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Elucidation of the biological roles of Wnt5a signaling in follicle development

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Résumé

La santé folliculaire est déterminée par un nombre de facteurs endocriniens, paracrines et autocrines. Les gonadotrophines hypophysaires sont les principaux moteurs du développement du follicule, mais leurs actions sont modulées localement par les hormones et des facteurs de croissance. Les glycoprotéines de la famille des WNTs représentent une grande famille de molécules impliquées dans différentes voies de signalisation. Ils sont sécrétés dans le but de moduler et coordonner la réponse des follicules aux gonadotrophines, et leurs activités sont indispensables à la fonction ovarienne et à la fertilité féminine. Les WNTs sont généralement classés en fonction de la (des) voie(s) qu'ils activent. Le rôle des membres de la voie canonique WNT et de ses composants tels que CTNNB1, WNT4, WNT2, FZD1 et FZD4 est bien établi au cours du développement du follicule chez les rongeurs. Un rôle similaire des WNTs dans les espèces mono-ovulatoires demeure essentiellement inconnu. De plus, le rôle des WNT non canoniques dans l'ovaire de rongeurs est méconnu.

Les objectifs de cette thèse sont (1) d'élucider la régulation hormonale de l'expression de WNT5A et le rôle physiologique de WNT5A dans les cellules de la granulosa bovine *in vitro* et (2) d'identifier les rôles physiologiques de WNT5A dans l'ovaire de souris par inactivation génique conditionnelle. Chacun de ces objectifs a mené à la publication d'un article à partir des résultats obtenus au cours de cette thèse.

Dans le premier article, le rôle de WNT5A dans les cellules de la granulosa bovine a été étudié *in vitro*. Nous avons constaté que WNT5A est un régulateur négatif de la stéroïdogenèse stimulée par la FSH issue des cellules de la granulosa, et qu'il agit en supprimant l'activité de signalisation des WNTs canoniques tout en induisant la voie de signalisation MAPK8/JUN.

Dans le deuxième article, afin d'examiner le rôle de deux WNTs non-canoniques, WNT5A et WNT11, à différents stades de développement folliculaire, nous avons généré des modèles de souris knock-out conditionnels ciblant les cellules de la granulosa pour chacun de ces WNTs. Les résultats obtenus ont permis de mettre en évidence que WNT5A est nécessaire pour assurer la fertilité normale chez la femelle, le développement folliculaire et la stéroïdogenèse ovarienne. Il est aussi un antagoniste de la réponse aux gonadotrophines, agissant par l'intermédiaire de la suppression de la signalisation canonique des WNTs. Chez les souris knock-out pour WNT11, nous ne constatons aucun défaut important dans la fertilité des femelles.

L'ensemble de notre travail met en évidence que WNT5A est essentiel pour le développement normal du follicule et qu'il agit pour inhiber la différenciation des cellules de la granulosa. En résumé, nous avons fourni une étude novatrice et approfondie, utilisant plusieurs modèles et techniques pour déterminer les mécanismes par lesquels WNT5A régule le développement des follicules.

Mots clés: WNT5A, développement folliculaire, cellules de la granulosa, stéroïdogenèse ovarienne, vache, souris knock-out conditionnel.

Abstract

Follicle health is determined by an array of endocrine, paracrine and autocrine factors. Pituitary gonadotropins (LH and FSH) are major drivers of follicle development, but their actions are modulated by local hormones and growth factors. The WNT family of secreted glycoproteins are signaling molecules that act to modulate and coordinate follicular responses to the gonadotropins, and whose activities are indispensable for ovarian function and female fertility. WNTs are normally categorized according to pathway(s) via which they signal. The role of canonical WNT members and components such as CTNNB1, WNT4, WNT2, FZD4 and FZD1 is well established during follicle development in rodents. Whether WNTs play similar roles in monovular species remains essentially unknown. Moreover, the role of non-canonical WNTs in the rodent ovary is unclear.

The objectives of the present thesis were (1) to elucidate the hormonal regulation of WNT5a expression and the physiological role of WNT5a in bovine granulosa cells *in vitro* and (2) to identify the physiological roles of WNT5a in the mouse ovary by conditional gene inactivation.

The results of this thesis are presented in two articles. In the first article, the role of WNT5a in bovine granulosa cells was investigated *in vitro*. We found that WNT5A is a negative regulator of FSH-stimulated granulosa cell steroidogenesis, and that it acts by suppressing canonical WNT signaling activity and inducing the noncanonical MAPK8/JUN pathway. In the second article, we generated granulosa-specific knockout mouse models to examine the roles of two non-canonical WNTs, WNT5a and WNT11, in different stages of the follicular development. Our results showed that WNT5a is required for normal female fertility, follicle development and ovarian steroidogenesis, and is an

antagonist of gonadotropin responsiveness that acts via the suppression of canonical WNT signaling. However we did not observe any significant defects in fertility of WNT11 knockout mice.

Together, our work demonstrates that WNT5a is essential for normal follicle development and acts to inhibit differentiation in granulosa cells. In overview, we provided a novel and comprehensive investigation, using multiple models and techniques, to determine the mechanisms by which WNT5a regulates follicle development.

Key words: WNT5a, follicle development, granulosa cells, ovarian steroidogenesis, cow, conditional knockout mouse.

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List of abbreviations

ADAM: A disintegrin and metalloproteinase domain-containing protein 8

AKAPs: A-kinase anchor proteins

AMH: Anti-Mullerian hormone

AREG: Amphiregulin

APC: Adenomatosis polyposis coli

Bcl-2: B-cell lymphoma 2

BMP: Bone morphogenetic protein

BP: Binding protein

BTC: Betacellulin

CaMK: Calmodulin-dependent protein kinase

cAMP: Cyclic adenosine monophosphate

CC: Cumulus cells

CEEF: Cumulus expansion enabling factor

CK1a: Casein kinase 1a

CL: Corpus luteum

CNA: Calcineurin

COC: Cumulus oocyte complex

CRD: Cysteine-rich domain

CTNNB1: Beta-catenin

CYP11A1: Cytochrome P450 cholesterol side-chain cleavage

CYP17A1: Cytochrome P450 17β hydroxylase/C17–20 lyase

CYP19A1: Cytochrome P450 aromatase

DAG: Diacylglycerol

Dazla: Deleted in azoospermia

Dkk: Dickkopf

DMC1: Disrupted meiotic cDNA 1 homologue

ECM: Extracellular matrix

EGF: Epidermal growth factor

EGFR: Epidermal growth factor receptor

EREG: Epiregulin

Figla α: Factor in the germline alpha

FGF: Fibroblast growth factor

Foxl2: Forkhead box protein L2

Foxo3a: Forkhead box O3

FSH: Follicle-stimulating hormone

FSHr: FSH receptor

FZD: Frizzled

GDF: Growth and differentiation factor

GDNF: Glial cell-derived neurotrophic factor

GH: Growth hormone

GnRH: Gonadotropin-releasing hormone

GPCRs: G protein coupled receptors

GSK3: Glycogen synthase kinase 3

GVBD: Germinal vesicle breakdown

HSD17B1:17β-hydroxysteroid dehydrogenase

HSD3B2:3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase

IGF-I: Insulin-like growth factors

IGFBPs: IGF binding protein

IαI: Inter-α trypsin inhibitor

Int1: Integration 1

IP3: Inositol 1, 4, 5-triphosphate

ISH: *In situ* hybridization

IVF: *In vitro* fertilization

JNK: Jun N-terminal kinase

KL: Kit-ligand

LDL: Low-density lipoprotein

LEF: Lymphoid enhancer factor

LH: Luteinizing hormone

LHR: LH receptor

LIF: Leukemia inhibitory factor

LRPs: Low-density lipoprotein receptor-related protein

MI: Metaphase I

MII: Metaphase II

MAPK: Mitogen activated protein kinases

MGC: Mural granulosa cells

MIS: Müllerian-inhibitory substance

MMTV: Mouse mammary tumor virus

MMP: Metalloprotease matrix protein

MSH5: Muts homologue 5

NFAT: Nuclear factor of activated T-cells

NGF: Nerve growth factor

NLK: Nemo-like kinase

NOBOX: Newborn ovary homeobox protein

PCP: Planar cell polarity

PGC: Primordial germ cell

PGE: Prostoglandin

PI3K: Phosphatidylinositol-3-kinase

PKC: Protein kinase C

PLC: Phospholipase C

PR: Progesterone receptor

PRL: Prolactin

PTEN: Phosphatase and tensin homolog deleted on chromosome 10

Ror: Tyrosine kinase-like orphan receptor

Ryk: Typical tyrosine kinase receptor

SCP2: Sterol carrier protein

SF1: Steroidogenic factor 1

StAR: Steroidogenic acute regulatory protein

SPO11: Sporulation protein homology

TAK1: TGFβ activated kinase

Sgy: Soggy

sFRP: Secreted FZD-related protein

TCF: T-cell factor TGF: Transforming growth factor

TIAR: T-cell intracellular antigen-1-related

TMR: Transmembrane pass receptor

Tsc-1: Tumor suppressor tuberous sclerosis complex 1

TSG-6: Tumor necrosis factor stimulated gene 6

Wg: Wingless

ZP: Zona pellucida

This is dedicated to

my parents and my husband

for their love, support and encouragement in all my endeavours

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Introduction

Follicles are the basic functional units of mammalian ovaries and are composed of germ cells (oocyte) and somatic cells (granulosa, theca and stromal cells) (Hsueh et al., 2015). Follicle development initiates during fetal life (in humans) or after birth (in rodents) when primordial follicles are formed. Once follicles start to grow, the activated primordial follicle composed of a single layer of small granulosa cells surrounding the primordial oocyte develops into a primary, secondary and eventually an antral follicle (Richards and Pangas, 2010). The process of the follicular maturation, known as folliculogenesis, is mainly under control of the gonadotropins (FSH (follicle stimulating hormone) and LH (luteinizing hormone)), which are secreted from the anterior pituitary (Richards et al., 2002). Besides the gonadotropins, ovarian follicle growth is controlled by the production of intraovarian growth regulatory factors that act by autocrine, paracrine and intracrine mechanisms (Richards, 1994).

Members of WNT family of signaling molecules have been shown to impact ovarian cell function and follicle organization. WNTs are secreted signaling molecules that act locally to control different developmental processes including cell proliferation and differentiation (Cadigan and Nusse, 1997). Canonical and non-canonical WNTs transduce their signal via Frizzled (FZD) family receptors to activate diverse signaling cascades (Slusarski et al., 1997), which will be further detailed in the end of chapter 1 of this thesis. Recent reports have suggested roles for canonical WNT signaling in the adult ovary. Indeed, canonical WNTs are required for normal antral follicle development and act by regulating granulosa cell functions including steroidogenesis and proliferation (Boyer et al., 2010a, Boyer et al., 2010b, Wang et al., 2010). Whether WNTs play similar roles in monovular species remains essentially unknown. Likewise, little attention has

been paid to the potential roles of non-canonical signaling pathways in the ovary of mono- and polyovular species.

This thesis describes studies that took advantage of both cow and mouse models to investigate the roles of non-canonical WNT signaling in the female fertility. The bovine species is a mono-ovulatory species and serves as a suitable model for studying human reproduction, as the two species have a similar length of gestation and typically release one oocyte at ovulation (Fortune, 1994). The rodent is a widely used model for studying reproduction for several reasons, including cost, ease of maintenance and a short generation interval. Transgenic mouse models have been effective and beneficial in understanding female reproduction (Matzuk and Burns, 2012).

Novel findings in this thesis may provide new insight into the roles and mechanisms of action of the non-canonical WNT pathways in granulosa cells. This thesis may also provide new clues regarding the etiology of various ovarian disorders.

Chapter 1. Literature review

1. Ovaries

Gametogenesis is the result of a coordinated signaling network between the gonads, pituitary, and hypothalamus. The ovaries are gonads that often found in pairs as part of the vertebrate female reproductive system and are crucial structures for the survival of the species (McDonald and Pineda, 1989). The germinal components (originating from primordial germ cells) start to colonize the gonadal primordia after migration from the yolk sac. The gonadal primordia forms a paired thickening of the coelomic epithelium that lines the ventral–medial surface of the mesonephros (the mid-region of the embryonic kidney), and arises within the intermediate mesoderm between the pronephros (initial kidney) and metanephros (the definitive kidney) on embryonic day 34 in human (Oktem and Oktay, 2008). The ovary is composed of two types of cells; germ cells (oocytes) and somatic cells (granulosa, thecal, and stromal), whose interactions dictate formation of follicles, development of both oocytes and somatic cells as follicles and ovulation (Richards and Pangas, 2010).

The ovary has two fundamental physiological roles. First is the gametogenic function that is responsible for the differentiation and release of a mature oocyte for fertilization. The second is the endocrine function that is essential for follicle development, menstrual/estrous cyclicity, and maintenance of the reproductive tract and its function. These two complementary roles are necessary for successful reproduction (Barnett et al., 2006).

1.1 Functional and anatomy of ovarian follicle structure

Growth and development of the somatic and germ cell compartments of the ovarian follicle occur in a highly coordinated and mutually dependent manner. In

mammalian ovaries, the individual graafian=preovulatory follicles consist of layers of theca and granulosa cells surrounding the germ cell (Gilchrist et al., 2004).

From the morphologic standpoint, the ovarian follicle may be classified into three major groups that are different in their size, complexity, and responsiveness to circulating gonadotropin. Primordial follicles include an oocyte surrounded by a single layer of epithelial, flattened granulosa cells with irregularly shaped nuclei. Theca cells are not present at this stage of folliculogenesis and they lack a distinct vasculature system.

Primary follicle contains a small oocyte with a single layer of cuboidal granulosa cells. Secondary follicles leave the primary stage and begin growth, but not develop a theca layer or antrum cavity. These types of follicles have two or more layer of granulosa cells and zona pellucida surrounding the oocyte. Preovulatory or graafian follicles have a clearly visible antrum. The zona pellucida (ZP) is formed and two layers of theca cells appear in this stage (Figure 1).

The oocyte pool in the mammalian ovary becomes fixed early in life. The number of primordial follicles that undergoing folliculogenesis to reach the mature, graafian=preovulatory stage is very low, and most of the follicles are either under a process of regression (atresia) or remain as primordial follicles without signs of growth. In response to preovulatory gonadotropin surges during each reproductive cycle, the dominant(s) graafian=preovulatory follicle ovulates to release the mature oocyte for fertilization, whereas the remaining theca and granulosa cells undergo transformation to become the corpus luteum (Myers et al., 2004).

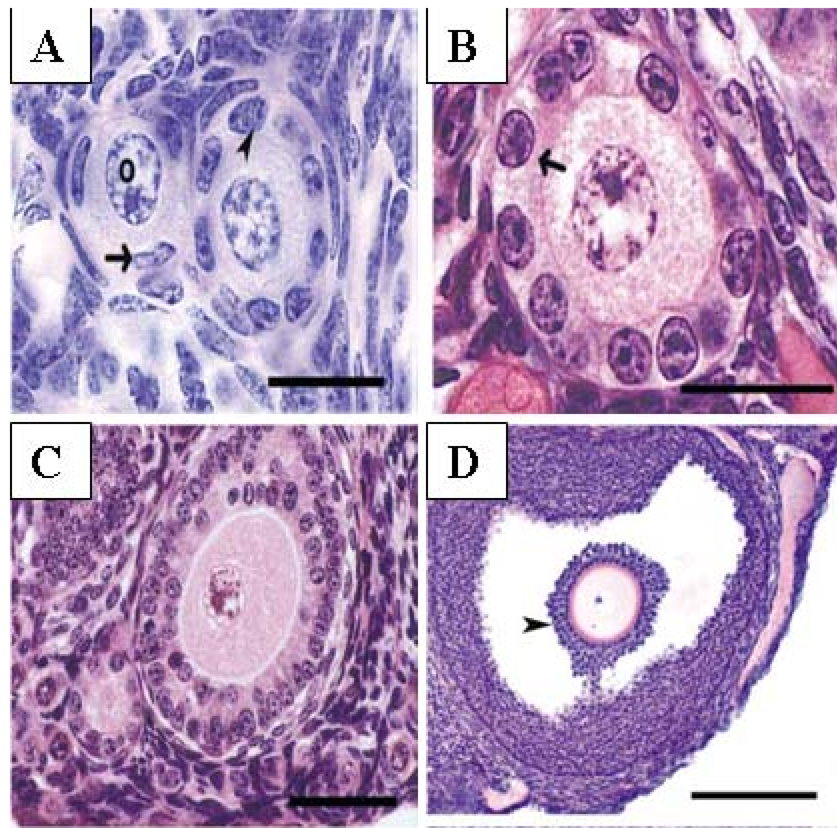

Figure 1. Follicular classification

(A) Primordial follicles are defined as an oocyte surrounded by a flat layer of granulosa cells. (B) Follicles with cuboidal granulosa cells around the oocyte are classified as primary. (C) Secondary follicles are surrounded by more than one layer of cuboidal granulosa cells, with no visible antrum. (D) Preovulatory follicles are the largest follicles and possess a defined cumulus cell layer. Taken from (Myers et al., 2004).

1.1.1 Oocyte

An oocyte is a female gametocyte or germ cell and is produced in the ovary during gametogenesis. During oogenesis, a primordial germ cell (PGC) undergoes meiosis to

form an oogonium, which becomes a primary oocyte (McDonald and Pineda, 1989). A relatively thick extracellular coat called the zona pellucida (ZP) surrounds the primary oocyte. The width of the ZP increases as the oocyte grows. Far from being a static structure, the ZP plays an important role in facilitating the interaction of sperm and oocyte at the time of fertilization. The ZP first appears as fibrillar material in the space between the oocyte and the cuboidal granulosa cells. As oocyte growth continues, the ZP becomes a denser and thicker network of connected filaments that completely surrounds the oocyte and largely separates it from the granulosa cells. Close contact continues to be maintained between the oocyte and surrounding granulosa cells via gap junction complexes within the zona until the completion of oocyte maturation (Conner et al., 2005).

Meiotic arrest of oocytes and cumulus cell expansion with germinal vesicle breakdown (GVBD) are signs of oocyte maturation. In mammalian follicles, primary oocytes enter meiosis but are arrested at the diplotene stage of prophase I. The oocytes stay in this state for months or years until the preovulatory stage. While the oocyte progresses through the maturation process in response to the preovulatory dose of LH surge, the germinal vesicle of the oocytes in preovulatory follicles undergoes GVBD, which is followed by chromatin condensation and the formation of meiotic spindles. The transition from metaphase I (MI) to metaphase II (MII) includes the extrusion of the first polar body. The maturing oocyte is the site of phosphorylation events that activate or deactivate the proteins involved in the progression of the cell cycle (Chian et al., 2003). Generally, meiotic arrest is controlled by the level of cAMP (cyclic adenosine monophosphate) in the oocyte. Recently, molecular studies have identified G-protein coupled receptors (GPCR) including GPR3 and GPR12, which control cAMP production and inhibit meiotic maturation in the oocytes of antral follicles. In fact, oocytes themselves

express a receptor that allows these cells to produce their own cAMP (Hinckley et al., 2005). In addition, AKAPs (PKA anchor proteins) can regulate the cAMP/PKA pathway and consequently meiosis in the mammalian ovary (Burton and McKnight, 2007).

Many of the effects of oocytes on follicular cells can be mimicked by members of the transforming growth factor (TGF) superfamily, including TGFβ1 and growth differentiation factor 9 (GDF-9) (Li et al., 2000). Genetic disruption of *Gdf9* in mice causes infertility and abnormal follicle development with arrest at the primary follicle stage. Similar to GDF9, GDF-9B (also called bone-morphogenetic factor-15 (BMP-15)) is an oocyte-derived growth factor that regulates granulosa cell proliferation and differentiation. There is a species-to-species difference in the role of BMP15. *Bmp15*-null mice are sub-fertile, have defects in cumulus-oocte complex (COC) formation and show a decrease in ovulation rate (Richards and Pangas, 2010). Mutations of *GDF9* and *BMP15* have been found to cause premature ovarian failure in women (Simpson, 2008).

Oocyte-secreted factors regulate folliculogenesis by modulating a large number of processes associated with granulosa cell growth and differentiation, including cumulus cell mucification and hyaluronic acid production, cellular proliferation, steroid synthesis and luteinization inhibitors (Gilchrist et al., 2004). Experimental ablation of oocytes leads to failure of folliculogenesis. Removing oocytes from preovulatory rabbit follicles results in spontaneous luteinization (el-Fouly et al., 1970). It has been shown that oocytes secrete a potent mitogenic factor(s) that promotes mural granulosa and cumulus cell DNA synthesis and cell proliferation. Importantly, this oocyte mitogen(s) interacts with key known granulosa cell regulators such as FSH, insulin-like growth factors (IGF-I) and androgens, and increase their growth promoting activities (Eppig et al., 1997). Oocytes also promote the primary to secondary and preantral to antral follicle transitions, granulosa

cell proliferation, and differentiation before the LH surge, and cumulus expansion and ovulation after the LH surge. It appears that oocyte plays a dominant role in this kind of communication, and the rate of ovarian follicular development is coordinated by the oocyte's developmental program (Su et al., 2009).

1.1.2 Granulosa and cumulus cells

As follicles grow and the antral cavity forms, granulosa cells separate into two anatomically and functionally distinct subtypes: the cumulus granulosa cells (CC), those surrounding and in contact with the oocyte, and the mural granulosa cells (MGC), which cover the follicle wall forming an epithelium with the basal lamina (Figure 2). Apart from anatomical differences, cumulus cells and MGC are functionally distinct (Albertini et al., 2001, Eppig et al., 1997).

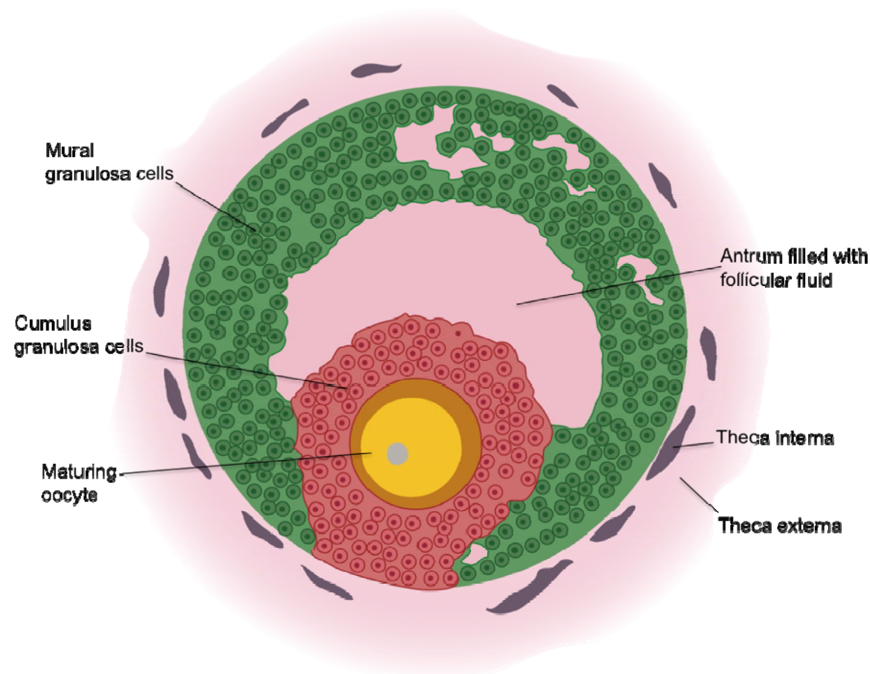

Figure 2. Antral follicle structure with different granulosa cell types surrounding the oocyte.

(http://lib.znate.ru/docs/index-236482.html?page=4)

In terms of function, CCs have a high rate of proliferation, low steroidogenic capacity, very low LHR (LH receptor) expression and high levels of insulin-like growth factor I compare to MGC, and they have the capacity to secrete hyaluronic acid and undergo mucification and expansion whereas MGC do not (Li et al., 2000). The expression of a variety of growth factors and hormone receptors also differ between MGC and CCs. Cumulus cells play an essential role in the normal growth and development of the oocyte, whereas MGCs serve a primarily endocrine function and support follicle growth. In addition, throughout ovulation, granulosa cells have an endocrine role by undergoing terminal differentiation to luteal cells, whereas cumulus cells mucify and are

ovulated with the oocyte, permitting ovum pickup and the sperm acrosome reaction (Li et al., 2000). Recently, many functional genomics studies have analyzed the importance of different genes by Cre/LoxP-mediated conditional deletion in granulosa cells. Two mouse strains expressing Cre recombinase (*Amhr2*-Cre and *Cyp19*-Cre) have been widely used used to target gene inactivation to granulosa cells. *Amhr2*-Cre is a knock-in of Cre in the anti-Mullerian hormone receptor type II locus, which directs Cre expression at early stages of follicle development. On the other hand *Cyp19*-Cre is a transgenic strain in which the aromatase promoter drives Cre expression in granulosa cells during antral stages of follicle development (Habenicht and Aitken, 2010).

1.1.2.1 Steroidogenesis

One of the major roles of granulosa cells is the production of sex steroid hormones. Estrogens have a crucial role in controlling fertility and infertility in mammals. Sex steroids play important roles in the development of the ovulatory follicles, the generation of the preovulatory surge of gonadotropins, facilitating sperm transport by changing the consistency of cervical mucus, and preparing the endometrial layer of the uterus for implantation. Compared to cumulus cells, mural granulosa cells are more steroidogenically active, as indicated by higher levels of mRNA expression for steroidogenic enzymes including 17β-hydroxysteroid dehydrogenase (HSD17B1) and aromatase (CYP19A1). The abundance of *Cyp19a1* mRNA in ovarian granulosa cells is regulated by factors which stimulate adenylyl cyclase and increase cAMP.

In the ovary, FSH can lead to the activation of second-messenger signaling including cAMP, which drives the expression of CYP19A1 and HSD17B1 and thereby permits MGCs to produce estradiol by converting androgen which comes from the theca

cells (Figure 3) (Simpson et al., 2002). The two-cell, two-gonadotropin model describes the role of theca and granulosa cells in the production of steroids, highlighting the cooperation between the two cell types, which is necessary for estrogen production (Li et al., 2000). Other than sex steroids, granulosa cells are capable of producing growth factors including inhibin, activin, bone morphogenetic proteins 2 (BMP-2) and BMP-6. These molecules can communicate between the GCs and the oocyte and theca cells during their development. In addition, after ovulation the luteinized granulosa cells produce progesterone (Gilchrist et al., 2004, Pangas, 2012).

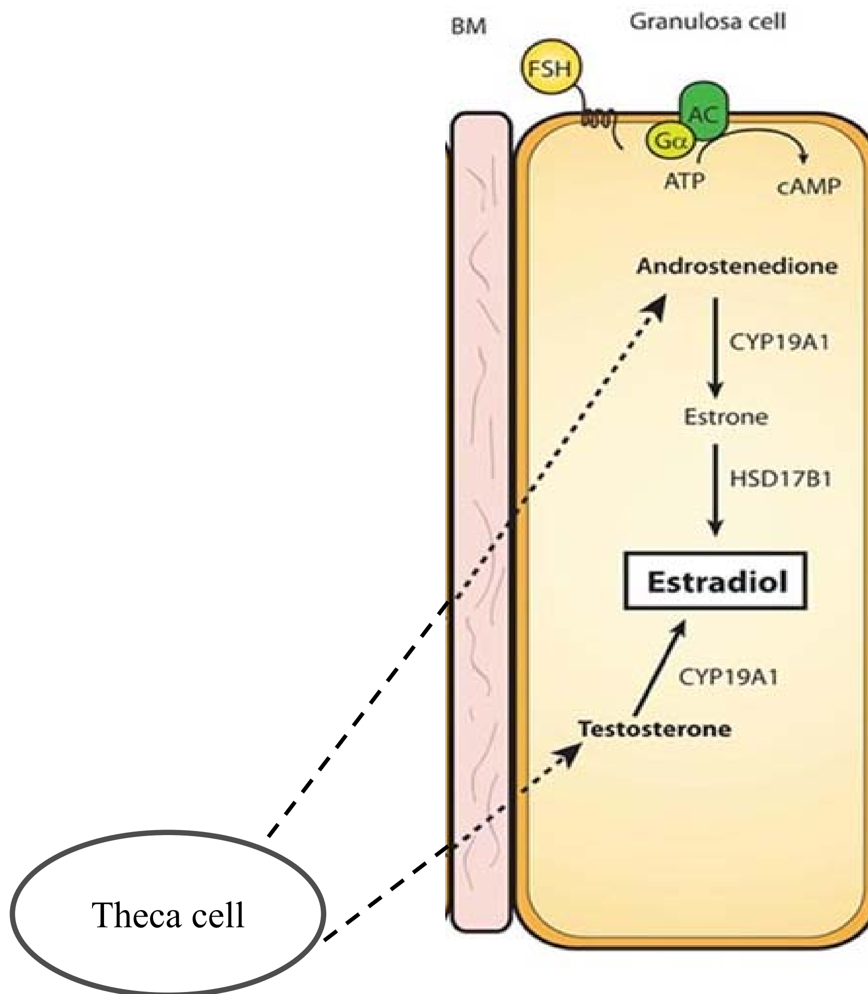

Figure 3. Steroidogenesis in mural granulosa cells.

In the growing follicle, androstenedione and testosterone can diffuse through the basement membrane. These androgens are then further transformed into estradiol in granulosa cells. Dotted arrows represent molecule translocations and plain arrows indicate an enzymatic conversion. Reproduced from reference (Lapointe and Boerboom, 2011).

