in regulation of mouse oocyte maturation', *Dev Biol*, 114: 453-62.

Bornslaeger, E. A., M. W. Wilde, and R. M. Schultz. 1984. 'Regulation of mouse oocyte maturation: involvement of cyclic AMP phosphodiesterase and calmodulin', *Dev Biol*, 105: 488-99.

Borum, K. 1961. 'Oogenesis in the mouse. A study of the meiotic prophase', *Exp Cell Res*, 24: 495-507.

Bouniol-Baly, C., L. Hamraoui, J. Guibert, N. Beaujean, M. S. Szollosi, and P. Debey. 1999. 'Differential transcriptional activity associated with chromatin configuration in fully grown mouse germinal vesicle oocytes', *Biol Reprod*, 60: 580-7.
Brambillasca, F., M. C. Guglielmo, G. Coticchio, M. Mignini Renzini, M. Dal Canto, and R. Fadini. 2013. 'The current challenges to efficient immature oocyte cryopreservation', *J Assist Reprod Genet*, 30: 1531-9.
Brandt, B., S. Meyer-Staeckling, H. Schmidt, K. Agelopoulos, and H. Buerger. 2006. 'Mechanisms of egfr gene transcription modulation: relationship to cancer risk and therapy response', *Clin Cancer Res*, 12: 7252-60.
Braude, P., H. Pelham, G. Flach, and R. Lobatto. 1979. 'Post-transcriptional control in the early mouse embryo', *Nature*, 282: 102-5.
Braw-Tal, R. 2002. 'The initiation of follicle growth: the oocyte or the somatic cells?', *Mol Cell Endocrinol*, 187: 11-8.
Brinster, R. L. 1968. 'Hexokinase activity in the preimplantation mouse embryo', *Enzymologia*, 34: 304-8.
Brinster, R. L. 1971. 'Oxidation of pyruvate and glucose by oocytes of the mouse and rhesus monkey', *J Reprod Fertil*, 24: 187-91.
Britt, K. L., A. E. Drummond, V. A. Cox, M. Dyson, N. G. Wreford, M. E. Jones, E. R. Simpson, and J. K. Findlay. 2000. 'An age-related ovarian phenotype in mice with targeted disruption of the Cyp 19 (aromatase) gene', *Endocrinology*, 141: 2614-23.
Britt, K. L., and J. K. Findlay. 2003. 'Regulation of the phenotype of ovarian somatic cells by estrogen', *Mol Cell Endocrinol*, 202: 11-7.
Brower, P. T., and R. M. Schultz. 1982. 'Intercellular communication between granulosa cells and mouse oocytes: existence and possible nutritional role during oocyte growth', *Dev Biol*, 90: 144-53.
Brunet, S., and B. Maro. 2005. 'Cytoskeleton and cell cycle control during meiotic maturation of the mouse oocyte: integrating time and space', *Reproduction*, 130: 801-11.
Brunet, S., and B. Maro. 2007. 'Germinal vesicle position and meiotic maturation in mouse oocyte', *Reproduction*, 133: 1069-72.
Buccione, R., A. C. Schroeder, and J. J. Eppig. 1990. 'Interactions between somatic cells and germ cells throughout mammalian oogenesis', *Biol Reprod*, 43: 543-7.
Buccione, R., B. C. Vanderhyden, P. J. Caron, and J. J. Eppig. 1990. 'FSH-induced expansion of the mouse cumulus oophorus in vitro is dependent upon a specific factor(s) secreted by the oocyte', *Dev Biol*, 138: 16-25.
Burghardt, R. C., and R. L. Matheson. 1982. 'Gap junction amplification in rat ovarian granulosa cells. I. A direct response to follicle-stimulating hormone', *Dev Biol*, 94: 206-15.
Burns, K. H., C. Yan, T. R. Kumar, and M. M. Matzuk. 2001. 'Analysis of ovarian gene expression in follicle-stimulating hormone beta knockout mice', *Endocrinology*, 142: 2742-51.
Byskov, A. G. 1974. 'Cell kinetic studies of follicular atresia in the mouse ovary', *J Reprod Fertil*, 37: 277-85.
Calafell, J. M., J. Badenas, J. Egozcue, and J. Santalo. 1991. 'Premature chromosome condensation as a sign of oocyte immaturity', *Hum Reprod*, 6: 1017-21.
Cameron, M. R., J. S. Foster, A. Bukovsky, and J. Wimalasena. 1996. 'Activation of mitogen-activated protein kinases by gonadotropins and cyclic adenosine 5'-monophosphates in porcine granulosa cells', *Biol Reprod*, 55: 111-9.

Camp, T. A., J. O. Rahal, and K. E. Mayo. 1991. 'Cellular localization and hormonal regulation of follicle-stimulating hormone and luteinizing hormone receptor messenger RNAs in the rat ovary', *Mol Endocrinol*, 5: 1405-17.

Campen, C. A., and W. Vale. 1988. 'Interaction between purified ovine inhibin and steroids on the release of gonadotropins from cultured rat pituitary cells', *Endocrinology*, 123: 1320-8.

Cantley, L. C., and B. G. Neel. 1999. 'New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway', *Proc Natl Acad Sci U S A*, 96: 4240-5.

Carabatsos, M. J., J. Elvin, M. M. Matzuk, and D. F. Albertini. 1998. 'Characterization of oocyte and follicle development in growth differentiation factor-9-deficient mice', *Dev Biol*, 204: 373-84.

Carabatsos, M. J., C. Sellitto, D. A. Goodenough, and D. F. Albertini. 2000. 'Oocyte-granulosa cell heterologous gap junctions are required for the coordination of nuclear and cytoplasmic meiotic competence', *Dev Biol*, 226: 167-79.

Carletti, M. Z., and L. K. Christenson. 2009. 'Rapid effects of LH on gene expression in the mural granulosa cells of mouse periovulatory follicles', *Reproduction*, 137: 843-55.

Carvalho, C. R., J. B. Carvalheira, M. H. Lima, S. F. Zimmerman, L. C. Caperuto, A. Amanso, A. L. Gasparetti, V. Meneghetti, L. F. Zimmerman, L. A. Velloso, and M. J. Saad. 2003. 'Novel signal transduction pathway for luteinizing hormone and its interaction with insulin: activation of Janus kinase/signal transducer and activator of transcription and phosphoinositol 3-kinase/Akt pathways', *Endocrinology*, 144: 638-47.

Casarini, L., E. Pignatti, and M. Simoni. 2011. 'Effects of polymorphisms in gonadotropin and gonadotropin receptor genes on reproductive function', *Rev Endocr Metab Disord*, 12: 303-21.

Castrillon, D. H., L. Miao, R. Kollipara, J. W. Horner, and R. A. DePinho. 2003. 'Suppression of ovarian follicle activation in mice by the transcription factor Foxo3a', *Science*, 301: 215-8.

Cattanach, B. M., C. A. Iddon, H. M. Charlton, S. A. Chiappa, and G. Fink. 1977. 'Gonadotrophin-releasing hormone deficiency in a mutant mouse with hypogonadism', *Nature*, 269: 338-40.

Cecconi, S., and R. Colonna. 1996. 'Influence of granulosa cells and of different somatic cell types on mammalian oocyte development in vitro', *Zygote*, 4: 305-7.

Cecconi, S., C. Tatone, R. Buccione, F. Mangia, and R. Colonna. 1991. 'Granulosa cell-oocyte interactions: the phosphorylation of specific proteins in mouse oocytes at the germinal vesicle stage is dependent upon the differentiative state of companion somatic cells', *J Exp Zool*, 258: 249-54.

Cetica, P., L. Pintos, G. Dalvit, and M. Beconi. 2002. 'Activity of key enzymes involved in glucose and triglyceride catabolism during bovine oocyte maturation in vitro', *Reproduction*, 124: 675-81.

Chabot, J. G., R. St-Arnaud, P. Walker, and G. Pelletier. 1986. 'Distribution of epidermal growth factor receptors in the rat ovary', *Mol Cell Endocrinol*, 44: 99-108.

Chaigne, A., C. Campillo, N. S. Gov, R. Voituriez, J. Azoury, C. Umana-Diaz, M. Almonacid, I. Queguiner, P. Nassoy, C. Sykes, M. H. Verlhac, and M. E. Terret. 2013. 'A soft cortex is essential for asymmetric spindle positioning in mouse oocytes', *Nat Cell Biol*, 15: 958-66.

Chakraborty, S., S. Mitra, M. M. Falk, S. H. Caplan, M. J. Wheelock, K. R. Johnson, and P. P. Mehta. 2010. 'E-cadherin differentially regulates the assembly of Connexin43 and Connexin32 into gap junctions in human squamous carcinoma cells', *J Biol Chem*, 285: 10761-76.

Chang, H. C., H. Liu, J. Zhang, J. Grifo, and L. C. Krey. 2005. 'Developmental incompetency of denuded mouse oocytes undergoing maturation in vitro is ooplasmic in nature and is associated with aberrant Oct-4 expression', *Hum Reprod*, 20: 1958-68.

Chang, H. M., J. C. Cheng, E. Taylor, and P. C. Leung. 2014. 'Oocyte-derived BMP15 but not GDF9 down-regulates connexin43 expression and decreases gap junction intercellular communication activity in immortalized human granulosa cells', *Mol Hum Reprod*, 20: 373-83.

Channing, C. P., T. Hillensjo, and F. W. Schaerf. 1978. 'Hormonal control of oocyte meiosis, ovulation and luteinization in mammals', *Clin Endocrinol Metab*, 7: 601-24.

Chen, J., C. Melton, N. Suh, J. S. Oh, K. Horner, F. Xie, C. Sette, R. Blelloch, and M. Conti. 2011. 'Genome-wide analysis of translation reveals a critical role for deleted in azoospermia-like (Dazl) at the oocyte-to-zygote transition', *Genes Dev*, 25: 755-66.

Chen, J., S. Torcia, F. Xie, C. J. Lin, H. Cakmak, F. Franciosi, K. Horner, C. Onodera, J. S. Song, M. I. Cedars, M. Ramalho-Santos, and M. Conti. 2013. 'Somatic cells regulate maternal mRNA translation and developmental competence of mouse oocytes', *Nat Cell Biol*, 15: 1415-23.

Chen, L., P. T. Russell, and W. J. Larsen. 1993. 'Functional significance of cumulus expansion in the mouse: roles for the preovulatory synthesis of hyaluronic acid within the cumulus mass', *Mol Reprod Dev*, 34: 87-93.

Chen, L., H. Zhang, R. W. Powers, P. T. Russell, and W. J. Larsen. 1996. 'Covalent linkage between proteins of the inter-alpha-inhibitor family and hyaluronic acid is mediated by a factor produced by granulosa cells', *J Biol Chem*, 271: 19409-14.

Chesnel, F., and J. J. Eppig. 1995a. 'Induction of precocious germinal vesicle breakdown (GVB) by GVB-incompetent mouse oocytes: possible role of mitogen-activated protein kinases rather than p34cdc2 kinase', *Biol Reprod*, 52: 895-902.

Chesnel, F., and J. J. Eppig. 1995b. 'Synthesis and accumulation of p34cdc2 and cyclin B in mouse oocytes during acquisition of competence to resume meiosis', *Mol Reprod Dev*, 40: 503-8.

Chiquoine, A. D. 1960. 'The development of the zona pellucida of the mammalian ovum', *Am J Anat*, 106: 149-69.

Cho, W. K., S. Stern, and J. D. Biggers. 1974. 'Inhibitory effect of dibutyryl cAMP on mouse oocyte maturation in vitro', *J Exp Zool*, 187: 383-6.

Choi, M., O. H. Lee, S. Jeon, M. Park, D. R. Lee, J. J. Ko, T. K. Yoon, A. Rajkovic, and Y. Choi. 2010. 'The oocyte-specific transcription factor, Nobox, regulates the expression of Pad6, a peptidylarginine deiminase in the oocyte', *FEBS Lett*, 584: 3629-34.

Choi, Y., D. J. Ballow, Y. Xin, and A. Rajkovic. 2008. 'Lim homeobox gene, lhx8, is essential for mouse oocyte differentiation and survival', *Biol Reprod*, 79: 442-9.

Choi, Y., and A. Rajkovic. 2006. 'Characterization of NOBOX DNA binding specificity and its regulation of Gdf9 and Pou5f1 promoters', *J Biol Chem*, 281: 35747-56.

Christians, E., M. Boiani, S. Garagna, C. Dessy, C. A. Redi, J. P. Renard, and M. Zuccotti. 1999. 'Gene expression and chromatin organization during mouse oocyte growth', *Dev Biol*, 207: 76-85.

Chun, S. Y., K. M. Eisenhauer, S. Minami, H. Billig, E. Perlas, and A. J. Hsueh. 1996. 'Hormonal regulation of apoptosis in early antral follicles: follicle-stimulating hormone as a major survival factor', *Endocrinology*, 137: 1447-56.

Clark, B. J., J. Wells, S. R. King, and D. M. Stocco. 1994. 'The purification, cloning, and expression of a novel luteinizing hormone-induced mitochondrial protein in MA-10 mouse Leydig tumor cells. Characterization of the steroidogenic acute regulatory protein (StAR)', *J Biol Chem*, 269: 28314-22.

Collado-Fernandez, E., H. M. Picton, and R. Dumollard. 2012. 'Metabolism throughout follicle and oocyte development in mammals', *Int J Dev Biol*, 56: 799-808.

Colonna, R., S. Cecconi, C. Tatone, F. Mangia, and R. Buccione. 1989. 'Somatic cell-oocyte interactions in mouse oogenesis: stage-specific regulation of mouse oocyte protein phosphorylation by granulosa cells', *Dev Biol*, 133: 305-8.

Colonna, R., and F. Mangia. 1983. 'Mechanisms of amino acid uptake in cumulus-enclosed mouse oocytes', *Biol Reprod*, 28: 797-803.

Combelles, C. M., M. J. Carabatsos, T. R. Kumar, M. M. Matzuk, and D. F. Albertini. 2004. 'Hormonal control of somatic cell oocyte interactions during ovarian follicle development', *Mol Reprod Dev*, 69: 347-55.

Comiskey, M., and C. M. Warner. 2007. 'Spatio-temporal localization of membrane lipid rafts in mouse oocytes and cleaving preimplantation embryos', *Dev Biol*, 303: 727-39.

Comizzoli, P., B. S. Pukazhenthi, and D. E. Wildt. 2011. 'The competence of germinal vesicle oocytes is unrelated to nuclear chromatin configuration and strictly depends on cytoplasmic quantity and quality in the cat model', *Hum Reprod*, 26: 2165-77.

Conley, A. J., H. J. Howard, W. D. Slanger, and J. J. Ford. 1994. 'Steroidogenesis in the preovulatory porcine follicle', *Biol Reprod*, 51: 655-61.

Conti, M., M. Hsieh, A. M. Zamah, and J. S. Oh. 2012. 'Novel signaling mechanisms in the ovary during oocyte maturation and ovulation', *Mol Cell Endocrinol*, 356: 65-73.

Corbett, H. E., C. D. Dube, S. Slow, M. Lever, J. M. Trasler, and J. M. Baltz. 2014. 'Uptake of betaine into mouse cumulus-oocyte complexes via the SLC7A6 isoform of y+L transporter', *Biol Reprod*, 90: 81.

Corrigan, A. Z., L. M. Bilezikjian, R. S. Carroll, L. N. Bald, C. H. Schmelzer, B. M. Fendly, A. J. Mason, W. W. Chin, R. H. Schwall, and W. Vale. 1991. 'Evidence for an autocrine role of activin B within rat anterior pituitary cultures', *Endocrinology*, 128: 1682-4.

Coskun, S., M. Uzumcu, Y. C. Lin, C. I. Friedman, and B. M. Alak. 1995. 'Regulation of cumulus cell steroidogenesis by the porcine oocyte and preliminary characterization of oocyte-produced factor(s)', *Biol Reprod*, 53: 670-5.

Cottom, J., L. M. Salvador, E. T. Maizels, S. Reierstad, Y. Park, D. W. Carr, M. A. Davare, J. W. Hell, S. S. Palmer, P. Dent, H. Kawakatsu, M. Ogata, and M. Hunzicker-Dunn. 2003. 'Follicle-stimulating hormone activates extracellular signal-regulated kinase but not extracellular signal-regulated kinase kinase through a 100-kDa phosphotyrosine phosphatase', *J Biol Chem*, 278: 7167-79.

Couse, J. F., M. M. Yates, B. J. Deroo, and K. S. Korach. 2005. 'Estrogen receptor-beta is critical to granulosa cell differentiation and the ovulatory response to gonadotropins', *Endocrinology*, 146: 3247-62.

Cowden, R. R. 1962. 'RNA and yolk synthesis in growing oocytes of the sea urchin, Lytechinus verigatus', *Exp Cell Res*, 28: 600-4.

Cran, D. G., R. M. Moor, and M. F. Hay. 1980. 'Fine structure of the sheep oocyte during antral follicle development', *J Reprod Fertil*, 59: 125-32.
Da Silva-Buttkus, P., G. S. Jayasooriya, J. M. Mora, M. Mobberley, T. A. Ryder, M. Baithun, J. Stark, S. Franks, and K. Hardy. 2008. 'Effect of cell shape and packing density on granulosa cell proliferation and formation of multiple layers during early follicle development in the ovary', *J Cell Sci*, 121: 3890-900.
Dalton, C. M., and J. Carroll. 2013. 'Biased inheritance of mitochondria during asymmetric cell division in the mouse oocyte', *J Cell Sci*, 126: 2955-64.
Dattatreyamurty, B., L. W. Figgs, and L. E. Reichert, Jr. 1987. 'Physical and functional association of follitropin receptors with cholera toxin-sensitive guanine nucleotide-binding protein', *J Biol Chem*, 262: 11737-45.
Davidson, J. S., and I. M. Baumgarten. 1988. 'Glycyrrhetinic acid derivatives: a novel class of inhibitors of gap-junctional intercellular communication. Structure-activity relationships', *J Pharmacol Exp Ther*, 246: 1104-7.
Davis, B. J., D. E. Lennard, C. A. Lee, H. F. Tiano, S. G. Morham, W. C. Wetsel, and R. Langenbach. 1999. 'Anovulation in cyclooxygenase-2-deficient mice is restored by prostaglandin E2 and interleukin-1beta', *Endocrinology*, 140: 2685-95.
De La Fuente, R. 2006. 'Chromatin modifications in the germinal vesicle (GV) of mammalian oocytes', *Dev Biol*, 292: 1-12.
De La Fuente, R., and J. J. Eppig. 2001. 'Transcriptional activity of the mouse oocyte genome: companion granulosa cells modulate transcription and chromatin remodeling', *Dev Biol*, 229: 224-36.
De Lesegno, C. V., K. Reynaud, C. Pechoux, S. Thoumire, and S. Chastant-Maillard. 2008. 'Ultrastructure of canine oocytes during in vivo maturation', *Mol Reprod Dev*, 75: 115-25.
De Smedt, V., and D. Szollosi. 1991. 'Cytochalasin D treatment induces meiotic resumption in follicular sheep oocytes', *Mol Reprod Dev*, 29: 163-71.
de Vant'ery, C., A. C. Gavin, J. D. Vassalli, and S. Schorderet-Slatkine. 1996. 'An accumulation of p34cdc2 at the end of mouse oocyte growth correlates with the acquisition of meiotic competence', *Dev Biol*, 174: 335-44.
Dean, J. 1992. 'Biology of mammalian fertilization: role of the zona pellucida', *J Clin Invest*, 89: 1055-9.
Debey, P., M. S. Szollosi, D. Szollosi, D. Vautier, A. Girousse, and D. Besombes. 1993. 'Competent mouse oocytes isolated from antral follicles exhibit different chromatin organization and follow different maturation dynamics', *Mol Reprod Dev*, 36: 59-74.
Dekel, N., and W. H. Beers. 1978. 'Rat oocyte maturation in vitro: relief of cyclic AMP inhibition by gonadotropins', *Proc Natl Acad Sci U S A*, 75: 4369-73.
Dekel, N., and W. H. Beers. 1980. 'Development of the rat oocyte in vitro: inhibition and induction of maturation in the presence or absence of the cumulus oophorus', *Dev Biol*, 75: 247-54.
Dekel, N., T. Hillensjo, and P. F. Kraicer. 1979. 'Maturational effects of gonadotropins on the cumulus-oocyte complex of the rat', *Biol Reprod*, 20: 191-7.
Dekel, N., and P. F. Kraicer. 1978. 'Induction in vitro of mucification of rat cumulus oophorus by gonadotrophins and adenosine 3',5'-monophosphate', *Endocrinology*, 102: 1797-802.
Dekel, N., and I. Sherizly. 1985. 'Epidermal growth factor induces maturation of rat follicle-enclosed oocytes', *Endocrinology*, 116: 406-9.

