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Developmental and molecular aberrations associated with deterioration of oocytes during FSH-receptor deficiency

par

Yinzhi YANG

Programme de sciences biomédicales

Faculté de médecine

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Université de Montréal

Faculté des études supérieures

Ce mémoire intitulé:

Developmental and molecular aberrations associated with deterioration of oocytes during FSH-receptor deficiency

Présenté par

Yinzhi YANG

A été évalué par un jury composé des personnes suivantes :

Puttaswamy Manjunath	**président-rapporteur**
M Ram Sairam	**directeur de recherche**
Gilles Bleau	**membre du jury**

Mémoire accepté le: ……………………

RÉSUMÉ

La suppression du gène codant pour le récepteur à la FSH (l'hormone folliculo-stimulante) chez la souris (FORKO) est très utile pour caractériser le processus dépendant des hormones glycoprotéiques FSH/FSHR dans l'ovaire. Cette modèle démontre un gène relié à un nouveau processus ovarien chez l'animal adulte. Les souris FORKO sont infertiles. Les hétérozygotes se retrouvent alors avec un system reproductif vieillissant de façon accélérée.

Le but de cette étude était d'examiner le développement et la communication entre les cellules ovocytes et les cellules de granulosa chez le souris FORKO. L'ovocyte des souris produit une zone pellucide d'épaisseur non uniforme. Tous ces changements morphologiques indiquent que les communications entre les ovocytes et les cellules de la granulosa sont compromisés.

Quelques marqueurs moléculaires tels le C-kit, le kit-ligand et le BMP-15 sont altérés. Chez les FORKO et hétérozygotes, l'expression des protéines ZP-A et ZP-B était manifestement diminuée alors que celle du ZP-C était accrue. Il est intéressant de noter que plusieurs de ces changements se sont aussi produits chez les souris haploinsuffisantes, mais à un degré moindre.

Ces résultats suggèrent que la perte de la signalisation via le FSH-R crée un environnement folliculaire altéré dans lequel la communication ovocyte-cellules de granulosa est perturbée, conduisant à la disparition complète d'un stage subséquent des oocytes dans l'ovaire. Nous croyons que ces données fournissent un modèle expérimental utile pour la compréhension des mécanismes responsables de la préservation de la structure et de la qualité des ovocytes à des âges différents.

Mots Clefs:

Ovocytes, follicule, souris FORKO, zone pellucide, Kit-ligand, C-kit, BMP-15

SUMMARY

Follitropin (FSH) plays an important role in supporting ovarian follicular development and endocrine function. FSH receptor gene knockout (FORKO) mice are infertile and FSH-R heterozygote females undergo accelerated reproductive aging. The loss of FSH-R creates hormonal imbalances leading to follicular degeneration. The purpose of this study was to examine some aspects of oocyte development and oocyte-granulosa cell communication under impaired FSH-R signaling.

Changes in ovarian structural parameters were observed in the ovaries of FORKO mice at different ages. By three months of age, oocyte growth was significantly retarded in the FORKOs. In addition, these mice develop uneven zona pellucida and some cumulus cells even cross the matrix to cover the oocyte. Another feature apparent is the advanced status of oocytes, represented by follicles possessing a large intact oocyte with only one layer of granulosa cells. In addition, aberrations such as two oocytes in one follicle were observed in the heterozygous mice.

Besides the morphological defects, some molecular markers, such as c-kit/kit-ligand, and BMP-15 are altered. These proteins were down regulated in the null oocytes. There were also extensive changes in the expression of ZP glycoproteins between FORKO and +/+ ovaries. In the FORKO mice, ZP-A and ZP-B protein expression was down regulated while ZP-C expression was enhanced. Interestingly all of these alterations also occurred in the haploinsufficient mice but to a lesser degree.

These results suggest that the loss of FSH-R creates altered follicular environment where oocyte-granulosa cells communication is perturbed, resulting in infertility in the null and early reproductive senescence in +/- animals. These data provide an experimental paradigm to understand the mechanisms responsible for preserving the structure and quality of oocytes at different ages.

Key Words: Oocyte, follicle, FORKO mouse, zona pellucida, growth factors, Kit-ligand, C-kit, BMP-15.

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LIST OF ABBREVIATIONS

3β-HSD	3β - hydroxysteroid dehydrogenase
BMP-15	Bone Morphogenetic Protein 15
CG	Chorionic gonadotropin
FIG-α	Factor In the Germline alpha
FORKO	Follitropin Receptor KnockOut;
FSH	Follicle-stimulating hormone (Follitropin)
FSH-R	FSH receptor
GC	Granulosa Cell;
GDF-9	Growth Differentiation Factor 9
IHC	Immunohistochemistry
KL	Kit-ligand
LH	Luteinizing hormone (Lutropin)
LH-R	Luteinizing hormone receptor
P450scc	P450 side chain cleavage enzyme
PCR	Polymerase chain reaction
StAR	Steroidogenic acute regulatory protein
TGF-β	Transforming growth factor-beta

TSH	Thyroid-stimulating hormone (Thyrotropin)
ZP	Zona Pellucida.
ZP-A	ZONA PELLUCIDA PROTEIN A
ZP-B	Zona Pellucida protein B
ZP-C	Zona Pellucida protein C

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1. INTRODUCTION

1.1 Ovarian Structure and Follicular Development

The mammalian ovary has two major functions. The first one is to produce mature oocytes for fertilization and for successful propagation of the species; it is an internal process, assisted by extra-ovarian hormones and growth factors, involving activation, growth, and development of ovarian follicles. The second one is to provide the proper environment for successful oocyte production, embryo implantation, and growth of the embryo and fetus. This involves the synthesis and secretion of various ovarian hormones such as estrogens, progesterone, activin and inhibin for regulating cyclic or pregnancy related changes in other organs throughout the body, especially the uterus, hypothalamus and pituitary. Additionally, the steroids produced by the ovary allow the development of female secondary sexual characteristics.

Morphologically, the human ovary has three structural regions: an outer cortex that contains the oocytes and represents most of the mass of the ovary. There are follicles at different stages of development or degeneration in this region. The inner medulla, formed by stromal cells and cells with steroid-producing characteristics is the middle region. Finally, the hilum is the point of entry of the nerves and blood vessels, which represents the attachment region of the gland to the mesovarium (Figure 1.1).

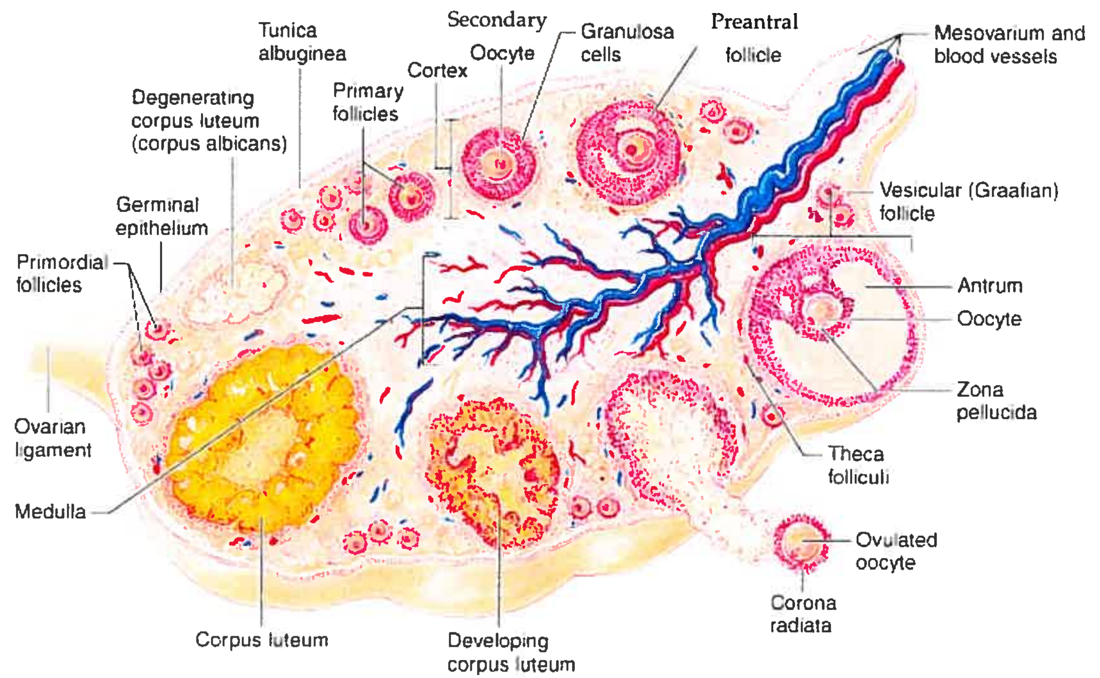


Figure 1.1 Structure of the human ovary

Different types of follicles are found in the ovarian cortex. The blood vessels and nerves enter the ovary through the hilum. The cells that fill the space between the follicles are called stromal cells.

In mammalian ovaries the follicles are the basic functional units. The individual follicles consist of an innermost oocyte surrounded by supporting granulosa cells, which are in turn surrounded by outer layers of theca cells in later stages of follicular development.

Folliculogenesis is a continuous developmental process. Growth of follicles is characterized not only by an increase in the number of somatic cells, but also by a considerable increase in the complexity of the follicular structure. The morphological alterations that reflect functional changes of follicles include granulosa cells becoming cuboidal and proliferative upon activation; formation of multiple layers of granulosa cells (Hirshfield, 1991). In addition, there is a development of a theca cell layer from the surrounding tissue, formation of a fluid-filled antrum and differentiation of the granulosa cell compartment into mural granulosa cells along the antrum side of the basement membrane and cumulus cells that are intimately associated with the oocyte. The oocyte also undergoes significant changes during follicle development. Oocytes grow in diameter depending upon the species. In the mouse it grows from 12-20 μm in diameter in primordial follicles to the fully-grown size of approximately 80-100μm in diameter in large antral follicles, which acquire full preimplantation character (Eppig et al., 1998; Hirshfield and Midgley, Jr., 1978; McGee and Hsueh, 2000). In the humans the diameter of the primary oocyte is 40μm and 100um in large antral follicles (Ferin et al. 1993). In response to preovulatory LH surges during each reproductive cycle, the dominant follicles ovulate to release

the mature oocyte for fertilization, whereas the remaining theca and granulosa cells undergo transformation to become the corpus luteum. Granulosa and theca cells work in concert to produce the steroids: progesterone, androstenedione, testosterone and 17-β estradiol (Eppig, 1996). However these ovulating follicles are the major source of the cyclic secretion of ovarian estrogens in reproductive-age females.

In addition, the appearance of the zona pellucida - an extracellular matrix surrounding the oocyte - is a biochemical marker of oocyte growth. Zona pellucida appearing at secondary stage, however the proteins that make up the zona pellucida were detected even earlier in the cytoplasm of primordial oocytes (Bousquet et al., 1981). The thickness of it is around 3 μm at secondary stage and in the early antrum stage it increases to 7 μm in the mouse (Bleil and Wassarman, 1980). The zona pellucida surrounding the growing oocytes, the ovulated eggs and preimplantation embryos in mammals is critical for species-specific fertilization, the subsequent block to polyspermy and passage of the early embryo through the oviduct prior to implantation (Yanagimachi, 1994). In addition it plays important roles during oogenesis, fertilization and preimplantation development (Wassarman, 1988; Wassarman and Mortillo, 1991).

In the female mouse, stages of folliculogenesis are reflected through granulosa cell differentiation and function (O'Shaughnessy et al., 1996). The

follicles develop through primordial, primary (one layer of cuboidal granulosa cells), secondary (two to four layers of granulosa cells), and preantral stages before acquiring an antral cavity (more than four layers of granulosa cells). At the antral stage, most follicles undergo atretic degeneration. This process is characterized by apoptosis of granulosa cells and degradation of the oocyte followed by hypertrophy of the theca cells (Tilly, 2001). Over 99% of follicles that are present at puberty become atretic and undergo programmed cell death during every stage of folliculogenesis, and only less than 1% of the oocytes present in the ovaries of mammals at birth ever ovulate (Reynaud and Driancourt, 2000; Tilly, 2001). Fig 1.1 depicts different stages of follicles seen in the human ovary.

Regulation of follicular development is a complex process. Follicles must be in the right place at the right time and receive the right signaling to maintain growth and to ultimately ovulate. Endocrine as well as paracrine factors control the fate of each follicle (Gougeon, 1996).

1.2 Regulation of Follicular Development

Folliculogenesis is regulated by the interplay of extraovarian factors including the pituitary gonadotropins – FSH, LH and introvarian factors such as steroids and other paracrine factors secreted by both the oocyte and granulosa cells (McGee and Hsueh, 2000; Eppig, 2001).

1.2.1 Communication between the oocyte and granulosa cell

Folliculogenesis involves orchestration of developmental programs in germ and somatic cells and the communication between them (Fig 1.2.). This communication between oocyte and granulosa cells is essential for follicle survival and development (Coskun et al., 1995; Eppig, 1991b; Eppig et al., 2002; Vanderhyden et al., 1992; Vanderhyden et al., 1993; Vanderhyden and Tonary, 1995; Eppig et al., 2002). The oocyte promotes granulosa cell proliferation, differentiation and function.

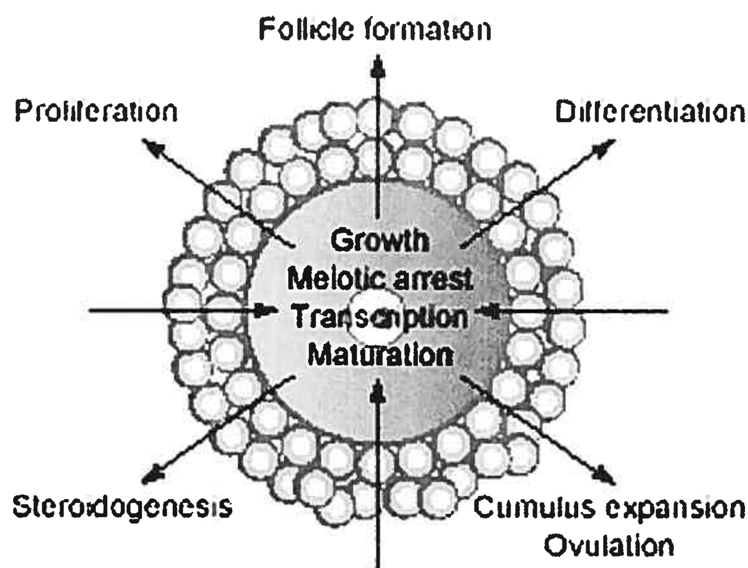


Figure 1.2 Bidirectional communication between oocyte and granulosa cells

This figure shows the processes in granulosa cells controlled by oocyte and the influences of granulosa cells on oocyte development. Factors secreted by the oocyte lead to differentiation and proliferation of the GCs, and affect steroidogenic processes in these cells; similarly GC secreted factors have various roles in oocyte development and maturation. Proper bi-directional communication between germ cell and somatic cells leads to follicle formation and development. (Reproduced from Eppig, JJ. 2001).

For instance, oocytes secrete paracrine signals that suppress progesterone production by granulosa cells, promote granulosa cells proliferation, enable cumulus cells to produce hyaluronic acid and undergo cumulus expansion in response to FSH stimulation (Buccione et al., 1990), and suppress LH receptor mRNA expression by granulosa cells (Eppig et al., 1997b). An interesting recent experiment done by Eppig et al demonstrated that the oocyte also controls the rate of follicle development (Eppig et al., 2002). In this experiment, mid-sized oocytes isolated from secondary follicles were transferred to somatic cells of primordial follicles. This transfer doubled the rate of follicular development and the differentiation of follicular somatic cells (Fig.1.3). These studies demonstrated that the oocytes orchestrate and coordinate the development of mammalian follicles and that the rate of follicular development is essentially based on a developmental program intrinsic to the oocyte. Thus oocytes control their microenvironment by regulating differentiation of the supporting cells. On the other hand, granulosa cells surrounding the developing oocyte provide a critical microenvironment for follicular growth. Therefore oocyte development in antral follicles is also highly dependent on communication with cumulus cells (Eppig et al., 1997a). In addition, granulosa cells signal to the surrounding theca cells, which help regulate growth and differentiation of the ovarian somatic cells (Eppig et al., 2002).