1.1.2.2 Cumulus expansion

In most mammals, the LH surge starts an ovulatory cascade by inducing cAMP signaling, which stimulates the expansion of the cumulus cells surrounding the oocytes, the rupture of the follicle wall, and the release of the fertilizable ovum. As mentioned above, one of the distinct differences between MGC and CC is the capacity of cumulus cells to undergo mucification and expansion. CCs secrete proteins creating a large round mass of expanded cumulus cells via an extensive rearrangement of the actin cytoskeleton and induction of hyaluronan synthesis, which transforms the tightly packed cumulus complex into a much larger volume of mucified cells. Hyaluronan is formed by various hyaluronan binding proteins including versican, inter-α trypsin inhibitor (IαI) and tumor necrosis factor stimulated gene 6 (TSG-6). This structure has viscoelastic properties which allow COCs to deform and easily pass through the ruptured follicle wall during ovulation (Nagyova, 2012). *In vitro*, cumulus expansion is induced by treatment with FSH, which stimulates cAMP signaling to produce a massive mucoid extracellular matrix. COCs expansion varies in different species, for instance mouse COC expansion in culture requires an oocyte secreted factor(s), termed the cumulus expansion enabling factor (CEEF). In comparison, FSH can induce rat, cow, and pig COC expansion in the absence of the oocyte (Gilchrist et al., 2004, Li et al., 2000).

Small molecules such as ions, metabolites and amino acids can be transferred via gap junctions between the oocyte and cumulus cells as well as between cumulus cells to help oocyte growth and development (Gilchrist et al., 2004).

1.1.3 Theca cells

Theca cells are vital components of the follicle, providing both structural support and the production of ovarian androgens for the growing follicle as it progresses through the developmental stages to produce a mature and fertilizable oocyte. Androgens are essential substrates for estradiol production in the neighbouring granulosa cells. Theca cells are recruited by factors secreted in activated primary follicles from surrounding stromal tissue (Magoffin, 2005). Theca cells are first observed follicles with two or more layers of granulosa cells. Before this stage, undifferentiated theca cells do not express LH receptors or steoidogenic enzymes. As theca cells are only associated with growing follicles, it is assumed that the follicle itself produces factors that signal to the stroma to recruit cells that form the theca. Theca cells have some necessary roles during folliculogenesis, such as the synthesis of androgens and provide crosstalk with the oocyte and granulosa cells during follicle development. Moreover, they play an important role in the establishment of a vascular system that creates communication with the pituitary gland throughout the reproductive cycle, and delivers essential nutrients to these highly active cells. During atresia, the theca cells are often the last cell type to undergo apoptosis, and after ovulation, theca cells luteinize and form cells of the corpus luteum (Magoffin, 2005, Young and McNeilly, 2010).

1.1.3.1 Steroidogenesis

Theca cells are capable of producing androgen from circulating cholesterol (high and low-density lipoproteins) in response to LH from the pituitary gland, which activates cAMP signaling via a G protein-coupled receptor. Pulsatile release of LH triggers an increase in androstenedione and estradiol secretion by the ovary in many species.

Androgens synthesized in theca cells are transported to the granulosa cells where cytochrome P450 aromatase enzyme converts these androgens to estrone and 17β-estradiol (Young and McNeilly, 2010). In theca cells, LH/cAMP induces the expression of the key steroidogenic enzymes cytochrome P450 cholesterol side-chain cleavage (CYP11A1), cytochrome P450 17β-hydroxylase/C17–20 lyase (CYP17A1), 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase (HSD3B2) and steroidogenic acute regulatory protein (STAR*)*. Cholesterol inside the theca cells is transported by sterol carrier protein 2 (SCP2) to the mitochondria. This internalization of cholesterol by the mitochondria is the rate-limiting step for steroidogenesis. STAR helps cholesterol to enter the inner mitochondrial membrane, where CYP11A1 converts it to pregnenolone. Pregnenolone can then be converted to progesterone by the enzyme 3β-hydroxysteroid dehydrogenase, or to 17α-hydroxypregnenolone by the enzyme CYP17A1 (Figure 4) (Conley and Bird, 1997, Lapointe and Boerboom, 2011).

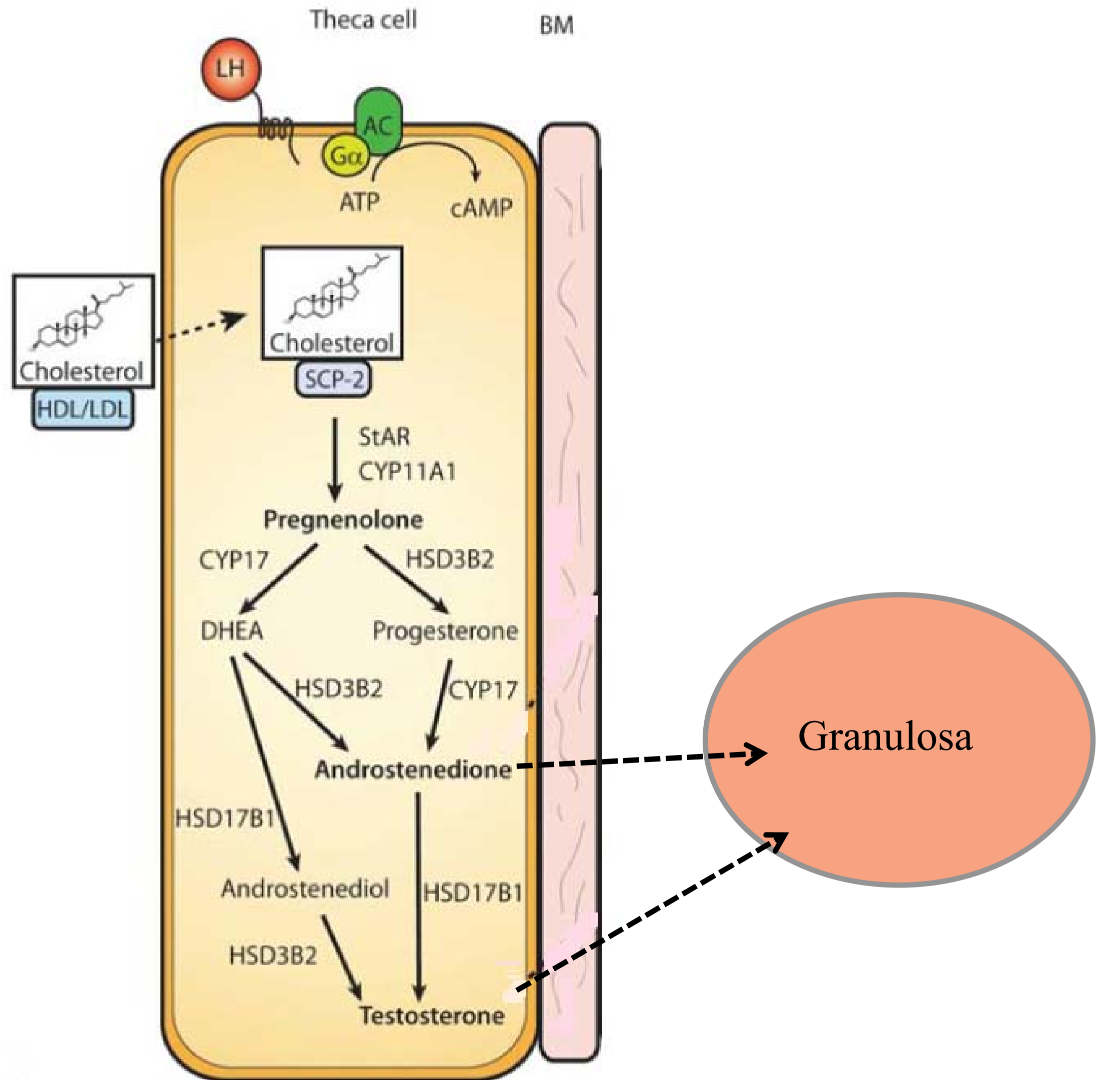

Figure 4. Enzymatic control of steroidogenesis in the theca cell.

LH can stimulate theca cells to convert cholesterol into androstenedione and testosterone, which can diffuse to the granulosa cell layer. Dashed arrows represent molecule translocations and plain arrows indicate an enzymatic conversion. Reproduced from reference (Lapointe and Boerboom, 2011).

1.1.4 Basement membrane

The basement membrane or lamina propria separates the theca and granulosa cell layers. The basal lamina creates a blood–follicle barrier between the two follicular cell compartments, and the presence of this extracellular matrix (ECM) boundary influences the interactions of follicular somatic cells. Some evidence supports the involvement of ECM components in regulating granulosa cell survival, proliferation, and steroidogenesis in the ovarian follicles of several species. A continuous remodelling of the basal lamina occurs during follicle development and its composition is changed during this process (Allegrucci et al., 2003). For instance, the laminin β1 chain was only detectable in the basal lamina of large preantral follicles, and type IV collagens α1 and -6 were reduced considerably in larger antral follicles (Rodgers et al., 2000).

1.2 Endocrine regulation of follicle growth and development

Folliculogenesis describes the progression of a number of small primordial follicles into large preovulatory follicles with the potential to either ovulate or undergo atresia. Regulation of follicle development is a complicated process that is controlled by several factors such as hormones, protein growth factors, and nutrition. Folliculogenesis can be coordinated by these factors via autocrine (affecting the cell type that produces the factor), paracrine (affecting cells adjacent to the one(s) producing the factor) and endocrine (affecting distant cells/organs) mechanisms (Armstrong and Webb, 1997, Webb et al., 2004).

1.2.1 Gonadotropins

LH and FSH are called gonadotropins because stimulate the gonads, the testes in males, and the ovaries in females. They are both are essential for steroidogenesis, spermatogenesis, folliculogenesis, and ovulation. They are non-covalently linked heterodimeric glycoproteins include a common α-subunit and specific β subunits. Distinct genes encode the gonadotropin subunits, which are synthesized as precursor proteins and processed and secreted by the pituitary gonadotropes of the anterior pituitary (Burger et al., 2004). Pituitary gonadotropins release is under the acute trophic control of the main reproductive system regulator; the hypothalamic decapeptide gonadotropin-releasing hormone (GnRH). GnRH is released and transported to the anterior pituitary in a pulsatile manner, where it binds to specific receptors and regulates gonadotropin biosynthesis and secretion. The pulse is modulated by a wide variety stimulatory and inhibitory feedback to the both hypothalamus and the pituitary.

Steroidal hormones can regulate gonadotropin levels by adjusting the GnRH pulse frequency at the levels of the in hypothalamus, and by acting directly on the pituitary gonadotrope cells. On the other hand, the members of the transforming growth factor-β superfamily, activin and inhibin, act at the level of the pituitary to control FSH secretion (Figure 5). Both LH and FSH exert their effects on ovarian somatic cells via G protein-coupled receptors that activate several signaling pathways (Burger et al., 2004, Dohler et al., 1977).

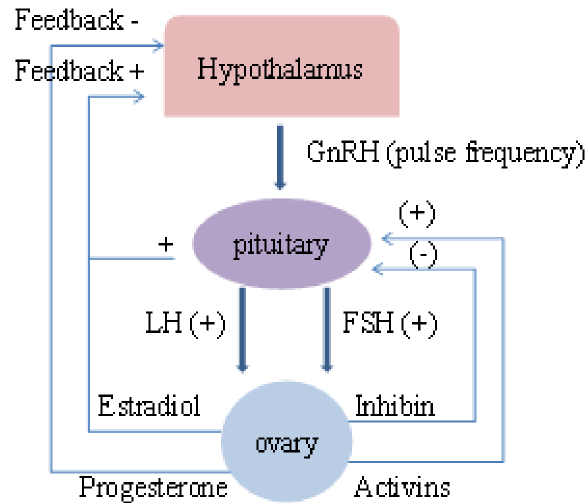

Figure 5. Regulation of gonadotropins

FSH and LH plasma concentrations above threshold levels lead to the emergence of greater inhibitory feedback, and thus a reduction in gonadotropin secretion. Estradiol increases GnRH pulse frequency through a positive feedback mechanism, and results in elevated LH secretion. However, progesterone has a negative feedback effect on pulse frequency; therefore LH secretion is suppressed in the presence of a functional corpus luteum. Moreover, activin, opposite to inhibin, can stimulate FSH release.

1.2.1.1 FSH

It is now well accepted that circulating FSH concentrations increase transiently with the emergence of each follicular wave during estrous. *FSHR* (FSH receptor) mRNA is detected in follicles with only one or two layers of granulosa cells in humans (Oktay et al., 1997).

Suppression of FSH secretion inhibits follicular waves in cattle (Turzillo and Fortune, 1990). One study in cattle shown that a single follicle continues to grow and

mature because it acquires increased sensitivity to FSH, while further development of other follicles in the same cohort with less sensitivity to FSH is inhibited. Follicles, growing under the FSH support, start producing more estradiol and inhibin, which act on the anterior pituitary to inhibit FSH secretion through a negative feedback mechanism. The decline in FSH concentrations is closely related with the selection of one follicle as dominant and others as its subordinates (Gibbons et al., 1997). Generally, FSH is responsible for oocyte maturation indirectly by inducing Lhcgr in GC and estrogen synthesis. In absence of FSH, follicle development fails and ovulation does not occur. FSH acts through G-protein couple receptors to transduce its signal. Binding of FSH to its receptor results in cAMP synthesis as a second messenger, resulting in stimulation of PKA and the p38 mitogen-activated protein kinase (MAPK) signaling pathway (Levallet et al., 1999).

1.2.1.2 LH

LH acts via G protein coupled receptors in the preovulatory follicle. LH receptors are first expressed on the cells of the theca interna at the tertiary stage of follicle development, and this expression is maintained until the preovulatory stage. In addition, granulosa cells of large estrogenic antral follicles develop LH receptors, as do luteal cells. (Ashkenazi et al., 2005). LH controls granulosa cell differentiation, cumulus expansion and progesterone production. Meiosis in the oocyte, arrested in the first meiotic division during embryogenesis, resumes following the luteinizing hormone surge. LH also up-regulates the expression of proteases that allow the follicle wall to rupture and release the oocyte. Luteinizing hormone increases steroidogenesis by promoting cholesterol transport to mitochondria and the stimulation of the expression of steroidogenic genes.

The LH surge induces a large increase in mRNA encoding epiregulin (*EREG*) and amphiregulin (*AREG*), which then activate the EGFR (epidermal growth factor receptor) and initiate the ovulatory cascade and induce oocyte maturation (Hsieh and Conti, 2005). The LH surge also results in increased synthesis of prostaglandins in the preovulatory follicle that are required for ovulation (Richards, 1994).

1.2.2 Estradiol and progesterone

Estradiol and progesterone biosynthesis within the ovary is critical for various physiological processes related to normal growth, development, and reproduction (follicle development, regulation of gonadotropin secretion for ovulation and maintenance of pregnancy) (Stormshak and Bishop, 2008).

FSH stimulates the production of estrogens by the granulosa cells of the ovarian follicles. Healthy ruminant follicles contain higher estradiol and lower progesterone concentrations than atretic follicles. It is well established that small follicles contain relatively little estradiol in their follicular fluid, and estradiol concentrations increase with follicle size in healthy growing follicles while decreasing in subordinate follicles. In cattle, once the dominant follicle reaches its maximum diameter, estradiol concentrations dramatically increase in follicular fluid and it decreases when follicle starts regressing. Thus, estradiol concentrations are a key biochemical indicator of health and atresia of ruminant follicles (Price et al., 1995).

Progesterone has a critical role in the regulation of ovulation. The physiological effects of progesterone are mediated by two nuclear receptors (PR-A and PR-B). Studies of progesterone receptor-null (*Pr*-KO) mice showed that PRs are required for LH-dependent follicle rupture (Conneely, 2010).

In general, the action of progesterone on the reproductive tract is to stimulate the proliferation of uterine stromal cells, the epithelial cells of the mammary gland, and to ensure the maintenance of pregnancy. Progesterone, in addition to estradiol, plays an important role in the regulation of LH secretion by exerting negative feedback on the hypothalamus-pituitary axis, thereby reducing the release of GnRH and LH (Murphy, 2000, Niswender et al., 2000).

1.2.3 Androstenedione and testosterone

Androstenedione is a 19 carbon steroid hormone produced in the adrenal glands and the gonads as an intermediate product that can be converted to testosterone or estradiol. Testosterone is the primary mammalian androgen. It is the mainly known as a male sex hormone and an anabolic steroid. Testosterone is primarily secreted in the testicles of males and the ovaries of females, although small amounts are also secreted by the adrenal glands. In theca cells, much cytochrome P450 17α-hydroxylase enzymatic activity is present, and so pregnenolone (the first steroid in the steroidogenesis pathway) is converted to 17α-hydroxypregnenolone. This 17α-hydroxypregnenolone is then converted to androstenedione by P450 17α- hydroxylase (CYP17 or P45017-OH) and 3β-hydroxysteroid dehydrogenase (3β-HSD or HSD3B2). Ruminant theca cells convert limited amounts of androstenedione to testosterone with the enzyme 17β-hydroxysteroid dehydrogenase, and both androstenedione and testosterone can then diffuse to neighbouring granulosa cells for further conversion to estrogens (Gibbons et al., 1997).

1.2.4 Intraovarian regulators

Ovarian folliculogenesis is a dynamic process controlled by gonadotropins. However, intraovarian regulators also play key roles in granulosa cell biology, maturation of the oocyte, and neovascularization. These factors can act in an autocrine or paracrine manner through specific cell membrane receptors. The early stages of follicular growth are gonadotropin-independent, and these stages may therefore be controlled by intraovarian signaling (Ben-Ami et al., 2006).

1.2.4.1 IGF

The IGF family members are polypeptides similar to insulin. The IGF system includes two ligands (IGF-I and -II), 6 IGF binding protein (IGFBPs) and two receptors (IGF1R and -2R).

IGFs are regulated by gonadotropins and growth factors such as growth hormone (GH) and oxytocin in ovarian follicles, corpora lutea, and oocytes. In ruminants and rodents, IGF-I plays a key role in reproduction, whereas in human IGF-II appears more important. Whereas IGF-I probably has no effects on primordial follicles, IGF-I and IGF-II stimulate the growth of secondary follicles *in vivo* and *in vitro*. In antral follicles, IGF-I stimulates granulosa cell proliferation and synergizes with gonadotropins to promote differentiation of follicle cells and inhibit apoptosis (Beg and Ginther, 2006). IGFs increase the expression of FSH and LH receptors and the response of granulosa cells, theca cells, and oocytes to gonadotropins. Moreover, IGFs are considered to be one of the main local mediators of many hormones, including gonadotropins and GH (Quirk et al., 2004).

IGF-I and IGF-II are produced in large amounts in the corpus luteum to support luteal cell development and function (Beg and Ginther, 2006). Both IGF-I and IGF-II in mice promoted nuclear maturation of oocytes, with stimulation of proliferation and inhibition of apoptosis in the surrounding cumulus oophorus, resulting in increased developmental capacity of the oocytes and blastocyst production after *in vitro* fertilization (IVF) (Demeestere et al., 2004).

In addition, it is possible that IGF-I and IGF-II mediate effects of nutrients and nutrition-dependent metabolic hormones on reproductive processes (Sirotkin, 2010, Webb et al., 2004). IGF1 seems to stimulate estradiol and progesterone secretion and can stimulate PI3K (phosphoinositide 3-kinase) and PKC (protein kinase C) to increase the expression of CYP19A1 and other steroidogenic enzymes in bovine granulosa cells, and regulate secretion of progesterone and androstenedione in theca cells (Silva and Price, 2002, Stocco, 2008b).

The IGFBPs consist of a family of six non-glycosylated peptides with relatively low molecular weights, which serve to regulate the bioavailability of IGFs within the follicle. IGFBP2, 4 and 5 serve as IGF carriers in the serum, thereby extending their half-lives by preventing their degradation. IGFBPs also regulate the effects of IGFs by preventing their access to cell membrane surface receptors (Ben-Ami et al., 2006).

1.2.4.2 TGF-β superfamily

The TGF-β superfamily of extracellular signaling molecules includes over 35 structurally related but functionally diverse proteins. These proteins are widely distributed throughout the body and acts as extracellular ligands involved in numerous physiological processes during both prenatal and postnatal life. This big superfamily has been divided

into several subfamilies which include the TGF-β (including TGF-β1, TGF-β2 and TGF-β3), bone morphogenetic protein (BMP, with 20 members), growth and differentiation factor (GDF) (at least nine members), activin/inhibin (including activins A, AB and B and inhibins A and B), and glial cell-derived neurotrophic factor (GDNF) (including GDNF, artemin and neuturin) subfamilies, as well as other members including anti-Mullerian hormone (AMH) and nodal (Knight and Glister, 2006). Several members of this superfamily are expressed by oocytes, granulosa cells and theca cells, and have been implicated in granulosa and theca cell proliferation, atresia, steroidogenesis, oocyte maturation, ovulation and luteinization (Knight and Glister, 2003).

The TGF-β superfamily members GDF-9 and BMP-15 are expressed in the oocyte from an early stage of development, and play key roles in promoting follicle growth to the primary stage. Studies on later stages of follicle development have indicated important roles for activin, BMP-2, 5 and 6, theca cell-derived BMP-2, 4 and 7 and oocyte-derived BMP-6 in promoting granulosa cell proliferation, follicle survival and the prevention of premature luteinization and/or atresia (Figure 6). TGF-β increases LH receptor production in granulosa cells in response to FSH stimulation, whereas it inhibits androgen production in theca cells. In monovular species, dominant follicle selection may depend on the sensitivity of the growing cohort of small antral follicles to FSH. Changes in the intrafollicular concentrations of molecules including activins, AMH, GDF-9 and several BMPs may contribute to this selection process by controlling both FSH- and IGF-dependent signaling pathways in granulosa cells (Knight and Glister, 2003, 2006). Activin may also play a positive role in oocyte maturation and competency, and can increase FSH receptor expression in granulosa cells during the preantral to antral transition. Follicles with greater capacity for activin signaling are more sensitive to FSH, and show better

progression to the antral stage (Findlay et al., 2001). One study suggested that inhibin production by large antral follicles is a mechanism for the selection of the dominant follicle and the induction of apoptosis in small subordinate follicles (Vitale et al., 2002).

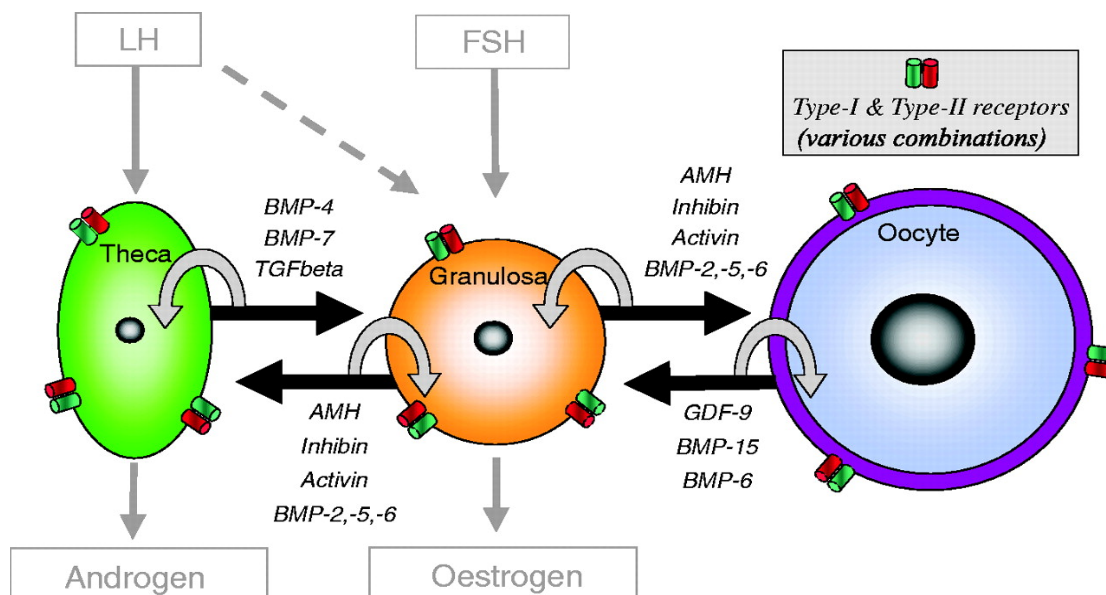

Figure 6. TGF-β superfamily members and their receptors and binding proteins are expressed in different follicle cells.

TGF-β superfamily members including GDF-9, inhibins, activins, TGF-β, BMPs 2, 4, 5, 6 and -7 and AMH are expressed in theca cells, granulosa cells and oocytes. These molecules serve as as intrafollicular autocrine (white arrows) and paracrine (black arrows) signaling molecules. Reproduced from reference (Knight and Glister, 2006).

1.2.4.3 FGFs

The fibroblast growth factor (FGF) superfamily is one of the largest families of growth factors that have been implicated in follicle cell function. There are 22 known

FGFs classed into 7 subfamilies. Most of the members of this family act through single-pass transmembrane tyrosine kinase receptors (FGFR) which are encoded by four separate genes and give rise to four distinct receptor proteins, FGFR1–4 (Schams et al., 2009). Fibroblast growth factors are most famous for their critical roles in organogenesis in the developing embryo (Schams et al., 2009). In the ovary, FGFs are predominantly expressed in oocytes and/or theca cells but are absent from the granulosa layer. FGFs are well known to stimulate granulosa cell proliferation. A consequence of increased proliferation is a decrease in differentiation and estradiol secretion (Buratini et al., 2007). In the reproductive system, FGF2, FGF9, FGF7, and FGF18 play roles in gonadal development and sex differentiation (Portela et al., 2010, Schams et al., 2009). Moreover, FGF7 and FGF10, which belong to the same FGF subfamily, are expressed predominantly in theca cells, and their main receptor (FGFR2) is located on granulosa cells. FGFs are therefore important for paracrine signaling in the ovarian follicle (Portela et al., 2010). FGF2 is one of the members of this family that can regulate angiogenesis in the theca. FGF2 also acts on granulosa cells to promote granulosa cell proliferation, inhibit apoptosis, and decrease steroidogenesis (Jiang et al., 2011). FGF9 is also expressed predominantly in theca cells and stimulates progesterone secretion by granulosa cells. FGF8 cooperates with BMP15 to stimulate glycolysis in cumulus cells (Portela et al., 2010).

FGFs can activate different interacellular pathways including 1) MAPK, widely accepted to be responsible for the mitogenic responses of cells to FGFs, 2) phospholipase C (PLC), which activates PKC and calcium signaling by catalysing the production of diacylglycerol and inositol-1,4,5-triphosphate, and 3) PI3K and AKT (Jiang et al., 2011).

1.2.4.4 EGF

Epidermal growth factor (EGF) is a protein with 53 amino acid residues and three intramolecular disulfide bonds, and plays crucial roles in reproduction. Members of the EGF-like family have highly similar structural and functional characteristics. Aside from EGF, other family members include TGF-α, amphiregulin, epiregulin, betacellulin (BTC), epigen, neuregulins and heparin-binding EGF-like growth factor. These proteins can work through four types of transmembrane receptors (Ashkenazi et al., 2005, Ben-Ami et al., 2006).

Because cumulus and oocyte cells do not have LH receptors, it has been proposed that factors released by mural granulosa cells function in an autocrine and paracrine manner to transduce the effects of LH within the follicle. Intrafollicular release of members of the EGF-like family may fulfil this role (Park et al., 2004). It is now known that the EGF-like growth factors AREG, EREG and BTC, rather than EGF, are rapidly and transiently induced in the somatic cells of the preovulatory follicle by LH (Ashkenazi et al., 2005, Hsieh and Conti, 2005, Park et al., 2004). LH/hCG induction of these EGF-like growth factors has been reported in multiple species (Chen et al., 2008, Fru et al., 2007, Hsieh et al., 2007, Wang et al., 2007).

The up-regulation of EGF ligands was initially proposed to occur selectively in mural granulosa cells, from which the ligands would be shed by members of the matrix metalloprotease (MMP) or a disintegrin and metalloprotease (ADAM) families to enable paracrine signaling to the cumulus layer (Conti et al., 2006). The mature growth factors bind to members of the EGFR (also called ERBB1) family, leading to receptor dimerization and autophosphorylation, and activation of downstream signaling cascades

including the ERK1/2, AKT, MAPK (via Gγ-c-Src) and PKC pathways to elicit distinct biological effects (Conti et al., 2012).

1.3 Folliculogenesis and the ovarian cycle

The major role of the ovary is the production of mature oocytes for fertilization. Folliculogenesis, the growth and development of ovarian follicles from primordial to preovulatory, is a prolonged process dependent on interactions between the oocyte and the somatic cells including the granulosa and theca cells. The ovarian reserve is determined by the number of primordial follicles in the ovary. Primordial follicles are activated for growth and pass through stages of development until they reach the antral stage. A group of antral follicles is then recruited for further growth, which is followed by the selection of a dominant follicle in monovular species. These processes are under the control of endocrine as well as paracrine factors in the ovary (Fortune et al., 2004, McGee and Hsueh, 2000). In response to preovulatory LH surges during each reproductive cycle, the dominant follicle ovulates and releases the mature oocyte for fertilization, while the remaining theca and granulosa cells will be transformed into corpus luteum through luteinization (Figure 7) (McGee and Hsueh, 2000).