Demeestere, I., A. K. Streiff, J. Suzuki, S. Al-Khabouri, E. Mahrous, S. L. Tan, and H. J. Clarke. 2012. 'Follicle-stimulating hormone accelerates mouse oocyte development in vivo', *Biol Reprod*, 87: 3, 1-11.

Deng, J., S. Lang, C. Wylie, and S. R. Hammes. 2008. 'The Xenopus laevis isoform of G protein-coupled receptor 3 (GPR3) is a constitutively active cell surface receptor that participates in maintaining meiotic arrest in X. laevis oocytes', *Mol Endocrinol*, 22: 1853-65.

Devoto, L., L. K. Christenson, J. M. McAllister, A. Makrigiannakis, and J. F. Strauss, 3rd. 1999. 'Insulin and insulin-like growth factor-I and -II modulate human granulosa-lutein cell steroidogenesis: enhancement of steroidogenic acute regulatory protein (StAR) expression', *Mol Hum Reprod*, 5: 1003-10.

Diaz, F. J., M. J. O'Brien, K. Wigglesworth, and J. J. Eppig. 2006. 'The preantral granulosa cell to cumulus cell transition in the mouse ovary: development of competence to undergo expansion', *Dev Biol*, 299: 91-104.

Diaz, F. J., K. Wigglesworth, and J. J. Eppig. 2007a. 'Oocytes are required for the preantral granulosa cell to cumulus cell transition in mice', *Dev Biol*, 305: 300-11.

Diaz, F. J., K. Wigglesworth, and J. J. Eppig. 2007b. 'Oocytes determine cumulus cell lineage in mouse ovarian follicles', *J Cell Sci*, 120: 1330-40.

Dierich, A., M. R. Sairam, L. Monaco, G. M. Fimia, A. Gansmuller, M. LeMeur, and P. Sassone-Corsi. 1998. 'Impairing follicle-stimulating hormone (FSH) signaling in vivo: targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormonal imbalance', *Proc Natl Acad Sci U S A*, 95: 13612-7.

DiLuigi, A., V. N. Weitzman, M. C. Pace, L. J. Siano, D. Maier, and L. M. Mehlmann. 2008. 'Meiotic arrest in human oocytes is maintained by a Gs signaling pathway', *Biol Reprod*, 78: 667-72.

Dissen, G. A., C. Garcia-Rudaz, and S. R. Ojeda. 2009. 'Role of neurotrophic factors in early ovarian development', *Semin Reprod Med*, 27: 24-31.

Dissen, G. A., D. F. Hill, M. E. Costa, C. W. Les Dees, H. E. Lara, and S. R. Ojeda. 1996. 'A role for trkA nerve growth factor receptors in mammalian ovulation', *Endocrinology*, 137: 198-209.

Dissen, G. A., C. Romero, A. N. Hirshfield, and S. R. Ojeda. 2001. 'Nerve growth factor is required for early follicular development in the mammalian ovary', *Endocrinology*, 142: 2078-86.

Dixit, H., L. K. Rao, V. V. Padmalatha, M. Kanakavalli, M. Deenadayal, N. Gupta, B. Chakrabarty, and L. Singh. 2006. 'Missense mutations in the BMP15 gene are associated with ovarian failure', *Hum Genet*, 119: 408-15.

Donahue, R. P., and S. Stern. 1968. 'Follicular cell support of oocyte maturation: production of pyruvate in vitro', *J Reprod Fertil*, 17: 395-8.

Dong, J., D. F. Albertini, K. Nishimori, T. R. Kumar, N. Lu, and M. M. Matzuk. 1996. 'Growth differentiation factor-9 is required during early ovarian folliculogenesis', *Nature*, 383: 531-5.

Dorrington, J. H., Y. S. Moon, and D. T. Armstrong. 1975. 'Estradiol-17beta biosynthesis in cultured granulosa cells from hypophysectomized immature rats; stimulation by follicle-stimulating hormone', *Endocrinology*, 97: 1328-31.

Downs, S. M. 1989. 'Specificity of epidermal growth factor action on maturation of the murine oocyte and cumulus oophorus in vitro', *Biol Reprod*, 41: 371-9.

Downs, S. M., and J. Chen. 2008. 'EGF-like peptides mediate FSH-induced maturation of cumulus cell-enclosed mouse oocytes', *Mol Reprod Dev*, 75: 105-14.
Downs, S. M., and M. Hunzicker-Dunn. 1995. 'Differential regulation of oocyte maturation and cumulus expansion in the mouse oocyte-cumulus cell complex by site-selective analogs of cyclic adenosine monophosphate', *Dev Biol*, 172: 72-85.
Downs, S. M., and A. M. Mastropolo. 1994. 'The participation of energy substrates in the control of meiotic maturation in murine oocytes', *Dev Biol*, 162: 154-68.
Downs, S. M., and A. M. Utecht. 1999. 'Metabolism of radiolabeled glucose by mouse oocytes and oocyte-cumulus cell complexes', *Biol Reprod*, 60: 1446-52.
Dragovic, R. A., L. J. Ritter, S. J. Schulz, F. Amato, D. T. Armstrong, and R. B. Gilchrist. 2005. 'Role of oocyte-secreted growth differentiation factor 9 in the regulation of mouse cumulus expansion', *Endocrinology*, 146: 2798-806.
Dragovic, R. A., L. J. Ritter, S. J. Schulz, F. Amato, J. G. Thompson, D. T. Armstrong, and R. B. Gilchrist. 2007. 'Oocyte-secreted factor activation of SMAD 2/3 signaling enables initiation of mouse cumulus cell expansion', *Biol Reprod*, 76: 848-57.
Dube, J. L., P. Wang, J. Elvin, K. M. Lyons, A. J. Celeste, and M. M. Matzuk. 1998. 'The bone morphogenetic protein 15 gene is X-linked and expressed in oocytes', *Mol Endocrinol*, 12: 1809-17.
Duffy, D. M. 2003. 'Growth differentiation factor-9 is expressed by the primate follicle throughout the periovulatory interval', *Biol Reprod*, 69: 725-32.
Dumesic, D. A., D. R. Meldrum, M. G. Katz-Jaffe, R. L. Krisher, and W. B. Schoolcraft. 2015. 'Oocyte environment: follicular fluid and cumulus cells are critical for oocyte health', *Fertil Steril*, 103: 303-16.
Dunning, K. R., L. N. Watson, V. J. Zhang, H. M. Brown, A. K. Kaczmarek, R. L. Robker, and D. L. Russell. 2015. 'Activation of Mouse Cumulus-Oocyte Complex Maturation In Vitro Through EGF-Like Activity of Versican', *Biol Reprod*, 92: 116.
Dupont, J., A. Musnier, J. Decourtye, T. Boulo, C. Lecureuil, H. Guillou, S. Valet, S. Fouchecourt, J. L. Pitetti, S. Nef, E. Reiter, and P. Crepieux. 2010. 'FSH-stimulated PTEN activity accounts for the lack of FSH mitogenic effect in prepubertal rat Sertoli cells', *Mol Cell Endocrinol*, 315: 271-6.
Dupont, S., A. Krust, A. Gansmuller, A. Dierich, P. Chambon, and M. Mark. 2000. 'Effect of single and compound knockouts of estrogen receptors alpha (ERalpha) and beta (ERbeta) on mouse reproductive phenotypes', *Development*, 127: 4277-91.
Dworkin, M. B., and E. Dworkin-Rastl. 1991. 'Carbon metabolism in early amphibian embryos', *Trends Biochem Sci*, 16: 229-34.
Edwards, R. G. 1965. 'Maturation in vitro of mouse, sheep, cow, pig, rhesus monkey and human ovarian oocytes', *Nature*, 208: 349-51.
Egbert, J. R., L. C. Shuhaibar, A. B. Edmund, D. A. Van Helden, J. W. Robinson, T. F. Uliasz, V. Baena, A. Geerts, F. Wunder, L. R. Potter, and L. A. Jaffe. 2014. 'Dephosphorylation and inactivation of NPR2 guanylyl cyclase in granulosa cells contributes to the LH-induced decrease in cGMP that causes resumption of meiosis in rat oocytes', *Development*, 141: 3594-604.
Eisenbach, M. 1999. 'Mammalian sperm chemotaxis and its association with capacitation', *Dev Genet*, 25: 87-94.

el-Danasouri, I., A. Frances, and L. M. Westphal. 1993. 'Immunocytochemical localization of transforming growth factor-alpha and epidermal growth factor receptor in human fallopian tubes and cumulus cells', *Am J Reprod Immunol*, 30: 82-7.
el-Fouly, M. A., B. Cook, M. Nekola, and A. V. Nalbandov. 1970. 'Role of the ovum in follicular luteinization', *Endocrinology*, 87: 286-93.
El-Hayek, S., and H. J. Clarke. 2015. 'Follicle-Stimulating Hormone Increases Gap Junctional Communication Between the Somatic and Germ-Line Follicular Compartments During Murine Oogenesis', *Biol Reprod*.
El-Hayek, S., I. Demeestere, and H. J. Clarke. 2014. 'Follicle-stimulating hormone regulates expression and activity of epidermal growth factor receptor in the murine ovarian follicle', *Proc Natl Acad Sci U S A*.
Elvin, J. A., A. T. Clark, P. Wang, N. M. Wolfman, and M. M. Matzuk. 1999. 'Paracrine actions of growth differentiation factor-9 in the mammalian ovary', *Mol Endocrinol*, 13: 1035-48.
Elvin, J. A., C. Yan, P. Wang, K. Nishimori, and M. M. Matzuk. 1999. 'Molecular characterization of the follicle defects in the growth differentiation factor 9-deficient ovary', *Mol Endocrinol*, 13: 1018-34.
Emmen, J. M., J. F. Couse, S. A. Elmore, M. M. Yates, G. E. Kissling, and K. S. Korach. 2005. 'In vitro growth and ovulation of follicles from ovaries of estrogen receptor (ER){alpha} and ER{beta} null mice indicate a role for ER{beta} in follicular maturation', *Endocrinology*, 146: 2817-26.
Emori, C., K. Wigglesworth, W. Fujii, K. Naito, J. J. Eppig, and K. Sugiura. 2013. 'Cooperative effects of 17beta-estradiol and oocyte-derived paracrine factors on the transcriptome of mouse cumulus cells', *Endocrinology*, 154: 4859-72.
Eppig, J. J. 1976. 'Analysis of mouse oogenesis in vitro. Oocyte isolation and the utilization of exogenous energy sources by growing oocytes', *J Exp Zool*, 198: 375-82.
Eppig, J. J. 1979a. 'A comparison between oocyte growth in coculture with granulosa cells and oocytes with granulosa cell-oocyte junctional contact maintained in vitro', *J Exp Zool*, 209: 345-53.
Eppig, J. J. 1979b. 'Gonadotropin stimulation of the expansion of cumulus oophori isolated from mice: general conditions for expansion in vitro', *J Exp Zool*, 208: 111-20.
Eppig, J. J. 1980. 'Regulation of cumulus oophorus expansion by gonadotropins in vivo and in vitro', *Biol Reprod*, 23: 545-52.
Eppig, J. J. 1981. 'Prostaglandin E2 stimulates cumulus expansion and hyaluronic acid synthesis by cumuli oophori isolated from mice', *Biol Reprod*, 25: 191-5.
Eppig, J. J. 1989. 'The participation of cyclic adenosine monophosphate (cAMP) in the regulation of meiotic maturation of oocytes in the laboratory mouse', *J Reprod Fertil Suppl*, 38: 3-8.
Eppig, J. J. 1991. 'Intercommunication between mammalian oocytes and companion somatic cells', *Bioessays*, 13: 569-74.
Eppig, J. J., M. Hosoe, M. J. O'Brien, F. M. Pendola, A. Requena, and S. Watanabe. 2000. 'Conditions that affect acquisition of developmental competence by mouse oocytes in vitro: FSH, insulin, glucose and ascorbic acid', *Mol Cell Endocrinol*, 163: 109-16.
Eppig, J. J., M. J. O'Brien, F. L. Pendola, and S. Watanabe. 1998. 'Factors affecting the developmental competence of mouse oocytes grown in vitro: follicle-stimulating hormone and insulin', *Biol Reprod*, 59: 1445-53.

Eppig, J. J., F. L. Pendola, K. Wigglesworth, and J. K. Pendola. 2005. 'Mouse oocytes regulate metabolic cooperativity between granulosa cells and oocytes: amino acid transport', *Biol Reprod*, 73: 351-7.
Eppig, J. J., and A. C. Schroeder. 1989. 'Capacity of mouse oocytes from preantral follicles to undergo embryogenesis and development to live young after growth, maturation, and fertilization in vitro', *Biol Reprod*, 41: 268-76.
Eppig, J. J., R. M. Schultz, M. O'Brien, and F. Chesnel. 1994. 'Relationship between the developmental programs controlling nuclear and cytoplasmic maturation of mouse oocytes', *Dev Biol*, 164: 1-9.
Eppig, J. J., and M. L. Steckman. 1976. 'Comparison of exogenous energy sources for in vitro maintenance of follicle cell-free Xenopus laevis oocytes', *In Vitro*, 12: 173-9.
Eppig, J. J., K. Wigglesworth, F. Pendola, and Y. Hirao. 1997. 'Murine oocytes suppress expression of luteinizing hormone receptor messenger ribonucleic acid by granulosa cells', *Biol Reprod*, 56: 976-84.
Erickson, G. F., C. Wang, and A. J. Hsueh. 1979. 'FSH induction of functional LH receptors in granulosa cells cultured in a chemically defined medium', *Nature*, 279: 336-8.
Escamilla-Hernandez, R., L. Little-Ihrig, K. E. Orwig, J. Yue, U. Chandran, and A. J. Zeleznik. 2008. 'Constitutively active protein kinase A qualitatively mimics the effects of follicle-stimulating hormone on granulosa cell differentiation', *Mol Endocrinol*, 22: 1842-52.
Espey, L. L. 1980. 'Ovulation as an inflammatory reaction--a hypothesis', *Biol Reprod*, 22: 73-106.
Fagbohun, C. F., and S. M. Downs. 1992. 'Requirement for glucose in ligand-stimulated meiotic maturation of cumulus cell-enclosed mouse oocytes', *J Reprod Fertil*, 96: 681-97.
Fairchild, C. L., and M. Barna. 2014. 'Specialized filopodia: at the 'tip' of morphogen transport and vertebrate tissue patterning', *Curr Opin Genet Dev*, 27: 67-73.
Falender, A. E., R. Lanz, D. Malenfant, L. Belanger, and J. S. Richards. 2003. 'Differential expression of steroidogenic factor-1 and FTF/LRH-1 in the rodent ovary', *Endocrinology*, 144: 3598-610.
Fan, C. H., C. L. Lu, J. L. Yang, X. Q. Hu, Y. Ren, H. Cai, M. Chen, Z. Y. Hu, F. Gao, and Y. X. Liu. 2010. 'Signal pathway of GnRH-III inhibiting FSH-induced steroidogenesis in granulosa cells', *Front Biosci (Elite Ed)*, 2: 1218-26.
Fan, H. Y., Z. Liu, N. Cahill, and J. S. Richards. 2008. 'Targeted disruption of Pten in ovarian granulosa cells enhances ovulation and extends the life span of luteal cells', *Mol Endocrinol*, 22: 2128-40.
Fan, H. Y., Z. Liu, M. Shimada, E. Sterneck, P. F. Johnson, S. M. Hedrick, and J. S. Richards. 2009. 'MAPK3/1 (ERK1/2) in ovarian granulosa cells are essential for female fertility', *Science*, 324: 938-41.
Farookhi, R., C. S. Geng, C. D. MacCalman, and O. W. Blaschuk. 1997. 'Hormonal regulation of N-cadherin mRNA levels in rat granulosa cells', *Ann N Y Acad Sci*, 816: 165-72.
Faure, M. O., L. Nicol, S. Fabre, J. Fontaine, N. Mohoric, A. McNeilly, and C. Taragnat. 2005. 'BMP-4 inhibits follicle-stimulating hormone secretion in ewe pituitary', *J Endocrinol*, 186: 109-21.
Felici, M. D., A. D. Carlo, M. Pesce, S. Iona, M. G. Farrace, and M. Piacentini. 1999. 'Bcl-2 and Bax regulation of apoptosis in germ cells during prenatal oogenesis in the mouse embryo', *Cell Death Differ*, 6: 908-15.

Fierro-Gonzalez, J. C., M. D. White, J. C. Silva, and N. Plachta. 2013. 'Cadherin-dependent filopodia control preimplantation embryo compaction', *Nat Cell Biol*, 15: 1424-33.
FitzHarris, G., and J. M. Baltz. 2009. 'Regulation of intracellular pH during oocyte growth and maturation in mammals', *Reproduction*, 138: 619-27.
Fitzpatrick, S. L., and J. S. Richards. 1991. 'Regulation of cytochrome P450 aromatase messenger ribonucleic acid and activity by steroids and gonadotropins in rat granulosa cells', *Endocrinology*, 129: 1452-62.
Fleming, W. N., and R. G. Saacke. 1972. 'Fine structure of the bovine oocyte from the mature graafian follicle', *J Reprod Fertil*, 29: 203-13.
Foote, R. H. 1987. 'In vitro fertilization and embryo transfer in domestic animals: applications in animals and implications for humans', *J In Vitro Fert Embryo Transf*, 4: 73-88.
Fortune, J. E., and D. T. Armstrong. 1977. 'Androgen production by theca and granulosa isolated from proestrous rat follicles', *Endocrinology*, 100: 1341-7.
Franchi, L. L. 1960. 'Electron microscopy of oocyte-follicle cell relationships in the rat ovary', *J Biophys Biochem Cytol*, 7: 397-8.
Franciosi, F., G. Coticchio, V. Lodde, I. Tessaro, S. C. Modina, R. Fadini, M. Dal Canto, M. M. Renzini, D. F. Albertini, and A. M. Luciano. 2014. 'Natriuretic peptide precursor C delays meiotic resumption and sustains gap junction-mediated communication in bovine cumulus-enclosed oocytes', *Biol Reprod*, 91: 61.
Freudzon, L., R. P. Norris, A. R. Hand, S. Tanaka, Y. Saeki, T. L. Jones, M. M. Rasenick, C. H. Berlot, L. M. Mehlmann, and L. A. Jaffe. 2005. 'Regulation of meiotic prophase arrest in mouse oocytes by GPR3, a constitutive activator of the Gs G protein', *J Cell Biol*, 171: 255-65.
Fuhrer, F., B. Mayr, K. Schellander, M. Kalat, and W. Schleger. 1989. 'Maturation competence and chromatin behaviour in growing and fully grown cattle oocytes', *Zentralbl Veterinarmed A*, 36: 285-91.
Fulop, C., R. V. Kamath, Y. Li, J. M. Otto, A. Salustri, B. R. Olsen, T. T. Glant, and V. C. Hascall. 1997. 'Coding sequence, exon-intron structure and chromosomal localization of murine TNF-stimulated gene 6 that is specifically expressed by expanding cumulus cell-oocyte complexes', *Gene*, 202: 95-102.
Fulop, C., A. Salustri, and V. C. Hascall. 1997. 'Coding sequence of a hyaluronan synthase homologue expressed during expansion of the mouse cumulus-oocyte complex', *Arch Biochem Biophys*, 337: 261-6.
Fulop, C., S. Szanto, D. Mukhopadhyay, T. Bardos, R. V. Kamath, M. S. Rugg, A. J. Day, A. Salustri, V. C. Hascall, T. T. Glant, and K. Mikecz. 2003. 'Impaired cumulus mucification and female sterility in tumor necrosis factor-induced protein-6 deficient mice', *Development*, 130: 2253-61.
Gall, L., N. Chene, M. Dahirel, S. Ruffini, and C. Boulesteix. 2004. 'Expression of epidermal growth factor receptor in the goat cumulus-oocyte complex', *Mol Reprod Dev*, 67: 439-45.
Galloway, S. M., K. P. McNatty, L. M. Cambridge, M. P. Laitinen, J. L. Juengel, T. S. Jokiranta, R. J. McLaren, K. Luiro, K. G. Dodds, G. W. Montgomery, A. E. Beattie, G. H. Davis, and O. Ritvos. 2000. 'Mutations in an oocyte-derived growth factor gene (BMP15) cause increased ovulation rate and infertility in a dosage-sensitive manner', *Nat Genet*, 25: 279-83.