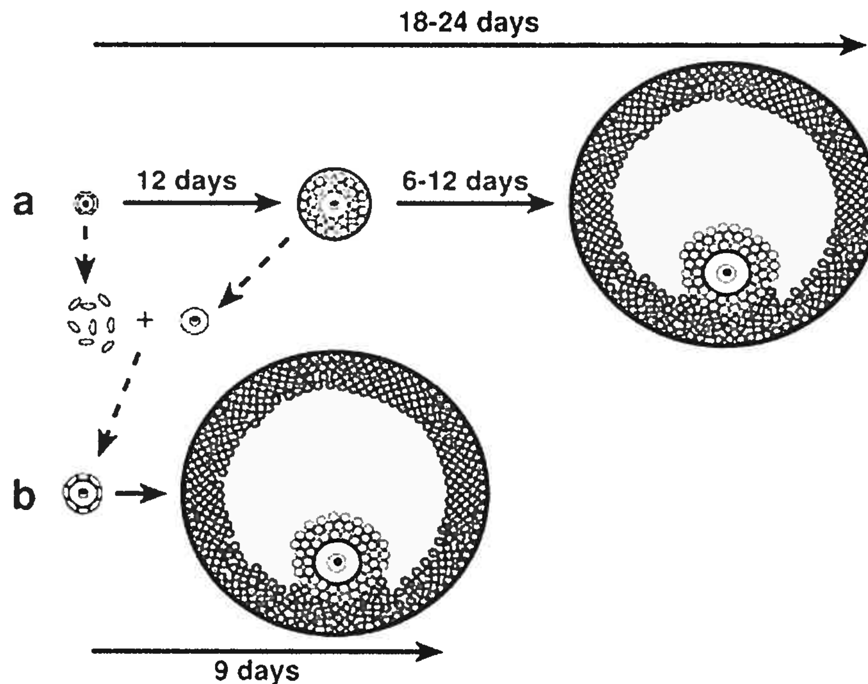


Figure 1.3 The oocyte orchestrates and coordinates the development of follicles

(a) The normal progression of follicular development in neonatal mice from the primordial to the secondary stage requires 10-12 days; development to the ovulatory follicle requires 6-12 days. The entire process requires 18-24 days.

(b) The experimental follicles develop to ovulatory stage in only 9 days, because oocyte secreted factors accelerated development. (Reproduced from Eppig. JJ. 2002).

1.2.2 Paracrine Factors

The communication between oocytes and granulosa cells is realized by paracrine factors derived throughout follicular development (Eppig, 1991b); (Eppig, 2001) (Fig.1.4.). There are a number of recently characterized gene

products, which are expressed or secreted by the oocyte, such as BMP-15 (Otsuka et al., 2001; Galloway et al., 2000), GDF-9 (Carabatsos et al., 1998), FIG-α (Soyal et al., 2000; Liang et al., 1997). In addition there are GC products which interact with the oocyte secreted products, such an example is the kit-ligand/c-kit system (Horie et al., 1991).

The Kit-ligand, also called stem cell factor (Zsebo et al., 1990), mast cell factor (Anderson et al., 1990) or steel factor is expressed in a wide range of cell types. In the mammalian ovary, it is a granulosa cell derived factor. Kit-ligand mRNA expression occurs in granulosa cells at all stages of follicle development in mice, although expression is low in primordial follicles and cumulus cells (Manova et al., 1993). In kit-ligand mutant female mice follicular growth is arrested at the one-layered granulosa cell stage of the primordial follicle (Kuroda et al., 1988), while less severe mutations that result in reduced production of the kit-ligand allow a few follicles to grow to the antral stage. In addition, these animals ovulate sporadically and show limited fertility. These studies suggest that kit-ligand is required at the early period of folliculogenesis (Huang et al., 1993). C-kit is the kit ligand receptor produced by the oocytes and theca cells (Horie et al., 1991). It is a tyrosine kinase receptor of the platelet-derived growth factor receptor family. C-kit mRNA is expressed in primordial, growing and fully-grown oocytes and during early stages of embryogenesis (Motro et al., 1991). Treatment of neonatal mice with a neutralizing antibody against the c-kit receptor caused apparent disturbances in initial follicle recruitment, primary follicle growth and

antrum formation in larger follicles (Kuroda et al., 1988). In addition to controlling oocyte growth and theca cell differentiation during early folliculogenesis in the normal state, the Kit-ligand/c-kit also protects preantral follicles from apoptosis. Reynaud's experiment concluded that the kit-ligand mutation affects the expression of c-kit and kit-ligand proteins resulting in alterations in granulosa cell proliferation and/or oocyte growth in preantral follicles (Reynaud et al., 2001). Moreover, formation of an antral cavity also requires a functional c-kit /Kit-ligand system (Vanderhyden et al., 1992; Packer et al., 1994). These findings suggest that the interaction of GC-derived Kit ligand and oocyte c-kit is indispensable for normal fertility.

Growth differentiation factor-9 (GDF-9) and BMP-15 (also called GDF 9b) are members of the transforming growth factor-β (TGF-β) super-family, selectively expressed in the oocytes within the ovary (Galloway et al., 2002; McGrath et al., 1995). GDF-9 expression in the mouse, rat and human is confined exclusively to the oocyte of primary and larger follicles, and is absent in primordial follicles (Dong et al., 1996; Aaltonen et al., 1999). However, in ovine and bovine ovaries, GDF-9 message could be detected as early as the primordial follicle stage (Bodensteiner et al., 1999). GDF-9 promotes granulosa cell proliferation and theca cells differentiation, but inhibits FSH-induced differentiation (Mazerboug and Hsueh, 2003). It plays multifunctional roles in oocyte–granulosa cell communication and regulation of follicle function,

specifically stimulating cumulus cell expansion (Dong et al., 1996). BMP-15 is expressed beginning at the one-layer primary follicle stage and continuing through ovulation (Dube et al., 1998). It appears to be involved in granulosa cell mitosis (Otsuka and Shimasaki, 2002). *In vitro* cell culture experiments show that BMP-15 has two functions: the inhibition of FSH-induced granulosa cell cytodifferentiation through the inhibition of FSH receptor expression in granulosa cell, and stimulation of granulosa cell proliferation (Otsuka et al., 2001). In fact BMP-15 is most closely related to and shares a coincident expression pattern with the GDF-9 gene (Dube et al., 1998). Both GDF-9 and BMP-15 induce granulosa cell proliferation and differentiation to support follicular maturation (Knight and Clister, 2003) and it is necessary for female fertility (Galloway et al., 2000; Galloway et al., 2002; Elvin et al., 1999).

Factor **i**n the **G**ermline alpha (FIG-α) is a germ cell - specific basic helix-loop-helix (bHLH) transcription factor that has been implicated in coordinating the expression of the three zona pellucida glycoprotein — ZP-A, ZP-B and ZP-C (Liang et al., 1997). Consequently, mice that lack FIG- α are infertile as they fail to produce the three zona pellucida transcripts (Soyal et al., 2000). The mouse zona pellucida is comprised of three glycoproteins originally called ZP-1, ZP-2 and ZP-3 according to their apparent molecular weights on SDS-polyacrylamide gels (Bleil and Wassarman, 1980). Recently it was proposed to rank the zona proteins rather according to the length of their coding regions. This led to the use of a different nomenclature: ZP-A, ZP-B, and ZP-C (Harris et al., 1994). In the

mouse and human ZP-A thus corresponds to ZP-2, ZP-B to ZP-1 and ZP-C to ZP-3. Although the zona matrix physically separates the oocyte and the surrounding somatic granulosa cells, constant communication between oocyte and GCs occurs through specialized channels, the gap junctions, which span the zona matrix and allow paracrine interactions between the cells (Eppig, 1991a).

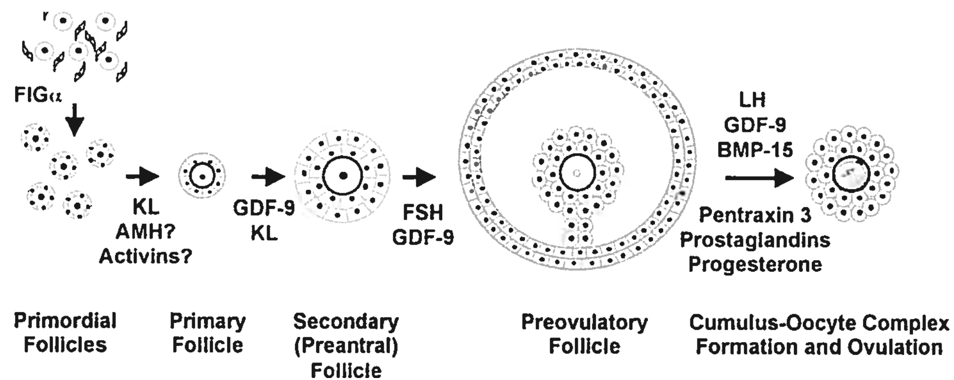


Figure 1.4 Diagrammatic representation of factors that affect follicle development

Even though many factors are secreted by the oocyte throughout folliculogenesis, gene disruption studies have demonstrated the need of particular factors at specific stages of follicle development. (Reproduced from Martin MM. 2002).

1.2.3 Endocrine signaling

In mammals, FSH and LH are two major gonadotropic hormones required for female reproductive health and gonadal development, maturation and reproductive function (Simoni and Nieschlag, 1995; Chappel and Howles, 1991). Of these the major endocrine signaling that is essential for normal

folliculogenesis is follicle stimulating hormone (FSH or follitropin) (Ulloa-Aguirre et al., 1995). FSH is crucial for maturation of follicles, normal antrum formation and control of recruitment of primordial follicles into the growing pool (Halpin et al., 1986). After its production and secretion by the anterior pituitary, FSH acts by binding to its receptor expressed exclusively on granulosa cells. The interaction of the hormone with its receptor isoforms results in activation of a variety of signaling pathways that initiate follicle development and induce steroidogenesis (Simoni et al., 1997; Babu et al., 2000).

1.3 FSH and the FSH-Receptor

FSH, also called follitropin, is a member of the heterodimeric glycoprotein family including luteinizing hormone (LH) and thyroid-stimulating hormone produced in the pituitary as well as chorionic gonadotropin (CG) synthesized by the primate placenta. This family of hormones contains two distinct subunits α and β held together by noncovalent bonds (Sairam et al., 1999; Sairam, 1999). The α-subunit is a common partner for all hormones, and the β-subunit confers hormone specificity and gives unique physiological activities (Combarnous, 1992; Pierce and Parsons, 1981; Ryan et al., 1988; Sairam et al., 1999; Sairam, 1999). The biochemical actions of these hormones are exerted by their binding to membrane receptors expressed in the target cells to stimulate the interaction of the intracellular domain of the receptor with G-proteins and initiate a cellular cascade of signal events, which result in the

induction of protein kinase A and other signaling pathways (Babu et al., 2000; Simoni et al., 1997; Crepieux et al., 2001; Richards, 2001).

FSH and LH also stimulate and maintain the biosynthesis of progesterone and estradiol in the female (Hsueh et al., 1994; Danilovich et al., 2000). FSH signaling induces some of the physiologically important genes including the P450 aromatase, luteinizing hormone receptor (LH-R), steroidogenic acute regulatory protein (StAR), P450 side chain cleavage enzyme (P450scc), 3β-hydroxysteroid dehydrogenase (3β-HSD), inhibin, and activin (Richards, 1994; Dierich et al., 1998). Aromatase is an enzyme of the cytochrome P450 gene family that converts testosterone to estradiol (Terashima et al., 1991). Aromatase activity is present in small antral follicles; however estrogen production at this stage of development is limited by an inability to produce the androgen substrate required for aromatization to estrogen. Growth beyond the small antral phase is therefore characterized by increased aromatase, androgen synthesis, and therefore follicular estrogen production.

Before ovulation is possible, the granulosa cells and theca cells of preantral and small antral follicles must undergo extensive proliferation and functional differentiation (Hsueh et al., 1984). Based on relative numbers of receptors for pituitary FSH and LH, granulosa cells of small antral follicles are thought to be primarily FSH dependent (Hsueh et al., 2000). As follicular development proceeds, the number of LH receptors on granulosa cells is

increased many fold and the number of FSH receptors is reduced. Throughout development of the ovulatory follicle, theca cells respond to LH only (Richards et al., 1976). The LH-R gene knockout animal model suggests a follicle arrest beyond the antral stage. These knockout females have preantral and antral follicles, but no prevulatory follicles or corpora lutea, so these mice are infertile (Zhang et al., 2001). Of the two gonadotropins, the functions of LH are quite straightforward and well understood, whereas FSH actions can be considered elusive.

FSH is an essential survival hormone for the prevention of the programmed demise of early antral follicles in rodents (Hirshfield, 1991; Hsueh et al., 1994). However, FSH is not only a survival factor for these follicles, but also a potent growth and differentiation factor for preantral follicles. It is necessary for the selection and growth of follicles beyond the early antrum phase (Hsueh et al., 2000). In the presence of LH, FSH increases estrogen secretion (Fitzpatrick and Richards, 1991). FSH is also important in the final differentiation of granulosa cells in antral and preovulatory follicles to allow the biosynthesis of estrogens and to prepare the preovulatory follicles for ovulation (Hsueh et al., 1994; O'Shaughnessy et al., 1997). Evidence from FSH-β knockout mice and from humans with FSH-β gene mutations suggests additional roles for FSH function. Female mice lacking the pituitary protein FSH-β are acyclic and infertile due to block at the preantral follicle stage. There are no preovulatory mature follicles or corpora lutea (Matzuk, 2000; Layman and McDonough,

2000). Clinical FSH-β deficient cases had primary amenorrhea, absent breast development, low FSH level, undetectable estradiol and elevated LH level (Matthews et al., 1993; Layman et al., 1997). Therefore, the clinical results confirmed that FSH is necessary for pubertal development and fertility in females.

The physiological effects of FSH are mediated by the FSH-receptor (Sprengel et al., 1990). The FSH-R belongs to the G-protein-coupled, seven-transmembrane receptor family (Nothacker and Grimmelikhuizen, 1993; Hauser et al., 1997; Hsu et al., 2000; Kudo et al., 2000). The FSH receptor is composed of a large extracellular domain (348-350 amino acids), a transmembrane domain and an intracellular carboxyterminus. The extracellular domain constitutes about 50% of the mature receptor protein. It traps the hormone that it meets from circulation (Sairam et al., 1999; Sairam, 1999). The transmembrane domain is the most conserved region. It is composed of seven hydrophobic transmembrane spanning domains that serve to firmly anchor the molecule on the cell surface (Sairam et al., 1999; Sairam, 1999). The last part of the receptor's intracellular carboxyterminus is connected with the inner machinery of the cell to bring about hormonal response (Sairam et al., 1999) (Fig.1.5. R1 FSH-R structure).

The FSH-R primary transcript gene also undergoes extensive alternative splicing to produce different structural motifs (Babu et al., 2001) that could couple to different signaling pathways.