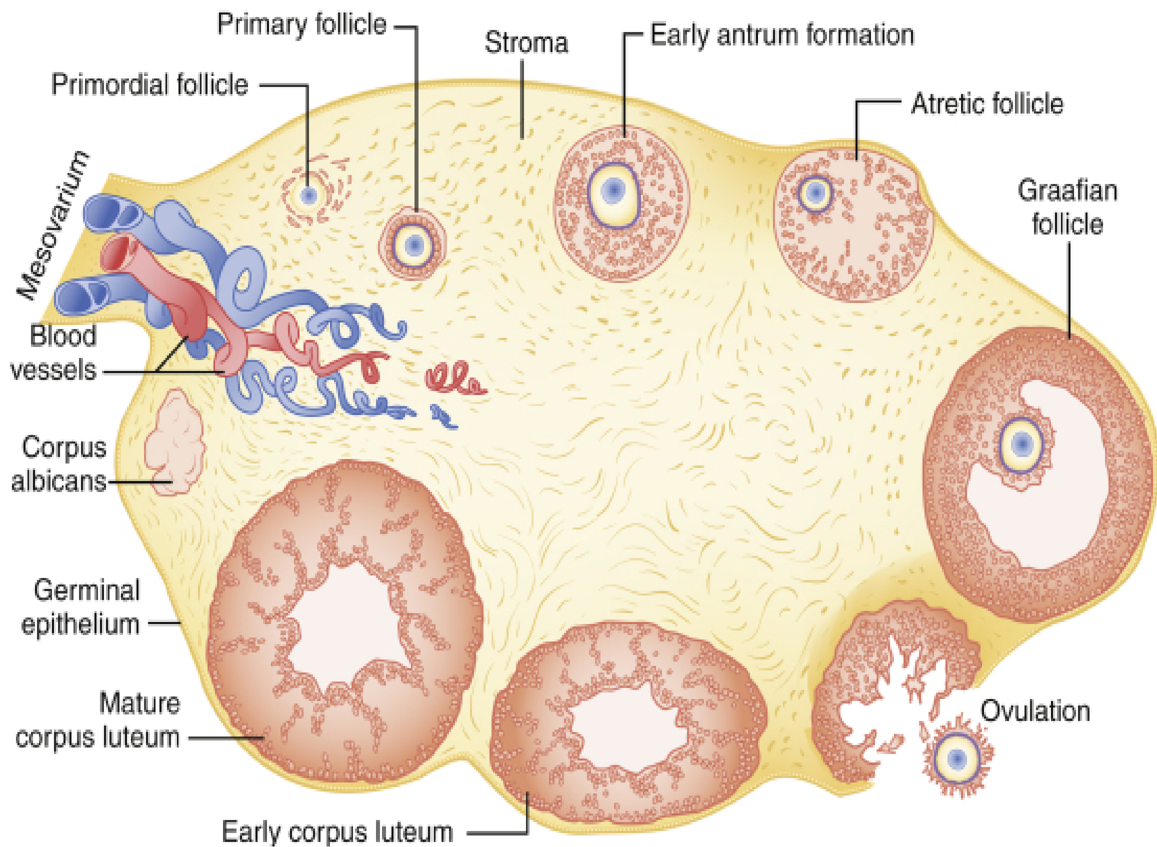

Figure 7. Schematic diagram of the ovary

The ovary is a dynamic organ that undergoes dramatic changes in structure and function. The follicle is the major endocrine and reproductive compartment of the ovary, and undergoes growth, rupture and formation of the corpus luteum during its life span. Taken from (McDonald and Pineda, 1989).

1.3.1 Primordial germ cells

The mammalian germ cell lineage is established early during development. Primordial germ cells (PGCs) originate in the proximal region of the epiblast, close to the extra embryonic endoderm, which during gastrulation clusters through the posterior part of the primitive line to become PGCs. These cells move into the endoderm of the yolk sac,

then proliferate and migrate via the yolk sac and hindgut endoderm at the caudal end of the embryo, and finally via the dorsal mesentery of hindgut to the genital ridges at the ventral sides of the mesonephros (Barnett et al., 2006). Active oogonia can recruit somatic pregranulosa cells from the surface epithelium at this stage. The oogonia then attach to pregranulosa cells and enter meiosis, during which at least 80% of the germ cells, but not their pregranulosa cells, undergo apoptosis (Scaramuzzi et al., 2011).

Primordial germ cells differentiate under the influence of signals from members of the transforming growth factor β superfamily, including BMP2, BMP4, and BMP8B. BMP2 originates from the endoderm, whereas BMP4 and -8b are from the extra-embryonic ectoderm. Loss of any of these signals prevents the formation of most or all of the primordial germ cells. (Barnett et al., 2006, Ying and Zhao, 2001). Conditional knockout mice have helped to identify genes required for primordial germ cell proliferation and migration. The c-kit receptor tyrosine kinase and its ligand (kit ligand, or stem cell factor) are required for primordial germ cell survival and migration. Expression of integrin β1 on the primordial germ cell surface is also required for successful migration to the genital ridge (Barnett et al., 2006).

1.3.2 Transition from oogonia to oocyte

PGCs are called oogonia once they reach to the gonads. Oogonia are connected to each other via intercellular cytoplasmic bridges. These oogonia are surrounded by mesonephros somatic cells, forming germ cell clusters or nests. These germ cell clusters break down before or after birth (according to species) to allow the formation of primordial follicles (Figure 8). Improper germ cell cluster breakdown may cause polyovular follicles (follicles with more than one oocyte).

Oogonia have higher mitotic activity compare to PGCs and undergo several divisions before starting meiosis. This mitotic activity is the major determinant of the size of the oocyte pool. On the other hand, a huge number of oogonia undergo atresia at this stage (approximately 60% in sows, 80% in rodents, 90% in humans and even more in sheep and cows (van den Hurk and Zhao, 2005). The end of reproductive life occurs when the follicle reserve is expended through follicle development and atresia. (Oktem and Urman, 2010).

Several genes are known to play roles in primordial follicle formation, including *Dazla* (deleted in azoospermia), which is essential for the differentiation of germ cells, and *Figla* (factor in the germline alpha), which is essential for follicle formation and the interaction between the oocyte and granulosa cells. Nerve growth factor (*Ngf*) is involved in primordial follicle assembly (Barnett et al., 2006).

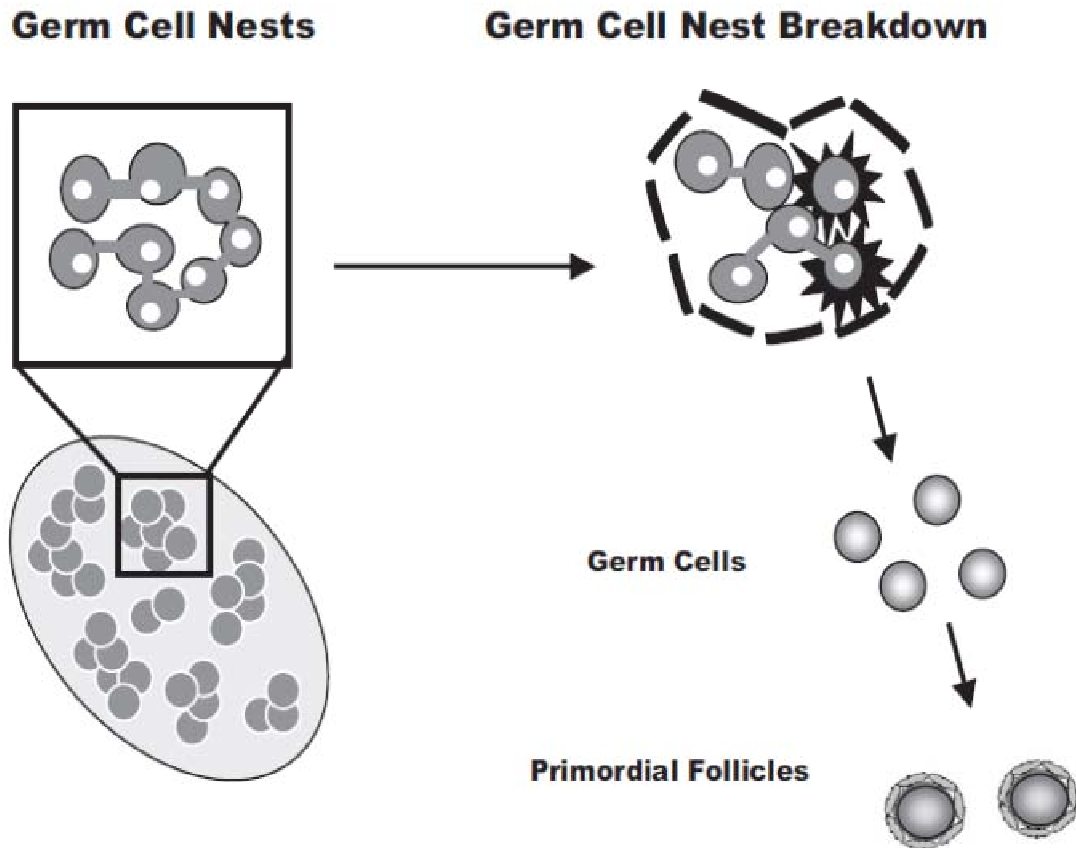

Figure 8. Germ cell cluster breakdown and formation of primordial follicles. Germ cells are present in the ovary during embryogenesis. Around birth, germ cell clusters start to break down and form primordial follicles. Several mechanisms including apoptosis are involved in this process. Taken from (Barnett et al., 2006).

1.3.3 Formation of primordial follicles

In term of morphology, primordial follicles in most species consist of small diplotene oocytes with a zona pellucida layer, surrounded by a flat layer of granulosa cells (Figure 9) (Rodgers and Irving-Rodgers, 2010). In primordial follicles, the granulosa cells and oocyte have receptors for some growth factors but not for gonadotropins. However, at this stage they do not require FSH and LH for their survival and development (Fortune et al., 2011). Around the initiation of follicle formation, estrogen levels are decreased

relative to the period of germ cells clusters, and low amounts of androgens and progestins are detectable. Steroid production could be important for primordial follicle formation, and seems to be independent of gonadotropin secretion (van den Hurk and Zhao, 2005).

A large number of factors including transcription factors, zona proteins, meiosis-specific enzymes and nerve growth factors are important for primordial follicle assembly (Oktem and Urman, 2010). Studies have identified a variety of genes involved in the formation of primordial follicles, which include sporulation protein homology (*Spo11*), disrupted meiotic cDNA 1 homologue (*Dmc1*) and muts homologue 5 (*Msh5*). All three genes are necessary for the initiation of double strand DNA breaks during meiosis and are involved in recombination (Barnett et al., 2006).

1.3.4 Transition of primordial follicles to primary follicles

Primary follicles are similar to primordial follicles in terms of size, but have more cubical-shaped granulosa cells. Primordial follicles first develop into primary follicles prior to or after birth (according to species), and this process continues postnatally until the ovarian reserve is depleted, which takes more than a year in rodents and several decades in women (Oktem and Oktay, 2008).

The transition of a primordial follicle into a primary follicle is more of a slow maturation than a growing process, as the diameter of its oocyte hardly changes (Rodgers and Irving-Rodgers, 2010). Communication among the oocytes, granulosa and certain extra cellular matrix components and autocrine/paracrine growth factors play important roles in this transition and subsequent growth of follicles (Oktem and Urman, 2010).

Inhibitory and stimulatory factors from different cells (oocytes, somatic cells, and stroma) control primary follicle development. Recently, it has been found that inhibitory

signals keep primordial follicles in the dormant state. For example, loss of function of some inhibitory molecules for follicular activation, including the tumor suppressor tuberous sclerosis complex 1 (*Tsc-1*), phosphatase and tensin homolog deleted on chromosome 10 (*Pten*), Forkhead box O3 (*Foxo3a*), newborn ovary homeobox protein (*Nobox*) and forkhead box protein L2 (*Foxl2)*, leads to premature activation of the primordial follicle pool (van den Hurk and Zhao, 2005).

According to studies on transgenic animal models and on the human ovary, several members of the TGF-β super family, such as AMH, activins, BMP-4, BMP-7, and GDF-9, play critical roles in the regulation of primary follicle activation. There are other growth factors and cytokines that act at a paracrine level in the formation of primary follicles, such as kit-ligand, fibroblast growth factor-7 and the leukemia inhibitory factor, which have been shown *in vitro* to promote follicle growth from the primordial to the primary stage, stimulate oocyte growth and the proliferation of theca cells (Barnett et al., 2006, Oktem and Oktay, 2008).

1.3.5 Follicle growth to pre-antral and antral stages

A follicle with two or more layers of granulosa cells is deemed a secondary or preantral follicle. At this stage, the oocyte enters its growth phase, its diameter increases, granulosa cells become more proliferative, and a theca layer develops around the granulosa cells. Moreover, at this stage the zona pellucida completely forms, providing a protective coat around the oocyte composed of three glycoproteins; ZP1, ZP2 and ZP3 (figure 9) (Eppig, 2001).

During oocyte growth, RNA and protein synthesis increases, and ribosomes, mitochondria and other organelles increase in number. Granulosa cells form gap junctions

with the oocyte to permit them to communicate, transfer nutrients, metabolic precursors (such as amino acids and nucleotides), informational molecules (neurotropins and growth factors) and inhibitory and stimulatory meiotic signals (van den Hurk and Zhao, 2005). At this stage the follicle begins to form a small antral cavity which fills with follicular fluid within the granulosa cell layers along with increased vascularization of the theca layer, continued growth of oocytes and proliferation of granulosa and theca cells (Oktem and Urman, 2010).

Development of a multi-layered secondary follicle from a primary follicle with a single layer of granulosa cells is a slow process that does not appear to be stimulated by the gonadotropins. However, the number of developing secondary follicles, their size, and rate of atresia are influenced by gonadotropins. FSH is the predominant survival factor, and acts to stimulate gap junction formation in granulosa cells. In addition, LH stimulates the synthesis of androgen by theca cells. Although preantral follicles are able to respond to gonadotropin hormones, they can develop to the antral stage with minimal circulating FSH and without FSH receptors (van den Hurk and Zhao, 2005).

Some locally produced members of the TGF-β super family including activins, BMP-4, BMP-7, TGF-β and BMP-15, play crucial roles in the growth of primary follicles into preantral follicles (Oktem and Urman, 2010). Other growth factors such as EGF, IGFs, FGF2 and FGF7 have been associated with the survival and development of secondary follicles in various rodent and domestic species. These growth factors suppress follicle cell apoptosis, while stimulating granulosa cell proliferation and differentiation under *in vitro* conditions. Also, AMH appears to have a negative effect on preantral follicle development beyond the primordial to primary transition (Oktem and Urman, 2010).

Further progression of the developing preantral follicle to the antral stage is characterized by formation of a fluid-filled cavity (Figure 9). Antral fluid is a source of essential substances derived from blood and secretions of the follicular cells, notably including gonadotropins, steroids, growth factors, enzymes, proteoglycans, and lipoproteins. The signals for antrum formation are not well characterized. *In vitro* studies with rodent follicles showed there is a possible role for FSH, LH, activin, KL (kit-ligand) and EGF in antrum formation. FSH becomes a critical determinant of further follicle growth and survival at this stage. Members of the TGF-β family, such as oocyte-derived GDF9, BMP15, BMP6 and granulosa cell-derived activin not only promote granulosa cell proliferation, but also modulate FSH-dependent follicle function in this stage (Oktem and Oktay, 2008).

Follicular development begins with the growth of a recruited cohort of small preantral follicles. From this pool, only a few follicles are selected to grow, while the remaining will undergo atresia. Follicular development from recruitment to maturity can occur during two or three follicular waves throughout one estrous cycle in the bovine species. These waves also occur before puberty and during pregnancy and resulting in depletion of primordial follicles.

1.3.6 Selection process of the dominant follicle

The selection process during a follicular wave is characterized by the continued growth of the developing dominant follicle and reduced growth of the subordinate follicles. The antral follicle selection process occurs as FSH levels decrease (with high pulsatile secretion of LH). Follicle selection in monovular species such as the human and the cow is the process whereby only one follicle is chosen from a wave of growing

follicles to continue to grow and ovulate. However, in polyovular species such as pigs and rats, multiple follicles are selected and grow synchronously until ovulation. During this phase, the follicles grow at a similar rate and each follicle has the capacity to becoming a dominant follicle (Fortune, 1994). An extensive network of endocrine, autocrine, and paracrine interactions between germ cells and somatic cells is required for a large antral follicle to reach preovulatory status. Gonadotropins and local factors produced by the antral follicle itself and by neighbouring small preantral follicles participate in the selection process (Oktem and Oktay, 2008).

There are different theories about the mechanisms of follicular dominance. One theory is that in both rodents and cattle, the selected antral follicle shows increased FSHR expression and expresses LHR in granulosa cells, and therefore becomes more sensitive to FSH and LH-responsive. This allows the continued development of a selected antral follicle during a period when FSH levels drop in response to high estrogen and inhibin output by the selected follicle. A second theory suggests follicle dominance is established early by an increase in IGF1 within the chosen follicle. A third theory postulates that IGF1 and FSH work synergistically to make the selected follicle grow faster and produce increased amounts of estradiol, consequently slowing down the growth of the other follicles (Lucy, 2007a). However, the exact mechanism of large follicle selection remains unclear.

TGF family members play important roles in follicle selection. Activin-A and TGF-β increase FSH receptor expression and upregulate FSH-induced aromatase activity and LH receptor expression, while downregulating theca androgen production and preventing premature luteinization or atresia. BMP15 and GDF9 also modulate FSH

action and inhibit FSH stimulated progesterone production. In addition, GDF9 induces CYP19A1 activity and FSH induced LHR expression. (Oktem and Urman, 2010).

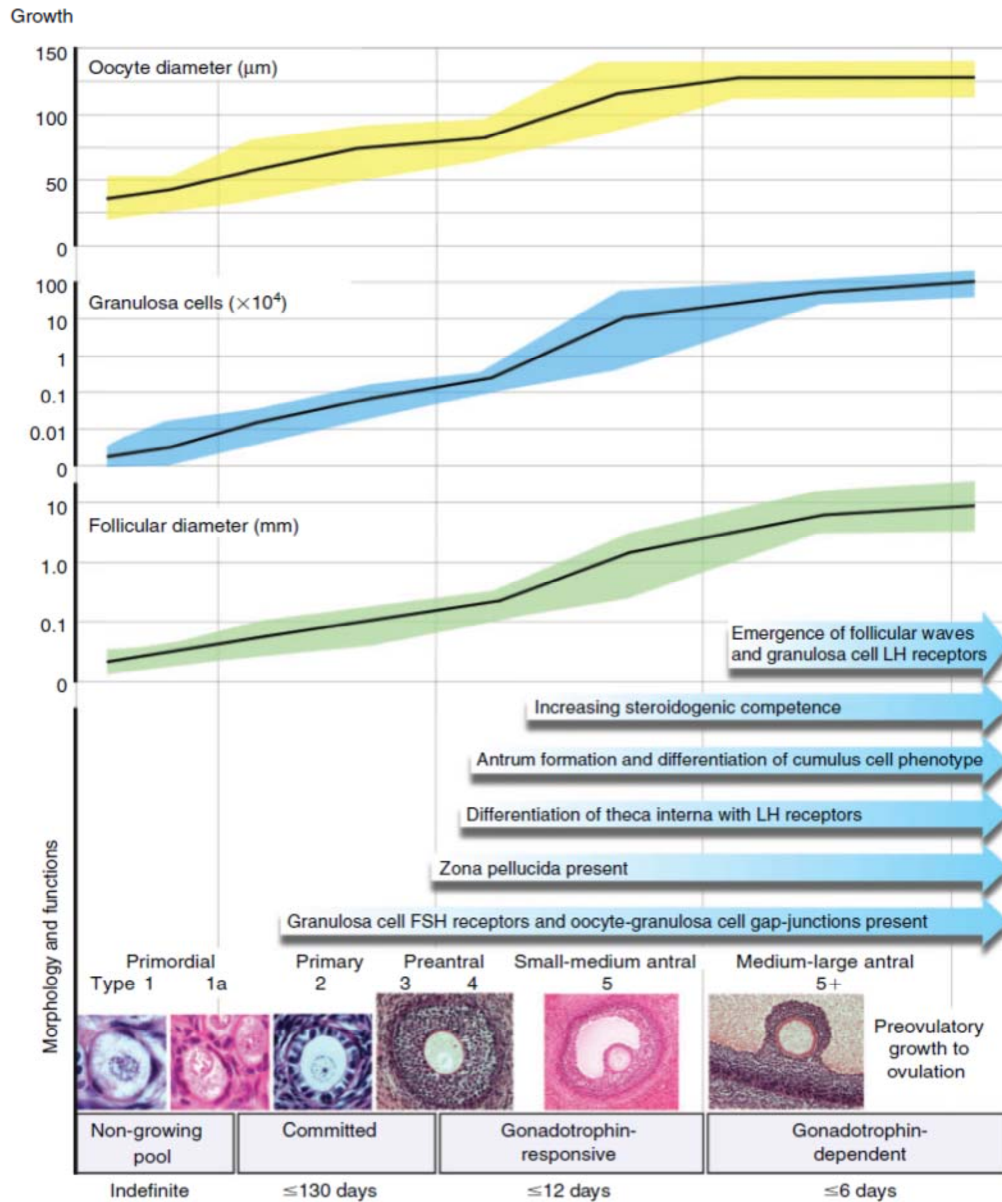

Figure 9. A summary of folliculogenesis in the ewe as an example for mono-ovular species.

The vertical axis of this diagram shows the growth of the follicle and oocyte and the number of granulosa cells, from primordial to ovulatory stages. The horizontal axis shows the morphological characteristics of follicles during growth and development and the presence of different layers and receptors. Taken from (Scaramuzzi et al., 2011).

1.3.7 Atresia

The fetal mammalian ovary contains millions of germ cells, which decrease in number by birth and decrease further postnatally with the onset of follicular development. Only limited numbers of primordial follicles are selected for ovulation while the rest of follicles undergo atresia (75–99.9%). It was found that atresia occurs in the ovary of all species (Wood and Van Der Kraak, 2001, Yu et al., 2004). Follicular atresia occurs by apoptosis, or programmed cell death, in both the somatic cells of the follicle and the oocyte. Apoptosis is an essential part of life for multicellular organisms, at a physiological level to eliminate unwanted cells in response to developmental signals or toxic stimulation, and it is tightly regulated and balanced against mitosis (Hengartner, 2000).

Characteristic features of apoptosis include condensation of nuclear chromatin, increased complexity of the nuclear and cellular outlines, fragmentation and budding of the cell, the production of the membrane-bound apoptotic bodies and their phagocytosis by macrophages, cytoplasmic vacuolization, cell contraction, and rapid nuclear DNA cleavage (Hussein, 2005).

Two general mechanisms activate apoptosis, one involves the binding of death molecules to cell surface receptors (death receptor pathway), and the other involves signals arising within the cell (mitochondrial pathway). Two important families of apoptosis regulators are the caspase family and the B-cell lymphoma 2 (BCL-2) family (Hengartner, 2000). Caspases include both 'initiator' caspases (8 and 9) and 'effector' caspases (3, 6 and 7). These proteases cleave enzymes and proteins essential to the cell's viability. Caspases participate in the formation of the apoptosome holoenzyme, which is a multi-protein complex that notably includes cytochrome c, APAF-1 and pro-caspase 9. Within this complex, pro-caspase 9 undergoes autoactivation to promote cleavage of

caspase-3. Caspase-3 then cleaves its target substrates to effect the changes associated with apoptosis (Hussein, 2005).

The BCL-2 family includes anti-apoptotic members, such as BCL-2 and BCL-XL, as well as proapoptotic members, including BAX, BID, BIK, BOD, BOK and BCL-XS. BCL-2 and BAX are expressed in granulosa cells of both fetal and adult ovaries, suggesting their possible role in atresia. The key function of BCL-2 family members is to regulate the release of pro apoptotic factors such as cytochrome c from the mitochondria into the cytosol. Expression of this gene family is related to gonadotropin levels, as higher levels of gonadotropins increase the expression of BCL-2 and decrease the expression of BAX (Hussein, 2005).

Successful follicle development depends on the presence of gonadotropins and survival factors that promote follicle growth and protect cells from apoptosis (Quirk et al., 2004). FSH and LH are important factors for the proliferation and survival of follicular somatic cells and the cyclic recruitment of antral follicles. Decreased levels of *Fshr* and *Lhr* mRNAs results in reduced granulosa cell response to gonadotropins, and consequently increased apoptosis in follicles. Elimination of serum gonadotropins after hypophysectomy leads to atresia and apoptosis of developing follicles (Tilly et al., 1992). IGF1 stimulates follicle survival, promotes proliferation, and inhibits apoptosis by stimulating the synthesis of estrogen, which is an apoptosis inhibitor (Billig et al., 1993). Paracrine factors produced by theca cells (including transforming growth factor α, keratinocyte growth factor and hepatocyte growth factor) bind to specific receptors expressed in granulosa cells and suppress apoptosis by endocrine factors. In the absence of survival factors, endogenous apoptosis pathways within the follicle become activated and lead to follicular atresia (Tilly, 1996).

1.3.8 Ovulation

The dominant follicle shifts its gonadotropin dependence from FSH to LH during selection and is able to continue to grow while the subordinates regress. LH can activate a complicated signalling pathway to promote ovulation. LH receptors are present in theca and granulosa cells, but few or no receptors are found in cumulus cells or the oocyte (Russell and Robker, 2007).

In preovulatory follicles, the LH surge stimulates pathways including the cAMP-dependent serine kinase protein kinase A, extracellular regulated kinase (ERK1/2 or MAPK3/1), phosphoinositide 3-kinase/AKT (PI3K/AKT) and mitogen-activated protein kinase 14 (MAPK14 or P38) pathways (Conti et al., 2006, Panigone et al., 2008). LH rapidly induces the expression of ADAMs family members, which cleave and shed preformed EGF-like growth factors from the surface of mural granulosa cells. EGF-like growth factors (EREG, AREG and BTC) bind to EGF receptors on granulosa cells and induce the expression of genes related to cumulus expansion such as *Has2*, *Ptgs2* and *Tnfaip6* (Panigone et al., 2008, Park et al., 2004). EGF receptor signaling is mediated mostly by activation of the ERK1/2 pathway in granulosa cells as well as in cumulus cells. Prostaglandin (PGE) and the EGF-like factors produced by granulosa cells activate specific PGE and EGF receptors in cumulus cells and increase the expression of genes involved in making and stabilizing the hyaluronan matrix, as well as other genes related to immune responses including *Cd34*, *Cd52*, *Alcam* and *Il6* (Shimada et al., 2006a, Shimada et al., 2006b).

1.3.9 Luteinization

Luteinization is one of the main physiological events induced by the LH surge. It is the process whereby granulosa and theca cells stop proliferating and undergo terminal differentiation, leading to the formation and function of the corpus luteum (CL) (Stocco et al., 2007b). Cells undergoing luteinization begin expressing a new set of genes that allowing luteal cells to survive in a different hormonal environment. Biochemical changes in granulosa cells associated with luteinization include a significant increase in CYP11A1 expression, the transient expression of progesterone receptors, as well as dramatic decreases in mRNAs encoding *Cyp19a1* and *Cyp17a1*. These changes cause a transition from estrogen production in the preovulatory follicle to progesterone synthesis in the corpus luteum (Figure 10) (Richards et al., 1998). In the event of pregnancy, the corpus luteum becomes a metabolically highly active structure with a high progesterone production rate. Depends on the species if the oocyte is not fertilized, the CL regresses, allowing a new cycle to begin. Implantation, mating, or even cervical stimulation in some mammals initiates a complex mechanism resulting in the maintenance of CL function, ensuring a continuous supply of progesterone needed for fetal survival (Stocco et al., 2007a). For example in a non-pregnant cow if there is no maternal recognition of pregnancy, PGF2α released from the endometrium which induces luteolysis, characterized by hypoxic cell death resulting from hyalinization of blood vessels (Ginther and Beg, 2012).

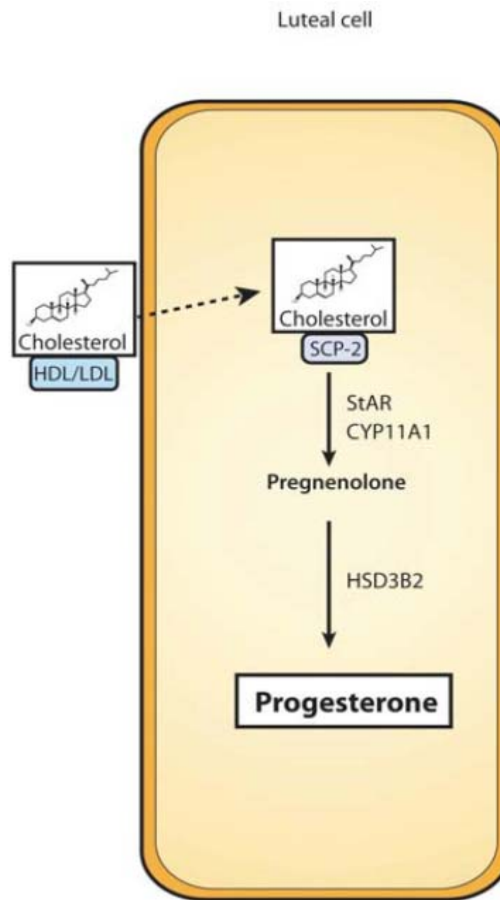

Figure 10. Steroidogenesis in the corpus luteum.

Luteal cells have the ability to transform cholesterol into pregnenolone and then progesterone. Taken from (Lapointe and Boerboom, 2011).

2. WNTs and WNT signaling

2.1 WNT overview

The WNT family is one of several important families of signaling molecules that have been identified over the past 20 to 30 years. The WNT genes encode a large family of secreted lipid-modified glycoproteins that have been identified in animals from hydra to humans. The WNT family plays crucial roles in the embryonic development of all animal species, and has also been implicated in adult tissue homeostasis. During development, WNTs have different roles in controlling cell fate, proliferation, migration, polarity, and apoptosis and are also implicated in a variety of human cancers (Cadigan and Liu, 2006, Logan and Nusse, 2004).

The first WNT gene was identified in 1982 by Nusse and Varmus as an integration site for the Mouse Mammary Tumor Virus (MMTV). MMTV integration at this site activated a gene, originally named integration 1 (*Int1*), which encodes a secreted cysteine rich protein. In drosophila, the wingless (*Wg*) gene was identified as a recessive mutation affecting wing development. In 1987, researchers found that *Wg* was the homologue to mammalian *Int1*, due to a common evolutionary origin evidenced by similar amino acid sequences of their encoded proteins. The name WNT comes from a combination of *Wg* in drosophila and *Int1* in mouse (Clevers, 2006, Wodarz and Nusse, 1998).

2.2 WNT genes and proteins

In humans and mice, 19 *Wnt* genes have been described. Human WNT genes are located very close to each other in the genome. Each WNT protein is characterized by an amino terminal signal for secretion, several sites for glycosylation, and a cysteine-rich segment (Miller, 2002).

WNT genes encode secreted glycoproteins of 350-400 amino acids (molecular weight from 39 kDa (WNT7a) to 46 kDa (WNT10a)). The majority of WNT proteins have around 35% similarity in amino acid sequence including a conserved pattern of 23-24 cysteine residues, in addition to other conserved amino acids. This sequence identity increases (from 58% to 83%) among members of subgroups, such as WNT3 and WNT3a, which have overlapping sites of expression (Cadigan and Nusse, 1997, Miller, 2002).