Garnett, K., J. Wang, and S. K. Roy. 2002. 'Spatiotemporal expression of epidermal growth factor receptor messenger RNA and protein in the hamster ovary: follicle stage-specific differential modulation by follicle-stimulating hormone, luteinizing hormone, estradiol, and progesterone', *Biol Reprod*, 67: 1593-604.
Gibert, M. A., J. Starck, and B. Beguet. 1984. 'Role of the gonad cytoplasmic core during oogenesis of the nematode Caenorhabditis elegans', *Biol Cell*, 50: 77-85.
Gieske, M. C., H. J. Kim, S. J. Legan, Y. Koo, A. Krust, P. Chambon, and C. Ko. 2008. 'Pituitary gonadotroph estrogen receptor-alpha is necessary for fertility in females', *Endocrinology*, 149: 20-7.
Gilchrist, R. B., M. Lane, and J. G. Thompson. 2008. 'Oocyte-secreted factors: regulators of cumulus cell function and oocyte quality', *Hum Reprod Update*, 14: 159-77.
Gilchrist, R. B., L. J. Ritter, and D. T. Armstrong. 2004. 'Oocyte-somatic cell interactions during follicle development in mammals', *Anim Reprod Sci*, 82-83: 431-46.
Gilula, N. B., M. L. Epstein, and W. H. Beers. 1978. 'Cell-to-cell communication and ovulation. A study of the cumulus-oocyte complex', *J Cell Biol*, 78: 58-75.
Gilula, N. B., O. R. Reeves, and A. Steinbach. 1972. 'Metabolic coupling, ionic coupling and cell contacts', *Nature*, 235: 262-5.
Gittens, J. E., and G. M. Kidder. 2005. 'Differential contributions of connexin37 and connexin43 to oogenesis revealed in chimeric reaggregated mouse ovaries', *J Cell Sci*, 118: 5071-8.
Gjorret, J. O., E. G. Crichton, N. M. Loskutoff, D. L. Armstrong, and P. Hyttel. 2002. 'Ultrastructure of oocyte maturation, fertilization, and early embryo development in vitro in the Siberian tiger (Panthera tigris altaica)', *Mol Reprod Dev*, 63: 79-88.
Glister, C., N. P. Groome, and P. G. Knight. 2003. 'Oocyte-mediated suppression of follicle-stimulating hormone- and insulin-like growth factor-induced secretion of steroids and inhibin-related proteins by bovine granulosa cells in vitro: possible role of transforming growth factor alpha', *Biol Reprod*, 68: 758-65.
Glister, C., D. S. Tannetta, N. P. Groome, and P. G. Knight. 2001. 'Interactions between follicle-stimulating hormone and growth factors in modulating secretion of steroids and inhibin-related peptides by nonluteinized bovine granulosa cells', *Biol Reprod*, 65: 1020-8.
Gmachl, M., S. Sagan, S. Ketter, and G. Kreil. 1993. 'The human sperm protein PH-20 has hyaluronidase activity', *FEBS Lett*, 336: 545-8.
Gonzalez-Robayna, I. J., A. E. Falender, S. Ochsner, G. L. Firestone, and J. S. Richards. 2000. 'Follicle-Stimulating hormone (FSH) stimulates phosphorylation and activation of protein kinase B (PKB/Akt) and serum and glucocorticoid-lnduced kinase (Sgk): evidence for A kinase-independent signaling by FSH in granulosa cells', *Mol Endocrinol*, 14: 1283-300.
Goritz, F., K. Jewgenow, and H. H. Meyer. 1996. 'Epidermal growth factor and epidermal growth factor receptor in the ovary of the domestic cat (Felis catus)', *J Reprod Fertil*, 106: 117-24.
Granot, I., and N. Dekel. 1997. 'Developmental expression and regulation of the gap junction protein and transcript in rat ovaries', *Mol Reprod Dev*, 47: 231-9.
Granot, I., and N. Dekel. 2002. 'The ovarian gap junction protein connexin43: regulation by gonadotropins', *Trends Endocrinol Metab*, 13: 310-3.
Green, D. P. 1997. 'Three-dimensional structure of the zona pellucida', *Rev Reprod*, 2: 147-56.
Grieshaber, N. A., C. Ko, S. S. Grieshaber, I. Ji, and T. H. Ji. 2003. 'Follicle-stimulating hormone-responsive cytoskeletal genes in rat granulosa cells: class I beta-tubulin, tropomyosin-4, and kinesin heavy chain', *Endocrinology*, 144: 29-39.

Griffin, J., B. R. Emery, I. Huang, C. M. Peterson, and D. T. Carrell. 2006. 'Comparative analysis of follicle morphology and oocyte diameter in four mammalian species (mouse, hamster, pig, and human)', *J Exp Clin Assist Reprod*, 3: 2.

Grummer, R., O. Traub, and E. Winterhager. 1999. 'Gap junction connexin genes cx26 and cx43 are differentially regulated by ovarian steroid hormones in rat endometrium', *Endocrinology*, 140: 2509-16.

Gui, L. M., and I. M. Joyce. 2005. 'RNA interference evidence that growth differentiation factor-9 mediates oocyte regulation of cumulus expansion in mice', *Biol Reprod*, 72: 195-9.

Guturi, K. K., T. Mandal, A. Chatterjee, M. Sarkar, S. Bhattacharya, U. Chatterjee, and M. K. Ghosh. 2012. 'Mechanism of beta-catenin-mediated transcriptional regulation of epidermal growth factor receptor expression in glycogen synthase kinase 3 beta-inactivated prostate cancer cells', *J Biol Chem*, 287: 18287-96.

Guzman, L., T. Adriaenssens, C. Ortega-Hrepich, F. K. Albuz, I. Mateizel, P. Devroey, M. De Vos, and J. Smitz. 2013. 'Human antral follicles <6 mm: a comparison between in vivo maturation and in vitro maturation in non-hCG primed cycles using cumulus cell gene expression', *Mol Hum Reprod*, 19: 7-16.

Haghighat, N., and L. J. Van Winkle. 1990. 'Developmental change in follicular cell-enhanced amino acid uptake into mouse oocytes that depends on intact gap junctions and transport system Gly', *J Exp Zool*, 253: 71-82.

Haisenleder, D. J., L. L. Burger, K. W. Aylor, A. C. Dalkin, H. E. Walsh, M. A. Shupnik, and J. C. Marshall. 2005. 'Testosterone stimulates follicle-stimulating hormone beta transcription via activation of extracellular signal-regulated kinase: evidence in rat pituitary cells', *Biol Reprod*, 72: 523-9.

Han, S. J., R. Chen, M. P. Paronetto, and M. Conti. 2005. 'Wee1B is an oocyte-specific kinase involved in the control of meiotic arrest in the mouse', *Curr Biol*, 15: 1670-6.

Harris, S. E., I. Adriaens, H. J. Leese, R. G. Gosden, and H. M. Picton. 2007. 'Carbohydrate metabolism by murine ovarian follicles and oocytes grown in vitro', *Reproduction*, 134: 415-24.

Harris, S. E., H. J. Leese, R. G. Gosden, and H. M. Picton. 2009. 'Pyruvate and oxygen consumption throughout the growth and development of murine oocytes', *Mol Reprod Dev*, 76: 231-8.

Hartshorne, G., S. Montgomery, and L. Klentzeris. 1999. 'A case of failed oocyte maturation in vivo and in vitro', *Fertil Steril*, 71: 567-70.

Hayashi, M., E. A. McGee, G. Min, C. Klein, U. M. Rose, M. van Duin, and A. J. Hsueh. 1999. 'Recombinant growth differentiation factor-9 (GDF-9) enhances growth and differentiation of cultured early ovarian follicles', *Endocrinology*, 140: 1236-44.

Hertig, A. T., and E. C. Adams. 1967. 'Studies on the human oocyte and its follicle. I. Ultrastructural and histochemical observations on the primordial follicle stage', *J Cell Biol*, 34: 647-75.

Herubel, F., S. El Mouatassim, P. Guerin, R. Frydman, and Y. Menezo. 2002. 'Genetic expression of monocarboxylate transporters during human and murine oocyte maturation and early embryonic development', *Zygote*, 10: 175-81.

Hess, K. A., L. Chen, and W. J. Larsen. 1998. 'The ovarian blood follicle barrier is both charge- and size-selective in mice', *Biol Reprod*, 58: 705-11.

Hickey, G. J., S. A. Chen, M. J. Besman, J. E. Shively, P. F. Hall, D. Gaddy-Kurten, and J. S. Richards. 1988. 'Hormonal regulation, tissue distribution, and content of aromatase

cytochrome P450 messenger ribonucleic acid and enzyme in rat ovarian follicles and corpora lutea: relationship to estradiol biosynthesis', *Endocrinology*, 122: 1426-36.
Hill, J. L., K. Hammar, P. J. Smith, and D. J. Gross. 1999. 'Stage-dependent effects of epidermal growth factor on Ca2+ efflux in mouse oocytes', *Mol Reprod Dev*, 53: 244-53.
Hinckley, M., S. Vaccari, K. Horner, R. Chen, and M. Conti. 2005. 'The G-protein-coupled receptors GPR3 and GPR12 are involved in cAMP signaling and maintenance of meiotic arrest in rodent oocytes', *Dev Biol*, 287: 249-61.
Hinrichs, K., A. L. Schmidt, P. P. Friedman, J. P. Selgrath, and M. G. Martin. 1993. 'In vitro maturation of horse oocytes: characterization of chromatin configuration using fluorescence microscopy', *Biol Reprod*, 48: 363-70.
Hirshfield, A. N. 1991. 'Development of follicles in the mammalian ovary', *Int Rev Cytol*, 124: 43-101.
Ho, C. C., and D. J. Bernard. 2009. 'Bone morphogenetic protein 2 signals via BMPR1A to regulate murine follicle-stimulating hormone beta subunit transcription', *Biol Reprod*, 81: 133-41.
Holt, J. E., J. Weaver, and K. T. Jones. 2010. 'Spatial regulation of APCCdh1-induced cyclin B1 degradation maintains G2 arrest in mouse oocytes', *Development*, 137: 1297-304.
Hope, J. 1965. 'The fine structure of the developing follicle of the rhesus ovary', *J Ultrastruct Res*, 12: 592-610.
Horner, K., G. Livera, M. Hinckley, K. Trinh, D. Storm, and M. Conti. 2003. 'Rodent oocytes express an active adenylyl cyclase required for meiotic arrest', *Dev Biol*, 258: 385-96.
Hosaka, T., W. H. Biggs, 3rd, D. Tieu, A. D. Boyer, N. M. Varki, W. K. Cavenee, and K. C. Arden. 2004. 'Disruption of forkhead transcription factor (FOXO) family members in mice reveals their functional diversification', *Proc Natl Acad Sci U S A*, 101: 2975-80.
Hreinsson, J. G., J. E. Scott, C. Rasmussen, M. L. Swahn, A. J. Hsueh, and O. Hovatta. 2002. 'Growth differentiation factor-9 promotes the growth, development, and survival of human ovarian follicles in organ culture', *J Clin Endocrinol Metab*, 87: 316-21.
Hsieh, M., D. Lee, S. Panigone, K. Horner, R. Chen, A. Theologis, D. C. Lee, D. W. Threadgill, and M. Conti. 2007. 'Luteinizing hormone-dependent activation of the epidermal growth factor network is essential for ovulation', *Mol Cell Biol*, 27: 1914-24.
Hsieh, M., K. Thao, and M. Conti. 2011. 'Genetic dissection of epidermal growth factor receptor signaling during luteinizing hormone-induced oocyte maturation', *PLoS One*, 6: e21574.
Hsu, C. J., and J. M. Hammond. 1987. 'Gonadotropins and estradiol stimulate immunoreactive insulin-like growth factor-I production by porcine granulosa cells in vitro', *Endocrinology*, 120: 198-207.
Huang, Q., A. P. Cheung, Y. Zhang, H. F. Huang, N. Auersperg, and P. C. Leung. 2009. 'Effects of growth differentiation factor 9 on cell cycle regulators and ERK42/44 in human granulosa cell proliferation', *Am J Physiol Endocrinol Metab*, 296: E1344-53.
Hussein, T. S., D. A. Froiland, F. Amato, J. G. Thompson, and R. B. Gilchrist. 2005. 'Oocytes prevent cumulus cell apoptosis by maintaining a morphogenic paracrine gradient of bone morphogenetic proteins', *J Cell Sci*, 118: 5257-68.
Hyttel, P., T. Greve, and H. Callesen. 1989. 'Ultrastructure of oocyte maturation and fertilization in superovulated cattle', *Prog Clin Biol Res*, 296: 287-97.
Ievoli, E., R. Lindstedt, A. Inforzato, A. Camaioni, F. Palone, A. J. Day, A. Mantovani, G. Salvatori, and A. Salustri. 2011. 'Implication of the oligomeric state of the N-terminal PTX3 domain in cumulus matrix assembly', *Matrix Biol*, 30: 330-7.

Igarashi, H., T. Takahashi, E. Takahashi, N. Tezuka, K. Nakahara, K. Takahashi, and H. Kurachi. 2005. 'Aged mouse oocytes fail to readjust intracellular adenosine triphosphates at fertilization', *Biol Reprod*, 72: 1256-61.

Irving-Rodgers, H. F., M. L. Mussard, J. E. Kinder, and R. J. Rodgers. 2002. 'Composition and morphology of the follicular basal lamina during atresia of bovine antral follicles', *Reproduction*, 123: 97-106.

Ishikawa-Ankerhold, H. C., R. Ankerhold, and G. P. Drummen. 2012. 'Advanced fluorescence microscopy techniques--FRAP, FLIP, FLAP, FRET and FLIM', *Molecules*, 17: 4047-132.

Ito, Y., M. Kihara, E. Nakamura, S. Yonezawa, and N. Yoshizaki. 2003. 'Vitellogenin transport and yolk formation in the quail ovary', *Zoolog Sci*, 20: 717-26.

Jaiswal, R., D. Breitsprecher, A. Collins, I. R. Correa, Jr., M. Q. Xu, and B. L. Goode. 2013. 'The formin Daam1 and fascin directly collaborate to promote filopodia formation', *Curr Biol*, 23: 1373-9.

Jamnongjit, M., A. Gill, and S. R. Hammes. 2005. 'Epidermal growth factor receptor signaling is required for normal ovarian steroidogenesis and oocyte maturation', *Proc Natl Acad Sci U S A*, 102: 16257-62.

Jensen, J. T., K. M. Schwinof, M. B. Zelinski-Wooten, M. Conti, L. V. DePaolo, and R. L. Stouffer. 2002. 'Phosphodiesterase 3 inhibitors selectively block the spontaneous resumption of meiosis by macaque oocytes in vitro', *Hum Reprod*, 17: 2079-84.

Jin, Y. X., H. S. Lee, X. J. Yin, X. S. Cui, I. K. Kong, and N. H. Kim. 2006. 'Chromatin, microtubule and microfilament configurations in the canine oocyte', *Reprod Fertil Dev*, 18: 849-56.

John, G. B., T. D. Gallardo, L. J. Shirley, and D. H. Castrillon. 2008. 'Foxo3 is a PI3K-dependent molecular switch controlling the initiation of oocyte growth', *Dev Biol*, 321: 197-204.

John, G. B., L. J. Shirley, T. D. Gallardo, and D. H. Castrillon. 2007. 'Specificity of the requirement for Foxo3 in primordial follicle activation', *Reproduction*, 133: 855-63.

Johnson, M. H. 1981. 'The molecular and cellular basis of preimplantation mouse development', *Biol Rev Camb Philos Soc*, 56: 463-98.

Johnson, M. L., D. A. Redmer, L. P. Reynolds, J. J. Bilski, and A. T. Grazul-Bilska. 2002. 'Gap junctional intercellular communication of bovine granulosa and thecal cells from antral follicles: effects of luteinizing hormone and follicle-stimulating hormone', *Endocrine*, 18: 261-70.

Johnson, M. T., E. A. Freeman, D. K. Gardner, and P. A. Hunt. 2007. 'Oxidative metabolism of pyruvate is required for meiotic maturation of murine oocytes in vivo', *Biol Reprod*, 77: 2-8.

Joyce, I. M., F. L. Pendola, M. O'Brien, and J. J. Eppig. 2001. 'Regulation of prostaglandin-endoperoxide synthase 2 messenger ribonucleic acid expression in mouse granulosa cells during ovulation', *Endocrinology*, 142: 3187-97.

Joyce, I. M., F. L. Pendola, K. Wigglesworth, and J. J. Eppig. 1999. 'Oocyte regulation of kit ligand expression in mouse ovarian follicles', *Dev Biol*, 214: 342-53.

Juengel, J. L., D. A. Heath, L. D. Quirke, and K. P. McNatty. 2006. 'Oestrogen receptor alpha and beta, androgen receptor and progesterone receptor mRNA and protein localisation within the developing ovary and in small growing follicles of sheep', *Reproduction*, 131: 81-92.