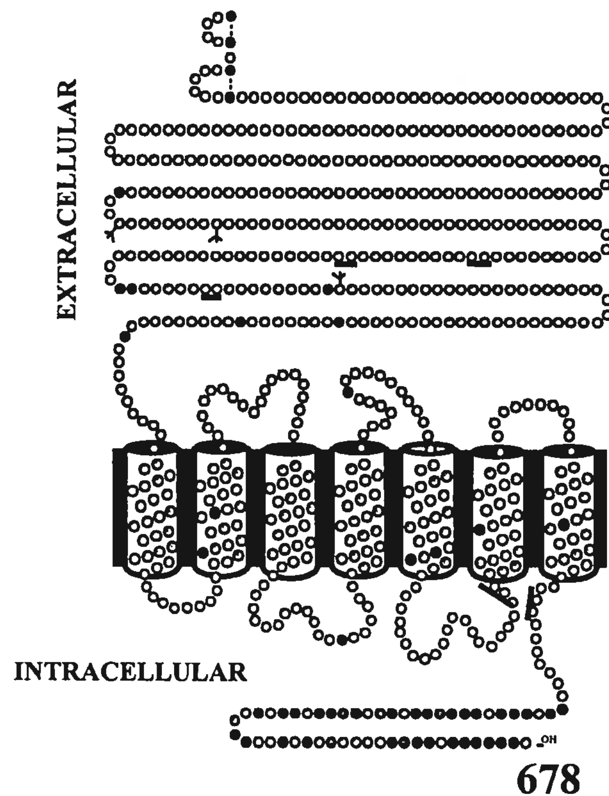


Figure 1.5 Primary sequence of the full length FSH-R protein showing the extracellular transmembrane and intracellular domain, FSH-R1.

Thus, studies from our laboratory have implicated the alternatively spliced growth factor type I receptor of FSH in cell proliferating functions of the hormone (Fig.1.6 R3).

Binding of FSH to the FSH receptor results in changes in the receptor protein that allow the activation of various G proteins and cAMP production

(O'Shaughnessy et al., 1994; Richards, 1994). However, more recent reports indicate its presence in oocytes as well (Patsoula et al., 2001; Meduri et al., 2002).

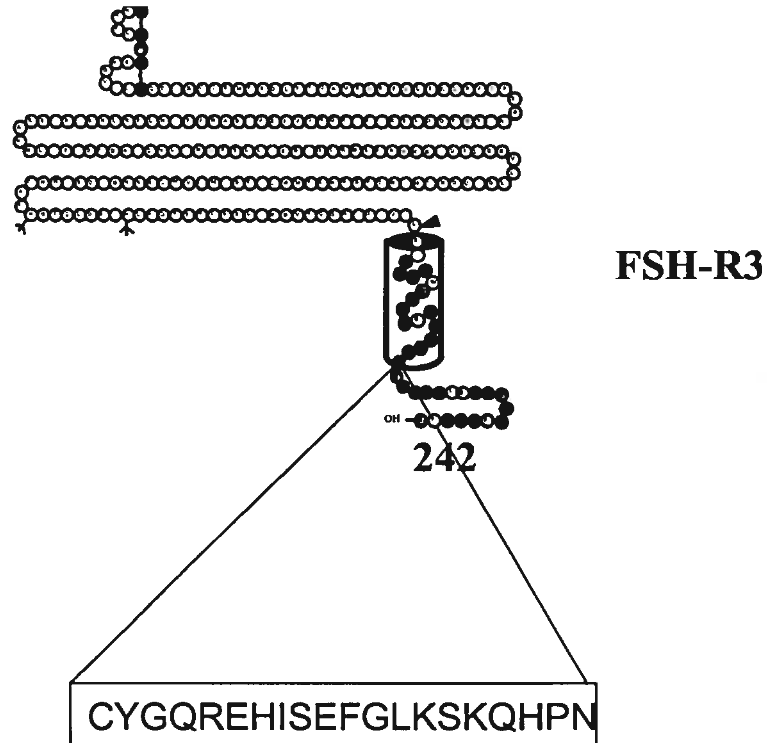


Figure 1.6 Primary sequence of one of the alternatively spliced forms of the receptor, FSH-R3

Ovarian function depends on perfect interaction between FSH and its receptor. Disruption of this interaction causes various reproductive deficits in humans and animals. Recent studies using FSH-R knockout models have

reinforced their critical roles not only in mammalian female reproduction (Kumar et al., 1997; Kumar et al., 1998; Dierich et al., 1998; Abel et al., 2000) but also in other systems (Danilovich et al., 2000).

1.4 FSH-R Knockout Mouse (FORKO) Model and its phenotypes

Human FSH-R mutation cases were first reported in Finnish women (Aittomäki et al., 1995). The frequency of FSH-R gene mutation carriers is quite common in Finland: about 1% of 2000 Finnish individuals examined in a study were heterozygous for the mutation. An inactivating mutation of the FSH-R gene was discovered to be associated with hypergonadotropic ovarian failure (Aittomäki et al., 1995; Tapanainen et al., 1998). This inactivating mutation found in females with pure ovarian dysgenesis leads to a defect characterized by high gonadotropins, and streaky gonads associated with primary amenorrhea (Aittomäki et al., 1995; Gromoll et al., 1996). The mutation mainly affects the folding and trafficking of the receptor protein to the cell membrane. All affected individuals displaying the disease were homozygous for the mutation, and all parents that could be studied were shown to be heterozygous. The heterozygous FSH-R gene mutations in these patients produced a partial deficient state. Upon more detailed clinical study, all affected women were found to have primary amenorrhea; of these, only half had normal breast development and histological sections from affected women showed primordial follicles in some patients, while in others, preantral, antral and even mature follicles were identified. There were no corpora lutea in the women with FSH-R gene mutations, indicating that

ovulation did not occur (Aittomäki et al., 1995). These are interesting correlates that appear to occur in heterozygous FSH-R mutant mice (Danilovich et al., 2000; Danilovich and Sairam, 2002).

Knockout mice are generated by disruption of certain genes resulting in the loss of specific gene products. This disruption and deletion of selected genes in mice is widely used to understand the physiological relevance of various systems (Camper et al., 1995). Our laboratory has generated and studied mice lacking the FSH-R by homologous recombination (Danilovich et al., 2000; Dierich et al., 1998).

Since FSH action is necessary for gonadal stimulation at puberty and gamete production during the fertile phase of life, it provides clear endpoints to compare the spontaneous human mutations with the phenotype of animal models that are available for study. The **Fo**llitropin **R**eceptor **K**nock**o**ut (FORKO) mouse model generated by a disruption strategy to delete 648 bp of the promoter that also includes the coding region, from the translation initiation site to the end of exon 1, assured the elimination of all alternatively spliced forms of FSH receptor (Dierich et al., 1998) that normally arise from a single large gene (Sairam et al., 1999; Simoni et al., 1997). The important role of FSH-R in ovarian function has been confirmed by genetic studies in our laboratory (Danilovich et al., 2000). Targeted disruption of FSH-R causes a gene dose-related endocrine and gametogenic abnormality in female mice. The resulting FORKO mutant is

acyclic, has no cycles, ovulatory defects, atrophic uterus and high FSH and LH levels, like postmenopausal women; the heterozygous animals which lost one allele of the FSH-receptor gene show reduced fertility, undergo early reproductive senescence and stop breeding altogether, like premature menopausal women.

FSH stimulates estradiol biosynthesis by granulosa cells. Estrogen plays an important role in reproductive organs as well as in the other systems, such as skeletal system, cardiovascular system and central nervous system. The FORKO mouse model reveals the importance of FSH-R signaling not only in the reproductive but also in many other systems (Danilovich et al., 2000) as a consequence of hormonal imbalances. Lack of FSH-R signaling in females causes severe ovarian dysfunction and other major symptoms producing chronic estrogen deficiency. Loss of estrogen in the null mutants leads to obesity and skeletal abnormalities that intensify with age, producing a hunchback appearance. Both these changes also become apparent in heterozygous mice, where early loss of estrogen is coincident with early reproductive senescence.

The knockout mice and human mutations of FSH-R have displayed surprisingly similar phenotypes. The FORKO mice provide a model to study human menopause and hypergonadotropic hypogonadism that is characterized by failure of follicular development, lack of ovarian response, and elevated levels of circulating gonadotropins. The reproductive senescence of the heterozygous

females may also be of potential interest in understanding premature ovarian failure (POF). This syndrome in women with premature menopause causes early depletion of the follicles from the ovary. This is now a major cause of infertility in middle-aged women (Aittomaki et al., 1996; van Kasteren et al., 1999; van Kasteren and Schoemaker, 1999).

The FORKO mice are infertile due to acyclicity and failure of ovulation. Histological analysis showed that these mice have primordial, primary, secondary and preantral follicles, but no mature follicles. This indicates that impaired follicular maturation causes the infertility. Of interest, heterozygote mice had estrous cycles similar to the wild type mice but displayed early reproductive senescence (Danilovich et al., 2000; Danilovich and Sairam, 2002). So far, little is known about oocyte function in the FORKO model. We are therefore interested in elucidating the characteristics of the oocyte and the zona pellucida in FORKO null and heterozygote female mice.

1.5 Research hypothesis and Experimental approach

As described above, studies done previously in our laboratory showed that FORKO mice are acyclic and infertile and the heterozygous mice exhibit reduced fertility and fecundity. The decline in fertility of heterozygous mice from 7 months of age results in premature reproductive senescence. In contrast, the wild type mice continue to reproduce to the age of 15 months. Based on this, we hypothesize that in the FORKO and heterozygous mice, there could be deficits in oocyte development. Thus, the overall goal of this research was to characterize the

development of oocytes and follicles in the FORKO and heterozygous mice. As mentioned earlier, development of the oocyte involves a complex interaction with granulosa cells mediated by endocrine, paracrine, and autocrine signaling. This prompts several interesting questions that can be probed in the model. Do oocyte or granulosa cell secreted factors change? How does the FSH-R signaling affect the oocyte and zona pellucida development and function? Our work focused on the effects of complete and partial FSH-receptor depletion at the morphological and molecular level in the ovary, especially on the oocyte and its extracellular matrix mass: the zona pellucida.

The specific aims of this study are

- Analyze the growth trend of whole follicles by measuring follicle and oocyte diameter in different genotypes. On the same histological sections, analyze the differences in zona pellucida thickness around the oocyte in all three genotypes.

- Using histological analysis, establish the morphological changes in the oocyte, zona pellucida and the whole follicle in different genotypes.

- Investigate the mechanism of communication between follicle and oocyte in FORKO and heterozygous female mice by using molecular markers, such as oocyte-derived factors BMP-15, granulosa cell

derived factor Kit-ligand and its receptor C-kit, and study protein expression and localization in the ovaries of different genotypes by using immunohistochemiscal staining and Western blotting.

- To evaluate the expression of the zona pellucida proteins, ZP-A, ZP-B and ZP-C in the ovary by immunohistochemistry and Western blotting.

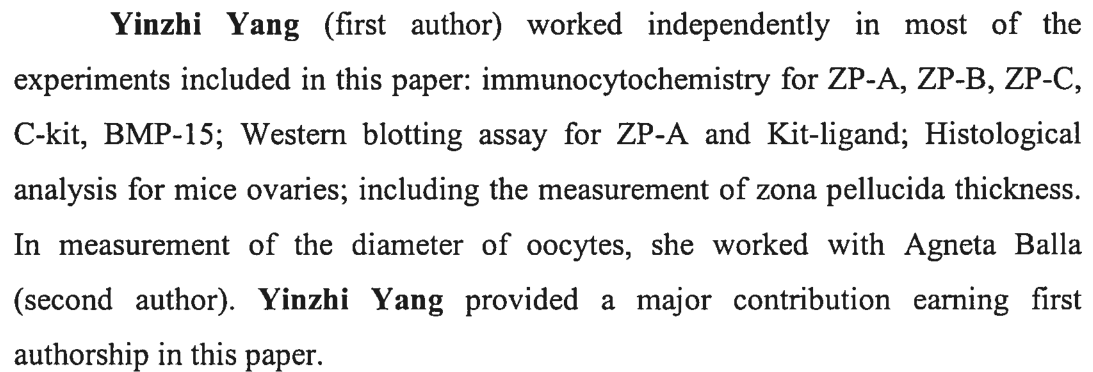
2. Developmental and Molecular Aberrations Associated with Deterioration of Oogenesis during Complete or Partial Follitropin-Receptor Deficiency in Mice (2003)

Yinzhi Yang, Agneta Balla, Natalia Danilovich and M. Ram Sairam, Biology of Reproduction In Press 69: 00-00 Published on line June 11, 2003

2.1 Abstract

Targeted disruption of the mouse follitropin receptor gene (FSH-R) that mediates the action of the hormone follitropin results in a gene dose related ovarian phenotype in the developing as well as the adult animal. While null females (FORKO) are sterile, the haploinsufficient mice experience early reproductive senescence. The purpose of this study was to first record changes in oocyte development in the null FORKO and haploinsufficient mice. Oocyte growth is significantly retarded in the null mutants with thinner zona pellucida in pre-antral follicles, but thicker zona pellucida in secondary follicles. This morphometric change indicates developmental aberrations in coordination of the germ cell (oocyte) and the somatic granulosa cell compartments. Markers for primordial germ cell proliferation and oocyte growth, such as the C-kit/Kit-Ligand and bone morphogenetic protein-15 (BMP-15) were down regulated in both null and +/- ovaries suggesting disrupted communication between oocyte and GCs. Extensive changes in the expression of other oocyte specific gene products like the zona pellucida glycoproteins (zona pellucida A, -B, and -C) indicate major alteration in the extracellular matrix surrounding the germ cells. This led to leaky germ cells that allowed infiltration of somatic cells. These results show that the loss of FSH-R signaling creates altered follicular environment where oocyte-granulosa interactions are perturbed creating an out of phase germ cell and somatic cell development. We believe that these data provide an experimental paradigm to explore the mechanisms responsible for preserving the structural integrity and quality of oocytes at different ages.

2.2 Contribution of Authors



Yinzhi Yang (first author) worked independently in most of the experiments included in this paper: immunocytochemistry for ZP-A, ZP-B, ZP-C, C-kit, BMP-15; Western blotting assay for ZP-A and Kit-ligand; Histological analysis for mice ovaries; including the measurement of zona pellucida thickness. In measurement of the diameter of oocytes, she worked with Agneta Balla (second author). **Yinzhi Yang** provided a major contribution earning first authorship in this paper.

2.3 Introduction

Folliculogenesis is a continuous developmental process wherein the oocytes grow steadily as the surrounding somatic layers of granulosa cells (GC) proliferate and differentiate and subsequently other layers of theca cells develop outside the follicle at defined stages of ovarian development [1, 2]. The ultimate goal of folliculogenesis is to produce a mature egg for ovulation and fertilization. Each mammalian ovary has a fixed number of primordial follicles at birth that later develop to primary, secondary and preantral/antral follicles. For example, the newborn mouse has about 15,000 oocytes that within 2 days complete the first meiotic division and stay arrested until puberty. At puberty under the cyclic influence of pituitary gonadotropins, signaling cascades are triggered stimulating follicular growth, oocyte maturation including release from meiotic arrest and ovulation. Only those follicles that enter this route are rescued while a majority of preantral/antral follicles undergo a degenerative process called atresia through apoptotic elimination. Besides external endocrine signals, local paracrine and autocrine mechanisms within follicular environment interact to determine optimal oocyte and GC development and polarization. This is accomplished by two-way intercellular communication that includes factors secreted by both the oocyte and GCs that act upon the juxtaposed cells [2]. Among the gene products recently characterized to play a significant role in this interaction are the C-kit/kit-ligand system [3], factor in the germ line alpha (FIG-α) [4, 5], growth differentiation factor 9 (GDF-9) [6, 7], and bone morphogenetic protein 15 (BMP-15) [8-10].