All WNT proteins are relatively hydrophobic molecules because of several post translational modifications including glycosylation, palmitoylation and palmitoleoylation that are essential for WNT secretion and function. For example, glycosylation is crucial for WNT protein folding, secretion and biological activities. These proteins have a conserved structure, which depends on the glycosylation and formation of several intramolecular disulfide bonds (Ching et al., 2008, Logan and Nusse, 2004, Miller, 2002).

2.3 WNT receptors

The frizzled (FZD) family of seven transmembrane-pass receptors (7TMR) are structurally similar to GPCR and are found exclusively at the plasma membrane of WNT responsive cells (Huang and Klein, 2004). In humans and mice, ten members of this family have been identified. Sequencing analysis suggests that the ten human frizzled genes can be divided into four groups: FZD1, FZD2 and FZD7 share approximately 75% amino acid identity, FZD5 and FZD8 share 70% identity, FZD4, FZD9 and FZD10 share 65% identity, and FZD3 and FZD6 share 50% identity (Huang and Klein, 2004). The length of frizzled receptors varies from 500 - 700 amino acids. These receptors have three extracellular and three intracellular loops. Molecular characterization of FZDs showed the amino terminus is extracellular and contains a cysteine-rich domain (CRD) followed by a

hydrophilic region of 40-100 amino acids. The presence of the hydrophobic domains in the C terminal part of FZD receptors suggests that these receptors are related to the GPCR superfamily (Wodarz and Nusse, 1998).

In addition to FZD proteins, the canonical WNT/β-catenin (CTNNB1) signaling pathway requires single-span transmembrane proteins that belong to a subfamily of low-density lipoprotein (LDL) receptor-related proteins (LRPs). Mutations in *Lrp6* in mice results in developmental defects similar to those seen in mice deficient for several individual WNT genes (Pinson et al., 2000). LRPs have relatively large extracellular and short cytosolic domains. Frizzled receptors activate β-catenin-dependent (canonical) as well as β-catenin-independent (non-canonical) pathways, whereas LRP5/6 function more specifically in the WNT/β-catenin pathway (He et al., 2004). Recent studies have identified other types of receptor molecules which can mediate non-canonical WNT signaling. One of them is the flamingo family of nonclassical cadherin molecules. Flamingos bind to frizzleds to form a protein complex that mediates non-canonical WNT signaling (Sugimura and Li, 2010). Moreover, receptor tyrosine kinase-like orphan receptor 1 and 2 (ROR1, 2), and a typical tyrosine kinase receptor (RYK), have been shown to act as WNT receptors (Kikuchi et al., 2009).

2.4 WNT inhibitors

WNT signaling is modulated by endogenous secreted substances, including two classes of antagonists: the members of the Dickkopf (DKK) and secreted FZD related protein (sFRP) families. Dickkopf proteins inhibit the canonical WNT signaling by binding to the LRP5/LRP6 component of the WNT receptor complex. Members of the sFRP family bind directly to WNTs or to the WNT receptor complex, therefore sFRPs can

inhibit both canonical and non-canonical pathways (Hsieh et al., 2002). In humans, the sFRP family consists of five members (sFRP1 to sFRP5) and orthologues of these genes have been found in all vertebrate species. WNT4 is one of the WNT family members that is regulated by both sFRP1 and 2 (Kawano and Kypta, 2003).

The DKK family includes four members (DKK1, -2, -3 and -4) and a unique DKK3-related protein named Soggy (SGY). All these secreted proteins except SGY have two CRDs at their N terminus. DKK1, -2 and -4 have been reported to inhibit the WNT/CTNNB1 pathway whereas DKK3 does not show an affinity for LRP6 or an effect on WNT signaling (Figure 11B) (De, 2011, Kawano and Kypta, 2003).

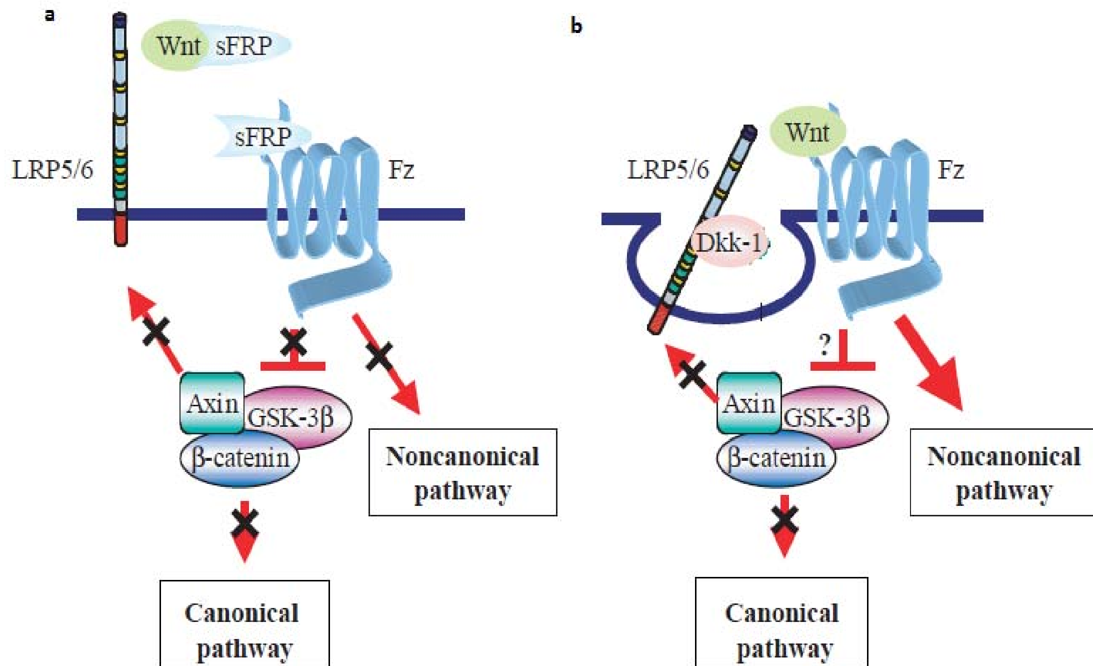

Figure 11. Regulation of WNT signalling by physiological inhibitors.

a) sFRPs prevent WNT from binding to its receptors or inhibit WNT signaling by binding to Frizzled. In this case, both the canonical and the noncanonical pathways are inactivated. b) DKK-1 interacts with LRP5/6, thereby preventing formation of the LRP5/6/WNT/Frizzled complex and consequently β-catenin stabilization. Axin brings together the proteins that promote β-catenin phosphorylation, enabling β-catenin degradation and inhibition of the canonical pathway. The WNT/FZD complex can still activate non-canonical WNT signaling in presence of DKK. Reproduced from (Kawano and Kypta, 2003).

2.5 Intracellular signal transduction

WNTs interact with the CRD of FZDs, or with accessory proteins or coreceptors, to activate specific downstream signaling events and exert physiological effects. It is still not well known which WNT(s) bind to which FZD(s) (or co-receptor) to exert their

function. It is now believed that certain WNT proteins such as WNT5a and WNT11 have a tendency to activate non-canonical signaling, whereas other WNT proteins including WNT1 and WNT8 are known to preferentially activate canonical signaling (Cadigan and Liu, 2006).

2.5.1 The canonical pathway

Canonical WNT signaling controls the stability of the multifunctional protein β-catenin. In the absence of WNT, a large multiprotein complex called the destruction complex is responsible of phosphorylation of β-catenin. This complex contains Axin, adenomatosis polyposis coli (APC) and glycogen synthase kinase 3β (GSK3β). β-catenin is phosphorylated by CK1 (casein kinase 1) at Ser45, followed by phosphorylation at Ser33, Ser37 and Thr41 by GSK3β. Phosphorylation results in the subsequent ubiquitination of the protein and its degradation by the cellular proteosomal machinery. In the resting state, the cytoplasmic β-catenin levels are thereby kept low (Rao and Kuhl, 2010).

The WNT/CTNNB1 pathway is activated by the binding of a WNT to a Frizzled family receptor and LDL receptor-related protein as a co-receptor (Cadigan and Liu, 2006). In the presence of WNT ligands, a signal is tranduced via disheveled that disrupts the destruction complex. This event allows β-catenin to escape the complex in an unphosphorylated form and to translocate to the nucleus. In the nucleus, CTNNB1 associates with various transcription factors that can modulate the transcriptional activity of different target genes (Figure 12A) (Boyer et al., 2010a).

2.5.2 The non-canonical pathways

Some WNTs activate β-catenin independent pathways, also known as non-canonical WNT pathways. The best-characterized non-canonical pathways are the WNT/Ca^{2+} and planar cell polarity (PCP) pathways (Kikuchi et al., 2009).

Several reports have shown that WNT5a, WNT11 and WNT4 can stimulate the WNT/Ca^{2+} pathway. WNT-FZD signaling activation leads to release of intracellular Ca^{2+} through G proteins to activate phospholipase C, which in turn generates diacylglycerol (DAG) and inositol 1,4,5 triphosphate (IP3) and increases intracellular Ca^{2+} concentrations that can stimulate several Ca^{2+} sensitive proteins including calcium/calmodulin-dependent protein kinase II (CaMKII) and PKC. CaMKII has been shown to activate the transcription factor nuclear factor of activated T-cells (NFAT) via activation of calcineurin (CNA), as well as TGFβ activated kinase (TAK1) and Nemo-like kinase (NLK) which can antagonize CTNNB1/TCF signaling (De, 2011). It has been shown that activation of the WNT/Ca^{2+} pathway can inhibit the activation of the Wnt/β-catenin pathway in Xenopus (Miller et al., 1999).

The WNT/JNK pathway has a high level of overlap with the planar cell polarity pathway originally described in Drosophila (Rao and Kuhl, 2010). PCP signaling involves the activation of several small Rho GTPases, which have different intracellular targets (Miller, 2002). Following the binding of WNT to FZDs, DVL can active the small GTPase Rho via DAAM1 (DVL associated activator morphogenesis 1) (Kishida et al., 2004). Another small GTPase of the Rho family that can be activated upon WNT/FZD binding and activation of DVL is RAC1. Activation of RAC1 stimulates the downstream effector c-Jun N-terminal kinase (JNK). JNK activation subsequently leads to the activation of transcription factors such as c-Jun (Figure 12) (Kikuchi et al., 2009). Studies

have shown that DAAM1 (dishevelled associated activator of morphogenesis 1), ROCK (rho-associated, coiled-coil-containing protein kinase 1) and JNK can act via different effectors mainly to modify the actin cytoskeleton, leading to cytoskeletal rearrangement and changes in cell morphology (Keller et al., 2003).

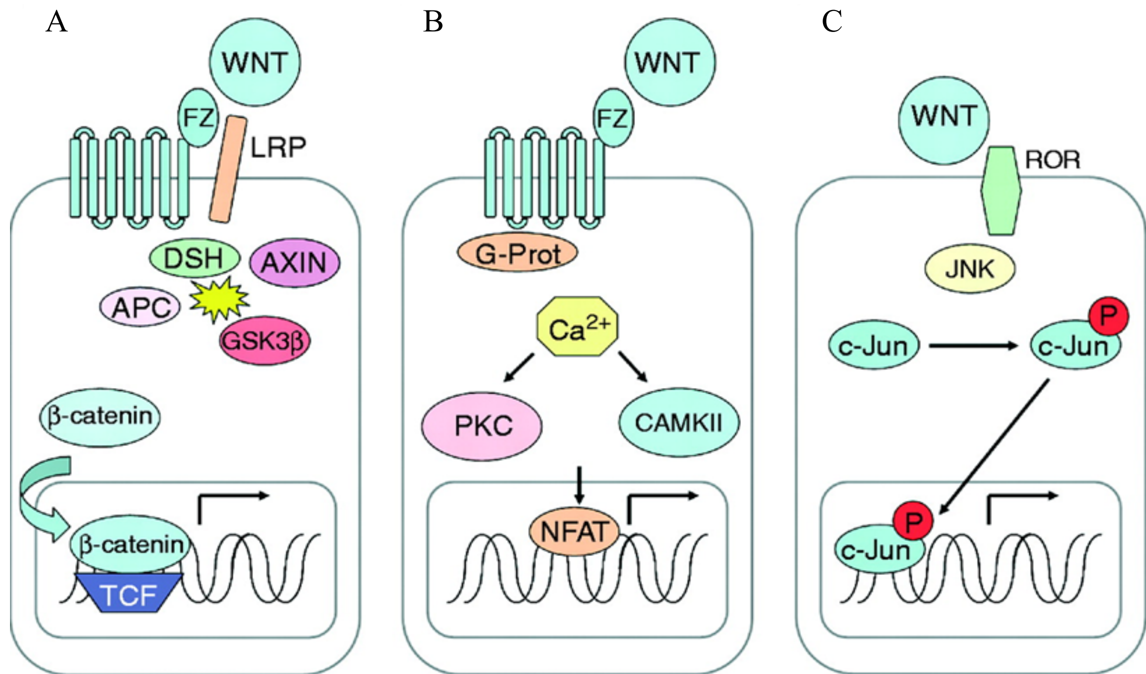

Figure 12. Multiple WNT signaling pathways.

In the canonical pathway, when WNT acts on its cell-surface receptor consisting of FZD and LRP5/6, β-catenin escapes from the degradation complex of APC protein, AXIN and GSK3β, resulting in the accumulation of cytosolic β-catenin and its translocation to the nucleus, where it binds to TCF/LEF transcription factors and thereby stimulates the transcription of various genes. In the β-catenin-independent pathways, specific WNT and FZD proteins can act to increase intracellular Ca^{2+} concentrations, which in turn activates CAMKII and PKC, thereby activating transcription via factors such NFAT and up-regulating target gene expression. Some FZDs stimulate the PCP pathway, by which small GTPases (Rac and Rho) and JNK are activated. JNK can phosphorylates c-JUN, which acts in conjunction with c-FOS to activate the transcription of target genes. Taken from (Laudes, 2011).

2.6 Role of WNT signaling in the ovary

Ovarian follicular growth and the regulation of steroidogenesis are controlled by intraovarian growth factors. Members of the WNT family of signaling molecules have been shown to regulate ovarian cell function and follicular organization. WNTs attracted the attention of ovarian biologist when it was reported that inactivation of *Wnt4* in mice results in partial female to male sex reversal and the loss of the majority of the oocyte reserve in the days prior to birth (Boyer et al., 2010a). Several WNTs and other components of WNT signaling pathways are expressed in the postnatal ovary and recently, studies using newer methods including transgenesis have helped to determine roles of some members of WNT pathway and its components (such as CTNNB1, WNT4 and FZD1, FZD4 and SFRP4) in folliculogenesis, steroidogenesis and female fertility (Boyer et al., 2010a).

2.6.1 CTNNB1

CTNNB1 has an essential role in cell adhesion and is a key signaling molecule in the canonical WNT pathway, which plays diverse and critical roles in embryonic development and in adulthood (Boyer et al., 2010a). CTNNB1, a transcriptional co-factor interacts with T-cell factor/lymphoid enhancer binding protein (TCF/LEF) to activate the transcription of target genes. In the absence of CTNNB1 signaling, TCF acts as a repressor for WNT target genes. When high levels of CTNNB1 are present in the nucleus, it converts TCF from a transcriptional repressor to an activator (Eastman and Grosschedl, 1999). Recently, two important roles have been suggested for CTNNB1 in steroidogenesis and follicular growth, as detailed below.

2.6.1.1 Steroidogenesis

As mentioned above, FSH induces estrogen biosynthesis by stimulating cAMP dependent signaling cascades to regulate transcription of the *Cyp19a1* gene. In rat granulosa cells, CTNNB1 was shown to bind the transcription factor NR5A1 (steroidogenic factor-1 (SF1)), resulting in the upregulation of CYP19A1 expression *in vitro*. Regulation of estrogen biosynthesis by FSH/cAMP requires a functional interaction between SF1 and CTNNB1 on the Cyp19a1 promoter. Decreasing β-catenin levels or disrupting the SF1 and β-catenin interaction decreases FSH/cAMP-induced *Cyp19a1* mRNA accumulation (Parakh et al., 2006). Challenging mouse and bovine granulosa cells with FSH could stimulate steroidogenic genes and enzymes via CTNNB1 activation (Castanon et al., 2012b, Fan et al., 2010b, Li et al., 2014).

Based on these studies, we can conclude that CTNNB1 is an essential transcriptional regulator of CYP19A1 expression and consequently estradiol secretion.

2.5.1.2 Follicle development

In a recent study, CTNNB1 was shown to facilitate FSH-induced follicular growth and decrease follicular atresia. FSH and CTNNB1 signaling can synergistically promote the expression of genes required for granulosa cell proliferation and estrogen biosynthesis such as *Cyp19a1*, *Nr5a1*, *Fshr* and *Ccnd2*, and decrease *Foxo1* which is a negative regulator of proliferation and steroidogenesis. On the other hand, CTNNB1 inhibited LH action and reduced fertility, ovulation, oocyte maturation, cumulus expansion, luteinization, and progesterone biosynthesis. These effects were mediated by the decreased the expression of genes involved in all of these processes, including *Areg* and

Ereg (Fan et al., 2010a). These findings indicate CTNNB1 can have profound effects on granulosa cell proliferation, differentiation, and survival.

2.5.2 WNT4

WNT4 activates different pathways according to cell type and physiological context. It has been observed to play a role in a wide variety of physiological and pathological events from embryogenesis to follicular development in the ovary (Franco et al., 2011).

2.5.2.1 Embryonic development

In mammals, the gonadal primordium is a unique tissue that is able to undergo two opposite fates to form either a testis or an ovary. It has been shown that WNT4 plays an important role in human and mouse sex determination (Hsieh et al., 2002). WNT4 is expressed in the early stages of embryonic development before differentiation in both female and male. However, its expression down regulated in the developing male gonad (11.5 days post-coitum in mice) and stays elevated in the developing ovary. Lack of WNT4 leads to masculinization of the XX embryo, which is characterized by including the persistence of the Wolffian duct.

It has been shown that WNT4 inhibits the migration of adrenal precursors and endothelial cells into the ovary (Jeays-Ward et al., 2003). *Wnt4*-null mice ovaries express many Leydig and Sertoli cell markers and secrete testosterone and Müllerian-inhibiting substance (MIS) (Hsieh et al., 2002). WNT4 has a crucial role during embrogenesis in males as well as in females. Ablation of *Wnt4* reduces the proliferation of the coelomic epithelium cells, decreasing the number of progenitors of Sertoli cells in XY mutant gonads, and leading to reduced numbers of Sertoli cells and the formation of a testis with

few seminiferous tubules. WNT4 is a regulator of cell proliferation in the early gonad regardless of its sex and has a specific role in ovarian differentiation (Chassot et al., 2012). In *Wnt4* mutant mouse ovaries the number of oocytes is abnormally low by the time of birth. WNT4 has not been shown to affect the number of primordial germ cells in the undifferentiated gonad, but it seems to act as an oocyte survival factor during female embryogenesis (Vainio et al., 1999).

One study has shown an essential role for WNT4 in normal postnatal uterine development and function. Ablation of *Wnt4* leads to subfertility with defects in embryo implantation and endometrial stromal cell survival, differentiation and responsiveness to progesterone signaling (Franco et al., 2011).

2.5.2.2 Follicle development

WNT4 is expressed in small preantral follicles, preovulatory follicles, and corpora lutea in the rodent ovary. *Wnt4* mRNA levels increase significantly in response to an ovulatory dose of hCG in rat and mouse granulosa cells, suggesting a role important in some aspects of late follicle development, ovulation, and/or luteinization (Hsieh et al., 2002).

Because *Wnt4*-null mice die shortly after birth due to kidney defects, granulosa cell-targeted conditional inactivation of *Wnt4* was used to study its role in the ovary. Results showed that WNT4 is required for normal antral follicle development and can regulate granulosa cell functions. *Wnt4*$^{(flox/-)}$;*Amhr2*$^{tm3(cre)Bhr/-}$ mice were born with normal numbers of follicles, however they had fewer healthy antral follicles at puberty compared to controls. In addition, WNT4 was shown to regulate the expression of genes related to ovulation, including *Adamts1* and *Ptgs2*, and the steroidogenic genes *Cyp19a1*, *Cyp11a1*

and *Star* (Boyer et al., 2010b). Another study showed that inactivation of *Wnt4* in granulosa cells significantly reduced folliculogenesis and female fertility, and led to premature ovarian failure. These deficiencies were related to cell polarity defects in the follicles. *Wnt4* deficiency reduces the expression of adherens junction and gap junction components and compromises follicular cell polarity. Therefore, WNT4 is important for maturation of ovarian follicles, cell survival, and polarized organization of the follicular cells (Prunskaite-Hyyrylainen et al., 2014).

2.5.3 WNT2

WNT2 is expressed in the developing pericardium, lung, and placenta. Ovarian steroids can modulate WNT2 expression in the endometrium and mammary gland. RT-PCR and *in situ* hybridization (ISH) analyses indicate that *Wnt2* is expressed in follicular granulosa cells in the immature rat ovary. Targeted disruption of the *Wnt2* gene in mice results in placentation defects however, *Wnt2*-null mice did not show fertility problems, suggesting that it may not be essential for follicular function. (Ricken et al., 2002).

Knockdown of WNT2 decreased DNA synthesis in granulosa cells, whereas WNT2 overexpression increased this process. WNT2 knockdown led to an accumulation of glycogen synthase kinase-3β in the cytoplasm and reduced the expression of CTNNB1. Conversely, WNT2 overexpression reduced the expression of GSK3β in the cytoplasm and induced β-catenin translocation from the membrane to the nucleus. CTNNB1 knockdown also inhibited DNA synthesis in granulosa cells and neutralized the effect of WNT2 overexpression. This study therefore showed that WNT2 stimulates granulosa cell proliferation *in vitro* via the CTNNB1 pathway (Wang et al., 2010). The same group reported the WNT2/CTNNB1 pathway regulates connexin43 expression and gap-

junctional intercellular communication in granulosa cells by modulating CTNNB1 stability and localization in adherens junctions (Wang et al., 2013)

In cultured b bovine granulosa cells, only *WNT2* mRNA was induced after 24 h of FSH treatment among the *WNT* genes that were tested. In addition, western blot analysis determined that CTNNB1 expression was regulated by FSH *in vitro*. Therefore, FSH-induced WNT2 signaling may be responsible for the up-regulation of CTNNB1 in bovine granulosa cells (Castanon et al., 2012a).

2.5.4 FZD1

Little is known about the functions of FZDs in the mammalian ovary. *Fzd1* mRNA expression increased in PMSG treated mouse ovaries followed with hCG for 12h. FZD1 is not present on the surface of primary or small antral follicles, but is present on the surface of growing oocytes in late secondary follicles. This could indicate that FZD1 is involved in regulating gene expression during specific stages of follicle development (Harwood et al., 2008). FZD1 expression is first up-regulated in theca cells and then in granulosa cells of the ovulatory follicle by LH, however it is not detected in the corpora lutea of PMSG-treated mice. The selective and transient induction of FZD1 in granulosa cells by LH suggested that, in the ovulating follicle, *Fzd1* could regulate genes that impact follicle rupture or luteinization (Hsieh et al., 2002). *Fzd1*$^{-/-}$ females produced almost one less pup per litter compared to controls, indicating a significant role for FZD1 in female fertility. However, the numbers of follicles, expression of genes related to steroidogenesis and steroid hormone level were normal in *Fzd1*-null females. RT-qPCR analysis determined that FZD1 regulates genes related to cumulus expansion (*Btc*, *Ptgs2*, *Sema3a*, *Ptx3*, *Il6*, *Nts* and *Alcam*) and oocyte maturation (*Zp3*, *Dppa3*, *Nlrp5* and *Bmp15*). However,

cumulus expansion was normal in *Fzd1*$^{-/-}$ COCs both *in vitro* and *in vivo* (Lapointe et al., 2012).

2.5.5 FZD4

Fzd4 is expressed in pregnant and postpartum mouse ovaries. *Wnt4* and *Fzd4* are coexpressed in corpora lutea, suggesting that FZD4 could be a receptor for WNT4, and WNT4/FZD4 signaling may be important for regulation of luteal cell functions (Hsieh et al., 2002). It seems FZD4 is essential for normal fertility because both male and female of *Fzd4*-null mice are infertile. The latter mice had normal follicular development, ovulation and released mature oocytes. However, implantation never happened in *Fzd4*-null mice, and they failed to form functional corpora lutea. Defective luteal cell function appeared to be associated with inadequate angiogenesis (Hsieh et al., 2005).

2.5.6 SFRP4

SFRP4 is one of the members of the secreted FZD related protein (sFRP) family. The exact function of sFRP4 is still unclear, although sFRPs have generally been described as WNT antagonists. *sFRP4* expression was detected in the ovary of mice and rats (Hsieh et al., 2002). In the rodent model, sFRP4 was shown to be induced by LH independent of the progesterone receptor, and was also upregulated by prolactin (PRL) in granulosa cells. Regulation of SFRP4 by LH and PRL may be essential for regulation of FZD1, which is known to be expressed in preovulatory follicles, and WNT4/FZD4, which are expressed in corpora lutea (Hsieh et al., 2003). One study reported that human sFRP4 decreased as follicles grow to the preovulatory stage and its expression was higher in cumulus cells than in mural granulosa cells. In addition, sFRP4 expression was inhibited

by LH/hCG stimulation *in vivo* and in cell culture. These results show that sFRP4 might have role in follicular development and ovulation (Maman et al., 2011).

Chapter 2. Hypotheses and objectives

2. 1 Hypotheses and objectives

Recent reports have suggested roles of canonical WNTs signaling in the adult ovary. Canonical WNTs appear to play central roles in normal follicle development, and act to regulate granulosa cell functions including steroidogenesis and proliferation. Most of the information available about WNT signaling in the ovary has been derived from rodents. Whether WNTs play similar roles in large monovular species such as the cow remains essentially unknown. Therefore, my first hypothesis is that WNT signaling may be important for follicular development and steroidogenesis in bovine granulosa cells.

In addition, little is known about the role of non-canonical WNT signaling in the ovary. WNT5a and WNT11 are the two best-known non-canonical WNT members and are expressed in mouse granulosa cells, however their roles in the adult ovary are unknown. Therefore, the second hypothesis is that WNT5a and WNT11 are critical for follicle development and act in granulosa cells via a non-canonical signaling pathway.

To test our hypotheses, we established the following objectives for the present thesis: 1) To elucidate the hormonal regulation of major WNTs and their physiological roles in bovine granulosa cells *in vitro*. The results corresponding to this objective are described in chapter 3.

2) To identify the physiological roles of WNT5a and WNT11 by conditional gene inactivation in mouse granulosa cells, as well as their mechanisms of action. The data related to this objective are described in chapter 4.

Chapter 3. Article 1

3.1 Article 1.

Non-canonical WNT5A is a potential regulator of granulosa cell function in cattle

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3.2 Abstract

The WNT family has been implicated in follicular development in rodents, however, the role of WNTs in the follicle of monovulatory species is poorly understood. The objective of this study was to determine the potential roles of WNTs in bovine granulosa cell function. Cells cultured in serum-free medium expressed mRNA encoding WNT2B, WNT5B and WNT5A. Levels of *WNT5A*, but not of *WNT2B* or *WNT5B* mRNA were down-regulated by FSH. Addition of WNT5A to cultured cells suppressed FSH-stimulated estradiol and progesterone secretion, and levels of mRNA encoding the steroidogenic enzymes CYP19A1, CYP11A1 and the FSH receptor, but had no effect on cell proliferation or apoptosis. Immunoblot experiments showed that WNT5A reduced activation of CTNNB1 and stimulated phosphorylation of MAPK8 and JUN proteins. We conclude that WNT5A is a negative regulator of FSH-stimulated granulosa cell steroidogenesis, and that it acts by suppressing canonical WNT signaling activity and inducing the non-canonical MAPK8/JUN pathway.

Key words: WNT5A, steroidogenesis, granulosa cells, bovine

3.3 Introduction

Although the gonadotropins, FSH and LH, are the main endocrine regulators of ovarian follicle growth and development, a number of paracrine and autocrine factors modulate the response of follicle cells to gonadotropin signals (Richards et al., 2002). These include the transforming growth factor β family, the insulin-like growth factor family and the fibroblast growth factor family (Buratini and Price, 2011). Another large group of factors that has been linked to the reproductive system is the wingless-type mouse mammary tumor virus integration site (WNT) family.

In mammals, 19 different WNT proteins have been described, and have been reported to control diverse developmental processes such as cell fate specification, proliferation, differentiation and apoptosis in a wide range of tissues including the ovary (Boyer et al., 2010a; Cadigan and Nusse, 1997; Wang et al., 2012). Secreted WNT proteins are hydrophobic and travel short distances within the extracellular matrix, and it has become increasingly apparent that WNTs are secreted in exosomes (Zhang and Wrana, 2014); it is thus likely that WNTs act in an autocrine or juxtacrine manner. WNTs transduce their signals by binding to G protein-coupled receptors of the Frizzled (FZD) family and the lipoprotein-related receptor proteins LRP5 and LRP6 that act as co-receptors at the cell surface (Lapointe et al., 2012). The WNT-FZD complex transduces a signal via disheveled (DVL) to activate at least three distinct intracellular signaling pathways known as the canonical pathway, the planar cell polarity (PCP) pathway, and the WNT/Ca2+ pathway (Boerboom et al., 2005; Karner et al., 2006; Kohn and Moon, 2005). The pathway that is activated by a given WNT is determined by cell type, the composition of the receptor complex and the WNT ligand itself (Kühl et al., 2000). In the canonical pathway, the binding of a WNT ligand to the receptor complex results in the

hypophosphorylation, stabilization and accumulation of the signaling effector β-catenin (CTNNB1) in the cytoplasm. CTNNB1 can then translocate to the nucleus, where it associates with a number of transcription factors to alter the transcriptional activity of target genes (Boyer et al., 2010a; Lapointe and Boerboom, 2011). In the PCP pathway, WNT binding activates two parallel pathways involving the small GTPases RHO and RAC (Wallingford and Habas, 2005). The RHO branch of signaling activates RHO-associated kinase (ROCK) (Marlow et al., 2002; Weiser et al., 2007), and the RAC signaling branch stimulates JUN kinase (MAPK8) activity (Habas et al., 2003; Li et al., 1999). According to the prevailing view, ROCK and MAPK8 then act via different effectors mainly to modify the actin cytoskeleton leading to cytoskeletal rearrangement (Keller et al., 2003). However, MAPK8-mediated phosphorylation of the transcription factor JUN also occurs in PCP pathway signaling in certain contexts, resulting in the transcriptional activation of target genes (Saadeddin et al., 2009; Semenov et al., 2007).