Juneja, S. C., K. J. Barr, G. C. Enders, and G. M. Kidder. 1999. 'Defects in the germ line and gonads of mice lacking connexin43', *Biol Reprod*, 60: 1263-70.
Kageyama, R., G. T. Merlino, and I. Pastan. 1988. 'Epidermal growth factor (EGF) receptor gene transcription. Requirement for Sp1 and an EGF receptor-specific factor', *J Biol Chem*, 263: 6329-36.
Kanatsu-Shinohara, M., R. M. Schultz, and G. S. Kopf. 2000. 'Acquisition of meiotic competence in mouse oocytes: absolute amounts of p34(cdc2), cyclin B1, cdc25C, and wee1 in meiotically incompetent and competent oocytes', *Biol Reprod*, 63: 1610-6.
Kawamura, K., Y. Cheng, N. Kawamura, S. Takae, A. Okada, Y. Kawagoe, S. Mulders, Y. Terada, and A. J. Hsueh. 2011. 'Pre-ovulatory LH/hCG surge decreases C-type natriuretic peptide secretion by ovarian granulosa cells to promote meiotic resumption of pre-ovulatory oocytes', *Hum Reprod*, 26: 3094-101.
Keel, B. A., J. M. Hildebrandt, J. V. May, and J. S. Davis. 1995. 'Effects of epidermal growth factor on the tyrosine phosphorylation of mitogen-activated protein kinases in monolayer cultures of porcine granulosa cells', *Endocrinology*, 136: 1197-204.
Kelley, L. C., L. L. Lohmer, E. J. Hagedorn, and D. R. Sherwood. 2014. 'Traversing the basement membrane in vivo: a diversity of strategies', *J Cell Biol*, 204: 291-302.
Kerber, M. L., and R. E. Cheney. 2011. 'Myosin-X: a MyTH-FERM myosin at the tips of filopodia', *J Cell Sci*, 124: 3733-41.
Kessel, R. G., H. N. Tung, R. Roberts, and H. W. Beams. 1985. 'The presence and distribution of gap junctions in the oocyte-follicle cell complex of the zebrafish, Brachydanio rerio', *J Submicrosc Cytol*, 17: 239-53.
Kezele, P., E. E. Nilsson, and M. K. Skinner. 2005. 'Keratinocyte growth factor acts as a mesenchymal factor that promotes ovarian primordial to primary follicle transition', *Biol Reprod*, 73: 967-73.
Khalid, M., W. Haresign, and M. R. Luck. 2000. 'Secretion of IGF-1 by ovine granulosa cells: effects of growth hormone and follicle stimulating hormone', *Anim Reprod Sci*, 58: 261-72.
Kidder, G. M., and B. C. Vanderhyden. 2010. 'Bidirectional communication between oocytes and follicle cells: ensuring oocyte developmental competence', *Can J Physiol Pharmacol*, 88: 399-413.
Kim, K., H. Lee, D. W. Threadgill, and D. Lee. 2011. 'Epiregulin-dependent amphiregulin expression and ERBB2 signaling are involved in luteinizing hormone-induced paracrine signaling pathways in mouse ovary', *Biochem Biophys Res Commun*, 405: 319-24.
Klinger, F. G., and M. De Felici. 2002. 'In vitro development of growing oocytes from fetal mouse oocytes: stage-specific regulation by stem cell factor and granulosa cells', *Dev Biol*, 244: 85-95.
Kornberg, T. B., and S. Roy. 2014. 'Cytonemes as specialized signaling filopodia', *Development*, 141: 729-36.
Kovanci, E., J. Rohozinski, J. L. Simpson, M. J. Heard, C. E. Bishop, and S. A. Carson. 2007. 'Growth differentiating factor-9 mutations may be associated with premature ovarian failure', *Fertil Steril*, 87: 143-6.
Kreeger, P. K., N. N. Fernandes, T. K. Woodruff, and L. D. Shea. 2005. 'Regulation of mouse follicle development by follicle-stimulating hormone in a three-dimensional in vitro culture system is dependent on follicle stage and dose', *Biol Reprod*, 73: 942-50.

Krege, J. H., J. B. Hodgin, J. F. Couse, E. Enmark, M. Warner, J. F. Mahler, M. Sar, K. S. Korach, J. A. Gustafsson, and O. Smithies. 1998. 'Generation and reproductive phenotypes of mice lacking estrogen receptor beta', *Proc Natl Acad Sci U S A*, 95: 15677-82.

Kumar, T. R., J. Agno, J. A. Janovick, P. M. Conn, and M. M. Matzuk. 2003. 'Regulation of FSHbeta and GnRH receptor gene expression in activin receptor II knockout male mice', *Mol Cell Endocrinol*, 212: 19-27.

Kumar, T. R., Y. Wang, N. Lu, and M. M. Matzuk. 1997. 'Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility', *Nat Genet*, 15: 201-4.

Laissue, P., S. Christin-Maitre, P. Touraine, F. Kuttenn, O. Ritvos, K. Aittomaki, N. Bourcigaux, L. Jacquesson, P. Bouchard, R. Frydman, D. Dewailly, A. C. Reyss, L. Jeffery, A. Bachelot, N. Massin, M. Fellous, and R. A. Veitia. 2006. 'Mutations and sequence variants in GDF9 and BMP15 in patients with premature ovarian failure', *Eur J Endocrinol*, 154: 739-44.

Lam, X., C. Gieseke, M. Knoll, and P. Talbot. 2000. 'Assay and importance of adhesive interaction between hamster (Mesocricetus auratus) oocyte-cumulus complexes and the oviductal epithelium', *Biol Reprod*, 62: 579-88.

Larson, S. M., H. J. Lee, P. H. Hung, L. M. Matthews, D. N. Robinson, and J. P. Evans. 2010. 'Cortical mechanics and meiosis II completion in mammalian oocytes are mediated by myosin-II and Ezrin-Radixin-Moesin (ERM) proteins', *Mol Biol Cell*, 21: 3182-92.

LaVoie, H. A., D. C. DeSimone, C. Gillio-Meina, and Y. Y. Hui. 2002. 'Cloning and characterization of porcine ovarian estrogen receptor beta isoforms', *Biol Reprod*, 66: 616-23.

Law, N. C., J. Weck, B. Kyriss, J. H. Nilson, and M. Hunzicker-Dunn. 2013. 'Lhcgr expression in granulosa cells: roles for PKA-phosphorylated beta-catenin, TCF3, and FOXO1', *Mol Endocrinol*, 27: 1295-310.

Layman, L. C., E. J. Lee, D. B. Peak, A. B. Namnoum, K. V. Vu, B. L. van Lingen, M. R. Gray, P. G. McDonough, R. H. Reindollar, and J. L. Jameson. 1997. 'Delayed puberty and hypogonadism caused by mutations in the follicle-stimulating hormone beta-subunit gene', *N Engl J Med*, 337: 607-11.

Ledent, C., I. Demeestere, D. Blum, J. Petermans, T. Hamalainen, G. Smits, and G. Vassart. 2005. 'Premature ovarian aging in mice deficient for Gpr3', *Proc Natl Acad Sci U S A*, 102: 8922-6.

Lee, D., R. S. Pearsall, S. Das, S. K. Dey, V. L. Godfrey, and D. W. Threadgill. 2004. 'Epiregulin is not essential for development of intestinal tumors but is required for protection from intestinal damage', *Mol Cell Biol*, 24: 8907-16.

Lee, K. B., V. Khivansara, M. M. Santos, P. Lamba, T. Yuen, S. C. Sealfon, and D. J. Bernard. 2007. 'Bone morphogenetic protein 2 and activin A synergistically stimulate follicle-stimulating hormone beta subunit transcription', *J Mol Endocrinol*, 38: 315-30.

Lee, K. B., M. Zhang, K. Sugiura, K. Wigglesworth, T. Uliasz, L. A. Jaffe, and J. J. Eppig. 2013. 'Hormonal coordination of natriuretic peptide type C and natriuretic peptide receptor 3 expression in mouse granulosa cells', *Biol Reprod*, 88: 42.

Lee, W. S., F. Otsuka, R. K. Moore, and S. Shimasaki. 2001. 'Effect of bone morphogenetic protein-7 on folliculogenesis and ovulation in the rat', *Biol Reprod*, 65: 994-9.

Leese, H. J., and A. M. Barton. 1985. 'Production of pyruvate by isolated mouse cumulus cells', *J Exp Zool*, 234: 231-6.

Lefevre, B. 2011. '[Follicular atresia: Its features as predictive markers for the outcome of assisted reproduction]', *Gynecol Obstet Fertil*, 39: 58-62.
Lefevre, B., A. Gougeon, F. Nome, and J. Testart. 1989. 'In vivo changes in oocyte germinal vesicle related to follicular quality and size at mid-follicular phase during stimulated cycles in the cynomolgus monkey', *Reprod Nutr Dev*, 29: 523-31.
Lei, Z. M., S. Mishra, W. Zou, B. Xu, M. Foltz, X. Li, and C. V. Rao. 2001. 'Targeted disruption of luteinizing hormone/human chorionic gonadotropin receptor gene', *Mol Endocrinol*, 15: 184-200.
Levran, D., J. Farhi, H. Nahum, M. Glezerman, and A. Weissman. 2002. 'Maturation arrest of human oocytes as a cause of infertility: case report', *Hum Reprod*, 17: 1604-9.
Lew, D. J., and S. Kornbluth. 1996. 'Regulatory roles of cyclin dependent kinase phosphorylation in cell cycle control', *Curr Opin Cell Biol*, 8: 795-804.
Li, J., K. Kawamura, Y. Cheng, S. Liu, C. Klein, S. Liu, E. K. Duan, and A. J. Hsueh. 2010. 'Activation of dormant ovarian follicles to generate mature eggs', *Proc Natl Acad Sci U S A*, 107: 10280-4.
Li, M., C. G. Liang, B. Xiong, B. Z. Xu, S. L. Lin, Y. Hou, D. Y. Chen, H. Schatten, and Q. Y. Sun. 2008. 'PI3-kinase and mitogen-activated protein kinase in cumulus cells mediate EGF-induced meiotic resumption of porcine oocyte', *Domest Anim Endocrinol*, 34: 360-71.
Li, M., S. Yin, J. Yuan, L. Wei, J. S. Ai, Y. Hou, D. Y. Chen, and Q. Y. Sun. 2008. 'Cdc25A promotes G2/M transition in oocytes', *Cell Cycle*, 7: 1301-2.
Li, R., and D. F. Albertini. 2013. 'The road to maturation: somatic cell interaction and self-organization of the mammalian oocyte', *Nat Rev Mol Cell Biol*, 14: 141-52.
Li, R., and J. P. Mather. 1997. 'Lindane, an inhibitor of gap junction formation, abolishes oocyte directed follicle organizing activity in vitro', *Endocrinology*, 138: 4477-80.
Li, R., R. J. Norman, D. T. Armstrong, and R. B. Gilchrist. 2000. 'Oocyte-secreted factor(s) determine functional differences between bovine mural granulosa cells and cumulus cells', *Biol Reprod*, 63: 839-45.
Li, X., S. K. Tripurani, R. James, and S. A. Pangas. 2012. 'Minimal fertility defects in mice deficient in oocyte-expressed Smad4', *Biol Reprod*, 86: 1-6.
Lin, C. J., F. M. Koh, P. Wong, M. Conti, and M. Ramalho-Santos. 2014. 'Hira-mediated H3.3 incorporation is required for DNA replication and ribosomal RNA transcription in the mouse zygote', *Dev Cell*, 30: 268-79.
Lin, Y., K. Mahan, W. F. Lathrop, D. G. Myles, and P. Primakoff. 1994. 'A hyaluronidase activity of the sperm plasma membrane protein PH-20 enables sperm to penetrate the cumulus cell layer surrounding the egg', *J Cell Biol*, 125: 1157-63.
Lincoln, A. J., D. Wickramasinghe, P. Stein, R. M. Schultz, M. E. Palko, M. P. De Miguel, L. Tessarollo, and P. J. Donovan. 2002. 'Cdc25b phosphatase is required for resumption of meiosis during oocyte maturation', *Nat Genet*, 30: 446-9.
Lindzey, J., F. L. Jayes, M. M. Yates, J. F. Couse, and K. S. Korach. 2006. 'The bi-modal effects of estradiol on gonadotropin synthesis and secretion in female mice are dependent on estrogen receptor-alpha', *J Endocrinol*, 191: 309-17.
Lintern-Moore, S., and G. P. Moore. 1979. 'The initiation of follicle and oocyte growth in the mouse ovary', *Biol Reprod*, 20: 773-8.

Liu, C., J. Peng, M. M. Matzuk, and H. H. Yao. 2015. 'Lineage specification of ovarian theca cells requires multicellular interactions via oocyte and granulosa cells', *Nat Commun*, 6: 6934.
Liu, K. C., D. T. Jacobs, B. D. Dunn, A. S. Fanning, and R. E. Cheney. 2012. 'Myosin-X functions in polarized epithelial cells', *Mol Biol Cell*, 23: 1675-87.
Liu, K., S. Rajareddy, L. Liu, K. Jagarlamudi, K. Boman, G. Selstam, and P. Reddy. 2006. 'Control of mammalian oocyte growth and early follicular development by the oocyte PI3 kinase pathway: new roles for an old timer', *Dev Biol*, 299: 1-11.
Liu, M., D. Sims, P. Calarco, and P. Talbot. 2003. 'Biochemical heterogeneity, migration, and pre-fertilization release of mouse oocyte cortical granules', *Reprod Biol Endocrinol*, 1: 77.
Liu, R., E. V. Linardopoulou, G. E. Osborn, and S. M. Parkhurst. 2010. 'Formins in development: orchestrating body plan origami', *Biochim Biophys Acta*, 1803: 207-25.
Lodde, V., F. Franciosi, I. Tessaro, S. C. Modina, and A. M. Luciano. 2013. 'Role of gap junction-mediated communications in regulating large-scale chromatin configuration remodeling and embryonic developmental competence acquisition in fully grown bovine oocyte', *J Assist Reprod Genet*, 30: 1219-26.
Lodde, V., S. Modina, C. Galbusera, F. Franciosi, and A. M. Luciano. 2007. 'Large-scale chromatin remodeling in germinal vesicle bovine oocytes: interplay with gap junction functionality and developmental competence', *Mol Reprod Dev*, 74: 740-9.
Lodde, V., S. Modina, P. Maddox-Hyttel, F. Franciosi, A. Lauria, and A. M. Luciano. 2008. 'Oocyte morphology and transcriptional silencing in relation to chromatin remodeling during the final phases of bovine oocyte growth', *Mol Reprod Dev*, 75: 915-24.
Luciano, A. M., F. Franciosi, S. C. Modina, and V. Lodde. 2011. 'Gap junction-mediated communications regulate chromatin remodeling during bovine oocyte growth and differentiation through cAMP-dependent mechanism(s)', *Biol Reprod*, 85: 1252-9.
Luciano, A. M., V. Lodde, M. S. Beretta, S. Colleoni, A. Lauria, and S. Modina. 2005. 'Developmental capability of denuded bovine oocyte in a co-culture system with intact cumulus-oocyte complexes: role of cumulus cells, cyclic adenosine 3',5'-monophosphate, and glutathione', *Mol Reprod Dev*, 71: 389-97.
Lucifero, D., C. Mertineit, H. J. Clarke, T. H. Bestor, and J. M. Trasler. 2002. 'Methylation dynamics of imprinted genes in mouse germ cells', *Genomics*, 79: 530-8.
Luetteke, N. C., T. H. Qiu, S. E. Fenton, K. L. Troyer, R. F. Riedel, A. Chang, and D. C. Lee. 1999. 'Targeted inactivation of the EGF and amphiregulin genes reveals distinct roles for EGF receptor ligands in mouse mammary gland development', *Development*, 126: 2739-50.
Lund, S. A., J. Murdoch, E. A. Van Kirk, and W. J. Murdoch. 1999. 'Mitogenic and antioxidant mechanisms of estradiol action in preovulatory ovine follicles: relevance to luteal function', *Biol Reprod*, 61: 388-92.
Lundy, T., P. Smith, A. O'Connell, N. L. Hudson, and K. P. McNatty. 1999. 'Populations of granulosa cells in small follicles of the sheep ovary', *J Reprod Fertil*, 115: 251-62.
Lunenfeld, B. 2004. 'Historical perspectives in gonadotrophin therapy', *Hum Reprod Update*, 10: 453-67.
Macaulay, A. D., I. Gilbert, J. Caballero, R. Barreto, E. Fournier, P. Tossou, M. A. Sirard, H. J. Clarke, E. W. Khandjian, F. J. Richard, P. Hyttel, and C. Robert. 2014. 'The gametic synapse: RNA transfer to the bovine oocyte', *Biol Reprod*, 91: 90.

Machell, N. H., and R. Farookhi. 2003. 'E- and N-cadherin expression and distribution during luteinization in the rat ovary', *Reproduction*, 125: 791-800.
Macklon, N. S., R. L. Stouffer, L. C. Giudice, and B. C. Fauser. 2006. 'The science behind 25 years of ovarian stimulation for in vitro fertilization', *Endocr Rev*, 27: 170-207.
Maeda, E., S. Ye, W. Wang, D. L. Bader, M. M. Knight, and D. A. Lee. 2012. 'Gap junction permeability between tenocytes within tendon fascicles is suppressed by tensile loading', *Biomech Model Mechanobiol*, 11: 439-47.
Mahrous, E., Q. Yang, and H. J. Clarke. 2012. 'Regulation of mitochondrial DNA accumulation during oocyte growth and meiotic maturation in the mouse', *Reproduction*, 144: 177-85.
Maizels, E. T., J. Cottom, J. C. Jones, and M. Hunzicker-Dunn. 1998. 'Follicle stimulating hormone (FSH) activates the p38 mitogen-activated protein kinase pathway, inducing small heat shock protein phosphorylation and cell rounding in immature rat ovarian granulosa cells', *Endocrinology*, 139: 3353-6.
Maller, J. L., and E. G. Krebs. 1977. 'Progesterone-stimulated meiotic cell division in Xenopus oocytes. Induction by regulatory subunit and inhibition by catalytic subunit of adenosine 3':5'-monophosphate-dependent protein kinase', *J Biol Chem*, 252: 1712-8.
Maller, J. L., and E. G. Krebs. 1980. 'Regulation of oocyte maturation', *Curr Top Cell Regul*, 16: 271-311.
Manova, K., E. J. Huang, M. Angeles, V. De Leon, S. Sanchez, S. M. Pronovost, P. Besmer, and R. F. Bachvarova. 1993. 'The expression pattern of the c-kit ligand in gonads of mice supports a role for the c-kit receptor in oocyte growth and in proliferation of spermatogonia', *Dev Biol*, 157: 85-99.
Marangos, P., and J. Carroll. 2004. 'The dynamics of cyclin B1 distribution during meiosis I in mouse oocytes', *Reproduction*, 128: 153-62.
Masciarelli, S., K. Horner, C. Liu, S. H. Park, M. Hinckley, S. Hockman, T. Nedachi, C. Jin, M. Conti, and V. Manganiello. 2004. 'Cyclic nucleotide phosphodiesterase 3A-deficient mice as a model of female infertility', *J Clin Invest*, 114: 196-205.
Mason, A. J., J. S. Hayflick, R. T. Zoeller, W. S. Young, 3rd, H. S. Phillips, K. Nikolics, and P. H. Seeburg. 1986. 'A deletion truncating the gonadotropin-releasing hormone gene is responsible for hypogonadism in the hpg mouse', *Science*, 234: 1366-71.
Matsuda, F., N. Inoue, N. Manabe, and S. Ohkura. 2012. 'Follicular growth and atresia in mammalian ovaries: regulation by survival and death of granulosa cells', *J Reprod Dev*, 58: 44-50.
Matthews, C. H., S. Borgato, P. Beck-Peccoz, M. Adams, Y. Tone, G. Gambino, S. Casagrande, G. Tedeschini, A. Benedetti, and V. K. Chatterjee. 1993. 'Primary amenorrhoea and infertility due to a mutation in the beta-subunit of follicle-stimulating hormone', *Nat Genet*, 5: 83-6.
Mattila, P. K., and P. Lappalainen. 2008. 'Filopodia: molecular architecture and cellular functions', *Nat Rev Mol Cell Biol*, 9: 446-54.
Mattson, B. A., and D. F. Albertini. 1990. 'Oogenesis: chromatin and microtubule dynamics during meiotic prophase', *Mol Reprod Dev*, 25: 374-83.
Matunis, E. L., R. R. Stine, and M. de Cuevas. 2012. 'Recent advances in Drosophila male germline stem cell biology', *Spermatogenesis*, 2: 137-44.
Mazerbourg, S., C. Klein, J. Roh, N. Kaivo-Oja, D. G. Mottershead, O. Korchynskyi, O. Ritvos, and A. J. Hsueh. 2004. 'Growth differentiation factor-9 signaling is mediated by the type I receptor, activin receptor-like kinase 5', *Mol Endocrinol*, 18: 653-65.