The Kit-ligand expressed by GCs of growing follicles interacts with c-kit, a tyrosine kinase receptor of the platelet-derived growth factor receptor family, produced by the oocytes and theca cells [3]. Kit ligand together with c-kit controls oocyte growth and theca cells differentiation and protects preantral follicles from apoptosis [11, 12]. FIG-α is a germ cell specific basic helix-loop-helix (bHLH) transcription factor that has been implicated in coordinate expression of three zona pellucida (ZP) glycoproteins [4]. Consequently, mice that lack FIG-α are infertile as they fail to produce the three ZP transcripts [5]. GDF 9 [13, 14] and BMP-15 (also called GDF 9b) are members of the transforming growth factor-β (TGF-β) super-family selectively expressed in the oocytes. Both of these proteins induce GC proliferation and differentiation and are necessary for female fertility (6,13). BMP-15 has two functions; the inhibition of FSH induced GC cytodifferentiation through the inhibition of FSH receptor expression in GCs; and stimulation of GC proliferation [9]. BMP-15 and KL are all expressed in the early stages of follicular development and appear to be involved in GC mitosis [10].

The mouse zona pellucida comprising three glycoproteins called ZP-1 (ZP-B), ZP-2 (ZP-A), ZP-3 (ZP-C) is an extracellular matrix that surrounds the growing oocytes and remains associated with it after ovulation and formation of early embryo [15]. Despite the fact that the zona matrix physically separates the oocyte and the surrounding somatic GCs, very close associations are continuously

maintained throughout folliculogenesis by means of gap junctions that [16] allow interaction of paracrine factors noted above.

The major endocrine signal that is essential for normal folliculogenesis is follitropin (Follicle stimulating hormone -FSH) [17] that acts by binding to its receptor expressed exclusively on GCs. More recent reports however, indicate its presence in oocytes suggesting additional sites of action in the ovary [18, 19]. Follitropin interacts with its receptor isoforms resulting in activation of a variety of signaling pathways to initiate follicle development and induce steroidogenesis [20, 21]. Targeted disruption of the mouse FSH-R gene [22] results in female sterility and induces a gene dose related novel ovarian phenotype in the adult animal [23]. FSH-R gene disruption causes complete loss of ovarian estrogen production creating steroid hormone imbalance [23-25]. The mutant females exhibit profound changes in ovarian structure and secondary sex organ deficiencies. They are sterile because of a block in folliculogenesis before antral follicle formation. Interestingly heterozygous female mice also undergo early ovarian senescence and lose fertility [24]. In consideration of such a phenotype this haploinsufficient animal has been dubbed the “Menopause mouse” [26]. The hormonal imbalances and other changes following loss of FSH-R signaling lead to follicular degeneration in both null and +/- mice.

The development of follicles in the mammalian ovary involves a bi-directional communication system between the follicular cells and oocyte [16].

Our accompanying communication established the perinatal developmental changes in the somatic cells of the follicle and noted that structural alterations in the ovary of null females are apparent at/or before two days of life [27]. In the present investigation, our aim was to characterize the developmental state of the oocytes during FSH-R deficiency and explore follicle relationship with known oocyte specific gene products. As poor oocyte quality is a major cause for the aging related decline in fertility in middle-aged women [28] and increase in the incidence of aneuploidy [29], we have taken advantage of the strong impact of receptor haploinsufficiency in inducing early reproductive senescence to examine some oocyte characteristics in this model. Our data reveal that FSH-R deletion produces major changes in oocyte structure (and function) disrupting intercellular communication in the follicle. This model provides opportunities for additional mechanistic investigations.

2.4 Materials and Methods

2.4.1 Animals

All experiments involving animals were performed according to institutionally approved and animal care guidelines. Mice were housed in five mice per cage under standard and approved laboratory conditions with 12 hours light: 12 hours dark at 22°C, with unrestricted access to food and water. Mice were genotyped by PCR from DNA extracted from tailpieces or toes. For all experiments in this study virgin 1-, 3-and 7-month-old female mice were used. All

animals were killed at random disregarding the stage of the estrous cycle with the exception that sections used for histology and IHC from +/+ and +/- are derived from mice killed on the morning of proestrous. In any case, this was not relevant for the 1-month-old mice or the FORKO (any age) that do not cycle.

2.4.2 Antibodies

The following antibodies were used in our study to perform either immunohistochemistry or Western blot analysis. Well characterized and specific ZP-A (ZP2), ZP-B (ZP1), and ZP-C (ZP3) antibodies were kindly donated by Dr. U. Eberspaecher (Schering AG Berlin, Germany) [30]. These investigators prepared the ZP-A rabbit antibody against synthetic peptides CGTRYKFEDDKVVVYE and NRDDPNIKLVLDDC that had no homology to ZP-B or ZP-C. Rabbit antisera against the latter two were prepared using highly purified recombinant proteins. It should be noted that the nomenclature of zona pellucida glycoproteins has been changed [31] to reflect the proteins according to their length rather than apparent molecular weights. The old nomenclatures are indicated in parenthesis for clarification. The BMP-15 antibody was the gift of Dr. S. Shimasaki (Univ. of California, San Diego). Antibodies to C-kit and kit-ligand were purchased form Santa Cruz Biotechnology, Inc.

2.4.3 Histological Analysis of Follicles

Ovaries were removed and cleaned of fat and fixed in 10% buffered formalin for 24 h at 4^0C, processed in a tissue processor for paraffin embedding. Then 5 μm sections were cut serially and stained by standard protocols with

hematoxylin and eosin. Histological examination of the ovaries was performed by light microscopy and photomicrographs were taken at same time using a Carl Zeiss microscope.

2.4.4 Follicle Development and Zona Pellucida Thickness

Follicles were classified into six groups as described by Balla et al. [27]. In brief, a primordial follicle was an intact oocyte surrounded by a single layer of flattened GCs. Primary follicles consisted of an intact enlarged oocyte and surrounded by a single layer of mixed squamous and cuboidal or a single layer of cuboidal GCs; secondary refers to a small preantral follicle with two layers of GCs. A Pre-antral follicle has with more than two layers of GCs. Antral structure is a follicle with a fluid filled antrum. A preovulatory follicle is one that is close to the stage of ovulation. As will be noted in the results, FORKO mice lack structures beyond the antral stage.

Follicle development at different ages was analyzed by measuring the diameter of oocytes using microscope coupled to a camera. More than 1500 follicles were examined to compile data on oocytes. There were 4-5 mice for each age and genotype and more than 20-80 follicles of each type were included in each case. Only those follicles sectioned through the oocyte nucleolus (the largest follicle cross-section) were analyzed. The longest and shortest follicular and oocyte diameter were recorded and their average was used [24]. Zona pellucida thickness was measured in 4 directions around the oocyte of each follicle. Data are plotted as mean oocyte, zona pellucida and follicle diameters ±SEM.

2.4.5 Immunohistochemistry

Mouse ovary sections (5μm) were deparaffinized and rehydrated using an immunoCruz kit (Santa Cruz Biotechnology Inc.), incubated in peroxidase blocking solution in order to quench endogenous peroxidase activity. To avoid any non-specific reactivity of the antibodies, the sections were pretreated with 3% normal rabbit serum for 1 hour and incubated with primary antisera (antibodies to ZP-A 1:400, ZP-B 1:250, ZP-C 1:1000, C-kit 1:50, BMP-15 1:3000 in normal rabbit serum) overnight at 4°C. The sections were washed 3 times for 5 minutes each with PBS before the biotinylated secondary antibody (goat anti-rabbit 1:1000 in serum) was added for 1 h at room temperature. This was followed by incubation with horseradish peroxidase-conjugated antibody. After a final wash in PBS, the immunoreactive sites were visualized with the peroxidase substrate DAB. The sections were counter-stained with hematoxylin and mounted with Permount (Fisher Scientific Company). Finally, slides were observed under microscope and photomicrographs were taken at the same time using a Carl Zeiss microscope and computer aided EclipseTM image analyzer. In these evaluations, sections processed by treating with normal serum instead of the primary antibody served as the negative control.

2.4.6 Western Blotting

Ovaries were homogenized in a lysis buffer (50 mM tris-HCl pH 6.8) with additives described previously [23]. Samples (60-100 μg protein) were diluted

with equal volume of reducing loading buffer (187 mmol/l Tris pH 6.8, 2% SDS, 2% ß -mercaptoethanol, 1% sucrose, 0.01% bromophenol blue) and boiled for 6 minutes. Proteins were separated by SDS-polyacrylamide gel electrophoresis on a 6-10% acrylamide gel in parallel with prestained protein molecular weight markers (BioRad, Richmond, CA) and blotted onto PDF membranes (Amersham Pharmacia, Buckinghamshire, UK) overnight using wet blot apparatus (Biorad). Membranes were then blocked for 2 h at room temperature in 0.02 mol/l TBS (pH 7.6) containing 5% weight/volume dry milk powder, and then washed in TBS with 0.1% Tween-20 (TBST) before being incubated for 1-2 h with primary antibody (anti-c-kit ligand goat polyclonal, 1: 750, Santa Cruz) in TBST with 5% dry milk or the ZP-A antibody (1:2500) as appropriate. Bound antibody was detected using a rabbit anti-goat (1:6000) or goat anti-rabbit (1:15,000) HRP linked secondary antibody) and the enhanced chemiluminiscence visualization system (Amersham Pharmacia Biotech, UK) ECL+ Plus according to the manufacturer's instructions. Quantitative comparisons were done using Image-Quant software (Molecular Dynamics).

2.4.7 Statistical Analysis

Data are presented as the mean ±SEM and were analyzed by Student t-test or ANOVA with a Fischer least square difference (LSD) post-hoc test using $P < 0.05$ as the level of significance.

2.5 Results

2.5.1 Patterns of Oocyte Growth in FSH-R deficient Mice

At the onset of there studies, there was no information on the potential influence of FSH-R signaling per se on oocyte growth and function. Therefore ovaries from mice of all three genotypes and at three different ages (24 days, 3-months and 7-months) were serially sectioned for morphometric analysis.

Comparing data on oocyte diameter, we found differences for different types of follicles in the three genotypes (Fig.2.1). In the immature +/+ mouse, the oocyte diameter steadily increased from a mean of 13µm in the primordial reaching about 62µm in the antral stage. The growth patterns for the +/- were similar to the wild type except at the primary stage where they were significantly larger. The size of -/- oocytes differed significantly from the WT at the primary (showing a decrease) and pre-antral (increase) stages. It should be noted that there are no antral follicles in the -/- ovary at any age. At 3 months of age, oocyte size was in general smaller in the FORKO ovary as compared to the wild type at all stages. For the heterozygous mice, significant differences were seen for the secondary and preantral follicles. At this age the wild type and +/- oocytes of antral follicles reached an average diameter of 108µm. At 7 months, there was no apparent difference among the groups and the maximum diameter in the wild type reached 74µm in the antral stage; considerably smaller than that seen at 3 months.

2.5.2 Thickness of Zona Pellucida Matrix

The zona pellucida is first observed as extracellular patches that come together and form a uniform matrix surrounding oocytes in primary follicles. This structure increases in width to about 7 μm surrounding fully-grown oocytes of early antral follicles in the mouse [32]. Thus, the thickness of zona pellucida reflects the local environment during folliculogenesis and serves as an indicator of oocyte development. Accordingly, zona pellucida thickness was measured in all follicle types of 3-month-old mice (Fig.2.2) as the site of oocytes was maximal at this age. This matrix gradually thickens with increasing follicular growth becoming maximal in the ovulatory follicle (8.3±0.06μm) in the wild type ovary. For this genotype differences were significant at each stage. Structural changes were evident in the +/- ovaries at two stages; the zona matrix was thinner in the antral and ovulatory follicles ($p<0.005$). In the FORKO mice, the average thickness of zona pellucida matrix in preantral follicles was smaller (4.57 ± 0.114 μm) than that of wild type mice (5.43 ± 0.148 μm) ($P<0.001$). However, in secondary follicles, the zona pellucida became thicker (4.58 ± 0.147 μm) than that of wild type (3.64 ± 0.284 μm) ($P<0.005$). This result confirms that in the FORKO mice, some oocytes developed earlier than surrounding GCs indicating developmental imbalance (see Fig.2.3F). As reported previously [23, 27], such follicles are absent in FORKO ovaries.

2.5.3 Histological Characterization of Zona Pellucida, Oocyte and Follicle Growth in FORKO and Heterozygous Mice

The normal oocyte at various stages of its development in a healthy follicle is a perfectly rounded structure. Usually the zona pellucida is present in the perivitelline space between the plasma membrane of the oocytes and the layer of surrounding GCs. However, during folliculogenesis we noted that the periphery of the oocyte in FORKO mice was not round and smooth as in the wild type (see Fig.2.3 A-C). Some irregularity from its normal shape was found in the zona pellucida of most -/- oocytes at 3 months. The zona pellucida matrix that appeared uneven in null mutant was also apparent in +/- animals. A random sampling of ovarian sections from different mice revealed that about 5% of follicles contained GCs between the zona pellucida and the oolemma in the FORKO females (Fig.2.3 E). It may be noted that we have excluded such follicles and oocytes from our estimates of size or thickness (Fig.2.2). This feature was unique to the null mutants since it was not seen in the +/- at any age examined. In our previous communication [27], we have pointed out that this aberration of infiltration is already evident at 24 days of age in the FORKO ovary. Fig.2.3E (see arrow) illustrates the appearance of breaks indicating discontinuity in the extracellular matrix that might allow the seepage of GCs (denoted by asterisks) into the oocyte. In contrast to this aberrant characteristic in null mice, the +/- ovaries showed a different feature; frequent appearance of two oocytes within one follicle at the secondary or pre antral stages. Although this was rarely seen in the control animals, their frequent presence in the +/- animals was unique to this genotype

(Fig. 2.3D) and occurred at all ages (from 24 days to 7 mo) that we have examined. Panel F depicts an example of an enlarged oocyte in a secondary follicle in the FORKO ovary.

2.5.4 Expression of Oocyte Markers C-kit, Kit-Ligand, and BMP-15

In order to gain some mechanistic insights into the above perturbations we evaluated some well-established candidate markers that participate in oocyte-GC communication. In the normal ovary, C-kit is expressed in the oocyte and kit ligand expression is confined to GCs in the ovary [3]. In the 24-day-old mouse ovary, C-kit was clearly detectable by immunohistochemistry. Clear positive staining was seen in the oocyte of +/+ ovary, but not in the FORKO ovary where it was barely detectable (Fig 2.4C). Ovarian sections from heterozygous mice showed positive staining that was intermediate; it was weaker than that of wild type but stronger than that of FORKO (Fig. 2.4 B, C). The antisera that were available to us only allowed quantitation by Western blotting of the kit-ligand in the ovarian extracts. By this analysis, expression in the FORKO ovary was 47% of wild type and in the heterozygous; it was 85% of wild type (Fig. 2.5A). BMP-15 expression in the 24-day ovary of FORKO and heterozygous mice were also weaker than that in wild type ovary (Fig.2. 4 D-F).