Most of the information available about WNT signaling in the ovary has been derived from rodents. Messenger RNA encoding several WNT proteins has been detected in the ovary, including *Wnt2*, *Wnt3a*, *Wnt4* and *Wnt5a* (Hsieh et al., 2002; Ricken et al., 2002). The canonical *Wnt4* is expressed in granulosa cells and corpora lutea in mice (Hsieh et al., 2002) and is essential for the development of the ovary and follicles (Boyer et al., 2010a). Granulosa cell-specific knockout of *Wnt4* decreased luteal progesterone synthesis and levels of mRNA encoding steroidogenic acute regulatory protein (*Star*), cytochrome P450 side-chain cleavage (*Cyp11a1*) and cytochrome P450 aromatase (*Cyp19a1*) and, conversely, overexpression of *Wnt4* in mouse granulosa cells increased abundance of mRNA of these same genes (Boyer et al., 2010b). *Wnt2* is also expressed in rat granulosa cells, and was shown to stimulate granulosa cell proliferation *in vitro* via the

CTNNB1 pathway (Ricken et al., 2002; Wang et al., 2010). (Finnson et al., 2012) but its role *in vivo* has not yet been established.

Much less is known about the expression and function of ovarian WNTs in monovulatory species. In humans, *WNT2* but not *WNT4* mRNA was detected in cumulus cells (Wang et al., 2009), whereas *WNT4* and *WNT5A* transcripts have recently been reported in luteinized granulosa cells (Sanchez et al., 2014). In luteinized bovine granulosa cells, *WNT2* mRNA was detected and upregulated by FSH (Castañon et al., 2012), and CTNNB1 has been shown to act downstream of LH to regulate *STAR* expression and progesterone synthesis (Roy et al., 2009). To our knowledge, there are no reports of *WNT* expression in non-luteinized granulosa cells of a monovulatory species. The objectives of the present report were to determine the expression profile of selected WNTs in non-luteinizing bovine granulosa cells, and to test the hypothesis that WNTs alter bovine granulosa cell physiology and steroidogenesis.

3.4 Material and methods:

3.4.1 Cell culture

All materials were obtained from Life Technologies Inc. (Burlington, ON, Canada) unless otherwise stated. Bovine granulosa cells were placed into a well-defined serum-free culture system in which the cells are estrogenic and respond to FSH but not to LH (Gutiérrez et al., 1997; Sahmi et al., 2004; Silva and Price, 2000). Briefly, bovine ovaries were collected at an abattoir from adult cows irrespective of stage of the estrous cycle, and transported to the laboratory at 30 ºC in phosphate-buffered saline (PBS) containing penicillin (100 IU/ml), streptomycin (100 mg/ml), and fungizone (1 mg/ml). Follicles between 2 and 5 mm in diameter were dissected from the ovary and each one was cut into six to eight pieces and granulosa cells recovered by rinsing the follicle wall with medium; cells were washed twice by centrifugation at 980×g for 20 min each, and the cell suspension was filtered through a 150 mesh steel sieve (Sigma–Aldrich Canada, Oakville, ON, Canada). The number of cells was counted with a hemocytometer and cell viability was assessed by the dye exclusion method using 0.4% Trypan Blue. Cells were placed into 24-well tissue culture plates (Sarstedt Inc., Newton, NC) at a density of 106 viable cells in 1 ml DMEM/F12 containing sodium bicarbonate (10 mmol/L), sodium selenite (4 ng/ ml), bovine serum albumin (BSA) (0.1%; Sigma–Aldrich), penicillin (100 U/ml), streptomycin (100 mg/ml), transferrin (2.5 mg/ml), nonessential amino acid mix (1.1 mmol/L), bovine insulin (10 ng/ml), androstenedione (10-7 M), and bovine FSH (1 ng/ml unless otherwise stated; lot AFP-5332B, NIDDK). Cultures were maintained at 37ºC in 5% CO2, 95% air for 6 days, with 70% of the culture medium being replaced every 2 days.

To study the effect of FSH on WNT expression, cells were treated without and with graded doses (0, 0.1, 0.5, 1 ng/ml) of FSH on day 2 of culture. The effects of WNT5A on granulosa cells was tested by culturing cells with 1 ng/ml FSH and adding graded doses of recombinant human WNT5A (R&D Systems, Minneapolis, MN). Medium samples were collected on day 6 and stored at −20 ◦C until steroid assay, and cells were collected in Trizol and stored at−80 ◦C until RNA extraction.

To determine target genes and pathways activated by WNT5A, cells were treated with WNT5A (500 ng/ml) on day 5 of culture for 0, 1, 2, 4 and 6 h (for RNA extraction) or for 0, 1, 5, 30 and 120 min (for protein extraction). Specific signaling inhibitors were also used to dissect the major signaling pathway activated by WNT5A. The inhibitors were AS601245 (Calbiochem) an inhibitor of MAPK8 and NSC23766 (R&D Systems, Minneapolis, MN) an inhibitor of Rac GTPase, which acts upstream of MAPK8. The first inhibitor was dissolved in DMSO and the second with water, then directly added to the medium. The cells were pretreated with inhibitors for 1 h before addition of WNT5A for a further 1 h for RNA extraction and 0.5 h for protein extraction. Controls were treated with DMSO and water. All series of cultures were performed on at least three different pools of cells collected on different occasions.

3.4.2 Assessment of granulosa cell proliferation and apoptosis

Cells were cultured as described above and treated with 0 or 125 ng/ml WNT5A for the 2 last days of culture. Cell cycle analysis was performed by resuspending the cells in Krishan's buffer (0.05 mg/ml propidium iodide, 0.3% nonidet (NP-40), 0.1% sodium citrate, and 0.02 mg/ml RNAse) for 1 h on ice, followed by flow cytometric analysis. For each sample, 10,000 propidium iodide stained cells were analysed on a FACSCalibur (BD

Biosciences) using Cellquest Pro acquisition software. The Annexin V-FITC Apoptosis assay (Sigma–Aldrich Canada, Oakville, ON, Canada) was used to determine the percentage of early and late apoptotic cells. Cells were washed twice with PBS and then resuspended in binding buffer and processed according to the manufacturer's instructions.

3.4.3 *In vivo* study

To determine if *WNT5A* expression differs between dominant and subordinate follicles *in vivo*, the emergence of a follicle wave was synchronized by an injection of prostaglandin F2 , and the ovaries were recovered at slaughter on days 2 to 4 of the wave. The two largest follicles were dissected from each pair of ovaries, their diameters were measured, and the follicular fluid was aspirated, centrifuged, and frozen for steroid assay. The antral cavity was repeatedly flushed with cold saline solution and granulosa cells recovered by centrifugation at 1200 x g for 1 min and pooled with the follicular fluid pellet. The dominant follicle in each animal was identified by size, follicular fluid estradiol concentration and evaluation of mRNA encoding *CYP19A1* in granulosa cells; the estradiol data and detailed methods have been previously reported (Portela et al., 2010a).

3.4.4 Total RNA extraction and real-time RT-PCR

After treatments, the culture medium was removed and total RNA was extracted using Trizol as instructed by the manufacturer. Total RNA (1 µg) was treated with 1 U DNase (Promega, Madison, WI, USA) at 37 ◦C for 5 min to digest any contaminating DNA, and then reverse-transcribed with 4 U Omniscript RTase (Qiagen, Mississauga, ON, Canada) in the presence of 1 mmol/l oligo(dT) primer, 0.25 mmol/l dideoxynucleotide

triphosphate (dNTP) mix and 19.33 U RNase Inhibitor (GE Healthcare, Baie D'Urfé, QC, Canada) in a volume of 20 µl at 37ºC for 1 h. The reaction was terminated by incubation at 93 ºC for 3 min. Real-time PCR was performed on a 7300 Real-Time PCR system (Applied Biosystems, Streetsville ON, Canada) with Power SYBR Green PCR Master Mix. Bovine-specific primers for *H2AFZ* (Portela et al., 2010b), *CYP19A1* (Hamel et al., 2005), *FSHR* (Luo and Wiltbank, 2006), *HSD17B1* (Portela et al., 2010a) and *FOS* (Jiang et al., 2013) were previous used by us, those for *CYP11A1*, *HSD3B1*, *STAR* (Orisaka et al., 2006) and *JUN* (Atli et al., 2012) were as described by others. Bovine-specific WNT primers were designed with Primer Express (Applied Biosystems, Foster City, CA) and are: *WNT4*, forward 5'-CATGAACCTCCACAACAACGA-3', reverse 5'-TCGCCAGCACGTCTTTACCT-3'; *WNT2*, forward 5'-CCAGAGCCCTGATGAATCTTCA-3', reverse 5'-TCACGCCATGACACTTGCA-3'; *WNT2B*, forward 5'- GACCGGGACCACACTGTCTT-3', reverse 5'-GACCACCCCTGCTGATGAGA-3'; *WNT5A*, 5'-TTCTCTCCTTCGCCCAGGTT-3', reverse 5'-AGAGAGGCTGCGCTCCTATG-3'; *WNT5B*, forward 5' CCGCGTGCTCATCATGAACCT-3', reverse, 5'-GAGACGCCATGGCATTTGC-3'. Common thermal cycling parameters (3 min at 95 ºC, 40 cycles of 15 s at 95ºC, 30 s at 59 ºC, and 30 s at 72ºC were used to amplify each transcript. Amplicons obtained from original primers were sequenced to confirm identity, and melting-curve analyses were performed in each assay. Samples were run in duplicate and were normalized to histone 2AFZ (*H2AFZ*). The RT-PCR data obtained with this method were presented as fold increases in gene expression relative to a reference sample for each gene analyzed. Data were normalized to a calibrator sample using the ΔΔCt method with correction for amplification efficiency (Pfaffl, 2001).

3.4.5 Immunoblotting

After treatment, granulosa cells were washed with ice-cold PBS and lysed in 50 µl/well ice-cold RIPA buffer (25 mM Tris– HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate, and protease inhibitor cocktail). The homogenate was centrifuged at 6000g for 5 min at 4 C and the supernatant stored at -80 ºC. Protein concentrations were determined by BCA protein assay (Pierce, Rockford IL). Samples (15 µg total protein) were loaded onto 10% SDS-polyacrylamide gels in SDS loading buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 12.5 mM EDTA, and 0.02% bromophenol blue), electrophoresed and electrotransferred to Hybond-P PVDF membrane (GE Amersham, Piscataway, NJ) in 39 mM glycine, 48 mM Tris-base, 1% SDS, 20% methanol, pH 8.3. After transfer, the membranes were blocked in 5% non-fat dry milk in TTBS (10 mM Tris–HCl, 150 mM NaCl, 0.1% Tween-20, pH 7.5) for 1 h. Membranes were incubated overnight with the primary against WNT5A (cat # ab72583 1:1000 , Abcam Inc, Toronto, Canada), active CTNNB, (cat # 8814, 1:1000, all the following antibodies from Cell Signaling Technology, Danvers, MA) total CTNNB, (cat # 8480, 1:1000), phospho-MAPK8, (cat # 9251, 1:1000), MAPK8, (cat # 9252, 1:1000), phospho-JUN (cat #2361, 1:500) or JUN, (cat # 9165, 1:500) diluted in 5% bovine serum albumin. After washing three times with TTBS, membranes were incubated for 1 h at room temperature with 1:10,000 anti-rabbit HRP-conjugated IgG (GE Healthcare Canada) diluted in 5% non-fat dry milk in TTBS. The protein bands were visualized by chemiluminescence (ECL; Millipore, Billerica MA) and quantified using a Vilber Lourmat Fusion Fx chemiluminescence detection system. Active CTNNB and phospho MAPK8 levels were expressed relative to total CTNNB and

MAPK8 levels, respectively, and phosphorylated and total JUN levels were expressed relative to ACTB levels.

3.4.6 Steroid Assay

Steroid concentrations in the culture medium were corrected for cell number by expressing the data per unit mass of total cell protein. Cells were lysed with 100 µl of 1N NaOH for 2 h and neutralized with 100µl of 1N HCl, and total cell protein was measured by the Bradford protein assay (Bio-Rad, Mississauga, ON, Canada). Estradiol was measured in follicle fluid and in conditioned medium in duplicate as described (Bélanger et al., 1990) without solvent extraction. Intra- and interassay coefficients of variation were 6% and 9%, respectively. Progesterone was measured in conditioned medium in duplicate as described (Lafrance and Goff, 1985), and mean intra- and interassay coefficients of variation were 7.2% and 18%, respectively. The sensitivity of these assays was 10 pg and 4 pg per tube for estradiol and progesterone, equivalent to 0.3 and 20 ng/µg protein, respectively.

3.4.7 Statistical analysis

All statistical analyses were performed with JMP software (SAS Institute, Cary, NC). Data were transformed to logarithms if they were not normally distributed (Shapiro–Wilk test). Proportions of cells at each stage of the cell cycle were arcsine transformed before analysis. ANOVA was used to test the main effects of treatments, and culture replicate was included as a random variable in the F-test. Differences between means were tested with Fisher's protected test. The data are presented as least square means ± SEM.

3.5 Results

3.5.1 WNT expression in bovine granulosa cells

We first surveyed WNT gene expression in bovine granulosa cells using a non-luteinizing culture model. Messenger RNA encoding *WNT2B* (average Ct value, 27), *WNT5B* (Ct, 28) and *WNT5A* (Ct, 27) were detected, but those encoding *WNT4* (Ct, 40) and *WNT2* (Ct, 38) were at minimum detectable levels. To examine the regulation of WNT expression, granulosa cells were cultured with graded doses of FSH (0, 0.1, 0.5, 1 ng/ml); *WNT2B* and *WNT5B* mRNA levels were not affected by FSH (data not shown), whereas *WNT5A* mRNA levels were significantly decreased by FSH (Fig 1A). Western blotting demonstrated that FSH also decreased WNT5A protein levels in granulosa cells (Fig 1A). We then determined whether altered *WNT5A* mRNA abundance occurs *in vivo* by assaying granulosa cells of well-characterized dominant compared to subordinate first-wave follicles: *WNT5A* mRNA levels were significantly lower in granulosa cells from dominant compared with subordinate follicles (Fig 1B).

3.5.2 WNT5A suppressed the effects of FSH on steroid secretion and steroidogenic enzyme gene expression.

As WNT5A was regulated during follicle development, we then focused on the actions of this WNT. Cultured bovine granulosa cells were challenged with FSH, recombinant human/mouse WNT5A (500 ng/ml), neither or both for 2 days. As expected, FSH significantly increased estradiol secretion and *CYP19A1* mRNA levels with minimal effects on progesterone secretion and *CYP11A1* and *FSHR* mRNA levels. Alone, WNT5A had no effect on estradiol secretion or *CYP19A1* mRNA levels, but potently suppressed the stimulatory effect of FSH on estradiol secretion and *CYP19A1* mRNA levels (Fig 2).

Addition of WNT5A alone significantly inhibited *FSHR* mRNA levels (Fig 2), and this was not overcome by the addition of FSH (Fig 2). Either FSH or WNT5A alone inhibited *STAR* mRNA levels, and combined there was no further reduction.

A subsequent dose-response experiment demonstrated that WNT5A inhibited FSH-stimulated steroid secretion at the lowest dose tested (62.5 ng/ml), and *CYP19A1* and *CYP11A1* mRNA levels were decreased with doses of 125 ng/ml and higher (Supplemental Fig 1). WNT5A had no significant effect on FSH-stimulated *STAR* or *HSD17β1* mRNA levels even at 500 ng/ml (Supplemental Fig 1).

3.5.3 WNT5A does not affect the cell cycle or apoptosis

The relatively high levels of *WNT5A* mRNA in granulosa cells of subordinate follicles and the ability of WNT5A to inhibit estradiol secretion prompted us to determine the effect of WNT5A on granulosa cell health. Addition of an effective dose of WNT5A (125 ng/ml) had no effect on progression through the cell cycle or on the incidence of apoptotic cells (Table 1). Further, WNT5A did not alter abundance of mRNA encoding the cell cycle marker *CCDN2*, or of the apoptosis-related genes *FASLG* or *GADD45B* (not shown).

3.5.4 WNT5A acts through the PCP pathway

Immunoblot experiments were performed to identify the predominant signaling pathways used by WNT5A in FSH-stimulated bovine granulosa cells. Abundance of active β-catenin was significantly reduced by the addition of WNT5A (Fig 3A), whereas WNT5A resulted in a transient increase in MAPK8 phosphorylation (Fig 3B) and in the abundance of both phosphorylated and total JUN proteins (Fig 3C).

The importance of the PCP pathway was investigated with the use of a competitive inhibitor of MAPK8 activity (AS601245) and by inhibition of Rac1 GDP/GTP exchange activity with NSC23766. Neither inhibitor alone altered total or phospho-JUN abundance, but each significantly reduced WNT5A-stimulated JUN phosphorylation without a significant effect on total JUN abundance (Fig 4). Inhibition of MAPK8 activity also increased β-catenin activation (Fig 4).

Targets of MAPK8 include *FOS* and *JUN* genes, therefore we determined whether WNT5A increases levels of mRNA encoding these transcription factors. WNT5A rapidly and transiently increased abundance of mRNA encoding *JUN* and *FOS* (Fig 5A). Consequently, we explored the role of PCP pathway on *JUN* and *FOS* mRNA abundance; inhibition of the MAPK8 pathway with AS601245 prevented WNT5A from stimulating *JUN* and *FOS* mRNA levels, and treatment with the Rac1 inhibitor NSC23766 inhibited the stimulatory effects of WNT5A on *JUN* and blunted the effect of WNT5A on *FOS* mRNA levels (Fig 5B).

3.6 Discussion

Canonical WNT signaling has been previously described in the rodent ovary. In the present study, we provide compelling evidence for a non-canonical WNT signaling pathway in bovine granulosa cells, and that this signaling regulates estradiol secretion and may be involved in follicle function. Our evidence suggests that WNT5A is one ligand that activates this pathway.

Messenger RNA encoding *WNT5A* has previously been detected in human granulosa cells (Sanchez et al., 2014), and we could readily detect mRNA and protein in bovine granulosa cells. Abundance of *WNT5A* mRNA was regulated by FSH, whereas *WNT2B* and *WNT5B* mRNA levels were not, which points to a specific regulation of *WNT5A* in the ovary. Other reports of WNT regulation in granulosa cells show that FSH stimulates abundance of mRNA encoding *Wnt2* and *Wnt4* (Castañon et al., 2012; Hsieh et al., 2002), therefore this is the first description of the negative regulation of a WNT by FSH. The reduction of *WNT5A* mRNA was reflected by a reduction of WNT5A protein levels, indicating that altered mRNA abundance has potential physiological relevance. This is supported by the increased levels of *WNT5A* mRNA in subordinate compared with dominant first-wave follicles; the dominant follicle avoids regression because it is able to maintain gonadotrophic signaling (through FSH and/or IGF1), and this signaling likely results in the lower *WNT5A* mRNA levels observed.

The main signaling pathway activated by WNT5A is a non-canonical pathway (reviewed in (Kikuchi et al., 2012)), although as WNT5A has been reported to activate CTNNB signaling in the presence of FZD4 (Mikels and Nusse, 2006) and as *FZD4* mRNA is present in granulosa cells (Ricken et al., 2002), we verified which pathway is activated by WNT5A in bovine granulosa cells. Western blot experiments clearly show

that WNT5A did not increase the abundance of active CTNNB protein, but increased MAPK8 and JUN phosphorylation and abundance of mRNA encoding the phospho-JUN transcriptional targets, *JUN* and *FOS*, which are components of the PCP pathway. The increase in *JUN* mRNA levels was reflected by an increase in JUN protein levels. These data are consistent with studies showing that knock-down of *WNT5A* decreased phospho-JUN levels in a sarcoma cell line (Jin et al., 2012) and that WNT5A upregulated phospho-JUN levels in NIH3T3 fibroblasts (Nomachi et al., 2008). Pharmacological inhibition of MAPK8 or Rac1 blocked the ability of WNT5A to stimulate phosphorylation of JUN and expression of *JUN* and *FOS* (present study). Collectively, these data demonstrate that WNT5A signals through the non-canonical PCP pathway in bovine granulosa cells.

Not only did WNT5A fail to activate CTNNB in granulosa cells, but it also decreased active CTNNB protein levels. Previous reports have demonstrated that treatment with WNT5A decreases total CTNNB protein levels and/or CTNNB1 signaling in luteinized human granulosa cells as well as other cell types (Pourreyron et al., 2012; Sanchez et al., 2014; Sato et al., 2009; Topol et al., 2003). Several mechanisms have been proposed to explain how non-canonical WNTs may antagonize the canonical pathway, including direct competition with canonical WNTs for binding to FZD receptor complexes (Bryja et al., 2009), increasing the expression of SIAH ubiquitin ligase and accelerating ubiquitination and degradation of CTNNB1 (Topol et al., 2003), and inhibiting coactivator recruitment to the promoters of target genes via a protein kinase C pathway (Lee et al., 2010). The inhibitor studies in the present experiment suggest that MAPK8 signaling is at least part of the mechanism by which WNT5A inhibits active CTNNB1 levels.

Addition of WNT5A to granulosa cells in culture decreased the secretion of estradiol and *CYP19A1* and *FSHR* mRNA levels, all of which are under positive

regulation by FSH. The exact mechanism used by WNT5A to suppress *FSHR*, *CYP19A1, CYP11A1 and STAR* mRNA levels remains unclear. Exogenous JUN protein binds to the human ovarian *CYP19A1* promoter and decreases transcriptional activity (Ghosh et al., 2005), whereas CTNNB1 is known to stimulate *Cyp19a1* expression and estrogen synthesis in rodent granulosa cells *in vitro* by binding to the transcription factor NR5A1 (Hernandez Gifford et al., 2009; Parakh et al., 2006); thus by suppressing CTNNB1 and stimulating JUN, WNT5A likely contributes to the suppression of estradiol synthetic capacity through both PCP and CTNNB1 signaling pathways. However, this may not hold true for the inhibition of *STAR* mRNA by WNT5A, as CTNNB1 and JUN have both been shown to stimulate *STAR* mRNA in bovine corpus luteum (Roy et al., 2009) and mouse Leydig cells, respectively (Manna and Stocco, 2008; Martin and Tremblay, 2009). This suggests that WNT5A-induced suppression of active CTNNB1 levels had a negative impact on *STAR* mRNA levels that was not compensated for by the increase in JUN activity.

Our PCR survey failed to detect *WNT4* mRNA in bovine granulosa cells, which is in agreement with a study in human cumulus cells (Wang et al., 2009) but in contrast to studies with human luteinized granulosa cells and rodent ovaries (Hsieh et al., 2002; Sanchez et al., 2014). We also failed to detect significant quantities of *WNT2* mRNA, unlike previous studies in bovine and rodent granulosa cells (Castañon et al., 2012; Ricken et al., 2002). One possible explanation for the discrepancy with the previous cow study is the differentiation status of the cultured cells, as Castañon et al. (Castañon et al., 2012) cultured cells with serum under luteinizing conditions.

In summary, the results of the present study point to a novel role for a non-canonical WNT pathway in follicle function. A possible scenario during the induction of

follicle atresia is that peripheral FSH concentrations decline, causing an increase in *WNT5A* expression. WNT5A decreases estradiol secretion and *CYP19A* mRNA levels in granulosa cells, likely through increased JUN and decreased CTNNB activities, and may act as an endogenous brake to steroidogenesis. A likely model to explain the role of WNT5A in the inhibition of canonical cascade and activation of non-canonical pathway is illustrated in the graphical abstract. Combined with a decline in factors supporting cell health (such as IGF1), WNT5A may thus contribute to the process of granulosa cell apoptosis and follicle regression.

3.7 Acknowledgements

We are grateful to Dr A.F. Parlow and National Hormone & Peptide Program, NIDDK for bovine FSH, and to the farm staff for care of the animals. This work was supported by Fonds de recherche du Québec - Nature et technologies.

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3. 8 Figures

Figure 1.

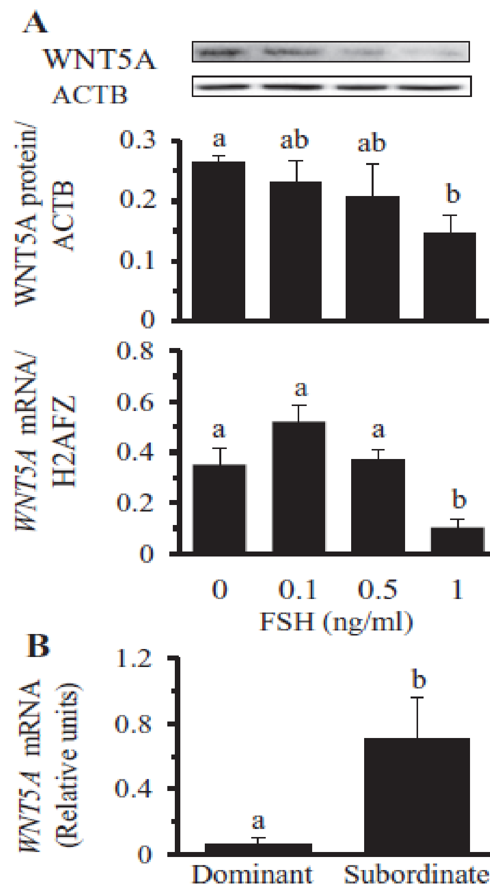


Figure 1. Regulation of WNT5A in granulosa cells.

(A) Effect of FSH on *WNT5A* mRNA and WNT5A protein levels in granulosa cells cultured under serum-free conditions (see Materials and Methods for details). (B) *WNT5A* mRNA levels in granulosa cells of dominant (n=4) or subordinate (n=4) follicles recovered on days 2-4 of an induced follicle wave. Steady-state mRNA levels were measured by real-time PCR and expressed relative to a calibrator sample with the ΔΔCt method. Protein abundance was measured by Western blot and expressed relative to ß-actin protein abundance. Culture data are means ± SEM of three independent cultures, and bars with no common letters are significantly different ($P<0.05$).

Figure 2.

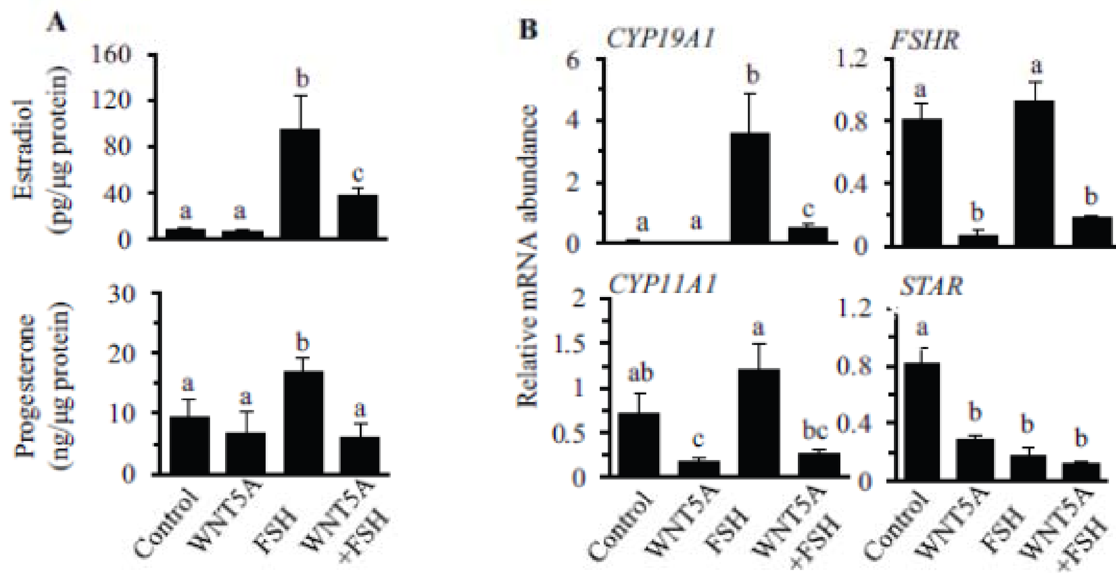


Figure 2. Effect of WNT5A on steroidogenesis and on steady-state levels of mRNA encoding steroidogenic proteins in bovine granulosa cells.

Granulosa cells from 2-5 mm follicles were cultured in serum-free medium for 6 days with and without FSH (1 ng/ml), and WNT5A (500 ng/ml) was added for the last 2 days of culture. Steady-state mRNA levels were measured by real-time PCR and expressed relative to a calibrator sample with the ΔΔCt method. Data are means ± SEM of three independent cultures, and bars with no common letters are significantly different ($P<0.05$).

Figure 3.