McArthur, M. E., H. F. Irving-Rodgers, S. Byers, and R. J. Rodgers. 2000. 'Identification and immunolocalization of decorin, versican, perlecan, nidogen, and chondroitin sulfate proteoglycans in bovine small-antral ovarian follicles', *Biol Reprod*, 63: 913-24.
McClure, N., A. M. Macpherson, D. L. Healy, N. Wreford, and P. A. Rogers. 1994. 'An immunohistochemical study of the vascularization of the human Graafian follicle', *Hum Reprod*, 9: 1401-5.
McGaughey, R. W., D. H. Montgomery, and J. D. Richter. 1979. 'Germinal vesicle configurations and patterns of polypeptide synthesis of procine oocytes from antral follicles of different size, as related to their competency for spontaneous maturation', *J Exp Zool*, 209: 239-54.
McGee, E. A., C. Sawetawan, I. Bird, W. E. Rainey, and B. R. Carr. 1996. 'The effect of insulin and insulin-like growth factors on the expression of steroidogenic enzymes in a human ovarian thecal-like tumor cell model', *Fertil Steril*, 65: 87-93.
McGrath, S. A., A. F. Esquela, and S. J. Lee. 1995. 'Oocyte-specific expression of growth/differentiation factor-9', *Mol Endocrinol*, 9: 131-6.
McLaren, A. 1988. 'Somatic and germ-cell sex in mammals', *Philos Trans R Soc Lond B Biol Sci*, 322: 3-9.
Means, A. R., and C. Huckins. 1974. 'Coupled events in the early biochemical actions of FSH on the Sertoli cells of the testis', *Curr Top Mol Endocrinol*, 1: 145-65.
Mehlmann, L. M., T. L. Jones, and L. A. Jaffe. 2002. 'Meiotic arrest in the mouse follicle maintained by a Gs protein in the oocyte', *Science*, 297: 1343-5.
Mehlmann, L. M., Y. Saeki, S. Tanaka, T. J. Brennan, A. V. Evsikov, F. L. Pendola, B. B. Knowles, J. J. Eppig, and L. A. Jaffe. 2004. 'The Gs-linked receptor GPR3 maintains meiotic arrest in mammalian oocytes', *Science*, 306: 1947-50.
Mehlmann, L. M., M. Terasaki, L. A. Jaffe, and D. Kline. 1995. 'Reorganization of the endoplasmic reticulum during meiotic maturation of the mouse oocyte', *Dev Biol*, 170: 607-15.
Meinecke, B., and S. Meinecke-Tillmann. 1979. 'Effects of gonadotropins on oocyte maturation and progesterone production by porcine ovarian follicles cultured in vitro', *Theriogenology*, 11: 351-65.
Miller, W. L. 1988. 'Molecular biology of steroid hormone synthesis', *Endocr Rev*, 9: 295-318.
Miller, W. L., D. C. Alexander, J. C. Wu, E. S. Huang, G. K. Whitfield, and S. H. Hall. 1983. 'Regulation of beta-chain mRNA of ovine follicle-stimulating hormone by 17 beta-estradiol', *Mol Cell Biochem*, 53-54: 187-95.
Mitra, J., and R. M. Schultz. 1996. 'Regulation of the acquisition of meiotic competence in the mouse: changes in the subcellular localization of cdc2, cyclin B1, cdc25C and wee1, and in the concentration of these proteins and their transcripts', *J Cell Sci*, 109 (Pt 9): 2407-15.
Monaco, M. E., E. I. Villecco, and S. S. Sanchez. 2007. 'Implication of gap junction coupling in amphibian vitellogenin uptake', *Zygote*, 15: 149-57.
Monniaux, D., and C. Pisselet. 1992. 'Control of proliferation and differentiation of ovine granulosa cells by insulin-like growth factor-I and follicle-stimulating hormone in vitro', *Biol Reprod*, 46: 109-19.
Montgomery, G. W., Z. Z. Zhao, A. J. Marsh, R. Mayne, S. A. Treloar, M. James, N. G. Martin, D. I. Boomsma, and D. L. Duffy. 2004. 'A deletion mutation in GDF9 in sisters with spontaneous DZ twins', *Twin Res*, 7: 548-55.

Moore, G. P., S. Lintern-Moore, H. Peters, and M. Faber. 1974. 'RNA synthesis in the mouse oocyte', *J Cell Biol*, 60: 416-22.
Moore, R. K., F. Otsuka, and S. Shimasaki. 2003. 'Molecular basis of bone morphogenetic protein-15 signaling in granulosa cells', *J Biol Chem*, 278: 304-10.
Mora, J. M., M. A. Fenwick, L. Castle, M. Baithun, T. A. Ryder, M. Mobberley, R. Carzaniga, S. Franks, and K. Hardy. 2012. 'Characterization and significance of adhesion and junction-related proteins in mouse ovarian follicles', *Biol Reprod*, 86: 153, 1-14.
Moreno, R. D., G. Schatten, and J. Ramalho-Santos. 2002. 'Golgi apparatus dynamics during mouse oocyte in vitro maturation: effect of the membrane trafficking inhibitor brefeldin A', *Biol Reprod*, 66: 1259-66.
Motlik, J., P. Sutovsky, J. Kalous, M. Kubelka, J. Moos, and R. M. Schultz. 1996. 'Co-culture with pig membrana granulosa cells modulates the activity of cdc2 and MAP kinase in maturing cattle oocytes', *Zygote*, 4: 247-56.
Motta, P. M., S. Makabe, T. Naguro, and S. Correr. 1994. 'Oocyte follicle cells association during development of human ovarian follicle. A study by high resolution scanning and transmission electron microscopy', *Arch Histol Cytol*, 57: 369-94.
Murdoch, W. J. 1998. 'Inhibition by oestradiol of oxidative stress-induced apoptosis in pig ovarian tissues', *J Reprod Fertil*, 114: 127-30.
Murphy, B. D., and S. D. Martinuk. 1991. 'Equine chorionic gonadotropin', *Endocr Rev*, 12: 27-44.
Musnier, A., D. Heitzler, T. Boulo, S. Tesseraud, G. Durand, C. Lecureuil, H. Guillou, A. Poupon, E. Reiter, and P. Crepieux. 2009. 'Developmental regulation of p70 S6 kinase by a G protein-coupled receptor dynamically modelized in primary cells', *Cell Mol Life Sci*, 66: 3487-503.
Nagyova, E., A. Camaioni, S. Scsukova, A. Mlynarcikova, R. Prochazka, L. Nemcova, and A. Salustri. 2011. 'Activation of cumulus cell SMAD2/3 and epidermal growth factor receptor pathways are involved in porcine oocyte-cumulus cell expansion and steroidogenesis', *Mol Reprod Dev*, 78: 391-402.
Nagyova, E., R. Prochazka, and B. C. Vanderhyden. 1999. 'Oocytectomy does not influence synthesis of hyaluronic acid by pig cumulus cells: retention of hyaluronic acid after insulin-like growth factor-I treatment in serum-free medium', *Biol Reprod*, 61: 569-74.
Nakamura, T., A. Iwase, B. Bayasula, Y. Nagatomo, M. Kondo, T. Nakahara, S. Takikawa, M. Goto, T. Kotani, T. Kiyono, and F. Kikkawa. 2015. 'CYP51A1 Induced by Growth Differentiation Factor 9 and Follicle-Stimulating Hormone in Granulosa Cells Is a Possible Predictor for Unfertilization', *Reprod Sci*, 22: 377-84.
Neal, P., and T. G. Baker. 1975. 'Response of mouse graafian follicles in organ culture to varying doses of follicle-stimulating hormone and luteinizing hormone', *J Endocrinol*, 65: 27-32.
Nechamen, C. A., R. M. Thomas, B. D. Cohen, G. Acevedo, P. I. Poulikakos, J. R. Testa, and J. A. Dias. 2004. 'Human follicle-stimulating hormone (FSH) receptor interacts with the adaptor protein APPL1 in HEK 293 cells: potential involvement of the PI3K pathway in FSH signaling', *Biol Reprod*, 71: 629-36.
Nekola, M. V., and A. V. Nalbandov. 1971. 'Morphological changes of rat follicular cells as influenced by oocytes', *Biol Reprod*, 4: 154-60.
Ng, S. T., T. H. Chang, and T. C. Wu. 1999. 'Prediction of the rates of fertilization, cleavage, and pregnancy success by cumulus-coronal morphology in an in vitro fertilization program', *Fertil Steril*, 72: 412-7.

Nicosia, S. V., D. P. Wolf, and M. Inoue. 1977. 'Cortical granule distribution and cell surface characteristics in mouse eggs', *Dev Biol*, 57: 56-74.
Nilsson, E. E., and M. K. Skinner. 2003. 'Bone morphogenetic protein-4 acts as an ovarian follicle survival factor and promotes primordial follicle development', *Biol Reprod*, 69: 1265-72.
Nogueira, D., R. Ron-El, S. Friedler, M. Schachter, A. Raziel, R. Cortvrindt, and J. Smitz. 2006. 'Meiotic arrest in vitro by phosphodiesterase 3-inhibitor enhances maturation capacity of human oocytes and allows subsequent embryonic development', *Biol Reprod*, 74: 177-84.
Norris, R. P., L. Freudzon, M. Freudzon, A. R. Hand, L. M. Mehlmann, and L. A. Jaffe. 2007. 'A G(s)-linked receptor maintains meiotic arrest in mouse oocytes, but luteinizing hormone does not cause meiotic resumption by terminating receptor-G(s) signaling', *Dev Biol*, 310: 240-9.
Norris, R. P., M. Freudzon, L. M. Mehlmann, A. E. Cowan, A. M. Simon, D. L. Paul, P. D. Lampe, and L. A. Jaffe. 2008. 'Luteinizing hormone causes MAP kinase-dependent phosphorylation and closure of connexin 43 gap junctions in mouse ovarian follicles: one of two paths to meiotic resumption', *Development*, 135: 3229-38.
Norris, R. P., M. Freudzon, V. O. Nikolaev, and L. A. Jaffe. 2010. 'Epidermal growth factor receptor kinase activity is required for gap junction closure and for part of the decrease in ovarian follicle cGMP in response to LH', *Reproduction*, 140: 655-62.
Norris, R. P., W. J. Ratzan, M. Freudzon, L. M. Mehlmann, J. Krall, M. A. Movsesian, H. Wang, H. Ke, V. O. Nikolaev, and L. A. Jaffe. 2009. 'Cyclic GMP from the surrounding somatic cells regulates cyclic AMP and meiosis in the mouse oocyte', *Development*, 136: 1869-78.
Nurnberg, A., T. Kitzing, and R. Grosse. 2011. 'Nucleating actin for invasion', *Nat Rev Cancer*, 11: 177-87.
O'Brien, J. K., D. Dwarte, J. P. Ryan, W. M. Maxwell, and G. Evans. 1996. 'Developmental capacity, energy metabolism and ultrastructure of mature oocytes from prepubertal and adult sheep', *Reprod Fertil Dev*, 8: 1029-37.
O'Brien, M. J., J. K. Pendola, and J. J. Eppig. 2003. 'A revised protocol for in vitro development of mouse oocytes from primordial follicles dramatically improves their developmental competence', *Biol Reprod*, 68: 1682-6.
O'Shaughnessy, P. J., K. Dudley, and W. R. Rajapaksha. 1996. 'Expression of follicle stimulating hormone-receptor mRNA during gonadal development', *Mol Cell Endocrinol*, 125: 169-75.
Oakberg, E. F. 1979. 'Follicular growth and atresia in the mouse', *In Vitro*, 15: 41-9.
Oatley, J. M., and R. L. Brinster. 2012. 'The germline stem cell niche unit in mammalian testes', *Physiol Rev*, 92: 577-95.
Ochsner, S. A., A. J. Day, M. S. Rugg, R. M. Breyer, R. H. Gomer, and J. S. Richards. 2003. 'Disrupted function of tumor necrosis factor-alpha-stimulated gene 6 blocks cumulus cell-oocyte complex expansion', *Endocrinology*, 144: 4376-84.
Ochsner, S. A., D. L. Russell, A. J. Day, R. M. Breyer, and J. S. Richards. 2003. 'Decreased expression of tumor necrosis factor-alpha-stimulated gene 6 in cumulus cells of the cyclooxygenase-2 and EP2 null mice', *Endocrinology*, 144: 1008-19.
Odor, D. L. 1960. 'Electron microscopic studies on ovarian oocytes and unfertilized tubal ova in the rat', *J Biophys Biochem Cytol*, 7: 567-74.

Odor, D. L., and R. J. Blandau. 1973. 'EGG transport over the fimbrial surface of the rabbit oviduct under experimental conditions', *Fertil Steril*, 24: 292-300.
Oh, J. S., S. J. Han, and M. Conti. 2010. 'Wee1B, Myt1, and Cdc25 function in distinct compartments of the mouse oocyte to control meiotic resumption', *J Cell Biol*, 188: 199-207.
Oktay, K., D. Briggs, and R. G. Gosden. 1997. 'Ontogeny of follicle-stimulating hormone receptor gene expression in isolated human ovarian follicles', *J Clin Endocrinol Metab*, 82: 3748-51.
Olsiewski, P. J., and W. H. Beers. 1983. 'cAMP synthesis in the rat oocyte', *Dev Biol*, 100: 287-93.
Orisaka, M., S. Orisaka, J. Y. Jiang, J. Craig, Y. Wang, F. Kotsuji, and B. K. Tsang. 2006. 'Growth differentiation factor 9 is antiapoptotic during follicular development from preantral to early antral stage', *Mol Endocrinol*, 20: 2456-68.
Otsuka, F., and S. Shimasaki. 2002. 'A novel function of bone morphogenetic protein-15 in the pituitary: selective synthesis and secretion of FSH by gonadotropes', *Endocrinology*, 143: 4938-41.
Otsuka, F., Z. Yao, T. Lee, S. Yamamoto, G. F. Erickson, and S. Shimasaki. 2000. 'Bone morphogenetic protein-15. Identification of target cells and biological functions', *J Biol Chem*, 275: 39523-8.
Packer, A. I., Y. C. Hsu, P. Besmer, and R. F. Bachvarova. 1994. 'The ligand of the c-kit receptor promotes oocyte growth', *Dev Biol*, 161: 194-205.
Palmer, J. S., Z. Z. Zhao, C. Hoekstra, N. K. Hayward, P. M. Webb, D. C. Whiteman, N. G. Martin, D. I. Boomsma, D. L. Duffy, and G. W. Montgomery. 2006. 'Novel variants in growth differentiation factor 9 in mothers of dizygotic twins', *J Clin Endocrinol Metab*, 91: 4713-6.
Pangas, S. A., Y. Choi, D. J. Ballow, Y. Zhao, H. Westphal, M. M. Matzuk, and A. Rajkovic. 2006. 'Oogenesis requires germ cell-specific transcriptional regulators Sohlh1 and Lhx8', *Proc Natl Acad Sci U S A*, 103: 8090-5.
Pangas, S. A., and M. M. Matzuk. 2005. 'The art and artifact of GDF9 activity: cumulus expansion and the cumulus expansion-enabling factor', *Biol Reprod*, 73: 582-5.
Pangas, S. A., W. Yan, M. M. Matzuk, and A. Rajkovic. 2004. 'Restricted germ cell expression of a gene encoding a novel mammalian HORMA domain-containing protein', *Gene Expr Patterns*, 5: 257-63.
Panigone, S., M. Hsieh, M. Fu, L. Persani, and M. Conti. 2008. 'Luteinizing hormone signaling in preovulatory follicles involves early activation of the epidermal growth factor receptor pathway', *Mol Endocrinol*, 22: 924-36.
Papkoff, H., and M. Ekblad. 1970. 'Ovine follicle stimulating hormone: preparation and characterization of its subunits', *Biochem Biophys Res Commun*, 40: 614-21.
Parakh, T. N., J. A. Hernandez, J. C. Grammer, J. Weck, M. Hunzicker-Dunn, A. J. Zeleznik, and J. H. Nilson. 2006. 'Follicle-stimulating hormone/cAMP regulation of aromatase gene expression requires beta-catenin', *Proc Natl Acad Sci U S A*, 103: 12435-40.
Parfenov, V., G. Potchukalina, L. Dudina, D. Kostyuchek, and M. Gruzova. 1989. 'Human antral follicles: oocyte nucleus and the karyosphere formation (electron microscopic and autoradiographic data)', *Gamete Res*, 22: 219-31.

Park, J. Y., F. Richard, S. Y. Chun, J. H. Park, E. Law, K. Horner, S. L. Jin, and M. Conti. 2003. 'Phosphodiesterase regulation is critical for the differentiation and pattern of gene expression in granulosa cells of the ovarian follicle', *Mol Endocrinol*, 17: 1117-30.
Park, J. Y., Y. Q. Su, M. Ariga, E. Law, S. L. Jin, and M. Conti. 2004. 'EGF-like growth factors as mediators of LH action in the ovulatory follicle', *Science*, 303: 682-4.
Park, Y., E. T. Maizels, Z. J. Feiger, H. Alam, C. A. Peters, T. K. Woodruff, T. G. Unterman, E. J. Lee, J. L. Jameson, and M. Hunzicker-Dunn. 2005. 'Induction of cyclin D2 in rat granulosa cells requires FSH-dependent relief from FOXO1 repression coupled with positive signals from Smad', *J Biol Chem*, 280: 9135-48.
Parker, K. L., and B. P. Schimmer. 1995. 'Transcriptional regulation of the genes encoding the cytochrome P-450 steroid hydroxylases', *Vitam Horm*, 51: 339-70.
Parr, E. L. 1974. 'Histological examination of the rat ovarian follicle wall prior to ovulation', *Biol Reprod*, 11: 483-503.
Parr, E. L. 1975. 'Rupture of ovarian follicles at ovulation', *J Reprod Fertil Suppl*: 1-22.
Parrott, J. A., and M. K. Skinner. 1999. 'Kit-ligand/stem cell factor induces primordial follicle development and initiates folliculogenesis', *Endocrinology*, 140: 4262-71.
Pascucci, T., J. Perrino, A. P. Mahowald, and G. L. Waring. 1996. 'Eggshell assembly in Drosophila: processing and localization of vitelline membrane and chorion proteins', *Dev Biol*, 177: 590-8.
Paul, S. J., G. A. Ortolano, D. J. Haisenleder, J. M. Stewart, M. A. Shupnik, and J. C. Marshall. 1990. 'Gonadotropin subunit messenger RNA concentrations after blockade of gonadotropin-releasing hormone action: testosterone selectively increases follicle-stimulating hormone beta-subunit messenger RNA by posttranscriptional mechanisms', *Mol Endocrinol*, 4: 1943-55.
Payne, C., and G. Schatten. 2003. 'Golgi dynamics during meiosis are distinct from mitosis and are coupled to endoplasmic reticulum dynamics until fertilization', *Dev Biol*, 264: 50-63.
Paynton, B. V., and R. Bachvarova. 1994. 'Polyadenylation and deadenylation of maternal mRNAs during oocyte growth and maturation in the mouse', *Mol Reprod Dev*, 37: 172-80.
Pelland, A. M., H. E. Corbett, and J. M. Baltz. 2009. 'Amino Acid transport mechanisms in mouse oocytes during growth and meiotic maturation', *Biol Reprod*, 81: 1041-54.
Peluffo, M. C., A. Y. Ting, A. M. Zamah, M. Conti, R. L. Stouffer, M. B. Zelinski, and J. D. Hennebold. 2012. 'Amphiregulin promotes the maturation of oocytes isolated from the small antral follicles of the rhesus macaque', *Hum Reprod*, 27: 2430-7.
Peng, J., Q. Li, K. Wigglesworth, A. Rangarajan, C. Kattamuri, R. T. Peterson, J. J. Eppig, T. B. Thompson, and M. M. Matzuk. 2013. 'Growth differentiation factor 9:bone morphogenetic protein 15 heterodimers are potent regulators of ovarian functions', *Proc Natl Acad Sci U S A*, 110: E776-85.
Peng, X. R., A. J. Hsueh, P. S. LaPolt, L. Bjersing, and T. Ny. 1991. 'Localization of luteinizing hormone receptor messenger ribonucleic acid expression in ovarian cell types during follicle development and ovulation', *Endocrinology*, 129: 3200-7.
Pepling, M. E., and A. C. Spradling. 2001. 'Mouse ovarian germ cell cysts undergo programmed breakdown to form primordial follicles', *Dev Biol*, 234: 339-51.
Pernasetti, F., V. V. Vasilyev, S. B. Rosenberg, J. S. Bailey, H. J. Huang, W. L. Miller, and P. L. Mellon. 2001. 'Cell-specific transcriptional regulation of follicle-stimulating hormone-