2.5.5 Distribution of Zona Pellucida Proteins (ZP-A, B, C) in the Oocyte

Mouse zona pellucida matrix is composed of three glycoproteins, ZP-A, ZP-B and ZP-C [15]. These glycoprotein are secreted by the growing mouse oocytes. ZP-A and ZP-C assemble into organized filaments that are cross-linked by ZP-B. The resulting extracellular matrix, called the zona pellucida is unique to the oocyte and is a thick coat that surrounds oocytes and plays a crucial function in oogenesis, fertilization and early embryogenesis [32]. In addition, ZP-A and ZP-C are believed to be essential for gamete recognition [33]. Thus, it was of interest to understand if the oocytes in the mutants would exhibit molecular changes that would affect their regulatory function. We were able to use formalin-fixed and paraffin-embedded sections of mouse ovaries to assess zona pellucida protein expression during different ages in the three genotypes. Using the specific antibodies (see methods) staining for all three ZP proteins was observed in the ovaries of normal and mutant mice. However, their expression patterns were greatly altered in a differential manner. For ZP-A and C proteins in the +/+, expression was confined to the adjacent extracellular area around the oocyte with virtually no staining in GCs. However, with anti ZP-B, expression was intense and localized to the whole oocyte but some weakly positive reaction was evident in the GCs. In this case, GCs of mutant ovaries showed more intense staining than normal ovaries. In the 24-day-old FORKO female, the ZP-A (Fig. 2.6C) and ZP-B (Fig. 2.6F) expressions were lower. We also noted lower expression for these proteins in haploinsufficient mice at this early age (Fig. 2.6 B and E). The pattern

of ZP-C expression was also markedly different. In this case, it was stronger in both heterozygous and FORKO mice compared to the wild type (Fig.2.6 G-I). Abundant expression in the FORKO ovary is clearly evident when compared to the +/+ or +/- states (compare Fig 2.6 I with H). Overall there was a clear imbalance in the expression of these important glycoproteins that was gene dosage dependent. We were able to quantify expression by western blotting only for ZP-A (see Fig.2.5B). ZP-A expression in the FORKO ovary was reduced to 49% of the wild type whereas in the heterozygote there was a 33% deficit. This confirmed the lower content that was seen by immunohistochemistry.

2.6 Discussion

Successful mammalian reproduction requires a healthy and competent egg that upon fertilization must be adequately protected and nurtured *in vivo* during gestation. The critical and dramatic changes that occur in this period are in part dictated by the competency of the egg that also determines unsuccessful events such as premature termination or reduced fertility or other developmental abnormalities. Many of these fall under the broad term called "miscarriage" that terminates a pregnancy. In mammalian ovaries, the individual follicles consist of an innermost oocyte representing the germ cell, surrounding GCs of the somatic type, and outer layers of thecal cells. The follicles develop through primordial, primary, secondary and preantral stages before acquiring an antral cavity that is filled with fluid bathing the cumulus cells as well as the oocytes. These developmental sequences are a prerequisite for GC differentiation and ovulation.

Under the influence of gonadotropins, a fluid filled structure called the antrum is formed and the selected antral follicles further increase in size converting to ovulatory follicles. The preovulatory surge of gonadotropin (luteinizing hormone, LH) stimulates oocyte maturation that results in the release of oocytes from meiotic arrest and allows for GC-cumulus expansion. The fate of the other large preantral follicles that are not selected for ovulation is to undergo atresia [2].

Follicle development depends on optimal communication between the oocyte and surrounding GCs [34, 35]. Such transcellular communication within the follicular compartment occurs as a result of direct physical contacts (intercellular junctions) and the local production of soluble factors that act in an autocrine or paracrine fashion [36]. This communication is bi-directional and occurs throughout follicular development [16, 37, 38]. These events must be tightly coordinated to ensure orderly development and completion of meiosis [34].

Our data of the present study reveals that this bi-directional communication is influenced in a quantitative manner by FSH-R signaling events and oocyte contribution to this process is greatly perturbed in the ovaries of mutant mice during the peri/postnatal period. Thus targeted disruption of the FSH-R gene provides new insight to explain the role played by receptor signaling in maintaining the follicular milieu in a state conducive for development. The phenomenon of gene haploinsufficiency in mutant animals derived from homologous recombination is of considerable experimental interest as shown by

the present studies because such animals have allowed us to investigate gene-dose related effects as they appear at different ages (data not shown). Even though the young +/- and -/- mutants were normal in outward appearance functional deficits were already apparent at 24 days. This emphasizes the need for careful developmental assessments to understand quantitative changes that could become apparent later in life. In our previous communication, we compared the postnatal developmental pattern of the ovary and showed that follicles from FORKO mice are structurally defective [27].

Previous reports from this laboratory on adult FSH-R mutants pointed out differences in follicular growth patterns [23, 24]. Extending this further we can now hypothesize that some of the molecular alterations detected in the current study might be a direct result of developmental asynchrony between the oocyte and GCs. However, additional investigations such as the transplantation of asynchronous and growth advanced mutant oocytes into normal early stage ovaries might help in establishing if their signals alter GC and folliculogenesis.

In addition several interesting features of the current report with respect to oocyte development merit attention. The incidence of multi oocyte follicles appeared to be unique to the +/- ovary (Fig. 2.3D), as they were extremely rare or not present in the +/+ or -/- ovaries. As this was already apparent in some animals at 1 month (not shown), we believe that such an abnormality takes effect quite early in development. This type of abnormal follicles has also been noted in

ovaries of mutant mice lacking GDF-9 or BMP-15, both of which are oocyte secreted growth factors [39, 40]. Several other deletions of genes expressed in GCs also produce this aberration as in ovaries of mice lacking the Ahch [41] which encodes the transcription factor Dax-1 involved in sex determination, or the Ca^{2+}/Calmodulin –dependent protein kinase IV knockout females that show reduced fertility [42] and in mice that over express the inhibin alpha gene [43]. In comparison to all of the above null mutants, the aberrations observed in our study are unique in that the multiple oocyte follicles appear only in the haploinsufficient state. Although the mechanisms underlying such abnormalities are complex, it is possible that early developmental events that lead to follicle organization are aberrant and incomplete in the +/- FSH R ovaries. Further work would be necessary to unravel the delicate imbalance of factors that prevails in the +/- state.

In contrast to the state in +/- FSH-R mice, the oocytes of null ovaries showed an infiltration of GCs in some preantral stage follicles (Fig. 2.3E), an event that never occurred in the +/+ ovary at any age (see below). The initial increases in zona thickness, only in secondary follicles but not at other stages of FORKO mice indicate faster progress and developmental imbalance in the interaction between the oocyte and GC. As such follicles also appeared in the GDF-9 knockout mice [7] we could speculate a deficit in GDF-9 secretion in the FSH-R deficient state pending further analysis of this important oocyte specific gene. However, based on drastic changes in many other markers (see below) we can suggest that oocyte structure and function is irreversibly altered in the ovaries

of our mutants. That some of these impairments occur as early as 1 month of age [27] indicates the strong impact of early establishment of communication in preserving the structural integrity of the oocyte-GC as a functional unit.

The Kit-ligand is a product of GCs specifically in ovarian follicles where its expression is hormonally regulated [12]. Its alteration as seen in the whole ovary (Fig.2.5A) coupled with drastic curtailment of the C-kit expression (Fig.2.4 A-C), can be taken as evidence of impaired or lack of communication between the adjacent germ cell and somatic compartments. In addition to controlling oocyte growth and theca cell differentiation during early folliculogenesis in the normal state, c-kit/Kit-ligand also protects preantral follicles from apoptosis. Moreover, formation of an antral cavity also requires a functional C-kit /Kit-ligand system [11, 12]. In the FSH-R mutant the ovarian follicle never advanced to these later developmental stages. Thus, as many steps of follicular development are controlled by the interactions between c-kit and kit-ligand the phenotypes observed in our study could be a direct result of its perturbation.

Drastic changes seen in BMP-15 protein expression in our mutants offer additional proof that oocyte development is impaired. BMP-15, the oocyte derived factor is a known physiological regulator of follicle cell proliferation and differentiation necessary for female fertility [8]. BMP -15 mRNA and protein are both co-expressed in oocytes throughout folliculogenesis and GCs are the first targets of BMP-15. Our observations on reduced (in +/- state) or virtual absence of

the protein in the -/- ovary (Fig. 2.4) is consistent with the reported data on BMP-15 regulation of proliferation and differentiation of GCs, inhibition FSH-R mRNA expression [9] as well as selective modulation of FSH action. This indicates that FSH-R and BMP-15 have a direct relationship, at least at the level of translation of the latter.

An unexpected and remarkable oocyte defect noted in the FSH-R mutants is the state of the zona pellucida that is normally formed during follicular development in the ovary. In the normal animal, this thick extracellular coat surrounding the growing oocytes, ovulated eggs and preimplantation embryos provides for oocyte survival, binding and activation of sperm cells leading to fertilization and early embryogenesis. It also prevents polyspermy [38]. The zona pellucida matrix consisting of 3 proteins (ZP-A, B & C) physically separates the oocyte and GCs, but maintains close associations throughout follicular development via paracrine factors and cellular processes. Several of the changes we have observed in these proteins (Fig.2.6) are reminiscent of alterations reported for mouse mutants lacking these individual proteins. For example, in ZP-B lacking mice the zona pellucida appears thinner than in normal mice, and there is an ectopic accumulation of GCs in the perivitelline space [44], as also seen in FORKO mice (Fig.2.3). Mice that lack ZP-A also produce a thin zona matrix but the abnormal zona matrix does not seem to affect the initial stage folliculogenesis. However, the numbers of antral follicles significantly decrease in these mice [45]. Mice without ZP-C do not form a zona pellucida matrix at all, even early in

oogenesis [45]. The over expression of this protein in both the +/- and -/- FSH-R mice is apparently insufficient to compensate for the loss in the other two as reproductive deficits occur in both states. This indicates that there is a different rate of change of the proteins that make up the ZP matrix in the FSH-R mutants. The FORKO mice develop uneven zona pellucida surrounding the growing oocyte and some cumulus cells even cross the zona pellucida matrix to cover the oocyte (Fig.2.3E). The matrix in the zona is visualized as a structure organized by filaments of heterodimeric repeats of ZP-A and ZP-C cross linked by ZP-B [30, 46]. Based on this, pathology similar to that seen in our mutants, in the ZP-B knockout mice was explained by the fragility and weakness of the ZP due to the mutation [44]. Thus, the ZP becomes leaky in the FSH-R ovary facilitating the entry of somatic cells that are themselves not organized very compactly. Indeed visual appearance of breaks in such oocytes in our study (Fig. 2.3 E) confirms that the extracellular matrix is not continuous; creating a dysfunctional assembly that lacks the required filament structure [46]. A more detailed study of these features is likely to reveal the regulatory aspects of ZP genes/proteins.

Normally, ZP thickness increases gradually during follicular development. The measurement of zona pellucida thickness in the different genotypes of 3-month-old mice shows that the zona pellucida thickness is less in the FORKO mice in the preantral follicles but thicker in the secondary follicles compared to the wild type mice. The thickness of secondary follicle zona pellucida in FORKO mice might be related to the advanced development of "secondary" oocytes. From

the present study we cannot say whether the thinness of the zona pellucida in our model simply reflects the different ratio of ZP-A, ZP--B, and ZP-C proteins or other unknown perturbations. Nevertheless, these data imply that infertility in the FORKO mice must also be related in part to changes in zona pellucida protein expression that will in turn influence the status of the entire follicle. However, it remains unclear where the initial molecular association of the three zona pellucida proteins occurs whether regulaton occurs at the secretion level or gene level and through what precise mechanism does FSH-R signaling control ZP protein expression. We conclude that reduced fertility and early reproductive senescence previously observed in haploinsufficient females [23, 24] must also be a consequence of the loss of the delicate balance of intercellular communication.

In conclusion our studies report for the first time that oocyte integrity and perhaps functional status are directly impaired in the absence of FSH-R signaling. Evidence emerging from haploinsufficient mice also suggests the existence of a clear gene dose related effect on the oocytes. These are related to alterations in many growth factor related genes and structural glycoproteins produced by the oocytes. Thus, collectively these data draw attention to the possible link between embryonic and/or immediate postnatal defects during FSH-R haploinsufficiency that need to be explored in future investigations. We believe that these phenomena may have relevance to premature ovarian failure and lower reproductive success in middle age women.

2.7 Acknowledgments

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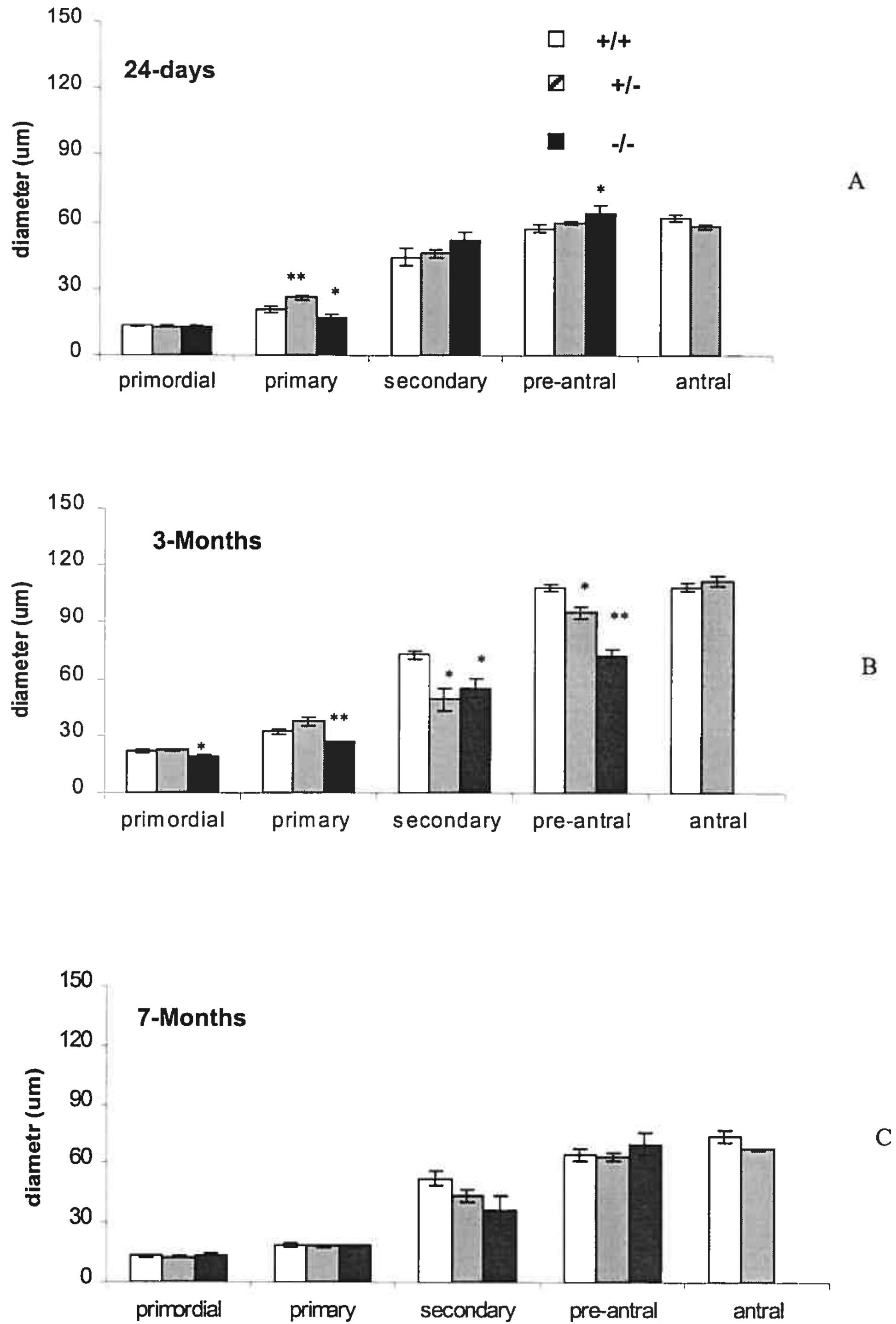
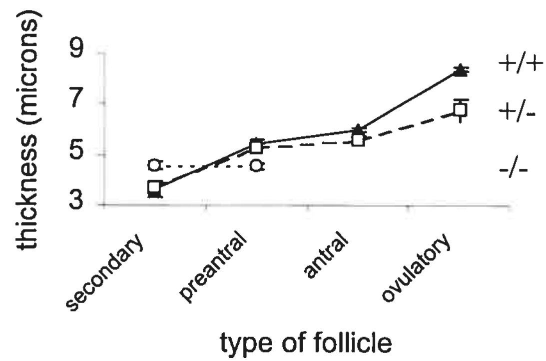


Figure 2.1: Developmental Influence on Oocyte Growth at Different Ages

Figure 2.1: Developmental influence on oocyte growth at different ages

In each panel the diameter of oocytes in follicles (20-80) of different sizes for all three genotypes are shown. 24-day-old mice. B. 3 months and C. 7 month. * denotes significant difference ($p<0.05$ when compared to +/+).