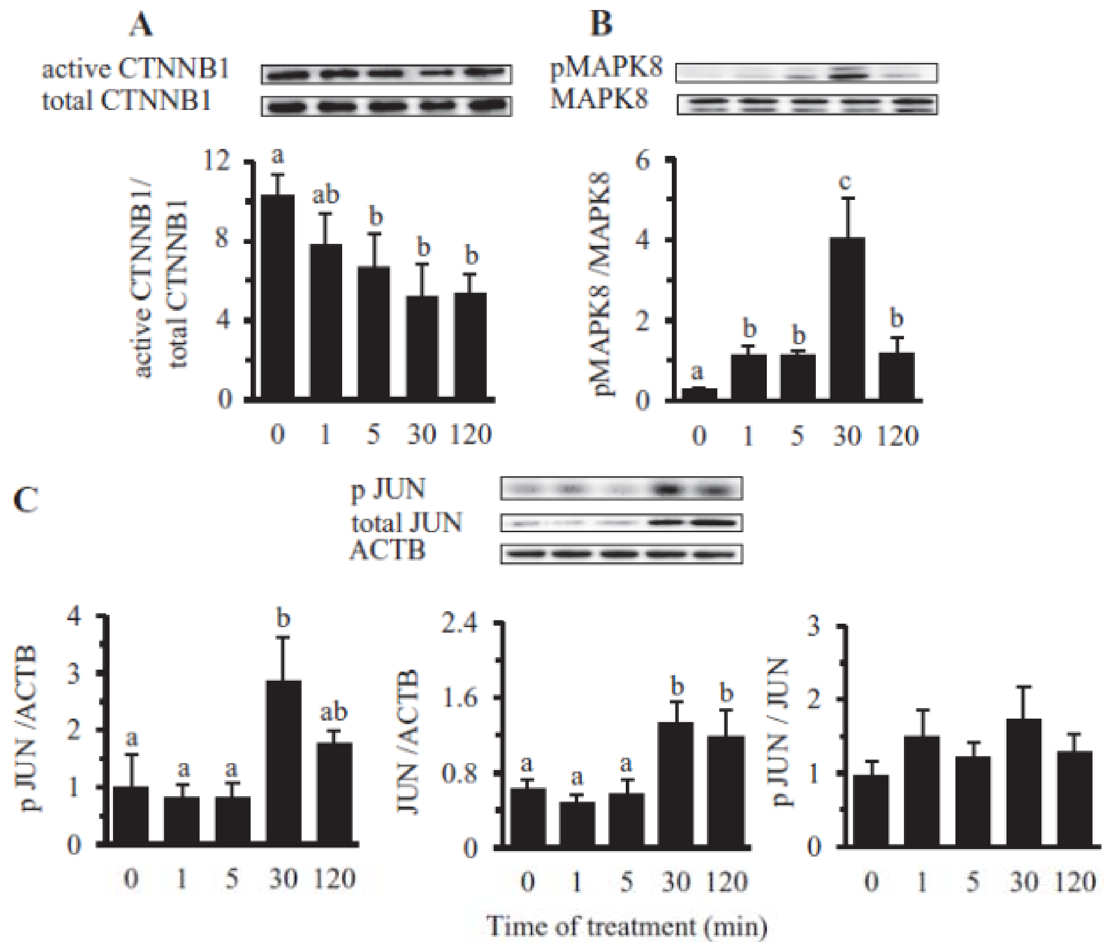


Figure 3. WNT5A modulates the activity of multiple signaling pathways in bovine granulosa cells. Cells were cultured in serum-free medium with FSH for 5 days, and challenged with WNT5A (500 ng/ml) for the times shown. Total cell protein was used to measure total and active β-catenin, total and phosphorylated JNK, and total and phosphorylated JUN by Western blot; a blot from one replicate is shown and the samples are in the same order as in the graph. Data are means ± SEM of three independent replicates. Means without common letters are significantly different ($P < 0.05$).

Figure 4.

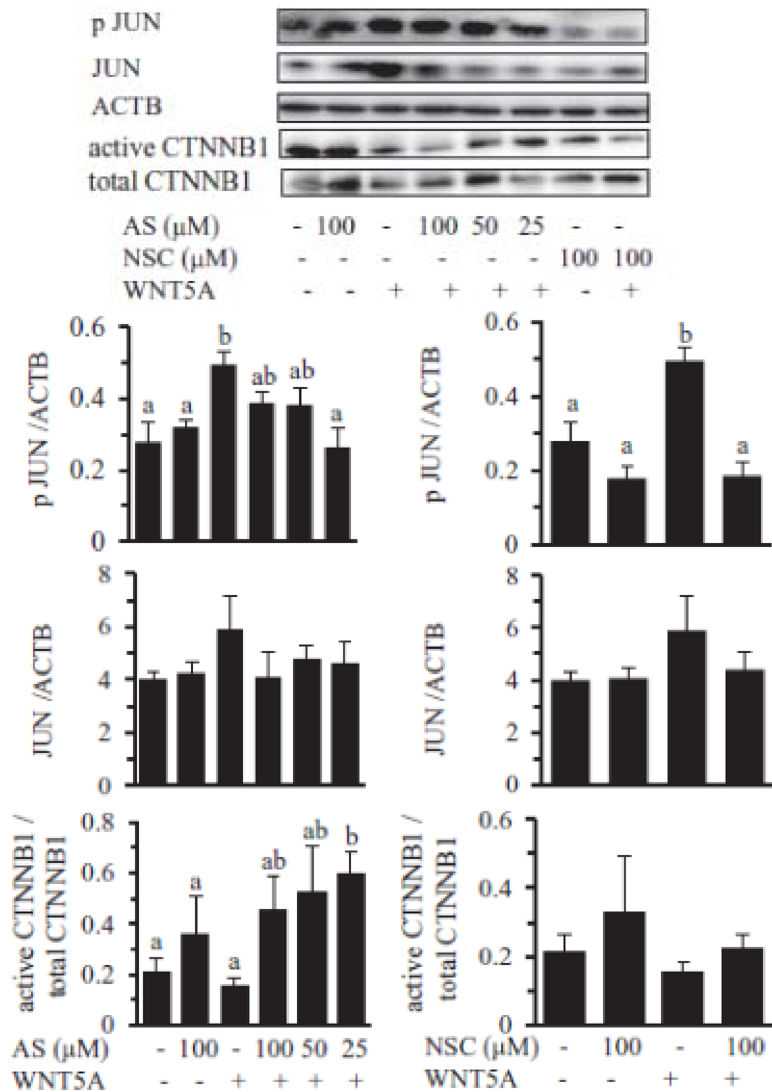


Figure 4. WNT5A induced JUN phosphorylation is dependent on JNK and Rac1 activity. Granulosa cells were treated with inhibitors of Rac1 (NSC23766; NSC) or JNK (AS601245; AS) for 1 h before challenge with WNT5A (500 ng/ml) for 30 min. Western blot analyses were performed to detect total and phosphorylated JUN. Data are means ± SEM of three independent replicates. Bars without common letters are significantly different ($P < 0.05$).

Figure 5.

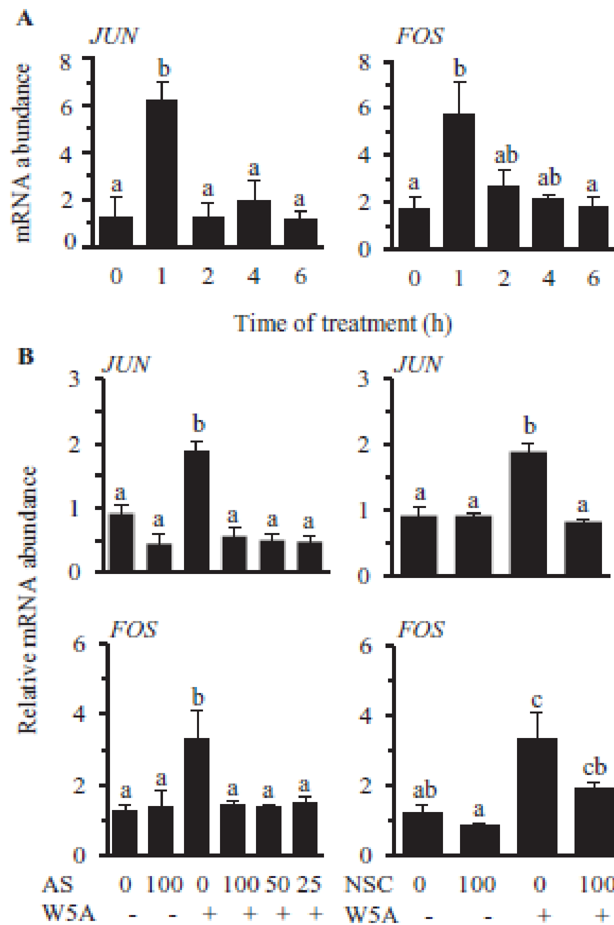
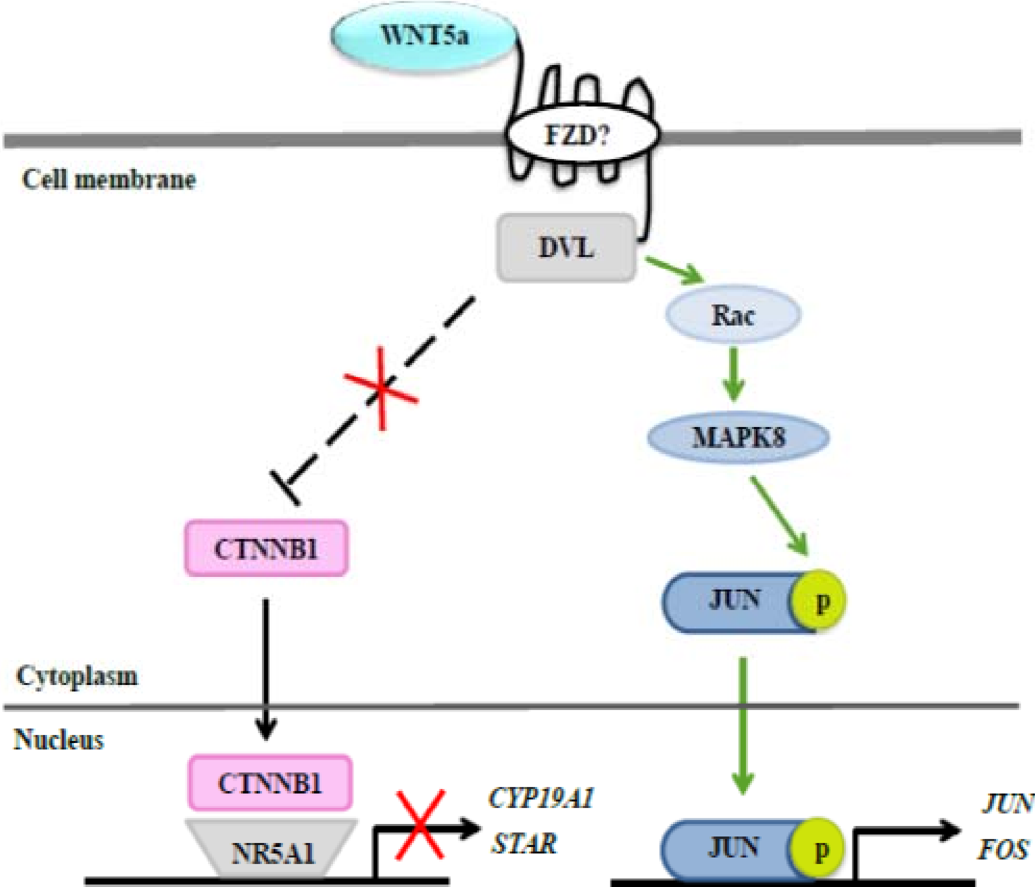


Figure 5. WNT5A upregulates *JUN* and *FOS* mRNA abundance in granulosa cells through the PCP pathway. (A) WNT5A transiently increased abundance of mRNA encoding *JUN* and *FOS*. (B) Inhibition of JNK with AS601245 (AS) or Rac1 with NSC23766 (NSC) abrogated the effect of WNT5A on *JUN* and *FOS* mRNA abundance. Cells were treated with inhibitors for 1 h before challenge with WNT5A (500 ng/ml) for 1 hour. Data are means ± SEM of three independent replicates. For each mRNA, means without common letters are significantly different ($P < 0.05$).

Figure 6. Graphical abstract (for review)



3.9 Supplemental figure

Figure S1.

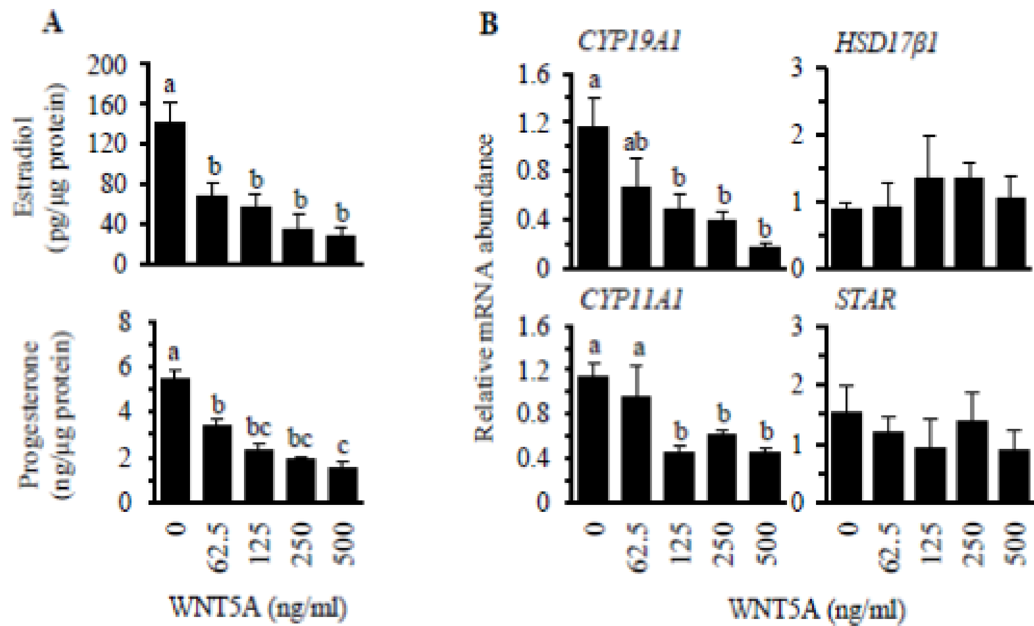


Figure S1. WNT5A inhibited steroid secretion and abundance of mRNA encoding *CYP19A1* and *CYP11A1* in a dose-dependent manner.

Bovine granulosa cells were cultured in serum-free medium with FSH (1 ng/ml), and challenged with graded doses of WNT5A for the 2 last days of culture. Abundance of mRNA was measured by real-time PCR. Data are means ± SEM of three independent replicates. For each mRNA, means without common letters are significantly different (P < 0.05). No letters indicates no significant effect of treatment.

3.10 Tables

Table 1. Primer sequences used in real-time PCR.

Gene	Forward primer (5´- 3´)	Reverse primer (3´- 5´)
WNT4	CATGAACCTCCACAACAACGA	TCGCCAGCACGTCTTTACCT
WNT2	CCAGAGCCCTGATGAATCTTCA	TCACGCCATGACACTTGCA
WNT2B	GACCGGGACCACACTGTCTT	GACCACCCCTGCTGATGAGA
WNT5B	CCGCGTGCTCATCATGAACCT	GAGACGCCATGGCATTTGC
WNT5A	TTCTCTCCTTCGCCCAGGTT	AGAGAGGCTGCGCTCCTATG

Table 2. Effect of WNT5a on the cell cycle, *CCND2* mRNA levels and apoptosis in bovine granulosa cells.

Variable	WNT5A (ng/ml)	
	0	125
Sub-G1 (%)	15 ± 1	18 ± 1
G0/G1 (%)	74 ± 2	72 ± 2
S (%)	6 ± 0.5	6 ± 0.5
G2/M (%)	4 ± 0.5	2 ± 0.5
CCND2 mRNA (relative units)	0.9 ± 0.1	1.1 ± 0.2
Apoptosis (%)*	24 ± 2	29.5 ± 2

* As measured by Annexin V-FITC staining.

Chapter 4. Article 2

4.1 Article 2

WNT5a is required for normal ovarian follicle development and antagonizes gonadotropin responsiveness in granulosa cells by suppressing canonical WNT signaling

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A.A., C.A.P., and D.B designed research; A.A., G.Z., E.L., M.P., C.T., and A.B perform research; A.A. analyzed data; K.H., H.H., and A.K provided mice; A.A., and D.B. wrote the paper.

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4.2 Abstract

Whereas the roles of the canonical WNT signaling pathway in the regulation of ovarian follicle growth and steroidogenesis are now established, non-canonical WNT signaling in the ovary has been largely overlooked. Non-canonical WNTs including WNT5a and WNT11 are expressed in granulosa cells and differentially regulated throughout follicle development, but their physiological roles remain unknown. Using conditional gene targeting, we found that granulosa cell-specific inactivation of *Wnt5a* (but not *Wnt11*) results in female infertility associated with increased follicular atresia and decreased rates of ovulation. Microarray analyses revealed WNT5a acts to down-regulate the expression of FSH-responsive genes *in vitro*, and corresponding increases in the expression of these genes were found in the granulosa cells of the conditional knockout mice. Unexpectedly, we found that WNT5a regulated its target genes not by signaling via the WNT/Ca^{2+} or planar cell polarity pathways, but rather by inhibiting the canonical pathway, causing both CTNNB1 and CREB protein levels to decrease via a GSK3β-dependent mechanism. We further found that WNT5a prevents FSH and LH from upregulating CTNNB1, CREB and their target genes, indicating that WNT5a functions as a physiological inhibitor of gonadotropin signaling. Together, these findings identify WNT5a as a key regulator of follicle development and gonadotropin responsiveness.

Key words: *Wnt5a,* granulosa cells, gonadotropins

4.3 Introduction

The pituitary gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH) represent the major endocrine regulators of ovarian function, and are essential for follicle development beyond the secondary stage (1, 2). In addition to the endocrine level of control, follicle development is also regulated by a number of paracrine and autocrine factors produced within the ovary itself. These factors notably include insulin-like growth factor-1, steroid hormones, prostaglandins, epidermal growth factor-like molecules and several transforming growth factor-β superfamily members, all of which are indispensable for normal ovarian function and female fertility. Importantly, the gonadotropins can impact the expression and function of these intraovarian factors; conversely these factors can modulate follicular responses to the gonadotropins (1-3).

Recent studies have established the WNT family of secreted glycoproteins as yet another class of signaling molecules that act to modulate and coordinate follicular responses to the gonadotropins, and whose activities are indispensable for ovarian function and female fertility. During FSH-stimulated follicle growth, the WNT/CTNNB1 signaling pathway (also known as the canonical pathway) appears to play a central role. Here, the binding of a WNT to a cognate frizzled (FZD) receptor results in the stabilization of the signaling effector CTNNB1 (β-catenin), which then translocates to the nucleus and associates with a number of transcription factors to alter the transcriptional activity of target genes (4-9). In granulosa cells, CTNNB1 was shown to bind the

transcription factor NR5A1 (steroidogenic factor-1), resulting in the up-regulation of *Cyp19a1* (aromatase) expression *in vitro* (10). Further *in vitro* experiments using the Cre/LoxP system to knock down CTNNB1 expression in primary cultured granulosa cells confirmed its requirement for FSH-induced estrogen synthesis (11). The identity of the WNT signaling molecule that acts upstream of CTNNB1 in this context remains unclear. WNT2 is expressed in granulosa cells, has been shown to stimulate proliferation *in vitro* and signals via the CTNNB1 pathway (12-14), but its *in vivo* role has not yet been established. Likewise, WNT4 is required for antral follicle development, and its overexpression results in increased *Cyp19a1* expression *in vitro*, however its conditional inactivation does not affect estrogen biosynthesis *in vivo* (7). Another possibility is that, in addition to (or perhaps instead of) by WNTs, the activation of CTNNB1 signaling in granulosa cells is caused by FSH itself. Indeed, FSH was found to induce the (ser552/ser665) phosphorylation of CTNNB1 in a PKA-dependent manner, resulting in an increase of *Lhcgr* transcriptional activity (15). Another recent study expanded upon the role of CTNNB1 during follicle growth using a transgenic mouse model in which CTNNB1 was constitutively activated in the granulosa cells of antral follicles (16). This resulted in a marked enhancement of FSH action, inducing an overexpression of FSH-responsive genes including *Cyp19a1*, *Nr5a1*, *Fshr* and *Ccnd2* and promoting follicle growth (16).

Considerable progress has also been made in the elucidation of the roles of WNT signaling in LH-regulated processes. LH induces the expression of a number of WNT signaling effectors in both granulosa and cumulus cells, including WNT4, the receptors FZD1 and FZD4, as well as secreted frizzled-related protein-2 (SFRP2) and SFRP4 (17). The conditional inactivation of *Wnt4* in granulosa cells *in vivo* blunts the induction of

genes involved in ovulation and steroidogenesis (*Adamts1*, *Ptgs2*, *Star*, *Cyp11a1*) in response to hCG/LH, and results in lower circulating progesterone levels (7). Female *Fzd4*-null mice are sterile at least in part due to abnormal development and function of the corpus luteum (CL), including decreased progesterone synthesis and altered morphology, gene expression and vasculogenesis (18). In addition, *Fzd1*-null mice are subfertile, and gene expression analyses of cumulus-oocyte complexes revealed a blunted response of both oocyte and cumulus genes to LH/hCG (19). As for FSH, LH may also activate CTNNB1 signaling independently of WNT/FZD. Using a bovine luteal cell model, Roy et al. observed that LH could induce the phosphorylation (and inactivation) of glycogen synthase kinase 3β (GSK3β) in a PKA-dependent manner. As phosphorylation of CTNNB1 by GSK3β normally causes its degradation, LH thereby stabilized CTNNB1, resulting in the transcriptional up-regulation of *STAR* and increased progesterone synthesis (20).

WNTs are frequently categorized according to pathway(s) via which they signal. Certain WNTs, such as WNT1 and WNT3a, preferentially (or exclusively) activate the canonical pathway, whereas WNT5a and WNT11 are associated with non-canonical pathways in mammalian cells. The two best-characterized non-canonical pathways are the planar cell polarity (PCP) and WNT/Ca^{2+} pathways (21, 22). In the latter, the WNT-FZD complex interacts with heterotrimeric G proteins to activate phospholipase C (PLC), resulting in increased intracellular Ca^{2+} concentrations and the activation of Ca^{2+}-dependent effectors including protein kinase C (PKC), calmodulin-dependent kinase II (CAMKII) and calcineurin (23-26). In the PCP pathway, the WNT-FZD complex activates two parallel pathways involving the small GTPases RHO and RAC (27), which in turn activate disheveled associated activator of morphogenesis 1 (DAAM1), RHO-associated

kinase (ROCK) and cJUN kinase (JNK) (28-31). DAAM1, ROCK and JNK then act via different effectors mainly to modify the actin cytoskeleton, leading to cytoskeletal rearrangement (32). However, JNK-mediated phosphorylation of the transcription factor cJUN also occurs in certain contexts, resulting in the transcriptional activation of target genes (22, 33).

Little is known of non-canonical WNT signaling in the ovary. *Wnt5a* and *Wnt11* are expressed in mouse granulosa cells (34), and *Wnt5a* transcripts have been detected in human luteinized granulosa cells and cumulus cells (35, 36). We have recently identified *Wnt5a* as gene whose expression is down-regulated by FSH, using a bovine primary granulosa cell culture model (37). We found that exogenous WNT5a suppressed FSH-stimulated estradiol and progesterone secretion and *CYP19A1* expression, but stimulated the expression of *FOS* and *JUN* by signaling via the PCP pathway. Although the physiological relevance of these findings is not clear, it was proposed that WNT5a may play a role in suppressing steroidogenesis during follicular atresia. The objectives of the present report were to determine the physiological roles of non-canonical WNTs in the mouse ovary, as well as their mechanisms of action. To this end, we used conditional targeting of WNT5a and WNT11 as a primary experimental approach.

4.4 Materials and methods

Animal models, fertility trials, ovulation rate and follicle counting

C57BL/6J mice (referred to herein as wild-type, WT) were obtained from the Jackson Laboratory (Bar Harbor, ME) and *Wnt5a*$^{tm1.1Homy}$ (RBRC04609, hereafter *Wnt5a*^{flox}) mice were obtained from RIKEN BRC (Tsukuba, Ibaraki, Japan). Genotyping analyses for the *Wnt5a* alleles were done by PCR using DNA from tail biopsies and the primers 5´- GTGAGGGACTGGAAGTTGCAGGA -3´, 5´-CAACGGAAACATCCGAGGTCTCT -3´and 5´-CCAGCCACTGGCTTTAGAGAAAGT -3´, using the following cycling conditions: 2 min at 95º C for one cycle, 30 sec at 95º C, 30 sec at 68º C, and 30 sec at 72º C for 35 cycles, and 7 min at 72º C for one cycle. Primers were designed to generate PCR products of 398 bp for the floxed allele, 335 bp for the Cre-recombined allele and 243 bp for the WT allele. Genotype analyses of *Wnt11*$^{tm1.1Khay}$ (38) (hereafter *Wnt11*^{flox}), *Amhr*2$^{tm3(cre)Bhr}$ (39) (hereafter *Amhr*2^{cre}) and Tg(*CYP19A1*-cre)1Jri (40) (hereafter *CYP19*-cre) mice were done as previously described. To assess female fertility, eight week-old females of experimental and control genotypes were housed with eight week-old WT males, and monitored daily for litters. Litter sizes were recorded at birth. Males were removed after 6 months, and the experiment was terminated 22d later to allow for the final litter. To determine ovulation rates, 8-10 week-old females of experimental and control genotypes were housed with WT males and monitored daily for the presence of a vaginal plug.

Females were then sacrificed, the oviducts removed and placed in sterile saline under a dissection microscope. Cumulus-oocyte complexes were released by tearing open the ampullae of the oviducts with forceps, and counted. Follicle counting was done on formalin-fixed, paraffin embedded ovaries. In all cases, only the left ovary from each animal was analyzed. Serial sections were prepared at a thickness of 5 µm and every 5th section was stained with hematoxylin and eosin. Follicles in which the oocyte nucleus was visible were counted, classified according to Pedersen's system and scored as healthy or atretic as previously described (7). All animal procedures were approved by the institutional animal care and use committee and conformed to the International Guiding Principles for Biomedical Research Involving Animals.

Granulosa cell isolation and culture

All materials were obtained from Life Technologies Inc. (Burlington, ON, Canada) unless otherwise stated. Ovaries were obtained from immature (21-26 day-old) mice 48 h after administration of equine chorionic gonadotropin (eCG, Folligon, Intervet, Kirkland, Québec, Canada, 5IU, IP) followed or not by human chorionic gonadotropin (hCG, Chorulon, Intervet, 5IU, IP) for 4-12 h. Granulosa cells (GCs) were isolated by placing the ovaries in Hanks balanced salt solution (HBSS) and puncturing with 26-gauge needles to release the GCs as previously described (41).

GCs for primary culture were obtained from eCG-treated immature mice as described above and plated in 48-well culture plates (each well receiving the GCs isolated from approximately 0.7 ovary) in MEM containing 1% fetal bovine serum, 0.25 nM sodium pyruvate, and 3 mM L-glutamate, for 3 h at 37ºC with 5% CO_2. Culture medium was then replaced with serum-free medium for 2h prior to addition of recombinant

WNT11 or WNT5a (R&D Systems, Minneapolis, MN). For some experiments, small molecule inhibitors (Tocris Bioscience, Bristol, United Kingdom) were added to the culture medium 1h prior to addition of WNT5a. These included SP600125 (cat # 1496, 100 µm), NSC23766 (cat # 2161, 100µm), KN-93 (cat # 1278, 2.5 µm), Xestrospongin C (cat # 1280, 5 µm), GF109203X (cat # 0741, 30 µm), and SB216763 (cat # 1616, 1 µm). To study the effects of WNT5a on gonadotropin signaling, GCs were pretreated with WNT5a (3.5 µg/ml) for 1 hour prior to addition (or not) of recombinant human FSH or LH (50 ng/ml, National Hormone and Peptide Program, Torrance, CA). Either immediately after isolation or following culture, GCs were lysed with RLT buffer (Qiagen) or Laemmli loading buffer and stored at -80ºC prior to RNA extraction or SDS-PAGE.

Real-time RT-PCR and microarray analyses

Total RNA from granulosa cells was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Reverse transcription was done using 200 ng of RNA and the SuperScript Vilo cDNA synthesis kit (Invitrogen). Real-time PCR was done using SsoAdvanced™ Universal SYBR® Green supermix (172-5274; Bio-Rad, Mississauga, Ontario, Canada) and a CFX96 Touch™ instrument (Bio-Rad). Each PCR reaction consisted of 7.5 µl of SsoAdvanced SYBR Green PCR Master Mix, 2.3 µl of water, 4 µl of cDNA sample, and 0.6 µl (10 pmol) of gene-specific primers (Supplemental Table 1). Cycling conditions were 3 min at 95º C followed by 40 cycles of 15 sec at 95º C, 30 sec at 60º C, and 30 sec at 72º C. To quantify relative gene expression, the Ct of target gene amplification was normalized to the expression level of a housekeeping gene (*Rpl19*) according to the ratio, $R = E^{Ct\ Rpl19}/E^{Ct\ target}$, where E is the amplification efficiency for each primer pair.

Microarray analyses were done in triplicate on RNA samples from cultured granulosa cells treated (or not) with 3.5 µg/ml recombinant WNT5a or WNT11 for 3h as described above, and using Agilent 8×60k Mouse Gene Expression Arrays (Agilent Technologies Inc., Mississauga, ON, Canada). All steps of RNA quality control, probe synthesis, hybridization, washing, and array scanning were performed by the McGill University (Genome Quebec) Innovation Center (Montreal, QC, Canada). Microarray data were analyzed using FlexArray 1.6.1 software (Genome Quebec). A *P* value threshold of 0.05 and 2.0-fold change cutoff values were used for identification of differentially expressed genes (WNT5a- or WNT11-treated *vs* untreated control). Gene ontology and pathway analyses were conducted online using the Database for Annotation, Visualization and Integrated Discovery (DAVID), v6.7 (NIAID, NIH).

Immunohistochemistry

Paraformaldehyde-fixed, paraffin-embedded ovaries were sectioned at a thickness of 4µm. Immunohistochemical analysis was done using an anti-cleaved caspase 3 antibody (9661S; Cell Signaling Technology) incubated at a 1:100 dilution overnight at 4°C. Subsequent detection steps were done using the Vectastain Elite ABC kit and the 3,3´ diaminobenzidine peroxidase substrate kit (Vector Laboratories, Burlingame, CA) as previously described (42). Slides were lightly counterstained with hematoxylin prior to mounting.