beta by activin and gonadotropin-releasing hormone in the LbetaT2 pituitary gonadotrope cell model', *Endocrinology*, 142: 2284-95.
Pescador, N., A. Houde, D. M. Stocco, and B. D. Murphy. 1997. 'Follicle-stimulating hormone and intracellular second messengers regulate steroidogenic acute regulatory protein messenger ribonucleic acid in luteinized porcine granulosa cells', *Biol Reprod*, 57: 660-8.
Pesce, M., A. Di Carlo, and M. De Felici. 1997. 'The c-kit receptor is involved in the adhesion of mouse primordial germ cells to somatic cells in culture', *Mech Dev*, 68: 37-44.
Peter, M., and J. M. Dubuis. 2000. 'Transcription factors as regulators of steroidogenic P-450 enzymes', *Eur J Clin Invest*, 30 Suppl 3: 14-20.
Phillips, C. L., L. W. Lin, J. C. Wu, K. Guzman, A. Milsted, and W. L. Miller. 1988. '17 Beta-estradiol and progesterone inhibit transcription of the genes encoding the subunits of ovine follicle-stimulating hormone', *Mol Endocrinol*, 2: 641-9.
Pi, X., R. Ren, R. Kelley, C. Zhang, M. Moser, A. B. Bohil, M. Divito, R. E. Cheney, and C. Patterson. 2007. 'Sequential roles for myosin-X in BMP6-dependent filopodial extension, migration, and activation of BMP receptors', *J Cell Biol*, 179: 1569-82.
Pierce, J. G., and T. F. Parsons. 1981. 'Glycoprotein hormones: structure and function', *Annu Rev Biochem*, 50: 465-95.
Pincus, G., and E. V. Enzmann. 1935. 'The Comparative Behavior of Mammalian Eggs in Vivo and in Vitro : I. The Activation of Ovarian Eggs', *J Exp Med*, 62: 665-75.
Piontkewitz, Y., K. Sundfeldt, and L. Hedin. 1997. 'The expression of c-myc during follicular growth and luteal formation in the rat ovary in vivo', *J Endocrinol*, 152: 395-406.
Piquette, G. N., P. S. LaPolt, M. Oikawa, and A. J. Hsueh. 1991. 'Regulation of luteinizing hormone receptor messenger ribonucleic acid levels by gonadotropins, growth factors, and gonadotropin-releasing hormone in cultured rat granulosa cells', *Endocrinology*, 128: 2449-56.
Pirino, G., M. P. Wescott, and P. J. Donovan. 2009. 'Protein kinase A regulates resumption of meiosis by phosphorylation of Cdc25B in mammalian oocytes', *Cell Cycle*, 8: 665-70.
Pluciennik, F., M. Joffre, and J. Deleze. 1994. 'Follicle-stimulating hormone increases gap junction communication in Sertoli cells from immature rat testis in primary culture', *J Membr Biol*, 139: 81-96.
Portela, V. M., A. Veiga, and C. A. Price. 2009. 'Regulation of MMP2 and MMP9 metalloproteinases by FSH and growth factors in bovine granulosa cells', *Genet Mol Biol*, 32: 516-20.
Pratt, H. P. 1982. 'Preimplantation mouse embryos synthesize membrane sterols', *Dev Biol*, 89: 101-10.
Pratt, H. P., J. Keith, and J. Chakraborty. 1980. 'Membrane sterols and the development of the preimplantation mouse embryo', *J Embryol Exp Morphol*, 60: 303-19.
Prochazka, R., M. Blaha, and L. Nemcova. 2012. 'Signaling pathways regulating FSH- and amphiregulin-induced meiotic resumption and cumulus cell expansion in the pig', *Reproduction*, 144: 535-46.
Prochazka, R., E. Nagyova, G. Brem, K. Schellander, and J. Motlik. 1998. 'Secretion of cumulus expansion-enabling factor (CEEF) in porcine follicles', *Mol Reprod Dev*, 49: 141-9.
Prochazka, R., E. Nagyova, Z. Rimkevicova, T. Nagai, K. Kikuchi, and J. Motlik. 1991. 'Lack of effect of oocytectomy on expansion of the porcine cumulus', *J Reprod Fertil*, 93: 569-76.

Prochazka, R., L. Nemcova, E. Nagyova, and J. Kanka. 2004. 'Expression of growth differentiation factor 9 messenger RNA in porcine growing and preovulatory ovarian follicles', *Biol Reprod*, 71: 1290-5.
Prochazka, R., V. Srsen, E. Nagyova, T. Miyano, and J. E. Flechon. 2000. 'Developmental regulation of effect of epidermal growth factor on porcine oocyte-cumulus cell complexes: nuclear maturation, expansion, and F-actin remodeling', *Mol Reprod Dev*, 56: 63-73.
Qu, J., P. A. Godin, M. Nisolle, and J. Donnez. 2000. 'Distribution and epidermal growth factor receptor expression of primordial follicles in human ovarian tissue before and after cryopreservation', *Hum Reprod*, 15: 302-10.
Quintana, J., R. W. Hipkin, J. Sanchez-Yague, and M. Ascoli. 1994. 'Follitropin (FSH) and a phorbol ester stimulate the phosphorylation of the FSH receptor in intact cells', *J Biol Chem*, 269: 8772-9.
Quirk, S. M., R. G. Cowan, and R. M. Harman. 2006. 'The susceptibility of granulosa cells to apoptosis is influenced by oestradiol and the cell cycle', *J Endocrinol*, 189: 441-53.
Rajah, R., E. M. Glaser, and A. N. Hirshfield. 1992. 'The changing architecture of the neonatal rat ovary during histogenesis', *Dev Dyn*, 194: 177-92.
Rajkovic, A., S. A. Pangas, D. Ballow, N. Suzumori, and M. M. Matzuk. 2004. 'NOBOX deficiency disrupts early folliculogenesis and oocyte-specific gene expression', *Science*, 305: 1157-9.
Ralph, J. H., E. E. Telfer, and I. Wilmut. 1995. 'Bovine cumulus cell expansion does not depend on the presence of an oocyte secreted factor', *Mol Reprod Dev*, 42: 248-53.
Ratchford, A. M., C. R. Esguerra, and K. H. Moley. 2008. 'Decreased oocyte-granulosa cell gap junction communication and connexin expression in a type 1 diabetic mouse model', *Mol Endocrinol*, 22: 2643-54.
Ratoosh, S. L., J. Lifka, L. Hedin, T. Jahsen, and J. S. Richards. 1987. 'Hormonal regulation of the synthesis and mRNA content of the regulatory subunit of cyclic AMP-dependent protein kinase type II in cultured rat ovarian granulosa cells', *J Biol Chem*, 262: 7306-13.
Reddy, P., L. Liu, D. Adhikari, K. Jagarlamudi, S. Rajareddy, Y. Shen, C. Du, W. Tang, T. Hamalainen, S. L. Peng, Z. J. Lan, A. J. Cooney, I. Huhtaniemi, and K. Liu. 2008. 'Oocyte-specific deletion of Pten causes premature activation of the primordial follicle pool', *Science*, 319: 611-3.
Reddy, P., L. Shen, C. Ren, K. Boman, E. Lundin, U. Ottander, P. Lindgren, Y. X. Liu, Q. Y. Sun, and K. Liu. 2005. 'Activation of Akt (PKB) and suppression of FKHRL1 in mouse and rat oocytes by stem cell factor during follicular activation and development', *Dev Biol*, 281: 160-70.
Reis, A., H. Y. Chang, M. Levasseur, and K. T. Jones. 2006. 'APCcdh1 activity in mouse oocytes prevents entry into the first meiotic division', *Nat Cell Biol*, 8: 539-40.
Reis, A., S. Madgwick, H. Y. Chang, I. Nabti, M. Levasseur, and K. T. Jones. 2007. 'Prometaphase APCcdh1 activity prevents non-disjunction in mammalian oocytes', *Nat Cell Biol*, 9: 1192-8.
Richard, F. J., A. Tsafriri, and M. Conti. 2001. 'Role of phosphodiesterase type 3A in rat oocyte maturation', *Biol Reprod*, 65: 1444-51.
Richard, S., and J. M. Baltz. 2014. 'Prophase I arrest of mouse oocytes mediated by natriuretic peptide precursor C requires GJA1 (connexin-43) and GJA4 (connexin-37) gap junctions in the antral follicle and cumulus-oocyte complex', *Biol Reprod*, 90: 137.

Richards, J. S. 1980. 'Maturation of ovarian follicles: actions and interactions of pituitary and ovarian hormones on follicular cell differentiation', *Physiol Rev*, 60: 51-89.
Richards, J. S. 1994. 'Hormonal control of gene expression in the ovary', *Endocr Rev*, 15: 725-51.
Richards, J. S. 2005. 'Ovulation: new factors that prepare the oocyte for fertilization', *Mol Cell Endocrinol*, 234: 75-9.
Richards, J. S., T. Jahnsen, L. Hedin, J. Lifka, S. Ratoosh, J. M. Durica, and N. B. Goldring. 1987. 'Ovarian follicular development: from physiology to molecular biology', *Recent Prog Horm Res*, 43: 231-76.
Richards, J. S., D. L. Russell, S. Ochsner, M. Hsieh, K. H. Doyle, A. E. Falender, Y. K. Lo, and S. C. Sharma. 2002. 'Novel signaling pathways that control ovarian follicular development, ovulation, and luteinization', *Recent Prog Horm Res*, 57: 195-220.
Rieger, D., and N. M. Loskutoff. 1994. 'Changes in the metabolism of glucose, pyruvate, glutamine and glycine during maturation of cattle oocytes in vitro', *J Reprod Fertil*, 100: 257-62.
Robinson, J. W., M. Zhang, L. C. Shuhaibar, R. P. Norris, A. Geerts, F. Wunder, J. J. Eppig, L. R. Potter, and L. A. Jaffe. 2012. 'Luteinizing hormone reduces the activity of the NPR2 guanylyl cyclase in mouse ovarian follicles, contributing to the cyclic GMP decrease that promotes resumption of meiosis in oocytes', *Dev Biol*, 366: 308-16.
Robker, R. L., and J. S. Richards. 1998. 'Hormone-induced proliferation and differentiation of granulosa cells: a coordinated balance of the cell cycle regulators cyclin D2 and p27Kip1', *Mol Endocrinol*, 12: 924-40.
Romero, S., and J. Smitz. 2010. 'Exposing cultured mouse ovarian follicles under increased gonadotropin tonus to aromatizable androgens influences the steroid balance and reduces oocyte meiotic capacity', *Endocrine*, 38: 243-53.
Rosenfeld, C. S., J. S. Wagner, R. M. Roberts, and D. B. Lubahn. 2001. 'Intraovarian actions of oestrogen', *Reproduction*, 122: 215-26.
Rudak, E., J. Dor, M. Kimchi, B. Goldman, D. Levran, and S. Mashiach. 1990. 'Anomalies of human oocytes from infertile women undergoing treatment by in vitro fertilization', *Fertil Steril*, 54: 292-6.
Rugg, M. S., A. C. Willis, D. Mukhopadhyay, V. C. Hascall, E. Fries, C. Fulop, C. M. Milner, and A. J. Day. 2005. 'Characterization of complexes formed between TSG-6 and inter-alpha-inhibitor that act as intermediates in the covalent transfer of heavy chains onto hyaluronan', *J Biol Chem*, 280: 25674-86.
Rushmer, R. A., and R. L. Brinster. 1973. 'Carbon dioxide production from pyruvate and glucose by bovine oocytes', *Exp Cell Res*, 82: 252-4.
Russell, D. L., K. M. Doyle, I. Gonzales-Robayna, C. Pipaon, and J. S. Richards. 2003. 'Egr-1 induction in rat granulosa cells by follicle-stimulating hormone and luteinizing hormone: combinatorial regulation by transcription factors cyclic adenosine 3',5'-monophosphate regulatory element binding protein, serum response factor, sp1, and early growth response factor-1', *Mol Endocrinol*, 17: 520-33.
Russo, V., A. Martelli, P. Berardinelli, O. Di Giacinto, N. Bernabo, D. Fantasia, M. Mattioli, and B. Barboni. 2007. 'Modifications in chromatin morphology and organization during sheep oogenesis', *Microsc Res Tech*, 70: 733-44.
Sainath, R., and G. Gallo. 2015. 'Cytoskeletal and signaling mechanisms of neurite formation', *Cell Tissue Res*, 359: 267-78.

Saito, T., M. Hiroi, and T. Kato. 1994. 'Development of glucose utilization studied in single oocytes and preimplantation embryos from mice', *Biol Reprod*, 50: 266-70.
Salustri, A., A. Camaioni, M. Di Giacomo, C. Fulop, and V. C. Hascall. 1999. 'Hyaluronan and proteoglycans in ovarian follicles', *Hum Reprod Update*, 5: 293-301.
Salustri, A., C. Garlanda, E. Hirsch, M. De Acetis, A. Maccagno, B. Bottazzi, A. Doni, A. Bastone, G. Mantovani, P. Beck Peccoz, G. Salvatori, D. J. Mahoney, A. J. Day, G. Siracusa, L. Romani, and A. Mantovani. 2004. 'PTX3 plays a key role in the organization of the cumulus oophorus extracellular matrix and in in vivo fertilization', *Development*, 131: 1577-86.
Salustri, A., S. Ulisse, M. Yanagishita, and V. C. Hascall. 1990. 'Hyaluronic acid synthesis by mural granulosa cells and cumulus cells in vitro is selectively stimulated by a factor produced by oocytes and by transforming growth factor-beta', *J Biol Chem*, 265: 19517-23.
Salustri, A., M. Yanagishita, and V. C. Hascall. 1989. 'Synthesis and accumulation of hyaluronic acid and proteoglycans in the mouse cumulus cell-oocyte complex during follicle-stimulating hormone-induced mucification', *J Biol Chem*, 264: 13840-7.
Salvador, L. M., M. P. Flynn, J. Avila, S. Reierstad, E. T. Maizels, H. Alam, Y. Park, J. D. Scott, D. W. Carr, and M. Hunzicker-Dunn. 2004. 'Neuronal microtubule-associated protein 2D is a dual a-kinase anchoring protein expressed in rat ovarian granulosa cells', *J Biol Chem*, 279: 27621-32.
Salvador, L. M., Y. Park, J. Cottom, E. T. Maizels, J. C. Jones, R. V. Schillace, D. W. Carr, P. Cheung, C. D. Allis, J. L. Jameson, and M. Hunzicker-Dunn. 2001. 'Follicle-stimulating hormone stimulates protein kinase A-mediated histone H3 phosphorylation and acetylation leading to select gene activation in ovarian granulosa cells', *J Biol Chem*, 276: 40146-55.
Sanchez, F., and J. Smitz. 2012. 'Molecular control of oogenesis', *Biochim Biophys Acta*, 1822: 1896-912.
Sanggaard, K. W., C. S. Sonne-Schmidt, T. P. Krogager, K. A. Lorentzen, H. G. Wisniewski, I. B. Thogersen, and J. J. Enghild. 2008. 'The transfer of heavy chains from bikunin proteins to hyaluronan requires both TSG-6 and HC2', *J Biol Chem*, 283: 18530-7.
Santiquet, N. W., Y. Develle, A. Laroche, C. Robert, and F. J. Richard. 2012. 'Regulation of gap-junctional communication between cumulus cells during in vitro maturation in swine, a gap-FRAP study', *Biol Reprod*, 87: 46.
Sasseville, M., N. Cote, C. Vigneault, C. Guillemette, and F. J. Richard. 2007. '3'5'-cyclic adenosine monophosphate-dependent up-regulation of phosphodiesterase type 3A in porcine cumulus cells', *Endocrinology*, 148: 1858-67.
Sasseville, M., L. J. Ritter, T. M. Nguyen, F. Liu, D. G. Mottershead, D. L. Russell, and R. B. Gilchrist. 2010. 'Growth differentiation factor 9 signaling requires ERK1/2 activity in mouse granulosa and cumulus cells', *J Cell Sci*, 123: 3166-76.
Sasson, R., A. Dantes, K. Tajima, and A. Amsterdam. 2003. 'Novel genes modulated by FSH in normal and immortalized FSH-responsive cells: new insights into the mechanism of FSH action', *FASEB J*, 17: 1256-66.
Sato, E., and S. S. Koide. 1984. 'Forskolin and mouse oocyte maturation in vitro', *J Exp Zool*, 230: 125-9.