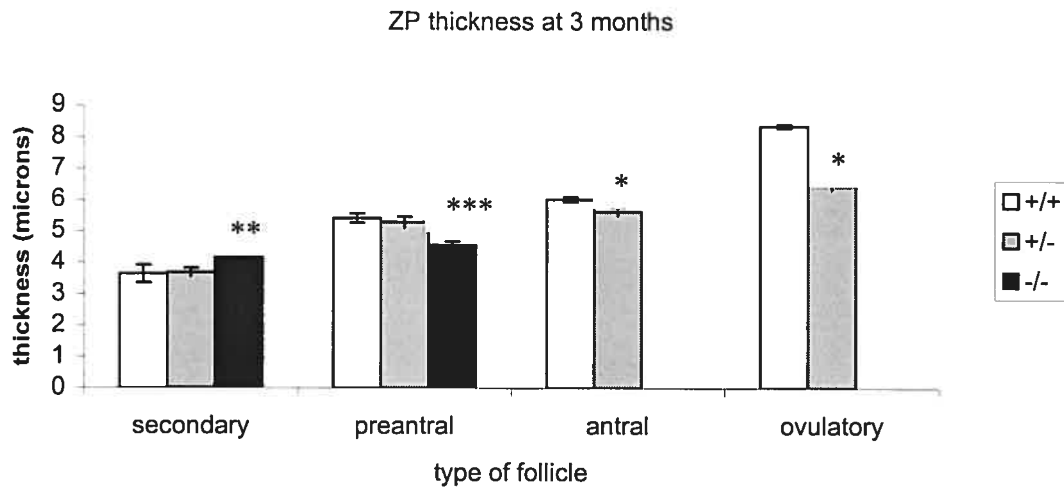


Figure 2.2 Zona pellucida development in 3-month-old mice

Figure 2.2: Zona pellucida development in 3-month-old mice.

The thickness of the zona is considered as an indication of the functional state of the follicle. The compilations are from comparisons of oocytes from the different types of follicles (n=20-80) in the three genotypes. For designation of follicle type, see methods. A preovulatory follicle is one, which appears to be in the final stages preparatory to the expulsion of the ovum. Note that the antral and preovulatory follicles are completely absent in null FORKO mice. Asterisks indicate statistically significant difference compared to the WT. * P, 0.05, ** $P < 0.005$, *** $P< 0.001$. As indicated in the inset the increase in ZP thickness in the +/+ is significantly different from the previous stage. Growth in the -/- is stagnant.

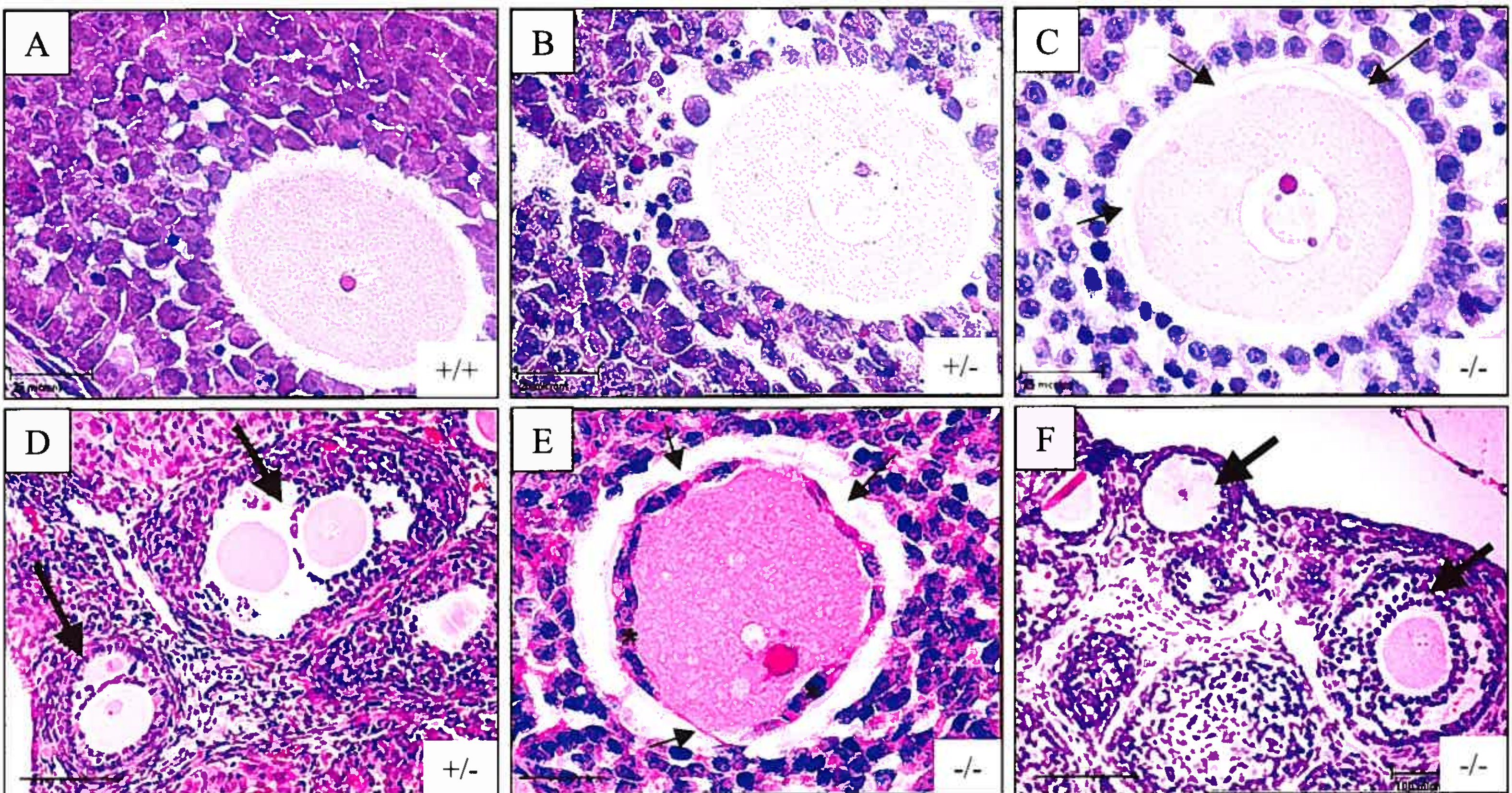


Figure2. 3: Patterns of histological change in oocytes of the FSH-R mutant ovaries and examples of early aberrations

Figure 2.3: Patterns of histological change in oocytes of the FSH-R mutant ovaries and examples of early aberrations.

The panels assemble examples that are typical of alterations observed in the FSH-R ovaries at the three different ages. Pictures shown are for 3 month-old ovaries. In the top panel comparable stage follicles are shown A. In the +/+ follicle the oocyte has smooth, rounded and evenly distributed zona pellucida. B. In the +/- follicle oocyte shape irregularity starts C. Oocyte in a FORKO ovary is not round and zona is uneven. Arrows indicate examples of variations. D. Ovarian follicles in a +/- ovary having two oocytes (see arrow). Degenerating GCs are also seen in these follicles. E. Example of a -/- oocyte in which the zona appears discontinuous and patchy (arrow) allowing infiltration of loose GCs (asterisks) into the oocyte. This was not found in the +/+ or +/- follicle. F. Example of a oocyte (arrow) big enough to be in preantral follicle but now found in association with only one to three layers of GCs. The scale bars are shown in each panel.

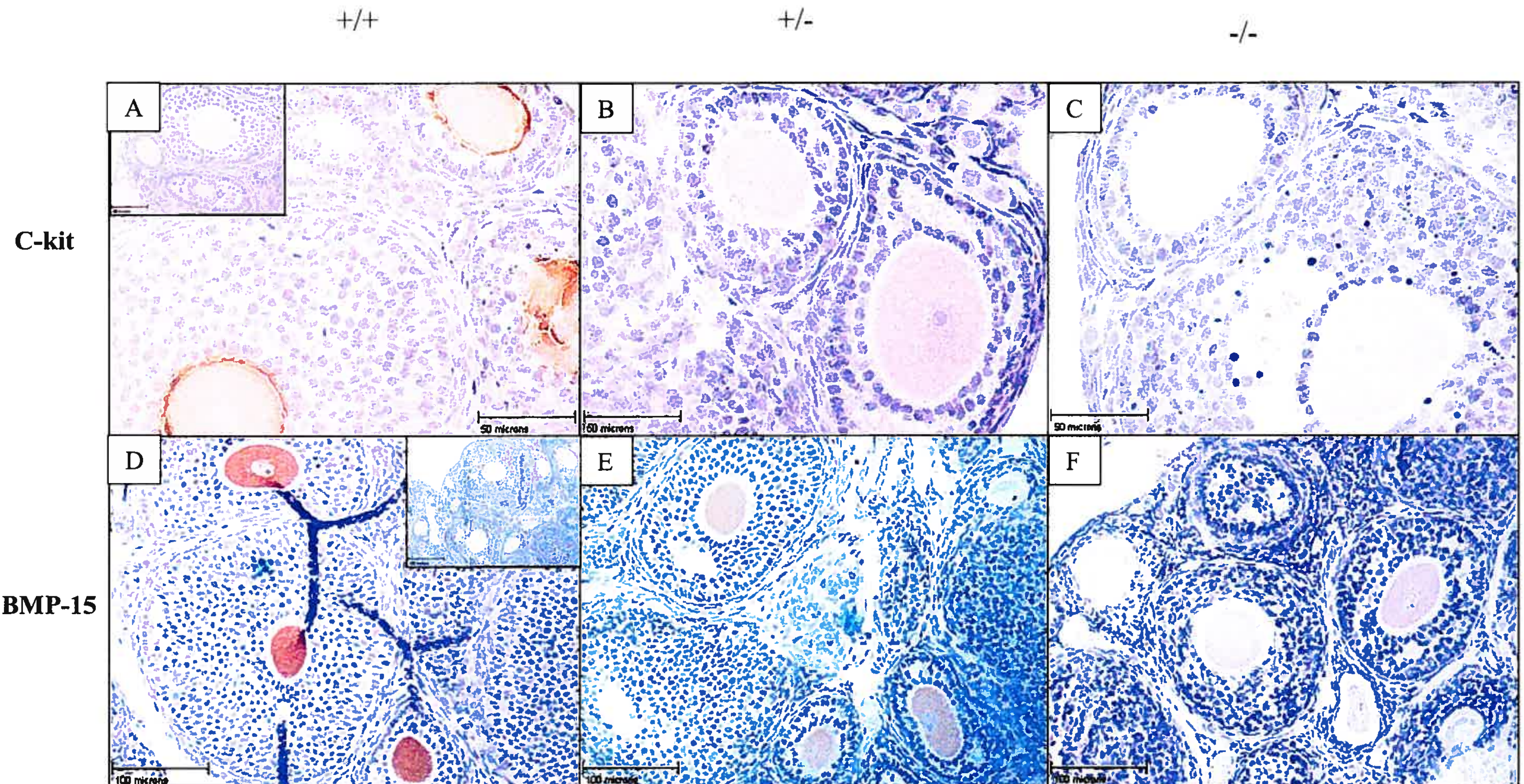


Figure 2.4: Immunohistochemical detection of c-kit and BMP-15 in the oocyte

Figure 2.4: Immunohistochemical detection of c-kit and BMP-15 in the oocyte.

The example shown is for a 24-day old ovary. A. Wild type ovaries express c-kit in the oocyte, where a strong reaction is evident. B. Section of a +/- ovary with weaker staining in a preantral follicle. C. Expression in a -/- follicle with very faint signal. D-F. BMP 15 localization for the +/+, +/- and -/- respectively. The scale bars are shown in each panel.

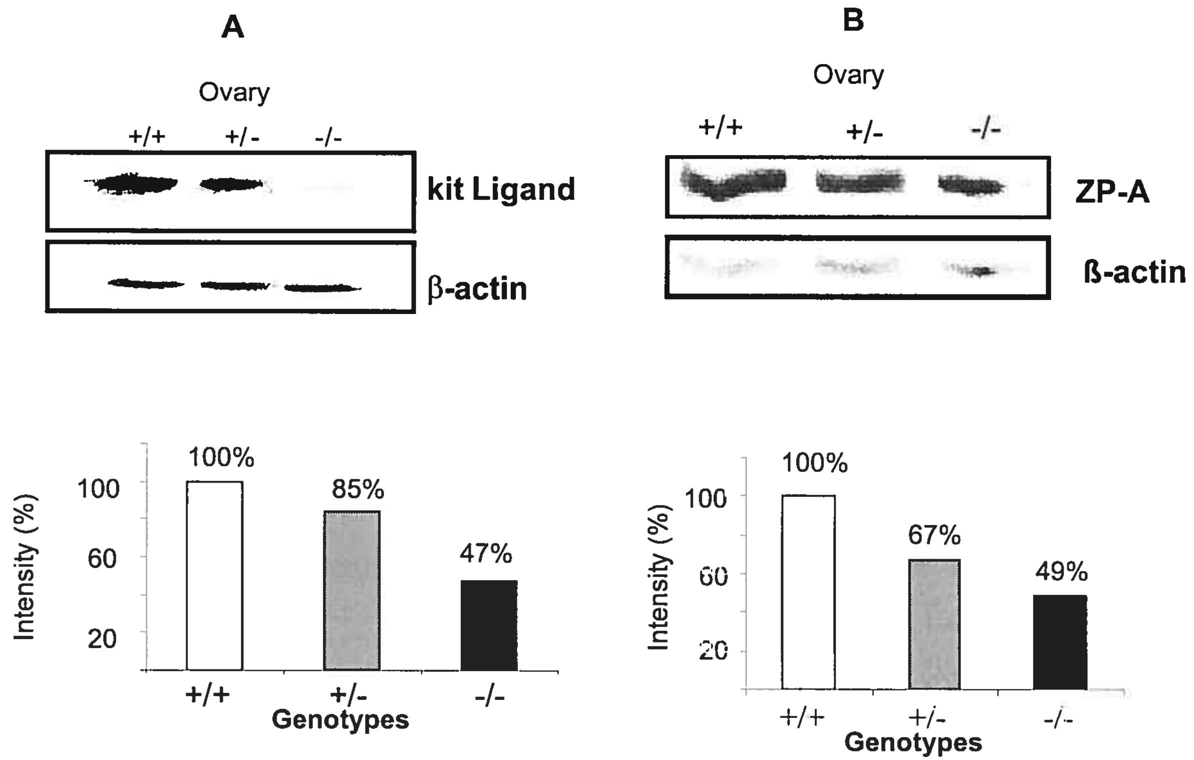
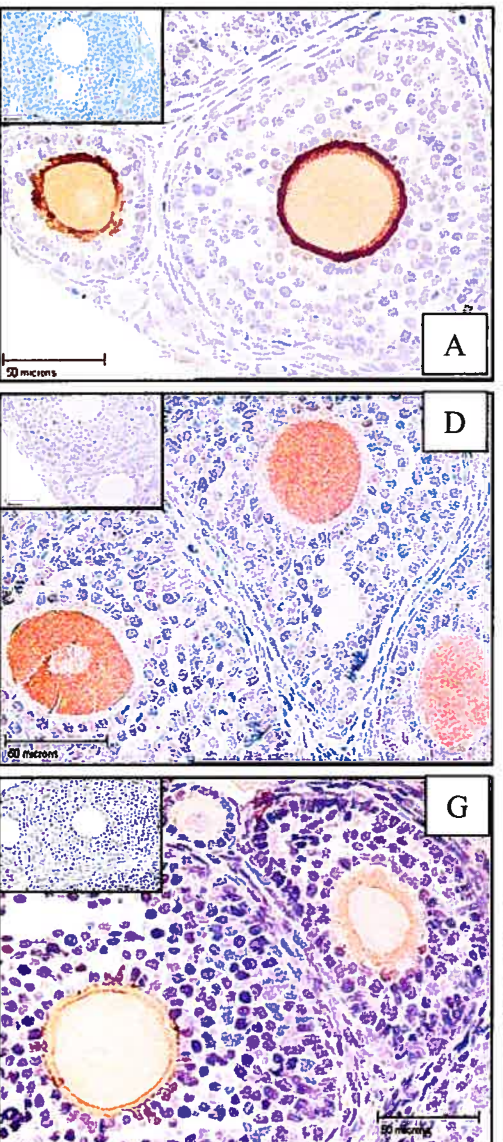


Figure 2.5. Western blotting comparing ovarian protein expression

Figure 2.5. Western blotting comparing ovarian protein expression.