Immunoblotting

Immunoblotting was done as previously described (43) using primary antibodies against non-phosphorylated (Ser33/37/Thr41) (i.e., active) CTNNB1 (catalog # 8814),

phospho (Ser552) CTNNB1 (# 9566s), total CTNNB1 (# 8480), phospho-CREB (# 9191), total CREB (# 9197), phospho-AKT (# 4060), total AKT (# 4691), phospho-JNK (# 9251), total JNK (# 9252), phospho-JUN (# 2361, 1:500), total JUN (# 9165, 1:500), phospho-CAMKII (# 3361), total CAMKII (# 4436), phospho-GSK3β (# 9323), total GSK3β (# 12456), phospho-CAPZIP (# 07-2190, Millipore, Billerica, MA) CAPZIP (# 07-2189, 1:250, Millipore) or ACTB (# sc-47778, 1:10000, Santa Cruz Biotechnology, Dallas, TX) diluted in 5% bovine serum albumin. All antibodies mentioned above were obtained from Cell Signaling Technology (Danvers, MA) and used at a 1:1000 dilution unless otherwise specified. After washing three times with TTBS, membranes were incubated for 1 h at room temperature with anti-rabbit HRP-conjugated IgG (GE Healthcare Life Sciences, Baie d'Urfé, Canada) diluted 1:10000 in 5% non-fat dry milk in TTBS. In some instances, membranes were sequentially stripped and re-probed with several antibodies. Protein bands were visualized by chemiluminescence (ECL, Millipore) and quantified using a ChemiDoc MP detection system (Bio-Rad) and Image Lab™ software.

Steroid Hormone Measurement

Blood samples were collected by cardiac puncture prior to euthanasia. Estradiol (E2) and progesterone (P4) levels in the serum were determined by ELISA and radioimmunoassay, respectively. All assays were performed by the Ligand Assay and Analysis Core Laboratory of the University of Virginia (Charlottesville, VA).

Statistical Analyses

All statistical analyses were performed using Prism 4.0a (GraphPad Software Inc., La Jolla, CA) software. All the data sets (ovary weights, follicle numbers, mRNA expression, protein expression, and steroid hormone levels) were subjected to the F-test to determine equality of variances. Data were transformed to logarithms if they were not normally distributed. Two-tailed t-tests were used when two experimental groups were compared, or ANOVA (with Tukey's multiple comparisons post-test) to compare three or more groups. All data are presented as means ± SEM.

4.5 Results

Wnt5a expression in granulosa cells is required for normal female fertility.

To study *Wnt5a* and *Wnt11* expression in granulosa cells and their regulation by gonadotropins *in vivo*, immature mice were treated with (FSH-mimetic) eCG for 48h to stimulate follicle growth, followed by (LH-mimetic) hCG to provoke ovulation/luteinization (ovulation occurs 12-14h post-hCG in this model). Results showed a gradual increase in *Wnt5a* mRNA levels throughout follicle development that reached statistical significance near the time of ovulation (Fig. 1). Conversely, eCG did not affect *Wnt11* expression, but hCG induced a transient increase in *Wnt11* mRNA that returned to basal levels by the time of ovulation. These results indicate that *Wnt5a* and *Wnt11* are differentially regulated by gonadotropins during follicle development.

To study the physiological functions of *Wnt5a* and *Wnt11* in granulosa cells, we generated conditional knockout (cKO) mice by mating strains bearing floxed alleles to both the *CYP19*-cre and *Amhr*2^{cre} strains. This strategy was designed to target distinct stages of follicle development, as the *Amhr*2^{cre} knock-in allele drives Cre activity in granulosa cells mainly from the secondary stage onwards (39), whereas the *CYP19*-Cre transgene drives *Cre* expression from the antral stage (40). RT-qPCR analyses of granulosa cells from eCG-treated immature mice showed a significant ($P<0.05$), ~4-6 fold reduction in *Wnt5a* and *Wnt11* mRNA levels in corresponding cKO mice relative to controls, using either the *Amhr*2^{cre} or the *CYP19*-Cre strains (Figs. 2 and S1). No compensatory overexpression of *Wnt11* was observed in the *Wnt5a* cKO model, or vice-versa. Six-month mating trials were then done to study the effects of *Wnt5a* and *Wnt11* loss on female fertility. Conditional inactivation of *Wnt5a* using the *Amhr*2^{cre} strain resulted in a ~52% decrease in average litter sizes, and ~20% when using the *CYP19*-Cre

($P<0.05$, Tables 1 and S2). Conversely, loss of *Wnt11* did not result in a loss of fertility with either Cre driver, and the concomitant inactivation of *Wnt5a* and *Wnt11* did not result in a greater loss of fertility than the inactivation of *Wnt5a* alone (Tables S3, S4, S5). These results indicate that *Wnt5a* expression in granulosa cells is required for normal female fertility, and that its loss at the preantral stage (i.e., driven by the *Amhr*2^{cre}) results in a more severe phenotype than when it is lost at later stages. *Wnt11* on the other hand appears dispensable for female fertility, and its expression in granulosa cells cannot compensate for the loss of *Wnt5a*.

Wnt5a$^{flox/-}$;*Amhr2*$^{cre/+}$ mice have defects in follicle development and ovulation

Histopathological examination of the ovaries and reproductive tracts were conducted in adult mice for all cKO models. No morphological defects were detected in the ovaries, uteri or oviducts of any genotype, except in the *Wnt5a*$^{flox/-}$;*Amhr2*$^{cre/+}$ and *Wnt5a*$^{flox/-}$;*Wnt11*$^{flox/-}$;*Amhr2*$^{cre/+}$ females, in which the ovaries appeared smaller and to have reduced numbers of antral follicles and corpora lutea (Fig. 3 and not shown). *Wnt5a*$^{flox/-}$;*Amhr2*$^{cre/+}$ mice (but none of the others) also had reduced ovary weights compared to controls ($P<0.05$, Table 2 and not shown).

To provide a quantitative analysis of folliculogenesis, follicle counting was done using serial histologic sections of ovaries from *Wnt5a*$^{flox/-}$;*Amhr2*$^{cre/+}$ mice at various ages. Five day-old *Wnt5a*$^{flox/-}$;*Amhr2*$^{cre/+}$ mice had normal numbers of primordial and primary follicles, indicative of a normal ovarian reserve and postnatal follicle formation (Fig. 4A). However, at 42d of age, numbers of primordial, healthy secondary and healthy antral follicles were decreased to 46%, 44% and 28% of controls, respectively ($P<0.05$) (Fig. 4B). Similar reductions in numbers of healthy secondary and antral follicles were

observed at 8 months of age, but numbers of primordial follicles further decreased to 20% of controls, and primary follicles were also significantly reduced (P<0.05, Fig. 4C). At 42d of age, fully 78% of antral follicles were atretic in the *Wnt5a*$^{flox/-}$;*Amhr2*$^{cre/+}$ group, versus 36% in controls. Increased atresia was also evidenced by activated caspase-3 immunohistochemistry, which showed a vastly greater incidence of granulosa cell apoptosis in secondary and antral follicles in ovaries from *Wnt5a*$^{flox/-}$;*Amhr2*$^{cre/+}$ mice (Fig. 3B).

Based on these results, we suspected that the infertility of *Wnt5a*$^{flox/-}$;*Amhr2*$^{cre/+}$ mice was due to the decrease in healthy antral follicle numbers, leading to a reduction in the number of ovulations occurring per cycle. To verify this, *Wnt5a*$^{flox/-}$;*Amhr2*$^{cre/+}$ females were sacrificed after mating and cumulus-oocyte complexes (COCs) retrieved from the oviducts and counted. Results showed a 59% decrease in recovered COCs in *Wnt5a*$^{flox/-}$;*Amhr2*$^{cre/+}$ females compared to controls (P<0.05, Table 3). Infertility in these mice was therefore attributed mainly to a reduction in the numbers of follicles attaining the ovulatory stage.

WNT5a down-regulates the expression of FSH-responsive genes

To determine the mechanism of action of WNT5a, we first aimed to identify its transcriptional targets. Granulosa cells from eCG-primed immature mice were placed in culture and treated (or not) with recombinant WNT5a or WNT11, and global changes in gene expression analyzed by microarray. WNT5a treatment increased the expression of 542 genes and decreased the expression of 1656 genes by at least 2-fold over control (Supplemental data, WNT5a microarray results Excel file 1). Conventional *in silico* gene ontology and pathway analyses failed to detect a significant association between the

putative WNT5a target genes and specific signaling pathways or biological functions/processes (not shown). Nonetheless, we observed that many of the genes most highly down-regulated by WNT5a were key FSH-responsive genes associated with granulosa cell steroidogenesis, differentiation and proliferation. The regulation of a subset of these by WNT5a was confirmed by RT-qPCR (Figure 5). The expression of many of these FSH-responsive genes was also found to be increased in granulosa cells from eCG-treated *Wnt5a*$^{flox/-}$;*Amhr2*$^{cre/+}$ mice (Fig. 6), confirming them as *bona fide* targets of WNT5a signaling *in vivo*. The increase in *Cyp19a1* expression was also accompanied by increased serum estradiol levels in these mice, as well as decreased progesterone levels (Table 4).

Unlike WNT5a, microarray analyses revealed a very small number of genes that were regulated by WNT11 *in vitro*; 51 and 22 genes were respectively up- or down-regulated at least two fold (Supplemental data, WNT11 microarray results Excel file 2). Only one gene, *Il1b*, was up-regulated by both WNT5a and WNT11 (Fig. 7). To further study potential functional redundancy between WNT5a and WNT11, granulosa cells were treated with graded doses of WNT11, and its effects on the mRNA levels of WNT5a target genes were determined by RT-qPCR. Although WNT11 was also able to down-regulate WNT5a target genes to some extent, it was generally much less potent than WNT5a itself (Fig. 7). Accordingly, the expression of WNT5a target genes was not changed in granulosa cells from eCG-treated *Wnt11a*$^{flox/-}$;*Amhr2*$^{cre/+}$ mice, nor were they further up-regulated in the granulosa cells of *Wnt*5a$^{flox/-}$;*Wnt11a*$^{flox/-}$;*Amhr2*$^{cre/+}$ relative to *Wnt*5a$^{flox/-}$;*Amhr2*$^{cre/+}$ mice (Fig. S2). WNT11 may therefore be able to signal via similar mechanisms as WNT5a, but does not seem to function in a redundant manner with WNT5a in the physiological context.

WNT5a suppresses the canonical pathway in granulosa cells

To determine the intracellular signaling mechanisms whereby WNT5a regulates its target genes, mouse granulosa cell primary cultures were treated with WNT5a on a time course, and the expression of mediators of the PCP signaling pathway was assessed. WNT5a did not affect JNK or cJUN expression or phosphorylation (Fig. S3). Phosphorylation of CAPZIP, another substrate of JNK, was similarly unaffected (Fig. S3). mRNA levels of cJUN transcriptional targets genes such as *Fos* and *Jun* did not change in response to WNT5a (Fig. 5), nor were they altered in the granulosa cells of *Wnt*5a$^{flox/-}$;*Amhr2*$^{cre/+}$ mice (Fig. 6). Furthermore, pretreatment of granulosa cells with the JNK inhibitor SP600125 or the RAC1 inhibitor NSC23766 did not prevent WNT5a from down-regulating the expression of its target genes (Fig. S4). Similar experiments were conducted to determine the effects of WNT5a on WNT/Ca^{2+} pathway activity. WNT5a did not affect the expression or phosphorylation of CAMKII (Fig. S3). Likewise, pretreatment of granulosa cells with the PKC inhibitor GF109203X, the CAMKII inhibitor KN-93 or with Xestrospongin C (which prevents Ca^{2+} release by blocking the IP3 receptor) had no effect on the ability of WNT5a to downregulate its target genes, although basal levels of *Cyp19a1* expression were affected in some cases (Fig. S4). Together, these results suggest that neither the PCP nor the WNT/Ca^{2+} pathway is a major mediator of WNT5a signaling in this model.

Beyond the activation of non-canonical signaling pathways, non-canonical WNTs are also known to suppress the canonical WNT pathway in certain contexts (26, 44-48). We therefore did a time course analysis of the effects of WNT5a on the expression of non-phosphorylated (Ser33/Ser37/Thr41)(i.e., active) CTNNB1 and total CTNNB1. The

expression of both forms showed a rapid and dramatic decrease in response to WNT5a (Fig. 8). Destabilization of CTNNB1 can be induced by phosphorylation at its N-terminus by GSK3β (15, 49, 50), and/or by loss of phosphorylation at the C-terminus (ser552/ser665) (15, 51-53). Although levels of phospho (Ser552) CTNNB1 decreased in response to WNT5a, the ratio of phospho (Ser552) CTNNB1:total CTNNB1 remained unchanged, suggesting that C-terminal phosphorylation was not affected (Fig. 8). Conversely, pretreatment of granulosa cells with the GSK3 inhibitor SB216763 prevented WNT5a from decreasing CTNNB1 expression (Fig. 9), suggesting that WNT5a destabilizes CTNNB1 via a GSK3-dependent mechanism. However, this mechanism did not involve a rapid WNT5a-induced alteration in the expression of either total or phospho (Ser9) GSK3β (Fig. 8).

WNT5a down-regulates CREB expression and inhibits gonadotropin responsiveness

As for CTNNB1, the transcription factor CREB is both a critical mediator of gonadotropin signaling and a substrate for GSK3β, and is degraded subsequent to GSK3β-mediated phosphorylation in certain contexts (51, 54-56). We therefore studied the effects of WNT5a on CREB expression in granulosa cells, and found that WNT5a down-regulated CREB protein levels in a manner similar to CTNNB1 (Fig. 8). Pretreatment with SB216763 prevented this effect, indicating that it is GSK3-dependent (Fig. 9).

As WNT5a was found to down-regulate FSH-responsive genes (Fig. 5) and destabilized CTNNB1 and CREB, both of which are key mediators of FSH and LH signaling (Figs 8, 9) (2, 10, 16), we hypothesized that WNT5a functions to antagonize gonadotropin responsiveness in granulosa cells. To test this, granulosa cells were pretreated (or not) with WNT5a for 1h prior to addition of FSH or LH, and the expression

of CTNNB1 and CREB and mRNA levels of key FSH and LH target genes were determined. Both FSH and LH induced an increase in total, active and phospho (ser552) CTNNB1 protein levels in cultured granulosa cells, and WNT5a vastly decreased both basal and gonadotropin-stimulated expression of all three forms (Fig. 10A). As previously reported (57), both FSH and LH also increased the levels of total and (Ser133) phosphorylated CREB. As for CTNNB1, WNT5a pretreatment drastically downregulated CREB expression in both untreated and gonadotropin-treated granulosa cells (Fig. 10A). WNT5a pretreatment also inhibited (or abrogated) the ability of both FSH and LH to increase the mRNA levels of their target genes (Fig. 10B). Importantly, the mRNA levels of the same LH target genes were increased in the granulosa cells of *Wnt*5a$^{flox/-}$;*Amhr2*$^{cre/+}$ mice specifically 12h post-hCG treatment (Fig. 6), indicating that WNT5a also antagonizes LH action *in vivo*. Together, these results indicate that WNT5a functions as a physiological antagonist of gonadotropin signaling.

4.6 Discussion

Whereas the gonadotropins are essential endocrine regulators of ovarian follicle development, their actions are dependent on a number of locally-produced ovarian factors that serve to modulate their effects. In this study, we identified WNT5a as a novel secreted regulatory molecule that acts to antagonize the follicular response to gonadotropins, based on our key findings that WNT5a prevented FSH and LH from inducing their transcriptional target genes *in vitro*, while an enhanced transcriptional response of these same targets to eCG/hCG was observed in *Wnt5a* cKO mice *in vivo*. *Wnt5a* may therefore function to prevent premature or excessive growth, limit steroid hormone synthesis, prevent premature luteinization, and otherwise temper the effects of FSH and LH as required to ensure the proper development of the follicle.

Our inability to detect activation of non-canonical signaling by WNT5a in murine granulosa cells was unexpected, particularly considering that we have recently reported that WNT5a signals via a RAC1/JNK/JUN pathway in cultured bovine granulosa cells (37). Although the basis for this discrepancy remains unknown, the culture systems used differed between the two studies. Notably, the bovine cells were cultured from early antral follicles, whereas the murine cells were cultured from the preovulatory follicles of eCG-primed immature animals. As the WNT receptor complexes and signaling machinery present in granulosa cells may vary considerably from one phase of follicle development to the next, the differences between the studies may indicate that WNT5a signals via different mechanisms (and hence may have different physiological functions) at specific stages of folliculogenesis. Consistent with this, we found that the follicle development phenotype of the *Wnt*5a$^{flox/-}$;*Amhr2*$^{cre/+}$ model (in which secondary follicles are targeted) was much more severe than that of the *Wnt*5a$^{flox/-}$;*Amhr2*$^{cre/+}$;*CYP19*-Cre model (in which

only antral stages are targeted), suggesting specific functions for WNT5a at the secondary stage of development. Further studies will be required to properly dissect the stage-specific roles and signaling mechanisms of WNT5a in granulosa cells.

Several mechanisms have been proposed to explain how non-canonical WNTs may act to decrease CTNNB1 levels. For instance, non-canonical WNTs have been shown to directly compete with canonical WNTs for binding to FZD receptor complexes, thereby attenuating the canonical CTNNB1 stabilization signal (44). WNT5a has been shown to increase the expression of SIAH ubiquitin ligase, resulting in the accelerated ubiquitination and degradation of CTNNB1 (45). WNT11 can also activate caspases that degrade CTNNB1 via an unknown mechanism (47). Although the mechanism whereby WNT5a causes CTNNB1 protein levels to decrease in granulosa cells was not determined in this study, we believe that the very rapid (i.e., minutes) nature of this effect suggests that competition with canonical WNTs for their receptor(s) is likely. Indeed, granulosa cells express several canonical WNTs (including WNT2 and WNT3a (58, 59)) and high levels of active CTNNB1, suggesting that a constitutive level of canonical WNT signaling normally occurs in the granulosa cells of growing follicles (see model, Fig. 11). Our data and previous studies suggest that FSH (and LH) signaling via PKA can enhance this CTNNB1 signaling in at least three ways. First, PKA can phosphorylate (and thereby inhibit) GSK3β, preventing it from promoting the degradation of CTNNB1 (52, 60). PKA can also directly phosphorylate CTNNB1 at ser552/ser665, enhancing its stability and transactivation activity (15). Finally, PKA also phosphorylates CREB, which then binds cAMP response elements present in the promoters of many of the CTNNB1 target genes identified in the current study (4, 52). Interestingly, CREB binding protein (CBP) has been shown to enhance the transcriptional properties of CTNNB1 in several cell types (16, 61,

62), suggesting an additional level of synergy between gonadotropin/PKA and canonical WNT signaling (Fig. 11). We propose that WNT5a disrupts these signaling processes by competing with canonical WNT(s) for their receptor(s). This results in the dissociation of the CTNNB1 destruction complex from the receptor complex and the activation of GSK3β, possibly due to the loss of an inhibitory interaction between GSK3β and the WNT co-receptor low-density lipoprotein receptor-related protein 5/6 (LRP5/6) (63). GSK3β then phosphorylates CTNNB1 and CREB, resulting in the proteosomal degradation of both, and effectively disrupting both the gonadotropin/PKA and canonical WNT pathways (Fig. 11). Gonadotropin signaling may therefore be regulated by the precise balance between WNT5a and canonical WNTs present within the follicular microenvironment.

The conditional knockout of *Wnt5a* in granulosa cells enhanced FSH signaling and resulted in an increase in the expression of FSH-responsive genes, many of which serve to promote follicle growth and survival. Despite this, a marked increase in follicular atresia occurred in the *Wnt*5a$^{flox/-}$;*Amhr2*$^{cre/+}$ model, causing a decreased ovulation rate and infertility, and leading to an accelerated depletion of the follicular reserve. Although we were unable to reconcile these apparently paradoxical findings, several explanations can be proposed. For instance, increased FSH responsiveness, estradiol synthesis and proliferation in granulosa cells may result in a loss of coordination of follicle development between the granulosa cells, theca cells and oocyte, ultimately compromising the viability of the follicle. Another possibility is that WNT5a produced in granulosa cells may be important for oocyte development, an idea that is supported by a recent study indicating that WNT5a plays a role in oocyte entry into meiosis during embryonic development (64). As suggested above, another possibility is that WNT5a plays roles during the secondary

(and/or earlier) stage(s) of follicle development that were not elucidated in our mechanistic studies. Indeed, our *in vitro* studies were conducted using gonadotropin-treated mice and were focused on late stages of follicle development; a mechanism of WNT5a action occurring specifically at the secondary stage would therefore have been overlooked in our study design. If this were true, increased atresia in the *Wnt*5a$^{flox/-}$;*Amhr2*$^{cre/+}$ model would be the result of a loss of WNT5a function other than its ability to suppress of CTNNB1 and gonadotropin signaling in growing follicles. Further studies will be required to test these ideas.

In summary, we report for the first time the crucial role of WNT5a in follicle development and normal female fertility. We found that WNT5a acts to suppress gonadotropin signaling by antagonizing the canonical WNT signaling pathway, thereby causing the degradation of CTNNB1 and CREB, two key transcriptional regulators of gonadotropin-responsive genes. This work provides new insight into the role of the non-canonical WNTs in granulosa cells, and elucidates new signaling mechanisms that modulate gonadotropin action.

4.7 Acknowledgments

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4. 9 Figures

Figure 1.

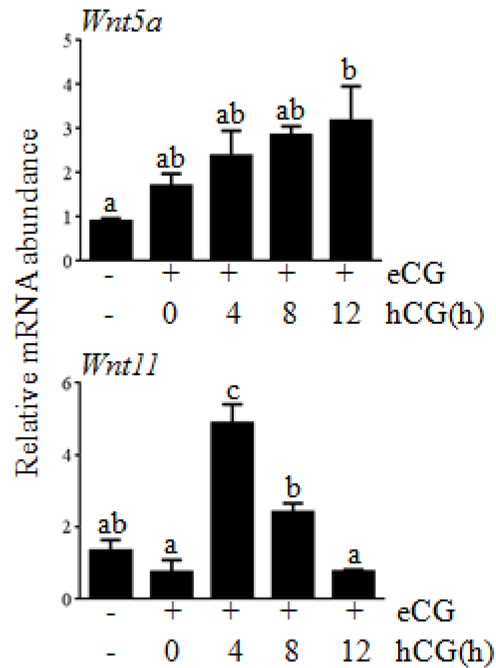


Figure 1. *In vivo* regulation of *Wnt5a* and *Wnt11* mRNA levels in granulosa cells by gonadotropins.

Immature (21-26 day-old) female mice were injected with eCG (5IU, IP) 44-48h prior to the administration of hCG. Granulosa cells were isolated by needle puncture. mRNA levels were determined by RT-qPCR and normalized to the housekeeping gene *Rpl19*. n=4 animals per time point. Columns = means, error bars = SEM, columns lacking common letters are significantly different (P<0.05).

Figure 2.

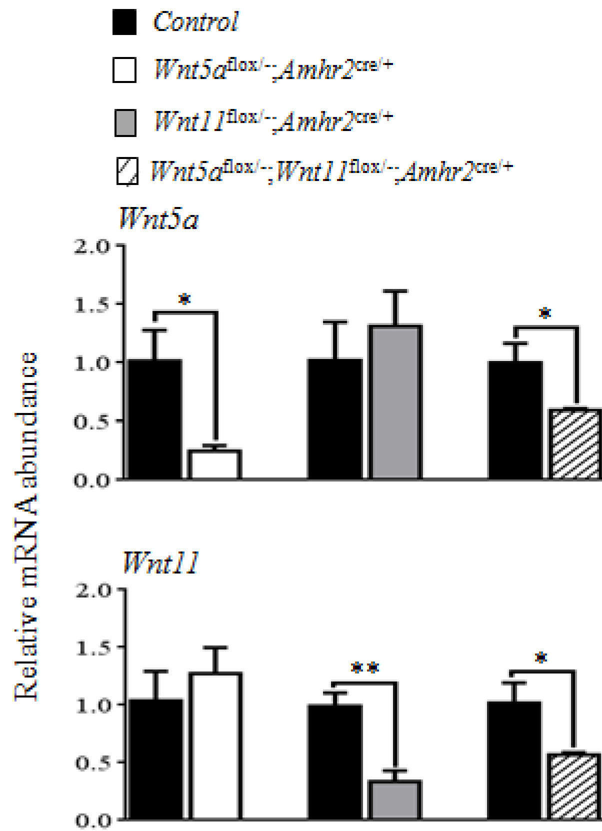
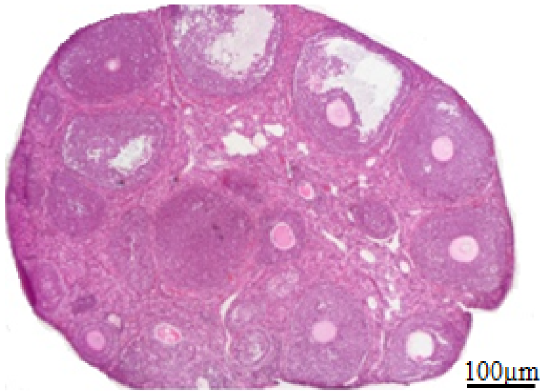


Figure 2. *Wnt5a* and *Wnt11* knockdown efficiency in the conditional knockout models. Expression of *Wnt5a* and *Wnt11* was determined by RT-qPCR in granulosa cells isolated from immature, eCG treated mice of the indicated genotypes. Expression of each transcript was normalized to the housekeeping gene *Rpl19*. Control = corresponding genotype lacking the *Amhr2*^{cre} allele. Values for each experimental genotype are expressed relative to their corresponding control. n=4 animals per genotype, columns = means, error bars = SEM, * = values are statistically different (P<0.05), ** = values are statistically different (P<0.01).

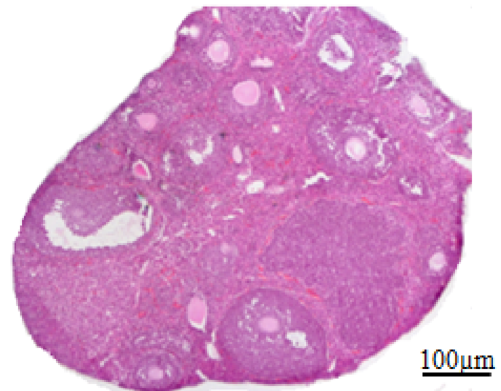
Figure 3.

A) Hematoxylin and eosin

Wnt5a$^{flox/-}$

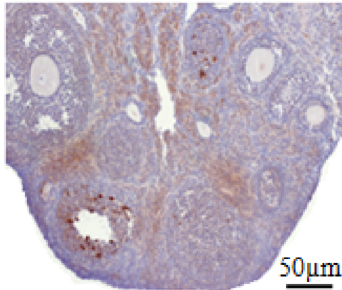


Wnt5a$^{flox/-}$;*Amhr2*$^{cre/+}$

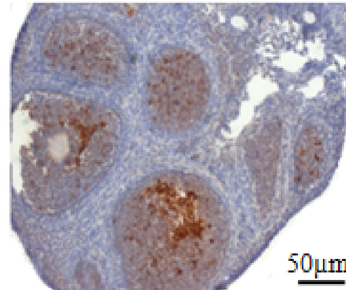


B) Caspase-3 immunohistochemistry

Wnt5a$^{flox/-}$



Wnt5a$^{flox/-}$;*Amhr2*$^{cre/+}$



Negative control

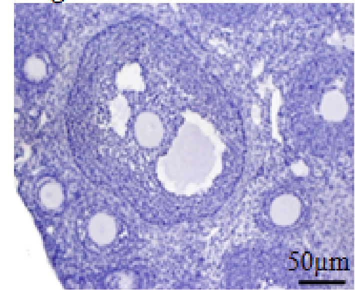
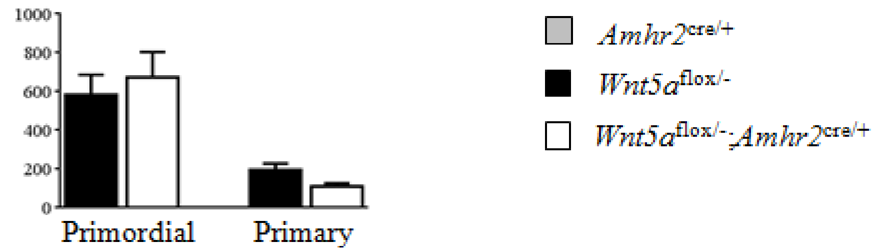


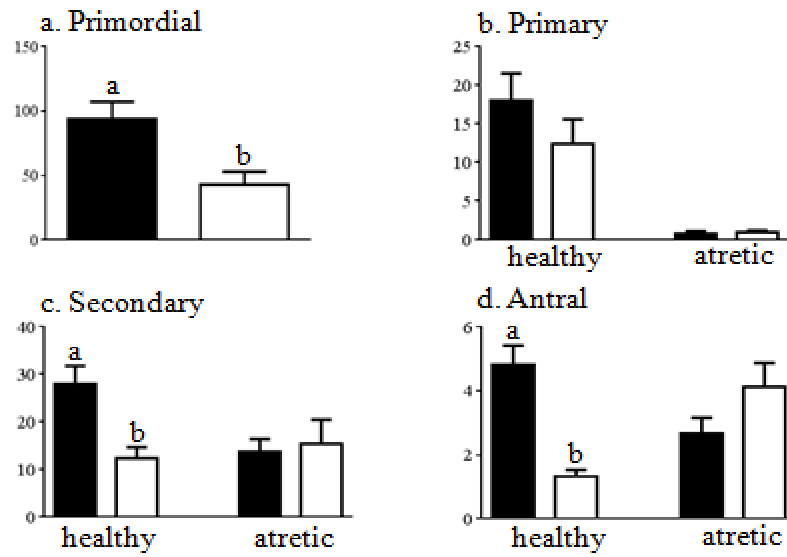
Figure 3. *Wnt*5a$^{flox/-}$;*Amhr2*$^{cre/+}$ mice have smaller ovaries and increased follicular atresia. A) Representative images of *Wnt*5a$^{flox/-}$;*Amhr2*$^{cre/+}$ (control) and *Wnt*5a$^{flox/-}$;*Amhr2*$^{cre/+}$ (mutant) ovaries from 42d-old animals. B) Activated caspase-3 immunohistochemistry done on ovaries from 6wk-old mice of the indicated genotypes. Negative control = control reaction in which the primary antibody was omitted.