Sato, H., S. Kajikawa, S. Kuroda, Y. Horisawa, N. Nakamura, N. Kaga, C. Kakinuma, K. Kato, H. Morishita, H. Niwa, and J. Miyazaki. 2001. 'Impaired fertility in female mice lacking urinary trypsin inhibitor', *Biochem Biophys Res Commun*, 281: 1154-60.
Sato, N., K. Kawamura, J. Fukuda, Y. Honda, T. Sato, H. Tanikawa, H. Kodama, and T. Tanaka. 2003. 'Expression of LDL receptor and uptake of LDL in mouse preimplantation embryos', *Mol Cell Endocrinol*, 202: 191-4.
Scarchilli, L., A. Camaioni, B. Bottazzi, V. Negri, A. Doni, L. Deban, A. Bastone, G. Salvatori, A. Mantovani, G. Siracusa, and A. Salustri. 2007. 'PTX3 interacts with inter-alpha-trypsin inhibitor: implications for hyaluronan organization and cumulus oophorus expansion', *J Biol Chem*, 282: 30161-70.
Schmidt, D., C. E. Ovitt, K. Anlag, S. Fehsenfeld, L. Gredsted, A. C. Treier, and M. Treier. 2004. 'The murine winged-helix transcription factor Foxl2 is required for granulosa cell differentiation and ovary maintenance', *Development*, 131: 933-42.
Schoppee, P. D., J. C. Garmey, and J. D. Veldhuis. 2002. 'Putative activation of the peroxisome proliferator-activated receptor gamma impairs androgen and enhances progesterone biosynthesis in primary cultures of porcine theca cells', *Biol Reprod*, 66: 190-8.
Schramm, R. D., and B. D. Bavister. 1995. 'Effects of granulosa cells and gonadotrophins on meiotic and developmental competence of oocytes in vitro in non-stimulated rhesus monkeys', *Hum Reprod*, 10: 887-95.
Schramm, R. D., M. T. Tennier, D. E. Boatman, and B. D. Bavister. 1993. 'Chromatin configurations and meiotic competence of oocytes are related to follicular diameter in nonstimulated rhesus monkeys', *Biol Reprod*, 48: 349-56.
Schroeder, T. E. 1981. 'Microfilament-mediated surface change in starfish oocytes in response to 1-methyladenine: implications for identifying the pathway and receptor sites for maturation-inducing hormones', *J Cell Biol*, 90: 362-71.
Schuetz, A. W. 1974. 'Role of hormones in oocyte maturation', *Biol Reprod*, 10: 150-78.
Schultz, R. M., G. E. Letourneau, and P. M. Wassarman. 1979. 'Program of early development in the mammal: changes in patterns and absolute rates of tubulin and total protein synthesis during oogenesis and early embryogenesis in the mouse', *Dev Biol*, 68: 341-59.
Schultz, R. M., R. R. Montgomery, and J. R. Belanoff. 1983. 'Regulation of mouse oocyte meiotic maturation: implication of a decrease in oocyte cAMP and protein dephosphorylation in commitment to resume meiosis', *Dev Biol*, 97: 264-73.
Schultz, R. M., and P. M. Wassarman. 1977. 'Biochemical studies of mammalian oogenesis: Protein synthesis during oocyte growth and meiotic maturation in the mouse', *J Cell Sci*, 24: 167-94.
Segaloff, D. L., H. Y. Wang, and J. S. Richards. 1990. 'Hormonal regulation of luteinizing hormone/chorionic gonadotropin receptor mRNA in rat ovarian cells during follicular development and luteinization', *Mol Endocrinol*, 4: 1856-65.
Sekar, N., J. C. Garmey, and J. D. Veldhuis. 2000. 'Mechanisms underlying the steroidogenic synergy of insulin and luteinizing hormone in porcine granulosa cells: joint amplification of pivotal sterol-regulatory genes encoding the low-density lipoprotein (LDL) receptor, steroidogenic acute regulatory (stAR) protein and cytochrome P450 side-chain cleavage (P450scc) enzyme', *Mol Cell Endocrinol*, 159: 25-35.
Sekiguchi, T., T. Mizutani, K. Yamada, T. Kajitani, T. Yazawa, M. Yoshino, and K. Miyamoto. 2004. 'Expression of epiregulin and amphiregulin in the rat ovary', *J Mol Endocrinol*, 33: 281-91.

Sela-Abramovich, S., E. Chorev, D. Galiani, and N. Dekel. 2005. 'Mitogen-activated protein kinase mediates luteinizing hormone-induced breakdown of communication and oocyte maturation in rat ovarian follicles', *Endocrinology*, 146: 1236-44.

Sela-Abramovich, S., I. Edry, D. Galiani, N. Nevo, and N. Dekel. 2006. 'Disruption of gap junctional communication within the ovarian follicle induces oocyte maturation', *Endocrinology*, 147: 2280-6.

Shimada, M., I. Hernandez-Gonzalez, I. Gonzalez-Robayna, and J. S. Richards. 2006. 'Paracrine and autocrine regulation of epidermal growth factor-like factors in cumulus oocyte complexes and granulosa cells: key roles for prostaglandin synthase 2 and progesterone receptor', *Mol Endocrinol*, 20: 1352-65.

Shimasaki, S., R. K. Moore, F. Otsuka, and G. F. Erickson. 2004. 'The bone morphogenetic protein system in mammalian reproduction', *Endocr Rev*, 25: 72-101.

Shiraishi, K., A. Okada, H. Shirakawa, S. Nakanishi, K. Mikoshiba, and S. Miyazaki. 1995. 'Developmental changes in the distribution of the endoplasmic reticulum and inositol 1,4,5-trisphosphate receptors and the spatial pattern of Ca2+ release during maturation of hamster oocytes', *Dev Biol*, 170: 594-606.

Shitsukawa, K., C. B. Andersen, F. J. Richard, A. K. Horner, A. Wiersma, M. van Duin, and M. Conti. 2001. 'Cloning and characterization of the cyclic guanosine monophosphate-inhibited phosphodiesterase PDE3A expressed in mouse oocyte', *Biol Reprod*, 65: 188-96.

Shuhaibar, L. C., J. R. Egbert, R. P. Norris, P. D. Lampe, V. O. Nikolaev, M. Thunemann, L. Wen, R. Feil, and L. A. Jaffe. 2015. 'Intercellular signaling via cyclic GMP diffusion through gap junctions restarts meiosis in mouse ovarian follicles', *Proc Natl Acad Sci U S A*, 112: 5527-32.

Sicinski, P., J. L. Donaher, Y. Geng, S. B. Parker, H. Gardner, M. Y. Park, R. L. Robker, J. S. Richards, L. K. McGinnis, J. D. Biggers, J. J. Eppig, R. T. Bronson, S. J. Elledge, and R. A. Weinberg. 1996. 'Cyclin D2 is an FSH-responsive gene involved in gonadal cell proliferation and oncogenesis', *Nature*, 384: 470-4.

Silva, J. M., and C. A. Price. 2002. 'Insulin and IGF-I are necessary for FSH-induced cytochrome P450 aromatase but not cytochrome P450 side-chain cleavage gene expression in oestrogenic bovine granulosa cells in vitro', *J Endocrinol*, 174: 499-507.

Simon, A. M., D. A. Goodenough, E. Li, and D. L. Paul. 1997. 'Female infertility in mice lacking connexin 37', *Nature*, 385: 525-9.

Simone, D. A., L. P. Chorich, and V. B. Mahesh. 1993. 'Mechanisms of action for an androgen-mediated autoregulatory process in rat thecal-interstitial cells', *Biol Reprod*, 49: 1190-201.

Simoni, M., J. Gromoll, and E. Nieschlag. 1997. 'The follicle-stimulating hormone receptor: biochemistry, molecular biology, physiology, and pathophysiology', *Endocr Rev*, 18: 739-73.

Simpson, E. R., M. S. Mahendroo, G. D. Means, M. W. Kilgore, M. M. Hinshelwood, S. Graham-Lorence, B. Amarneh, Y. Ito, C. R. Fisher, M. D. Michael, and et al. 1994. 'Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis', *Endocr Rev*, 15: 342-55.

Singh, B., J. M. Rutledge, and D. T. Armstrong. 1995. 'Epidermal growth factor and its receptor gene expression and peptide localization in porcine ovarian follicles', *Mol Reprod Dev*, 40: 391-9.

Sirois, J., and J. S. Richards. 1993. 'Transcriptional regulation of the rat prostaglandin endoperoxide synthase 2 gene in granulosa cells. Evidence for the role of a cis-acting C/EBP beta promoter element', *J Biol Chem*, 268: 21931-8.
Sirois, J., D. L. Simmons, and J. S. Richards. 1992. 'Hormonal regulation of messenger ribonucleic acid encoding a novel isoform of prostaglandin endoperoxide H synthase in rat preovulatory follicles. Induction in vivo and in vitro', *J Biol Chem*, 267: 11586-92.
Smyth, C. D., R. G. Gosden, A. S. McNeilly, and S. G. Hillier. 1994. 'Effect of inhibin immunoneutralization on steroidogenesis in rat ovarian follicles in vitro', *J Endocrinol*, 140: 437-43.
Solc, P., A. Saskova, V. Baran, M. Kubelka, R. M. Schultz, and J. Motlik. 2008. 'CDC25A phosphatase controls meiosis I progression in mouse oocytes', *Dev Biol*, 317: 260-9.
Sommersberg, B., A. Bulling, U. Salzer, U. Frohlich, R. E. Garfield, A. Amsterdam, and A. Mayerhofer. 2000. 'Gap junction communication and connexin 43 gene expression in a rat granulosa cell line: regulation by follicle-stimulating hormone', *Biol Reprod*, 63: 1661-8.
Sorensen, R. A., and P. M. Wassarman. 1976. 'Relationship between growth and meiotic maturation of the mouse oocyte', *Dev Biol*, 50: 531-6.
Sotelo, J. R., and K. R. Porter. 1959. 'An electron microscope study of the rat ovum', *J Biophys Biochem Cytol*, 5: 327-42.
Spicer, L. J., P. Y. Aad, D. T. Allen, S. Mazerbourg, A. H. Payne, and A. J. Hsueh. 2008. 'Growth differentiation factor 9 (GDF9) stimulates proliferation and inhibits steroidogenesis by bovine theca cells: influence of follicle size on responses to GDF9', *Biol Reprod*, 78: 243-53.
Spicer, L. J., C. S. Chamberlain, and S. M. Maciel. 2002. 'Influence of gonadotropins on insulin- and insulin-like growth factor-I (IGF-I)-induced steroid production by bovine granulosa cells', *Domest Anim Endocrinol*, 22: 237-54.
Sretarugsa, P., and R. A. Wallace. 1997. 'The developing Xenopus oocyte specifies the type of gonadotropin-stimulated steroidogenesis performed by its associated follicle cells', *Dev Growth Differ*, 39: 87-97.
St Johnston, D., W. Driever, T. Berleth, S. Richstein, and C. Nusslein-Volhard. 1989. 'Multiple steps in the localization of bicoid RNA to the anterior pole of the Drosophila oocyte', *Development*, 107 Suppl: 13-9.
Starich, T. A., D. H. Hall, and D. Greenstein. 2014. 'Two classes of gap junction channels mediate soma-germline interactions essential for germline proliferation and gametogenesis in Caenorhabditis elegans', *Genetics*, 198: 1127-53.
Sternlicht, A. L., and R. M. Schultz. 1981. 'Biochemical studies of mammalian oogenesis: kinetics of accumulation of total and poly(A)-containing RNA during growth of the mouse oocyte', *J Exp Zool*, 215: 191-200.
Stocco, D. M. 2001. 'Tracking the role of a star in the sky of the new millennium', *Mol Endocrinol*, 15: 1245-54.
Strassburger, D., S. Friedler, A. Raziel, E. Kasterstein, M. Schachter, and R. Ron-El. 2004. 'The outcome of ICSI of immature MI oocytes and rescued in vitro matured MII oocytes', *Hum Reprod*, 19: 1587-90.
Su, Y. Q., J. M. Denegre, K. Wigglesworth, F. L. Pendola, M. J. O'Brien, and J. J. Eppig. 2003. 'Oocyte-dependent activation of mitogen-activated protein kinase (ERK1/2) in cumulus

cells is required for the maturation of the mouse oocyte-cumulus cell complex', *Dev Biol*, 263: 126-38.
Su, Y. Q., K. Sugiura, and J. J. Eppig. 2009. 'Mouse oocyte control of granulosa cell development and function: paracrine regulation of cumulus cell metabolism', *Semin Reprod Med*, 27: 32-42.
Su, Y. Q., K. Sugiura, Q. Li, K. Wigglesworth, M. M. Matzuk, and J. J. Eppig. 2010. 'Mouse oocytes enable LH-induced maturation of the cumulus-oocyte complex via promoting EGF receptor-dependent signaling', *Mol Endocrinol*, 24: 1230-9.
Su, Y. Q., K. Sugiura, K. Wigglesworth, M. J. O'Brien, J. P. Affourtit, S. A. Pangas, M. M. Matzuk, and J. J. Eppig. 2008. 'Oocyte regulation of metabolic cooperativity between mouse cumulus cells and oocytes: BMP15 and GDF9 control cholesterol biosynthesis in cumulus cells', *Development*, 135: 111-21.
Su, Y. Q., K. Sugiura, Y. Woo, K. Wigglesworth, S. Kamdar, J. Affourtit, and J. J. Eppig. 2007. 'Selective degradation of transcripts during meiotic maturation of mouse oocytes', *Dev Biol*, 302: 104-17.
Su, Y. Q., K. Wigglesworth, F. L. Pendola, M. J. O'Brien, and J. J. Eppig. 2002. 'Mitogen-activated protein kinase activity in cumulus cells is essential for gonadotropin-induced oocyte meiotic resumption and cumulus expansion in the mouse', *Endocrinology*, 143: 2221-32.
Su, Y. Q., X. Wu, M. J. O'Brien, F. L. Pendola, J. N. Denegre, M. M. Matzuk, and J. J. Eppig. 2004. 'Synergistic roles of BMP15 and GDF9 in the development and function of the oocyte-cumulus cell complex in mice: genetic evidence for an oocyte-granulosa cell regulatory loop', *Dev Biol*, 276: 64-73.
Sugiura, K., F. L. Pendola, and J. J. Eppig. 2005. 'Oocyte control of metabolic cooperativity between oocytes and companion granulosa cells: energy metabolism', *Dev Biol*, 279: 20-30.
Sugiura, K., Y. Q. Su, F. J. Diaz, S. A. Pangas, S. Sharma, K. Wigglesworth, M. J. O'Brien, M. M. Matzuk, S. Shimasaki, and J. J. Eppig. 2007. 'Oocyte-derived BMP15 and FGFs cooperate to promote glycolysis in cumulus cells', *Development*, 134: 2593-603.
Sugiura, K., Y. Q. Su, and J. J. Eppig. 2009. 'Targeted suppression of Has2 mRNA in mouse cumulus cell-oocyte complexes by adenovirus-mediated short-hairpin RNA expression', *Mol Reprod Dev*, 76: 537-47.
Sugiura, K., Y. Q. Su, Q. Li, K. Wigglesworth, M. M. Matzuk, and J. J. Eppig. 2010. 'Estrogen promotes the development of mouse cumulus cells in coordination with oocyte-derived GDF9 and BMP15', *Mol Endocrinol*, 24: 2303-14.
Sui, H. S., Y. Liu, D. Q. Miao, J. H. Yuan, T. W. Qiao, M. J. Luo, and J. H. Tan. 2005. 'Configurations of germinal vesicle (GV) chromatin in the goat differ from those of other species', *Mol Reprod Dev*, 71: 227-36.
Sun, X., Z. Li, Y. Yi, W. Ding, J. Chen, J. F. Engelhardt, and G. H. Leno. 2009. 'Chromatin configurations in the ferret germinal vesicle that reflect developmental competence for in vitro maturation', *Reprod Domest Anim*, 44: 320-5.
Sutovsky, P., J. E. Flechon, B. Flechon, J. Motlik, N. Peynot, P. Chesne, and Y. Heyman. 1993. 'Dynamic changes of gap junctions and cytoskeleton during in vitro culture of cattle oocyte cumulus complexes', *Biol Reprod*, 49: 1277-87.
Sutovsky, P., J. E. Flechon, and A. Pavlok. 1995. 'F-actin is involved in control of bovine cumulus expansion', *Mol Reprod Dev*, 41: 521-9.

Suzumori, N., C. Yan, M. M. Matzuk, and A. Rajkovic. 2002. 'Nobox is a homeobox-encoding gene preferentially expressed in primordial and growing oocytes', *Mech Dev*, 111: 137-41.
Szollosi, D. 1967. 'Modification of the endoplasmic reticulum in some mammalian oocytes', *Anat Rec*, 158: 59-73.
Szollosi, D., P. Calarco, and R. P. Donahue. 1972. 'Absence of centrioles in the first and second meiotic spindles of mouse oocytes', *J Cell Sci*, 11: 521-41.
Szollosi, D., and R. H. Hunter. 1973. 'Ultrastructural aspects of fertilization in the domestic pig: sperm penetration and pronucleus formation', *J Anat*, 116: 181-206.
Szybek, K. 1972. 'In-vitro maturation of oocytes from sexually immature mice', *J Endocrinol*, 54: 527-8.
Tajima, K., A. Dantes, Z. Yao, K. Sorokina, F. Kotsuji, R. Seger, and A. Amsterdam. 2003. 'Down-regulation of steroidogenic response to gonadotropins in human and rat preovulatory granulosa cells involves mitogen-activated protein kinase activation and modulation of DAX-1 and steroidogenic factor-1', *J Clin Endocrinol Metab*, 88: 2288-99.
Talbot, P., B. D. Shur, and D. G. Myles. 2003. 'Cell adhesion and fertilization: steps in oocyte transport, sperm-zona pellucida interactions, and sperm-egg fusion', *Biol Reprod*, 68: 1-9.
Tao, Y. X., and D. L. Segaloff. 2009. 'Follicle stimulating hormone receptor mutations and reproductive disorders', *Prog Mol Biol Transl Sci*, 89: 115-31.
Teilmann, S. C. 2005. 'Differential expression and localisation of connexin-37 and connexin-43 in follicles of different stages in the 4-week-old mouse ovary', *Mol Cell Endocrinol*, 234: 27-35.
Tesoriero, J. V. 1981. 'Early ultrastructural changes of developing oocytes in the dog', *J Morphol*, 168: 171-9.
Themmen, A. P. N., and I. T. Huhtaniemi. 2000. 'Mutations of gonadotropins and gonadotropin receptors: elucidating the physiology and pathophysiology of pituitary-gonadal function', *Endocr Rev*, 21: 551-83.
Thibault, C., M. Gerard, and Y. Menezo. 1975. 'Preovulatory and ovulatory mechanisms in oocyte maturation', *J Reprod Fertil*, 45: 605-10.
Thomas, F. H., J. F. Ethier, S. Shimasaki, and B. C. Vanderhyden. 2005. 'Follicle-stimulating hormone regulates oocyte growth by modulation of expression of oocyte and granulosa cell factors', *Endocrinology*, 146: 941-9.
Thomas, F. H., R. S. Ismail, J. Y. Jiang, and B. C. Vanderhyden. 2008. 'Kit ligand 2 promotes murine oocyte growth in vitro', *Biol Reprod*, 78: 167-75.
Tian, X. C., A. K. Berndtson, and J. E. Fortune. 1995. 'Differentiation of bovine preovulatory follicles during the follicular phase is associated with increases in messenger ribonucleic acid for cytochrome P450 side-chain cleavage, 3 beta-hydroxysteroid dehydrogenase, and P450 17 alpha-hydroxylase, but not P450 aromatase', *Endocrinology*, 136: 5102-10.
Toda, K., K. Takeda, T. Okada, S. Akira, T. Saibara, T. Kaname, K. Yamamura, S. Onishi, and Y. Shizuta. 2001. 'Targeted disruption of the aromatase P450 gene (Cyp19) in mice and their ovarian and uterine responses to 17beta-oestradiol', *J Endocrinol*, 170: 99-111.
Tornell, J., M. Brannstrom, and T. Hillensjo. 1984. 'Different effects of cyclic nucleotide derivatives upon the rat oocyte-cumulus complex in vitro', *Acta Physiol Scand*, 122: 507-13.
Trigatti, B., H. Rayburn, M. Vinals, A. Braun, H. Miettinen, M. Penman, M. Hertz, M. Schrenzel, L. Amigo, A. Rigotti, and M. Krieger. 1999. 'Influence of the high density