A. Top panel shows a blot with equal amounts of protein extracts of the 3-month-old +/+, +/- and -/- ovaries probed with c-kit ligand antibody. ß actin is shown as a control following reprobing of the membrane with its antibody. Bottom panel compares ratios (densitometry) of c-kit ligand expression normalized to β actin. B. Blot for zona pellucida glycoprotein ZP-A with densitometric values normalized in the bottom panel. In these experiments pools the small size of the -/- ovaries required pooling of 6 or more ovaries per each experiment (n=3)


+/+
ZP-A
A
ZP-B
D
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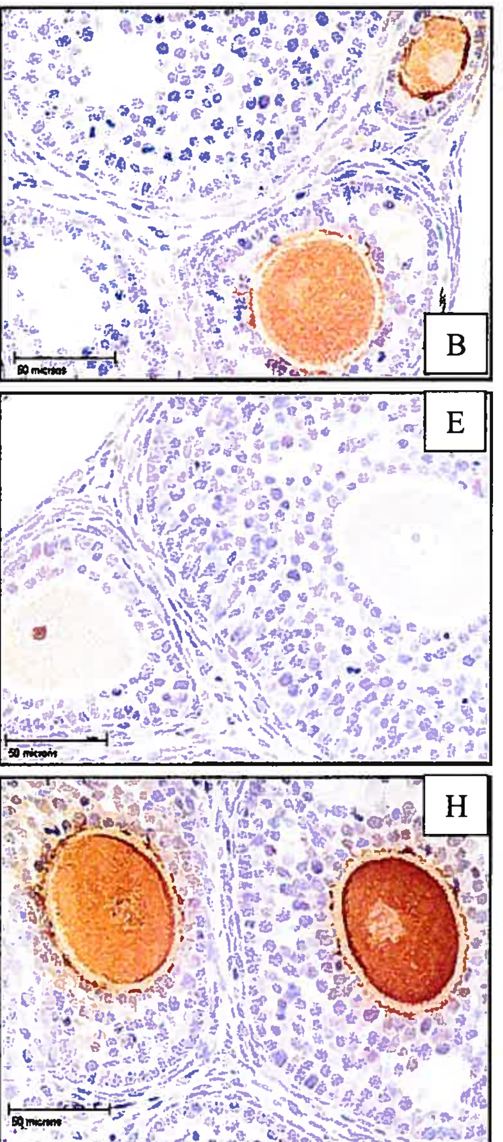
G
50 microns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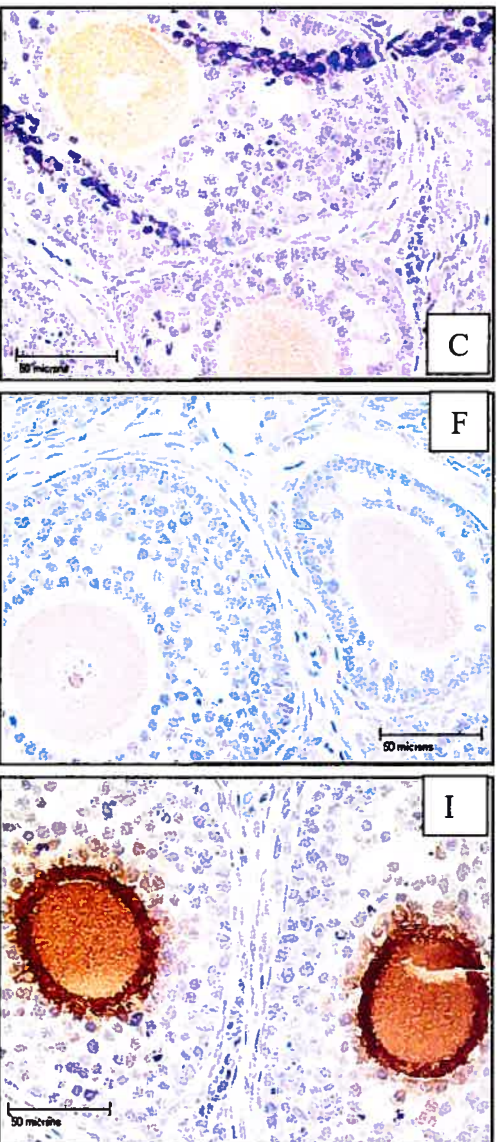
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Figure 2.6: Expression of oocyte zona glycoproteins revealed by immunohistochemistry.

All the examples shown are for the 24-day-old ovary of the 3 genotypes. Inset in each of the left panels show control omitting the primary antibody but including normal serum. A. Expression pattern using anti peptide ZP-A antibody. Protein is detected as a ring around the oocyte. Expression is lower in +/- and -/- ovaries. B. Antibody to recombinant ZP-B. This antibody detects intense reaction in the whole oocyte. C. Antibody to recombinant ZP-C. This antibody detects intense reaction as a rim around the oocyte. Very high expression is evident in oocytes of both the +/- and -/- ovarian sections with the latter being the strongest.

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3. General Discussion

In the present work, we have used the FSH-Receptor knockout mouse model – FORKO to characterize the oocyte development during complete or partial FSH-receptor deficiency. This work describes for the first time that there are deficits in oocyte development in both -/- and +/- mice. The experimental data demonstrated that lack of FSH receptor signalling could damage the communication between oocyte and GCs.

FSH signaling is essential for normal folliculogenesis and female fertility (Kumar et al. 1997; Kumar et al. 1998; Dierich et al.). FORKO mice gave us an opportunity to investigate the impairment of communication between oocyte and granulosa cells during FSH signaling deficiency. Previous reports from our laboratory showed that FORKO null mice are acyclic and infertile; the heterozygous mice exhibit reduced fertility and fecundity (Danilovich et al., 2000). Other studies show defects in follicular development in FORKO mice as early as 2 days after birth (Balla et al. 2003) . These results offer experimental evidence for the critical role of FSH-R signalling in ovarian follicular development. Extending these further, in this study we monitored the follicle development at the morphological and molecular mechanistic level in two genotypes: homozygous mice, which completely lack the FSH-receptor and heterozygous mice, which have only one allele of the gene.

3.1 Characterization of oocyte and follicle growth in the FORKO ovaries

In this investigation, we note that FORKO oocyte growth is signigicantly retarded in the 3-month-old mice (Fig.2.1). Analysis of follicles from 3-month-old +/+, +/- and -/- groups undertook a direct comparison of oocyte growth relative to follicle growth. In the follicle growth curves (Fig. 3.1), data from control group show that oocyte growth is linear over a follicle size of roughly 80-120μm. The growth trend of heterozygote oocyte was similar to that of wild type oocytes, but there was an arrest in follicle growth around 320 μm, whereas wild type follicles still grew bigger. In general, FORKO +/- ovaries demonstrated all stages of follicle development and appeared indistinguishable from control ovaries (Danilovich et al. 2000) , but the growth curve shows slight differences in follicular development. The oocyte in later stages secretes many factors that influence the proliferative and differentiative properties of granulosa cells (Buccione et al. 1990) . We assume that partial lack of FSH-R signalling affect these processes and lead to abnormal granulosa cell proliferation and differentiation. To further address the growth properties of follicles from +/- FORKO mice, besides using specific molecular markers, direct measurements of granulosa cell number at different stage of follicular development may provide useful information. In the null FORKO mice an arrest of both oocyte and follicular growth occurs when the oocyte grew to 80μm. This is in accordance with the fact that FORKO mice have all types of follicles except antral stages and beyond. Before the preantral stage, the follicle

growth occur by a process that is independent of extraovarian hormonal stimuli and it is likely that the bi-directional communication controls this early phase of folliculogenesis (Carabatsos et al. 1998). Complete lack of FSH-R signaling disturbs the communication between the oocyte and granulosa cells leading to an affect on the follicle growth in the early phases of development.

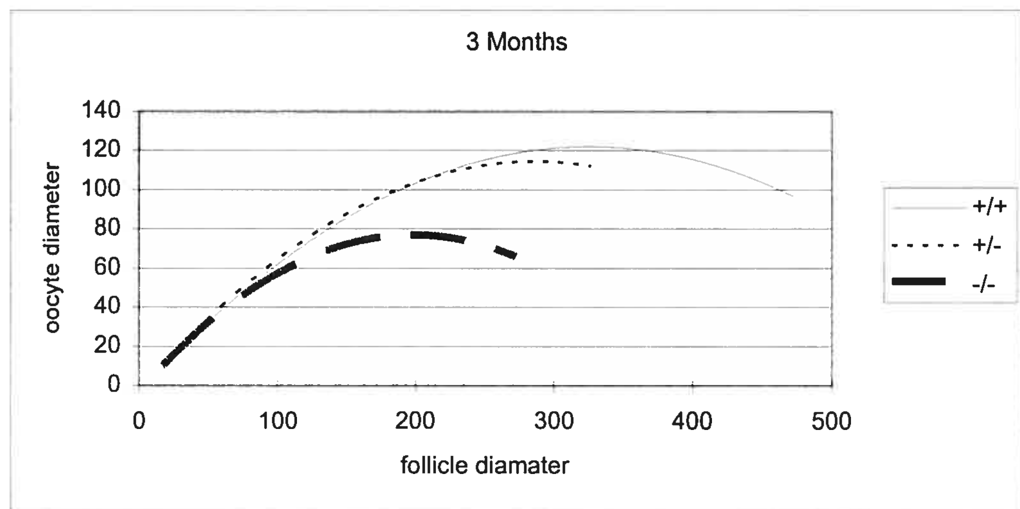

Figure 3.1 Growth curve of the oocyte versus follicle diameter in 3-month-old virgin mice.

These data are based on the analysis of more than four hundred follicles of 3 month- old mice.

3.2 Morphological and molecular changes in the FORKO ovaries.

Several interesting morphological and molecular features in FORKO mutants were discovered for the first time in this model.

3.2.1 Advanced follicle and GDF-9 expression

As described above, in FORKO null 3-month-old mice the majority of oocytes developments have retarded, but surprisingly some advanced development of "secondary" follicles appeared. As early as 1-month of age, some large oocytes appeared whose diameter corresponds to secondary follicles, but these follicles had only one layer of granulosa cells. This implies that the oocyte developed earlier than the granulosa cells because of a developmental asynchrony between oocyte and granulosa cells. Additional observations from our laboratory show an advanced follicular development present in the very young FORKO mice (Balla et al., 2003). In that study, Balla compared the postnatal developmental pattern of the ovary. In the wild type mice, 2-day-old ovaries contained a large number of naked oocytes, intermediate and primordial follicles, with only occasional appearance of primary follicles. The more interesting difference between wild type and FORKO ovary was the presence of secondary follicles in the FORKO null ovary, while wild type ovaries did not contain follicles of this type (Balla et al., 2003). Additional analysis of follicles from GDF-9 knockout mice have demonstrated that oocytes grow at a faster rate in the absence of this oocyte secreted protein (Elvin et al. 1999). In these mice, the follicles with intact big oocytes contain only one layer of granulosa cells blocked in the primary stage (Elvin et al.1999). Therefore, Elvin et al. hypothesized that lack of increase in granulosa cell number is due to either a lack of granulosa cell proliferation or to an increase in granulosa cell apoptosis resulting from impaired bi-directional communication between the oocyte and the GCs. This feature of the advanced follicles with intact big oocytes having only one

layer of granulosa cells suggests critical roles of multiple regulators of follicular growth through a mechanism involving bi-directional oocyte and somatic cell interactions. It will be interesting to investigate whether there are differences in GDF-9 expression in the FORKO mouse model.

3.2.2 Double oocyte in one follicle and BMP-15 expression

We observed that single follicles with two oocytes were a unique feature of the FORKO heterozygous mice (Fig 2.3D). Although the mechanisms are not clear at present, the dosage of FSH-R signaling appears to alter the development of the granulosa cell layers around individual oocytes, allowing the formation of these double oocyte follicles. This phenomenon also appears in the BMP-15 null mutant mice, BMP-15 -/- GDF-9 +/- double mutant mice and BMP-15 +/- GDF-9 +/- double mutant mice (Yan et al., 2001). BMP-15 -/- GDF-9 +/- double mutant mice have more multiple (double and triple) oocytes in one follicle than BMP-15 +/- GDF-9 +/- double mutant mice (Yan et al., 2001). Thus it indicates that BMP-15 participates somehow in the initial development of GC layers around the primordial germ cells. In addition, it seems that BMP-15 and GDF-9 have synergistic role.

BMP-15 null female mice are subfertile although they have all stages of follicle development and corpora lutea. They usually have decreased ovulation and fertilization rates producing fewer litters and smaller litter size than their heterozygous or wild type counterparts (Yan et al., 2001). However, sheep that are

naturally BMP-15 heterozygous show an increase in ovulation rate whereas homozygotes are infertile (Galloway et al., 2000). The null mutant sheep infertility is due to the inability of the follicles to grow beyond the primary stage. Without BMP-15, oocytes continue to grow in the absence of granulosa cell proliferation until they are unable to be supported by the residual granulosa cells, then the oocytes degenerate. On the other hand, inactivation of only one copy of BMP-15 increases ovulation rate and the occurrence of multiple pregnancies because a larger proportion of antral follicles contain granulosa cells that become responsive to LH. The discovery of these naturally mutations in sheep indicates that the different phenotypic effects appear to be related to dose, and show potential differences between mice and larger mammals. Moreover, in contrast to the BMP-15 null female mice, the BMP-15 mutant male mice are normal and fertile (Yan et al., 2001). These findings provided evidence for an essential role of BMP-15 as x-linked gene in female folliculogenesis and fertility (Dube et al. 1998) .

Based on the above studies, we can infer that the double oocytes that appeared in +/- FORKO are related to BMP-15 level in our mice. Indeed, the down regulation of BMP-15 protein in heterozygous animals and its virtual absence from the homozygous ovary supports these conclusions (Fig.2.4), BMP-15 is an important determinant of FSH action through its ability to inhibit FSH receptor expression (Otsuka et al., 2001b). It is a negative regulator of the major actions of FSH in the ovary because BMP-15 reduced the steady state mRNA levels of various proteins whose expression is induced by FSH. These include

StAR, P450scc, 3β-HSD, LH-R, inhibin/activin subunits, and the FSH-R (Otsuka et al. 2001a) . In addition, FSH induced steroidogenesis was suppressed by BMP-15 through the decrease of FSH-R message in both basal and FSH-stimulated cells (Otsuka et al., 2001b). Moreover, BMP-15 coordinates GC proliferation with FSH-dependent cytodifferentiation during follicle growth and development. Impairing FSH-R signalling affects the expression of BMP-15 protein and female fertility. Thus the loss of FSH-R signaling not only creates hormonal imbalances, but also generates the change of paracrine factors secreted by both the oocyte and granulosa cells leading to follicular abnormality.