lipoprotein receptor SR-BI on reproductive and cardiovascular pathophysiology', *Proc Natl Acad Sci U S A*, 96: 9322-7.
Tsafriri, A., S. Y. Chun, R. Zhang, A. J. Hsueh, and M. Conti. 1996. 'Oocyte maturation involves compartmentalization and opposing changes of cAMP levels in follicular somatic and germ cells: studies using selective phosphodiesterase inhibitors', *Dev Biol*, 178: 393-402.
Tsafriri, A., S. H. Pomerantz, and C. P. Channing. 1976. 'Inhibition of oocyte maturation by porcine follicular fluid: partial characterization of the inhibitor', *Biol Reprod*, 14: 511-6.
Tsutsumi, O., K. Satoh, Y. Taketani, and T. Kato. 1992. 'Determination of enzyme activities of energy metabolism in the maturing rat oocyte', *Mol Reprod Dev*, 33: 333-7.
Tsutsumi, O., T. Yano, K. Satoh, M. Mizuno, and T. Kato. 1990. 'Studies of hexokinase activity in human and mouse oocyte', *Am J Obstet Gynecol*, 162: 1301-4.
Turner, I. M., P. T. Saunders, S. Shimasaki, and S. G. Hillier. 1989. 'Regulation of inhibin subunit gene expression by FSH and estradiol in cultured rat granulosa cells', *Endocrinology*, 125: 2790-2.
Uda, M., C. Ottolenghi, L. Crisponi, J. E. Garcia, M. Deiana, W. Kimber, A. Forabosco, A. Cao, D. Schlessinger, and G. Pilia. 2004. 'Foxl2 disruption causes mouse ovarian failure by pervasive blockage of follicle development', *Hum Mol Genet*, 13: 1171-81.
Urner, F., W. L. Herrmann, E. E. Baulieu, and S. Schorderet-Slatkine. 1983. 'Inhibition of denuded mouse oocyte meiotic maturation by forskolin, an activator of adenylate cyclase', *Endocrinology*, 113: 1170-2.
Urner, F., and D. Sakkas. 1999. 'Characterization of glycolysis and pentose phosphate pathway activity during sperm entry into the mouse oocyte', *Biol Reprod*, 60: 973-8.
Vaccari, S., J. L. Weeks, 2nd, M. Hsieh, F. S. Menniti, and M. Conti. 2009. 'Cyclic GMP signaling is involved in the luteinizing hormone-dependent meiotic maturation of mouse oocytes', *Biol Reprod*, 81: 595-604.
Valdimarsson, G., P. A. De Sousa, and G. M. Kidder. 1993. 'Coexpression of gap junction proteins in the cumulus-oocyte complex', *Mol Reprod Dev*, 36: 7-15.
Van Blerkom, J., P. Davis, and S. Alexander. 2003. 'Inner mitochondrial membrane potential (DeltaPsim), cytoplasmic ATP content and free Ca2+ levels in metaphase II mouse oocytes', *Hum Reprod*, 18: 2429-40.
Van Blerkom, J., P. W. Davis, and J. Lee. 1995. 'ATP content of human oocytes and developmental potential and outcome after in-vitro fertilization and embryo transfer', *Hum Reprod*, 10: 415-24.
Van Soom, A., S. Tanghe, I. De Pauw, D. Maes, and A. de Kruif. 2002. 'Function of the cumulus oophorus before and during mammalian fertilization', *Reprod Domest Anim*, 37: 144-51.
Vanderhyden, B. C. 1993. 'Species differences in the regulation of cumulus expansion by an oocyte-secreted factor(s)', *J Reprod Fertil*, 98: 219-27.
Vanderhyden, B. C., P. J. Caron, R. Buccione, and J. J. Eppig. 1990. 'Developmental pattern of the secretion of cumulus expansion-enabling factor by mouse oocytes and the role of oocytes in promoting granulosa cell differentiation', *Dev Biol*, 140: 307-17.
Vanderhyden, B. C., J. N. Cohen, and P. Morley. 1993. 'Mouse oocytes regulate granulosa cell steroidogenesis', *Endocrinology*, 133: 423-6.
Vanderhyden, B. C., and E. A. Macdonald. 1998. 'Mouse oocytes regulate granulosa cell steroidogenesis throughout follicular development', *Biol Reprod*, 59: 1296-301.

Vanderhyden, B. C., E. A. Macdonald, E. Nagyova, and A. Dhawan. 2003. 'Evaluation of members of the TGFbeta superfamily as candidates for the oocyte factors that control mouse cumulus expansion and steroidogenesis', *Reprod Suppl*, 61: 55-70.
Vanderhyden, B. C., and A. M. Tonary. 1995. 'Differential regulation of progesterone and estradiol production by mouse cumulus and mural granulosa cells by A factor(s) secreted by the oocyte', *Biol Reprod*, 53: 1243-50.
Vandevoort, C. A., C. R. Shirley, D. L. Hill, and S. P. Leibo. 2008. 'Effects of cryoprotectants and cryopreservation on germinal vesicle-stage cumulus-oocyte complexes of rhesus monkeys', *Fertil Steril*, 90: 805-16.
Varnosfaderani Sh, R., S. Ostadhosseini, M. Hajian, S. M. Hosseini, E. A. Khashouei, H. Abbasi, P. Hosseinnia, and M. H. Nasr-Esfahani. 2013. 'Importance of the GDF9 signaling pathway on cumulus cell expansion and oocyte competency in sheep', *Theriogenology*, 80: 470-8.
Veitch, G. I., J. E. Gittens, Q. Shao, D. W. Laird, and G. M. Kidder. 2004. 'Selective assembly of connexin37 into heterocellular gap junctions at the oocyte/granulosa cell interface', *J Cell Sci*, 117: 2699-707.
Vignjevic, D., S. Kojima, Y. Aratyn, O. Danciu, T. Svitkina, and G. G. Borisy. 2006. 'Role of fascin in filopodial protrusion', *J Cell Biol*, 174: 863-75.
Vitt, U. A., M. Hayashi, C. Klein, and A. J. Hsueh. 2000. 'Growth differentiation factor-9 stimulates proliferation but suppresses the follicle-stimulating hormone-induced differentiation of cultured granulosa cells from small antral and preovulatory rat follicles', *Biol Reprod*, 62: 370-7.
Vitt, U. A., S. Mazerbourg, C. Klein, and A. J. Hsueh. 2002. 'Bone morphogenetic protein receptor type II is a receptor for growth differentiation factor-9', *Biol Reprod*, 67: 473-80.
Vitt, U. A., E. A. McGee, M. Hayashi, and A. J. Hsueh. 2000. 'In vivo treatment with GDF-9 stimulates primordial and primary follicle progression and theca cell marker CYP17 in ovaries of immature rats', *Endocrinology*, 141: 3814-20.
Vivarelli, E., M. Conti, M. De Felici, and G. Siracusa. 1983. 'Meiotic resumption and intracellular cAMP levels in mouse oocytes treated with compounds which act on cAMP metabolism', *Cell Differ*, 12: 271-6.
Vlahos, C. J., W. F. Matter, K. Y. Hui, and R. F. Brown. 1994. 'A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002)', *J Biol Chem*, 269: 5241-8.
Wade, M. H., J. E. Trosko, and M. Schindler. 1986. 'A fluorescence photobleaching assay of gap junction-mediated communication between human cells', *Science*, 232: 525-8.
Waksmonski, S. L., and R. I. Woodruff. 2002. 'For uptake of yolk precursors, epithelial cell-oocyte gap junctional communication is required by insects representing six different orders', *J Insect Physiol*, 48: 667-75.
Wang, H. X., C. Gillio-Meina, S. Chen, X. Q. Gong, T. Y. Li, D. Bai, and G. M. Kidder. 2013. 'The canonical WNT2 pathway and FSH interact to regulate gap junction assembly in mouse granulosa cells', *Biol Reprod*, 89: 39.
Wang, Q., M. M. Chi, T. Schedl, and K. H. Moley. 2012. 'An intercellular pathway for glucose transport into mouse oocytes', *Am J Physiol Endocrinol Metab*, 302: E1511-8.
Wang, Y., S. Chan, and B. K. Tsang. 2002. 'Involvement of inhibitory nuclear factor-kappaB (NFkappaB)-independent NFkappaB activation in the gonadotropic regulation of X-

linked inhibitor of apoptosis expression during ovarian follicular development in vitro', *Endocrinology*, 143: 2732-40.
Wassarman, P. M. 1988. 'Zona pellucida glycoproteins', *Annu Rev Biochem*, 57: 415-42.
Wassarman, P. M., and G. E. Letourneau. 1976. 'RNA synthesis in fully-grown mouse oocytes', *Nature*, 261: 73-4.
Wassarman, P. M., and S. Mortillo. 1991. 'Structure of the mouse egg extracellular coat, the zona pellucida', *Int Rev Cytol*, 130: 85-110.
Weakley, B. S. 1966. 'Electron microscopy of the oocyte and granulosa cells in the developing ovarian follicles of the golden hamster (Mesocricetus auratus)', *J Anat*, 100: 503-34.
Wert, S. E., and W. J. Larsen. 1989. 'Meiotic resumption and gap junction modulation in the cultured rat cumulus-oocyte complex', *Gamete Res*, 22: 143-62.
Whitelaw, P. F., C. D. Smyth, C. M. Howles, and S. G. Hillier. 1992. 'Cell-specific expression of aromatase and LH receptor mRNAs in rat ovary', *J Mol Endocrinol*, 9: 309-12.
Wickramasinghe, D., K. M. Ebert, and D. F. Albertini. 1991. 'Meiotic competence acquisition is associated with the appearance of M-phase characteristics in growing mouse oocytes', *Dev Biol*, 143: 162-72.
Wiesen, J. F., and A. R. Midgley, Jr. 1994. 'Expression of connexin 43 gap junction messenger ribonucleic acid and protein during follicular atresia', *Biol Reprod*, 50: 336-48.
Wigglesworth, K., K. B. Lee, C. Emori, K. Sugiura, and J. J. Eppig. 2015. 'Transcriptomic diversification of developing cumulus and mural granulosa cells in mouse ovarian follicles', *Biol Reprod*, 92: 23.
Wigglesworth, K., K. B. Lee, M. J. O'Brien, J. Peng, M. M. Matzuk, and J. J. Eppig. 2013. 'Bidirectional communication between oocytes and ovarian follicular somatic cells is required for meiotic arrest of mammalian oocytes', *Proc Natl Acad Sci U S A*, 110: E3723-9.
Willis, D., H. Mason, C. Gilling-Smith, and S. Franks. 1996. 'Modulation by insulin of follicle-stimulating hormone and luteinizing hormone actions in human granulosa cells of normal and polycystic ovaries', *J Clin Endocrinol Metab*, 81: 302-9.
Woodruff, T. K., H. Meunier, P. B. Jones, A. J. Hsueh, and K. E. Mayo. 1987. 'Rat inhibin: molecular cloning of alpha- and beta-subunit complementary deoxyribonucleic acids and expression in the ovary', *Mol Endocrinol*, 1: 561-8.
Worrad, D. M., P. T. Ram, and R. M. Schultz. 1994. 'Regulation of gene expression in the mouse oocyte and early preimplantation embryo: developmental changes in Sp1 and TATA box-binding protein, TBP', *Development*, 120: 2347-57.
Wright, C. S., D. L. Becker, J. S. Lin, A. E. Warner, and K. Hardy. 2001. 'Stage-specific and differential expression of gap junctions in the mouse ovary: connexin-specific roles in follicular regulation', *Reproduction*, 121: 77-88.
Wu, X., L. Chen, C. A. Brown, C. Yan, and M. M. Matzuk. 2004. 'Interrelationship of growth differentiation factor 9 and inhibin in early folliculogenesis and ovarian tumorigenesis in mice', *Mol Endocrinol*, 18: 1509-19.
Yamada, E.; Muta, T.; Motomura, A.; Koga, H. 1957. 'The fine structure of the oocyte in the mouse ovary studied with electron microscope', *The Kurume Medical Journal*, 4: 148-71.
Yamashita, Y., M. Hishinuma, and M. Shimada. 2009. 'Activation of PKA, p38 MAPK and ERK1/2 by gonadotropins in cumulus cells is critical for induction of EGF-like factor and TACE/ADAM17 gene expression during in vitro maturation of porcine COCs', *J Ovarian Res*, 2: 20.

Yan, C., P. Wang, J. DeMayo, F. J. DeMayo, J. A. Elvin, C. Carino, S. V. Prasad, S. S. Skinner, B. S. Dunbar, J. L. Dube, A. J. Celeste, and M. M. Matzuk. 2001. 'Synergistic roles of bone morphogenetic protein 15 and growth differentiation factor 9 in ovarian function', *Mol Endocrinol*, 15: 854-66.

Yang, Q., P. Allard, M. Huang, W. Zhang, and H. J. Clarke. 2010. 'Proteasomal activity is required to initiate and to sustain translational activation of messenger RNA encoding the stem-loop-binding protein during meiotic maturation in mice', *Biol Reprod*, 82: 123-31.

Yang, Y., A. Balla, N. Danilovich, and M. R. Sairam. 2003. 'Developmental and molecular aberrations associated with deterioration of oogenesis during complete or partial follicle-stimulating hormone receptor deficiency in mice', *Biol Reprod*, 69: 1294-302.

Yazawa, T., T. Mizutani, K. Yamada, H. Kawata, T. Sekiguchi, M. Yoshino, T. Kajitani, Z. Shou, and K. Miyamoto. 2003. 'Involvement of cyclic adenosine 5'-monophosphate response element-binding protein, steroidogenic factor 1, and Dax-1 in the regulation of gonadotropin-inducible ovarian transcription factor 1 gene expression by follicle-stimulating hormone in ovarian granulosa cells', *Endocrinology*, 144: 1920-30.

Yogo, K., T. Ogawa, M. Akiyama, N. Ishida, and T. Takeya. 2002. 'Identification and functional analysis of novel phosphorylation sites in Cx43 in rat primary granulosa cells', *FEBS Lett*, 531: 132-6.

Yoshida, H., N. Takakura, H. Kataoka, T. Kunisada, H. Okamura, and S. I. Nishikawa. 1997. 'Stepwise requirement of c-kit tyrosine kinase in mouse ovarian follicle development', *Dev Biol*, 184: 122-37.

Yoshimura, Y. 1998. 'Insulin-like growth factors and ovarian physiology', *J Obstet Gynaecol Res*, 24: 305-23.

Yoshimura, Y., S. A. Tischkau, and J. M. Bahr. 1994. 'Destruction of the germinal disc region of an immature preovulatory follicle suppresses follicular maturation and ovulation', *Biol Reprod*, 51: 229-33.

Yoshioka, S., S. Ochsner, D. L. Russell, T. Ujioka, S. Fujii, J. S. Richards, and L. L. Espey. 2000. 'Expression of tumor necrosis factor-stimulated gene-6 in the rat ovary in response to an ovulatory dose of gonadotropin', *Endocrinology*, 141: 4114-9.

Young, J. M., J. L. Juengel, K. G. Dodds, M. Laird, P. K. Dearden, A. S. McNeilly, K. P. McNatty, and T. Wilson. 2008. 'The activin receptor-like kinase 6 Booroola mutation enhances suppressive effects of bone morphogenetic protein 2 (BMP2), BMP4, BMP6 and growth and differentiation factor-9 on FSH release from ovine primary pituitary cell cultures', *J Endocrinol*, 196: 251-61.

Yu, F. Q., C. S. Han, W. Yang, X. Jin, Z. Y. Hu, and Y. X. Liu. 2005. 'Activation of the p38 MAPK pathway by follicle-stimulating hormone regulates steroidogenesis in granulosa cells differentially', *J Endocrinol*, 186: 85-96.

Yun, S. P., S. S. Park, J. M. Ryu, J. H. Park, M. O. Kim, J. H. Lee, and H. J. Han. 2012. 'Mechanism of PKA-dependent and lipid-raft independent stimulation of Connexin43 expression by oxytoxin in mouse embryonic stem cells', *Mol Endocrinol*, 26: 1144-57.

Zamah, A. M., M. Hsieh, J. Chen, J. L. Vigne, M. P. Rosen, M. I. Cedars, and M. Conti. 2010. 'Human oocyte maturation is dependent on LH-stimulated accumulation of the epidermal growth factor-like growth factor, amphiregulin', *Hum Reprod*, 25: 2569-78.

Zamboni, L. 1970. 'Ultrastructure of mammalian oocytes and ova', *Biol Reprod*, 2: Suppl 2:44-63.

Zamboni, L. 1974. 'Fine morphology of the follicle wall and follicle cell-oocyte association', *Biol Reprod*, 10: 125-49.
Zeleznik, A. J., A. R. Midgley, Jr., and L. E. Reichert, Jr. 1974. 'Granulosa cell maturation in the rat: increased binding of human chorionic gonadotropin following treatment with follicle-stimulating hormone in vivo', *Endocrinology*, 95: 818-25.
Zeleznik, A. J., D. Saxena, and L. Little-Ihrig. 2003. 'Protein kinase B is obligatory for follicle-stimulating hormone-induced granulosa cell differentiation', *Endocrinology*, 144: 3985-94.
Zhang, G., J. C. Garmey, and J. D. Veldhuis. 2000. 'Interactive stimulation by luteinizing hormone and insulin of the steroidogenic acute regulatory (StAR) protein and 17alpha-hydroxylase/17,20-lyase (CYP17) genes in porcine theca cells', *Endocrinology*, 141: 2735-42.
Zhang, H., S. Risal, N. Gorre, K. Busayavalasa, X. Li, Y. Shen, B. Bosbach, M. Brannstrom, and K. Liu. 2014. 'Somatic cells initiate primordial follicle activation and govern the development of dormant oocytes in mice', *Curr Biol*, 24: 2501-8.
Zhang, M., Y. Q. Su, K. Sugiura, K. Wigglesworth, G. Xia, and J. J. Eppig. 2011. 'Estradiol promotes and maintains cumulus cell expression of natriuretic peptide receptor 2 (NPR2) and meiotic arrest in mouse oocytes in vitro', *Endocrinology*, 152: 4377-85.
Zhang, M., Y. Q. Su, K. Sugiura, G. Xia, and J. J. Eppig. 2010. 'Granulosa cell ligand NPPC and its receptor NPR2 maintain meiotic arrest in mouse oocytes', *Science*, 330: 366-9.
Zhang, M., Y. Tao, G. Xia, H. Xie, H. Hong, F. Wang, and L. Lei. 2005. 'Atrial natriuretic peptide negatively regulates follicle-stimulating hormone-induced porcine oocyte maturation and cumulus expansion via cGMP-dependent protein kinase pathway', *Theriogenology*, 64: 902-16.
Zhang, M., Y. Tao, B. Zhou, H. Xie, F. Wang, L. Lei, L. Huo, Q. Sun, and G. Xia. 2005. 'Atrial natriuretic peptide inhibits the actions of FSH and forskolin in meiotic maturation of pig oocytes via different signalling pathways', *J Mol Endocrinol*, 34: 459-72.
Zhang, X., X. Q. Wu, S. Lu, Y. L. Guo, and X. Ma. 2006. 'Deficit of mitochondria-derived ATP during oxidative stress impairs mouse MII oocyte spindles', *Cell Res*, 16: 841-50.
Zhang, Y., Z. Zhang, X. Y. Xu, X. S. Li, M. Yu, A. M. Yu, Z. H. Zong, and B. Z. Yu. 2008. 'Protein kinase A modulates Cdc25B activity during meiotic resumption of mouse oocytes', *Dev Dyn*, 237: 3777-86.
Zhao, H., Y. Qin, E. Kovanci, J. L. Simpson, Z. J. Chen, and A. Rajkovic. 2007. 'Analyses of GDF9 mutation in 100 Chinese women with premature ovarian failure', *Fertil Steril*, 88: 1474-6.
Zhuo, L., M. Yoneda, M. Zhao, W. Yingsung, N. Yoshida, Y. Kitagawa, K. Kawamura, T. Suzuki, and K. Kimata. 2001. 'Defect in SHAP-hyaluronan complex causes severe female infertility. A study by inactivation of the bikunin gene in mice', *J Biol Chem*, 276: 7693-6.
Zuccotti, M., P. Giorgi Rossi, A. Martinez, S. Garagna, A. Forabosco, and C. A. Redi. 1998. 'Meiotic and developmental competence of mouse antral oocytes', *Biol Reprod*, 58: 700-4.
Zuccotti, M., R. H. Ponce, M. Boiani, S. Guizzardi, P. Govoni, R. Scandroglio, S. Garagna, and C. A. Redi. 2002. 'The analysis of chromatin organisation allows selection of mouse antral oocytes competent for development to blastocyst', *Zygote*, 10: 73-8.

Zuelke, K. A., and B. G. Brackett. 1992. 'Effects of luteinizing hormone on glucose metabolism in cumulus-enclosed bovine oocytes matured in vitro', *Endocrinology*, 131: 2690-6.