The next question that arises is why the +/- ovaries with lower BMP-15 protein expression have double-oocytes, and why FORKO null ovaries with virtual absence of the BMP-15 protein have advanced oocytes? The deficiencies in follicle growth during the absence of FSH-R signaling also raise questions about the status of communication and perhaps junctional communication between oocyte and granulosa cells. In the oocyte-GC communication system, there is an oocyte-GC feedback loop that regulates GC mitosis (Otsuka and Shimasaki, 2002). It involves the oocyte-derived factor BMP-15 and the GC-derived factor Kit ligand. These proteins are concomitantly expressed in the early stages of follicular development. Oocyte-derived BMP-15 acts through the BMP-15 receptor on GCs to stimulate GCs mitosis and KL

expression, whereas kit ligand acts through c-kit on the surface of the oocyte to inhibit BMP-15 expression. (Fig.3.2).

3.2.3 Oocyte-GC communication and the kit ligand/c-kit system

We show in this study that kit ligand and its receptor c-kit proteins had lower expression in the FORKO female mice. However, in the male FORKO mice, significant increase of kit-ligand was reported compared the wild type animals at 3 months of age (Krishnamurthy et al., 2000). Absence of FSH-receptor signaling in the male FORKO mice affect intercellular communication in the testis and therefore spermatogenesis. In addition, the observed changes in the hormonal profile in the male FORKOs is in many aspects the reverse of those observed in the females. For example, testosterone is reduced in the males, while it is increased in females. Therefore, it is possible that there are differential regulatory pathways in FORKO females and males, which lead to opposite changes in the microenvironment of the ovary and the testis.

Kit ligand together with its receptor c-kit is a crucial regulator of female reproduction by affecting oocyte-granulosa cell communication (Otsuka and Shimasaki, 2002; Motro and Bernstein, 1993; Laitinen et al., 1995; Ismail et al., 1996). Evidence from mouse mutants and *in vitro* experiments indicates that kit-ligand/c-kit control many steps of oogenesis and folliculogenesis in a paracrine manner (Yoshida et al., 1997; Parrott and Skinner, 1997) such as regulating early follicular morphogenesis and function by anti-apoptotic actions on primordial

germ cells, oogonia and oocytes (Motro et al., 1991). Fig.3.2 shows a suggested scheme of events in oocyte to granulosa cell interactions.

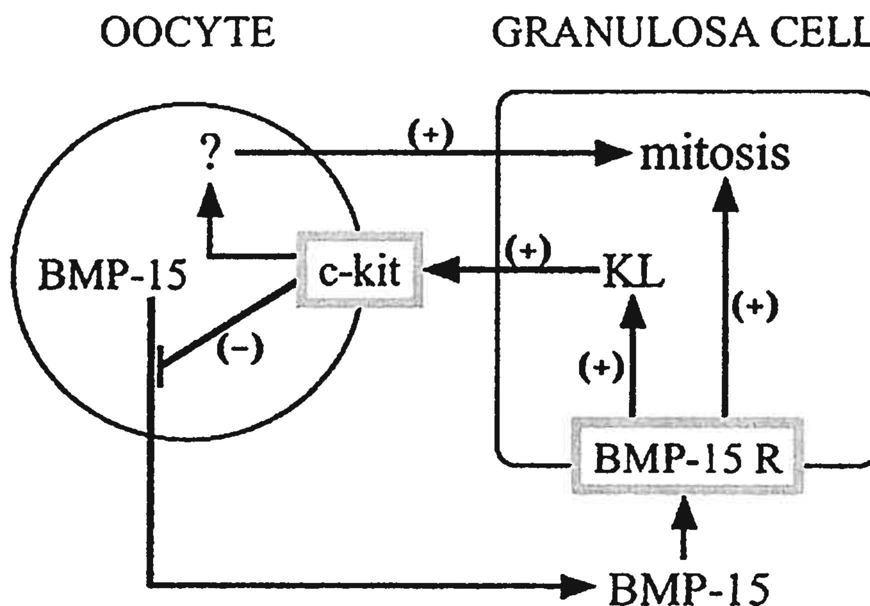

Figure 3.2 Proposed interactions of BMP-15 and KL in the regulation of GC mitosis.

A negative feedback system between oocyte BMP-15 and granulosa cell kit ligand. BMP-15 stimulates kit ligand expression in GCs. KL, in turn, acts through c-kit to inhibit BMP-15 expression, forming a negative feedback loop. KL also causes an increase in GC mitosis, presumably by stimulating the oocyte to secrete a currently unidentified mitogen denoted by "?". Reproduced from **Fumio Otsuka and Shunichi Shimasaki** 2002

In the later stages, the kit-ligand/c-kit interaction modulates the ability of the oocyte to undergo cytoplasmic maturation and theca cell differentiation (Packer et al., 1994; Vanderhyden et al., 1992). FSH can increase Kit-ligand mRNA levels in preantral granulosa cells (Joyce et al., 1999). In addition,

formation of an antral cavity requires a functional Kit-ligand/c-kit system (Driancourt et al., 2000). However, oocytes are able to both positively and negatively control granulosa cell kit ligand mRNA levels in a way that is characteristic of the stage of growth and development of the oocyte. (Joyce et al., 1999).

3.3 ZP features

During follicular development, the oocyte drives its own and granulosa cell development. Some changes observed in ZP at both the morphological and molecular levels in the FORKO ovaries are related to oocyte function. As seen in Fig.2.2, the zona pellucida thickness did not increase after the secondary stage in the null FORKO mice, although at this stage it is thicker than that of wild type mice (Fig.2.2). The thicker ZP in the secondary stage of FORKO null ovaries is probably a reflection of the "advanced" state of oocytes. In the other features such as the uneven and discontinuous zona pellucida as well as the unexpected infiltration of granulosa cells into oocytes (Fig.2-3.C E) in the FSH-R null mutants suggest functional deficits. Such ectopic follicles also appeared in the ZP-B null mice (Rankin et al., 1999). In the ZP-B null mice, about 10% of the follicles ectopic clusters of granulosa cells were juxtaposed between the inner aspect of the zona pellucida matrix and the plasma membrane. Therefore we propose that these ZP features in our model are probably related to the changes in ZP proteins. To address these issues, we performed immunohistochemical analysis of the mutant

ovaries. Changes in all ZP proteins significantly confirmed our predictions of oocyte aberrations.

Although deletions of individual ZP's have been performed the phenotypes vary from reduced fertility to sterility (Rankin et al., 1999; Rankin et al., 2001) and differing impact on follicular growth and development (Rankin and Dean, 1996), based on our data we suggest that impaired secretion of the ZP proteins altering their rations contributes to dysfunctionality of the oocytes in our animals, with subsequent effects on fertility, depending upon the extent of FSH-R gene deletion.

In summary, the FSH-R signaling is required to stimulate ovarian follicle development, granulosa cell function, and oocyte maturation and estrogen biosynthesis in females (Danilovich et al., 2000; Dierich et al., 1998; Danilovich et al., 2001). Based on our findings it is reasonable to conclude that there is damage in oocyte-granulosa cells communication following FSH-R signaling impairment. These morphological and molecular alterations detected in the current study might be a direct result of developmental imbalance between the oocyte and granulosa cells as depicted in Fig.3.3. These extend our knowledge of how FSH-R signaling and growth factors impact the growth of the follicle, especially the importance of the communication that controls oocyte-cumulus cell functions and granulosa-theca cell functions within the follicle, and how hormones, such as FSH impact these microenvironments.

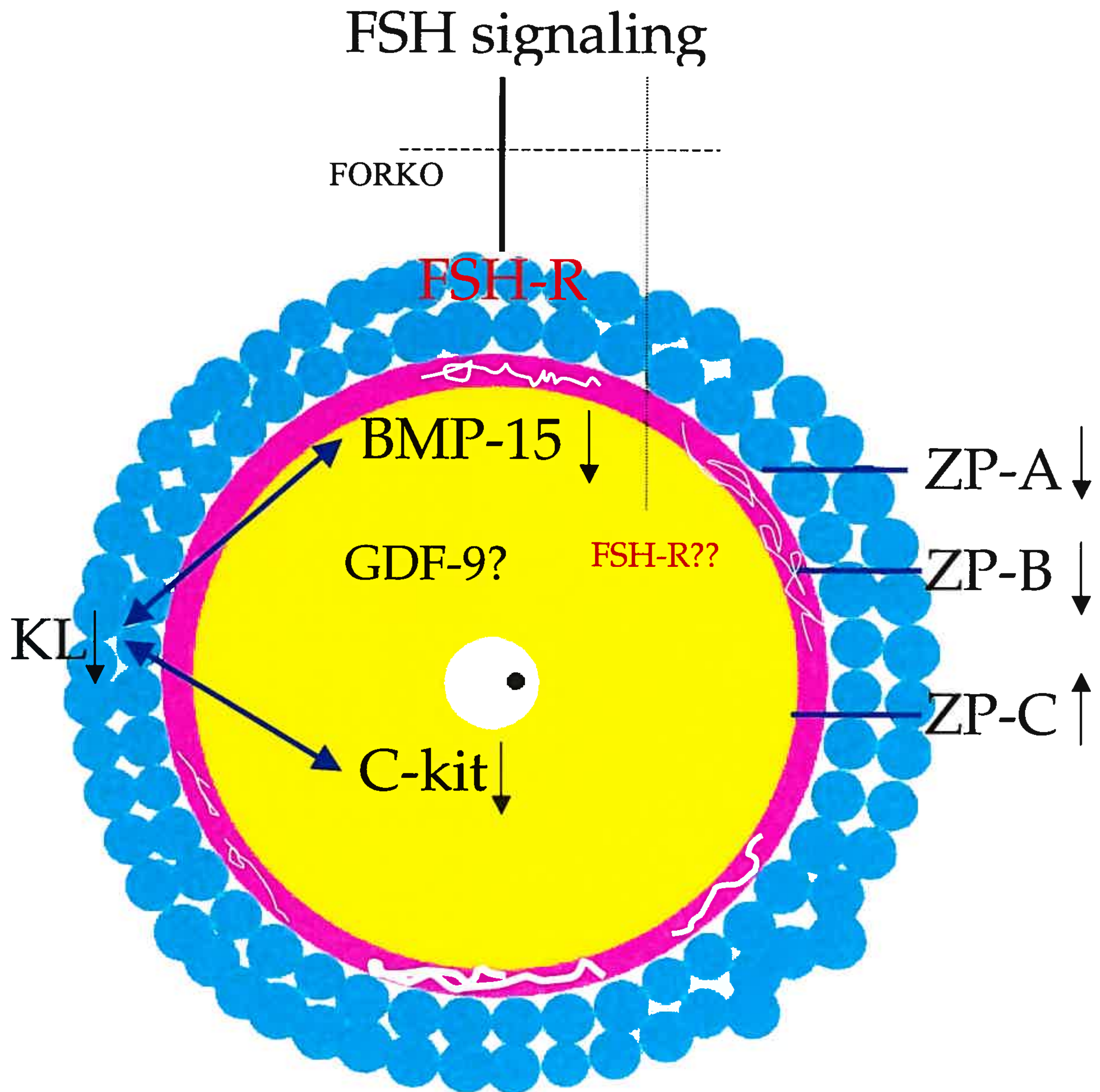

Figure. 3.3 Interaction of oocyte and GC secreted proteins in the FORKOs

Model of cellular signaling interactions between oocyte-granulosa cells during FSH-R impairment in the FORKO mice. Although this diagram depicts only the cumulus GCs, the mural GCs are also affected by oocyte factors.

3.4 Evaluation of the study

The significant contributions to research cited in the published paper presented in this thesis concentrate on characterization of the structural and molecular changes in the follicle, especially in the oocyte and zona pellucida matrix of mice lacking FSH receptor signaling. The study reveals that the communication between the oocyte and GCs was damaged contributing to deficiency in the follicular development in the FORKO mutants. This could be helpful for the understanding of mechanisms of infertility related to oocyte quality in early ovarian failure or pathological changes after menopause in women. We believe that further studies in this animal model may provide valuable insights in attempts to modify treatment of infertility due to oocyte quality and follicular microenvironment. But unfortunately in this work, we could not assess BMP-15 and c-kit due to lack of antibodies that permit quantitation by Western blotting. In addition, examining GDF-9 can perhaps provide additional proof to support our hypothesis on impaired bi-directional communication in the ovary and the quality of the oocyte.

3.5 Conclusion

- FSH-R signaling and growth factors affect the growth of follicles, especially the communication between oocyte and granulosa cells.

- Oocyte quality and its functional status is directly impaired in the complete or partial absence of FSH-R signaling.

- The status of follicles in FSH-R haploinsufficiency suggests a clear dose related effect on the oocytes.

3.6 Suggestions for future investigation

Among many interesting studies that are feasible the following might help extend my observations.

3.6.1 Transplantation

There are many follicles with one-layer granulosa cells and big oocyte "advanced" to the secondary stage in the FORKO null mice. This indicates faster progress and perhaps developmental asynchrony between the oocyte and granulosa cells. We know also that the oocyte derived signaling alters granulosa cell development and folliculogenesis during FSH-R impairment. In addition, as previously demonstrated the oocyte controls the rate of follicle development, the wild-type mice (Eppig et al., 2002). Based on these information it would be interesting to use transplant techniques to study the developmental stage of these large follicles. More specifically, oocytes isolated from the FORKO and/or heterozygous follicles could be transferred to somatic cells of wild type ovary. This experiment might help us understand if the rate of follicular development is accelerated or not in the mutant mice and the FORKO oocyte function change induced by normally GCs from wild type.

3.6.2 *In vitro* culture, maturation and fertilization

As we have found changes in oocyte quality in the FORKO and heterozygous mice, use of *in vitro* culture, maturation and fertilization would be helpful in establishing the patterns of oocyte aberrations from the functional aspect. Since +/- FORKO mice are subfertile, *in vitro* maturation of the oocytes could provide useful information on the exact time of the impairment. In addition, since FORKO animals do not ovulate, it is still not known whether these oocytes are fertilizable. IVF methods could shed some light on this. Finally, early embryonic studies could be performed to evaluate the development of one, two or four cell embryos. It could enhance our understanding of the role the oocyte quality in pregnancy and implantation in the mouse.

3.6.3 Ultrastructural evaluation in the oocytes of FORKO mice

In this study, morphological alterations have been described in the oocytes and ZP of FORKO mutants at the light microscopic level. Additional studies could involve more extensive evaluation at the ultrastructural level using the electron microscope. This would be helpful to understand the changes that occur in the follicles during the absence of FSH-R signaling. Since bi-directional communication in the ovaries is dependent on junctional proteins, such as gap junctions or adherence junctions, these could be analyzed in detail using the electron microscope. In addition, changes in organelle structure and/or number could reveal the origin of the functional abnormalities of FORKO mutants mice.

3.6.4 Expression of GDF-9 protein in the ovary

FORKO female mice have several characteristics similar to the GDF-9 knockout female mice including infertility, acyclicity, and large "egg-like" oocytes. GDF-9 plays multifunctional roles in oocyte–granulosa cell communication and regulation of follicle function. It is directly required for GC growth and differentiation and indirectly for oocyte meiotic competence and formation of a theca cells (Vitt et al. 2000). Given these roles of GDF-9, the FORKO model offers an excellent paradigm to define GDF's effect on GC and follicular development during lack of FSH-R signaling. Immunohistochemical detection of the protein levels, or RT-PCR to reveal mRNA levels could give an indication of the quantitative aspects of this oocyte marker. In addition, in case GDF-9 is reduced in FORKO mice, *in vitro* experiments where GDF-9 is externally added to mutant follicles could be performed. This would reveal the specific function of this protein on the oocyte and the surrounding GCs to understand the relationship between FSH-R and GDF-9 in the follicular development.

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