Figure 4.

A. 5 days



B. 42 days



C. 8 months

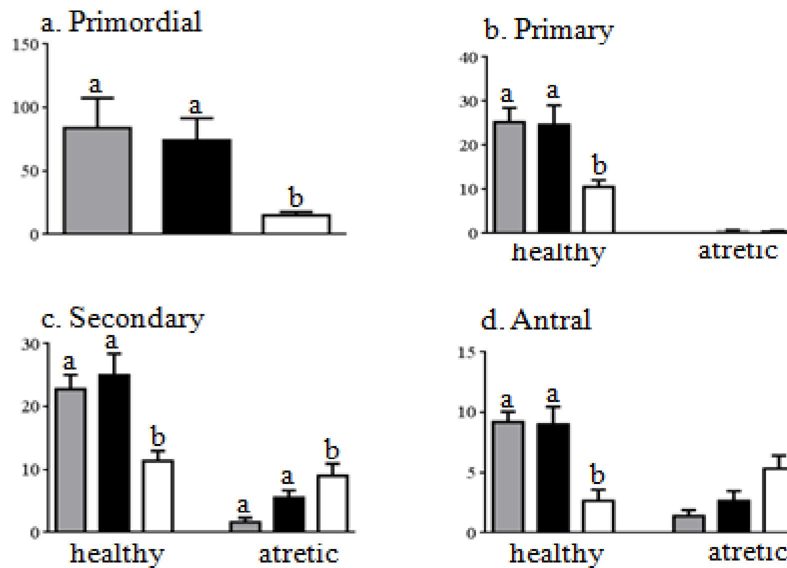


Figure 4. Progressive follicle loss and increased follicular atresia in the ovaries of *Wnt5*a$^{flox/-}$;*Amhr2*$^{cre/+}$ mice. Ovaries (n=6 per genotype at each age) were serially sectioned, and all follicles from every fifth section were counted, categorized as primordial, primary, secondary or antral, and scored as either healthy or atretic. Data represent raw follicle count numbers and were not adjusted to estimate the total ovarian follicle population. Columns = means, error bars = SEM, columns labeled with different letters are significantly different ($P<0.05$).

Figure 5.

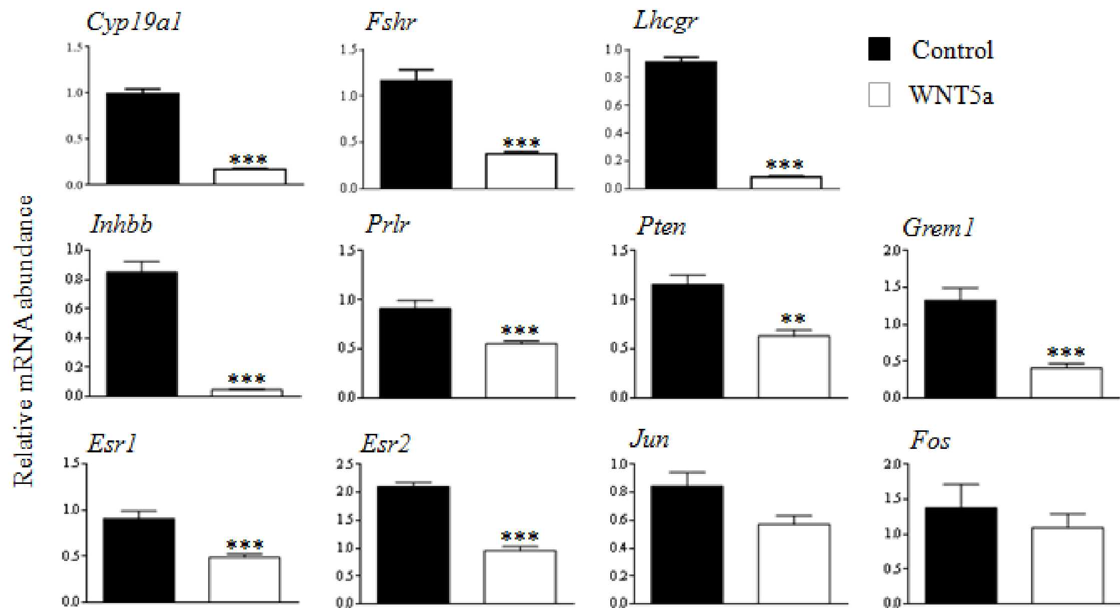


Figure 5. Quantitative RT-qPCR confirmation of microarray data. Primary cultured granulosa cells were treated or not with WNT5a (3.5µg/ml, 3h, n=4 samples/group), and mRNA levels of the indicated genes were determined by RT-qPCR and normalized to the housekeeping gene *Rpl19*. Columns = means, error bars = SEM, asterisks indicate a statistically significant (**: P<0.01, ***: P<0.001) effect of WNT5a treatment.

Figure 6.

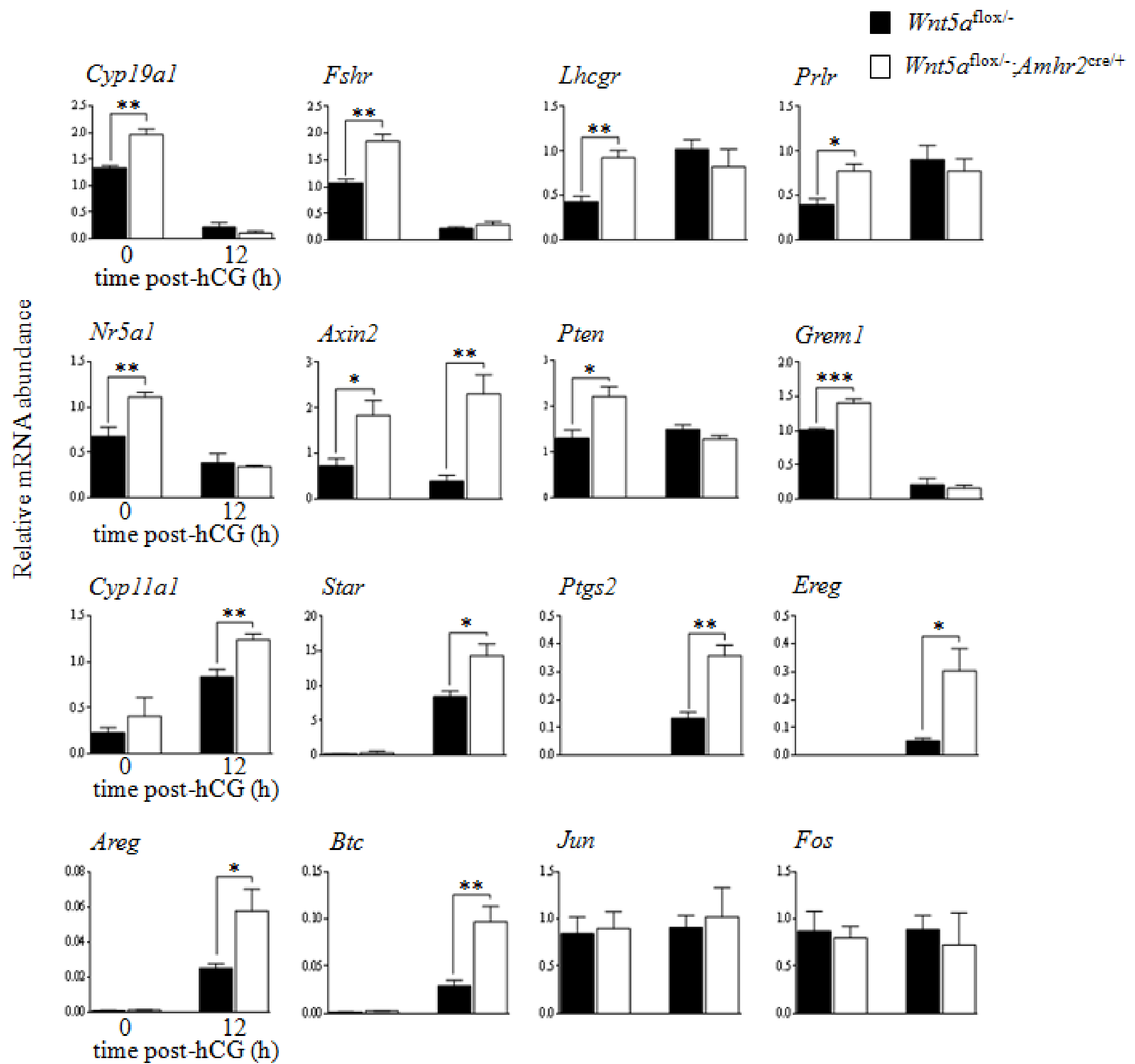


Figure 6. Expression of WNT5a target genes is altered in *Wnt*5a$^{flox/-}$;*Amhr2*$^{cre/+}$ mice. RT-qPCR analysis of the indicated genes was done on granulosa cell samples collected from immature mice of the indicated genotypes either immediately after eCG treatment (48h) or following an additional 12h treatment with hCG (n=4 animal/time point). All data were normalized to the housekeeping gene *Rpl19*. Columns = means, error bars = SEM, asterisks indicate a statistically significant (*: P<0.05, **: P<0.01, ***: P<0.001) difference vs control.

Figure 7.

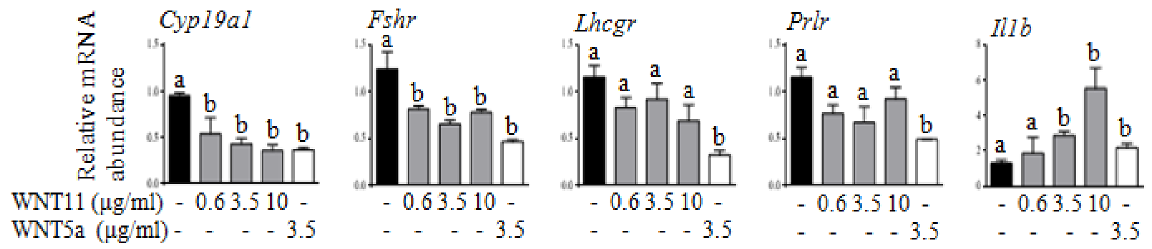


Figure 7. Regulation of WNT5a target genes by WNT11.

Primary cultured granulosa cells were treated or not with WNT11 or WNT5a at the indicated concentrations for 3h (n=4 samples/group), and mRNA levels of the indicated genes were determined by RT-qPCR and normalized to the housekeeping gene *Rpl19*. Columns = means, error bars = SEM, columns labeled with different letters are significantly different (P<0.05).

Figure 8.

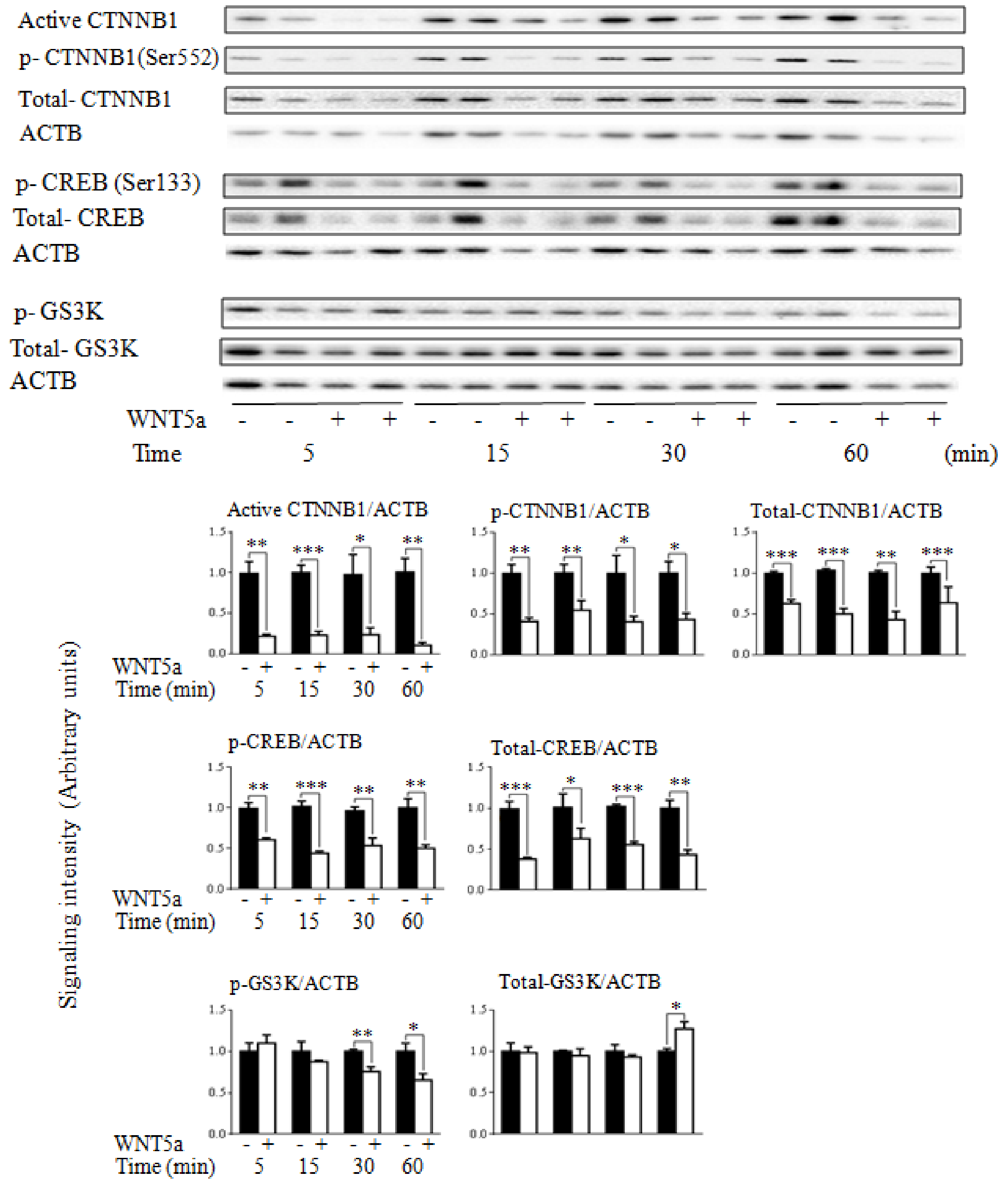


Figure 8. WNT5a downregulates canonical WNT signaling and CREB expression in granulosa cells *in vitro*.

Primary cultured granulosa cells were treated or not with WNT5a (3.5 µg/ml) for the indicated times, and the expression of the indicated proteins and phosphoproteins was studied by immunoblotting. Representative immunoblots showing 2 samples/treatment and time are shown in the upper panels, quantitative analyses of 4 samples/treatment and time are shown in the graphs. Columns = means, error bars = SEM, asterisks indicate a statistically significant (*: $P<0.05$, **: $P<0.01$, ***: $P<0.001$) effect of WNT5a treatment. Values for the WNT5a treated groups are expressed relative to their corresponding controls at each time point.

Figure 9.

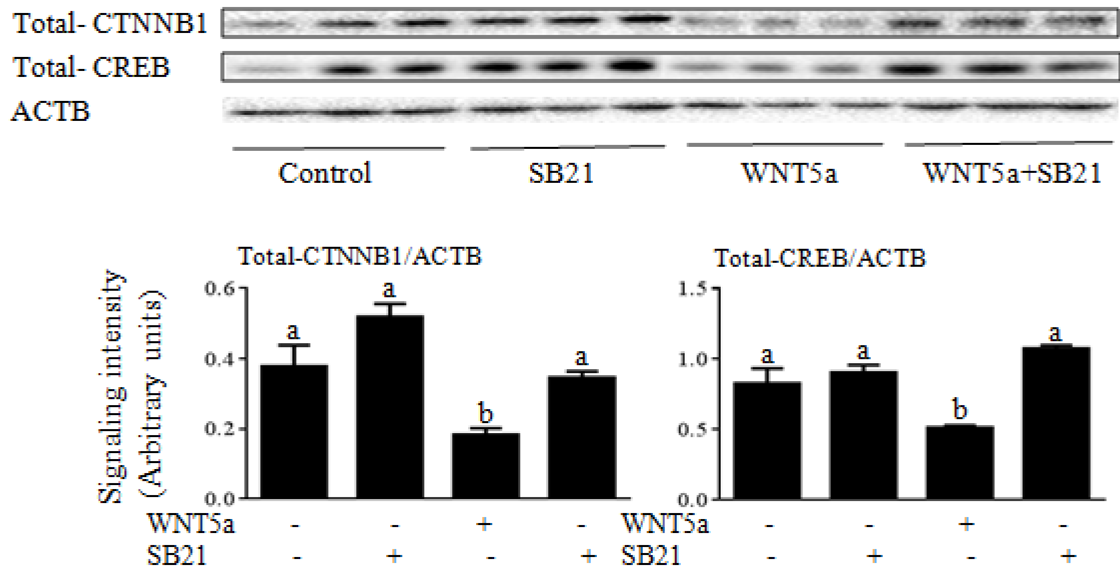


Figure 9. WNT5a downregulation of CTNNB1 and CREB is GSK3β-dependent. Primary cultured granulosa cells were treated with WNT5a (3.5 μg/ml) for 15 minutes, with or without pretreatment with SB216763 (1μM, 1h), and the expression of CTNNB1 and CREB was studied by immunoblotting (n = 3 samples/treatment). Immunoblots are shown in the upper panels, quantitative analyses of which are shown in the graphs. Columns = means, error bars = SEM, columns labeled with different letters are significantly different (P<0.05).

Figure 10.

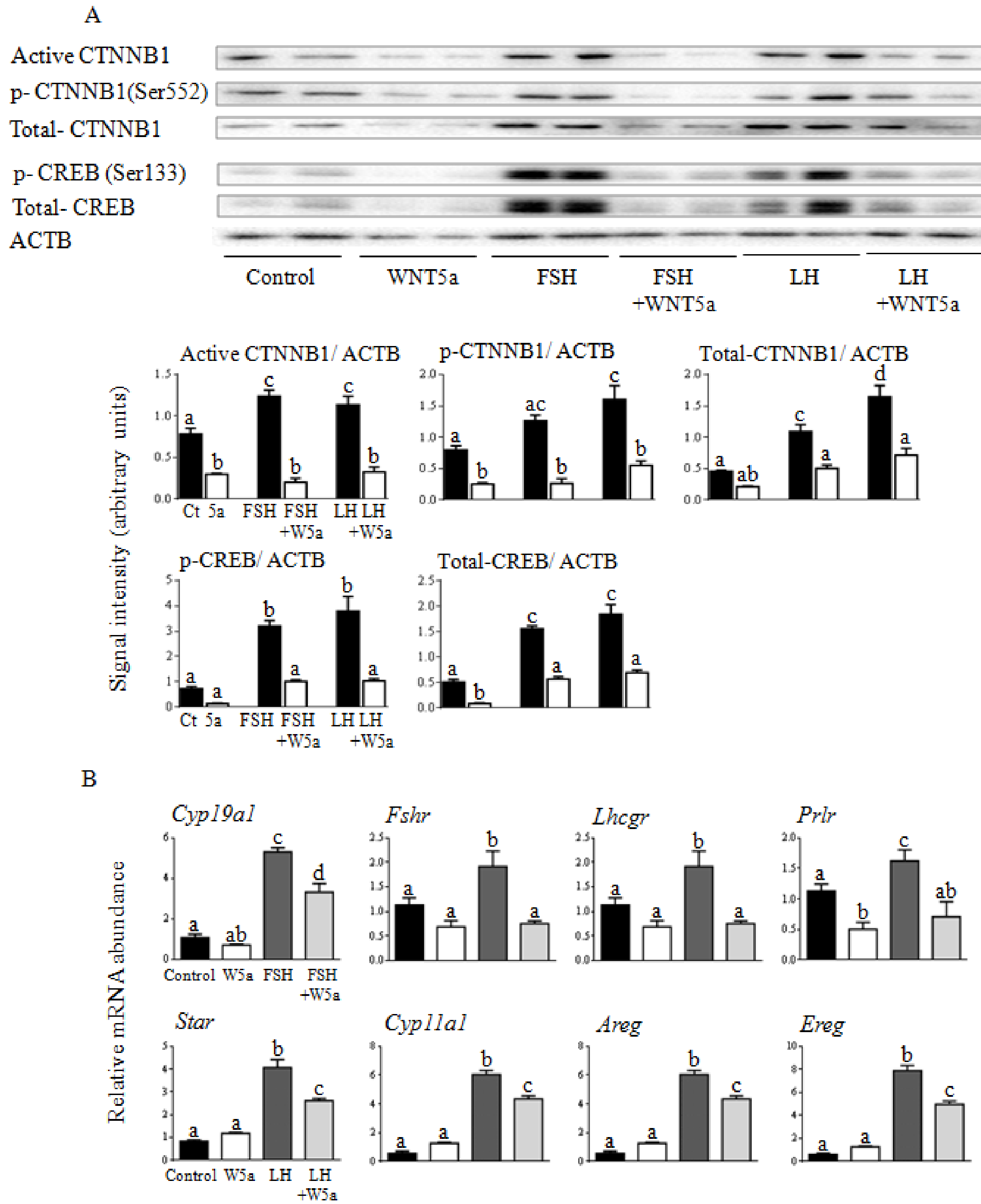


Figure 10. WNT5a suppresses gonadotropin signaling in granulosa cells.

A) Primary cultured granulosa cells were treated or not with LH or FSH (50 ng/ml, 1h), with or without pretreatment with WNT5a (3.5µM, 1h) and the expression of the indicated proteins and phosphoproteins was studied by immunoblotting. Representative immunoblots showing 2 samples/treatment and time are shown in the upper panels, quantitative analyses of 4 samples/treatment and time are shown in the graphs. B) Primary cultured granulosa cells were treated or not with LH or FSH (50 ng/ml, 2h), with or without pretreatment with WNT5a (3.5µM, 1h) and the mRNA levels of the indicated FSH and LH target genes were studied by RT-qPCR. Expression of each transcript was normalized to the housekeeping gene *Rpl19*, n = 4 samples/treatment. Columns = means, error bars = SEM, columns labeled with different letters are significantly different ($P<0.05$).

Figure 11.

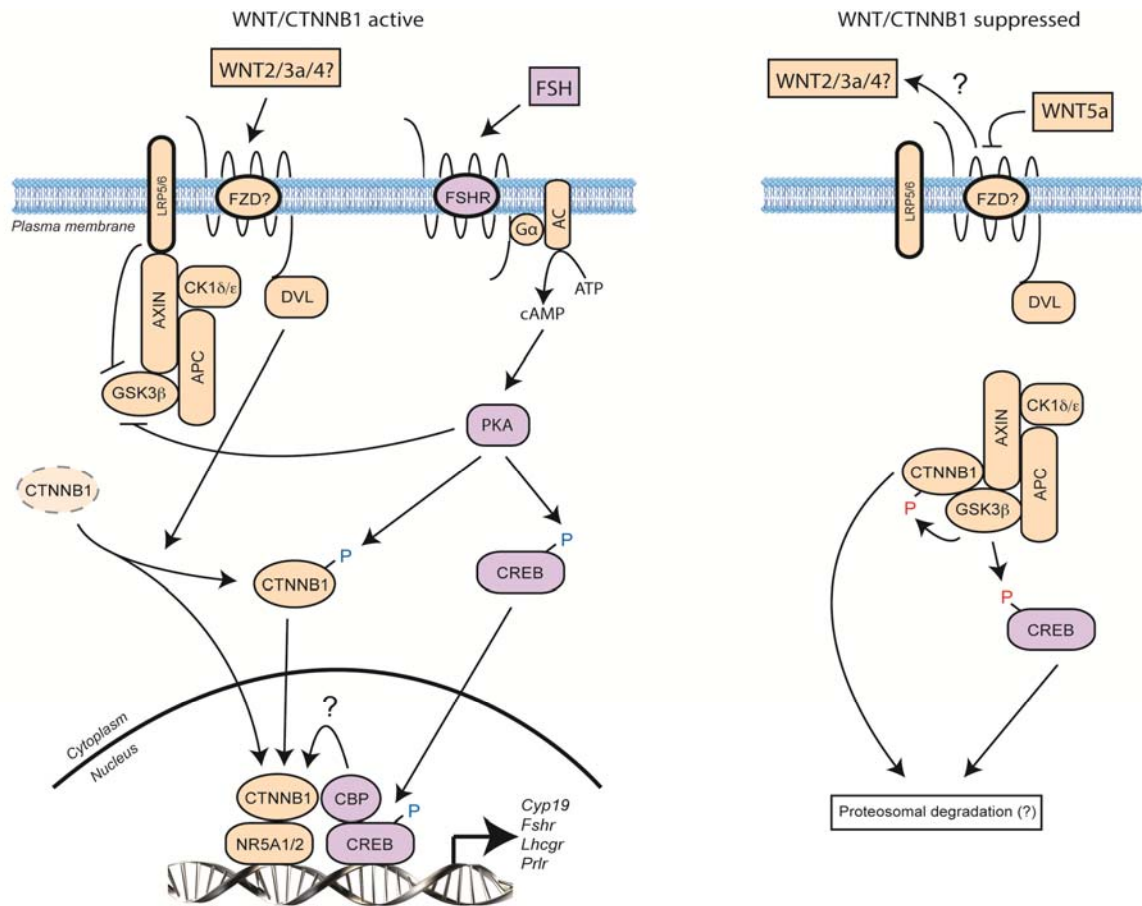


Figure 11. Working model of the mechanism of action of WNT5a in granulosa cells. Left: canonical WNTs act in tandem with gonadotropin/PKA signaling in the granulosa cells of growing follicles to stabilize CTNNB1 and CREB, resulting in the transcriptional activation of CTNNB1 and FSH/LH target genes. Right: WNT5a may antagonize canonical WNTs by competing for their receptor complex. The resultant disinhibition of GSK3β leads to the degradation of CTNNB1 and CREB, thereby antagonizing gonadotropin action.

4.10 Supplemental figures

Figure S1.

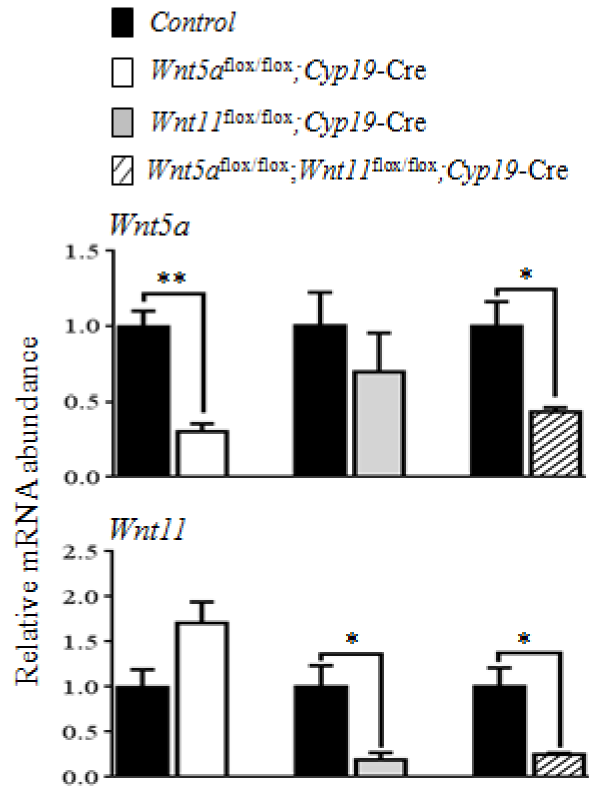


Figure S1. *Wnt5a* and *Wnt11* knockdown efficiency in the conditional knockout models. Expression of *Wnt5a* and *Wnt11* was determined by RT-qPCR in granulosa cells isolated from immature, eCG treated mice of the indicated genotypes. Expression of each transcript was normalized to the housekeeping gene *Rpl19*. Control = corresponding genotype lacking the *CYP19*-cre transgene. Values for each experimental genotype are expressed relative to their corresponding control. n=4 animals per genotype, columns = means, error bars = SEM, * = values are statistically different (P<0.05), ** = values are statistically different (P<0.01).

Figure S2.

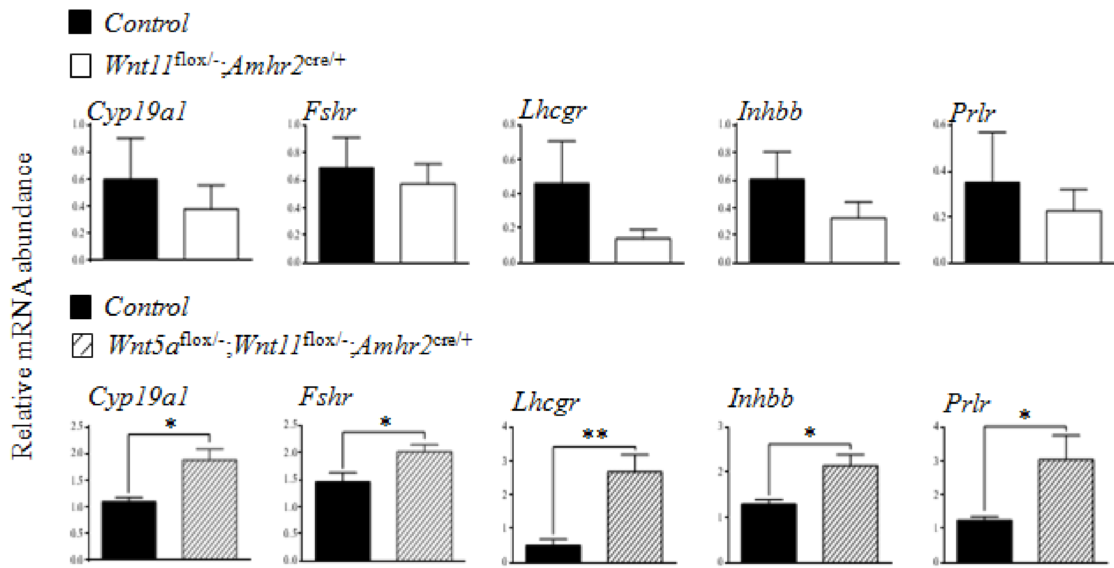
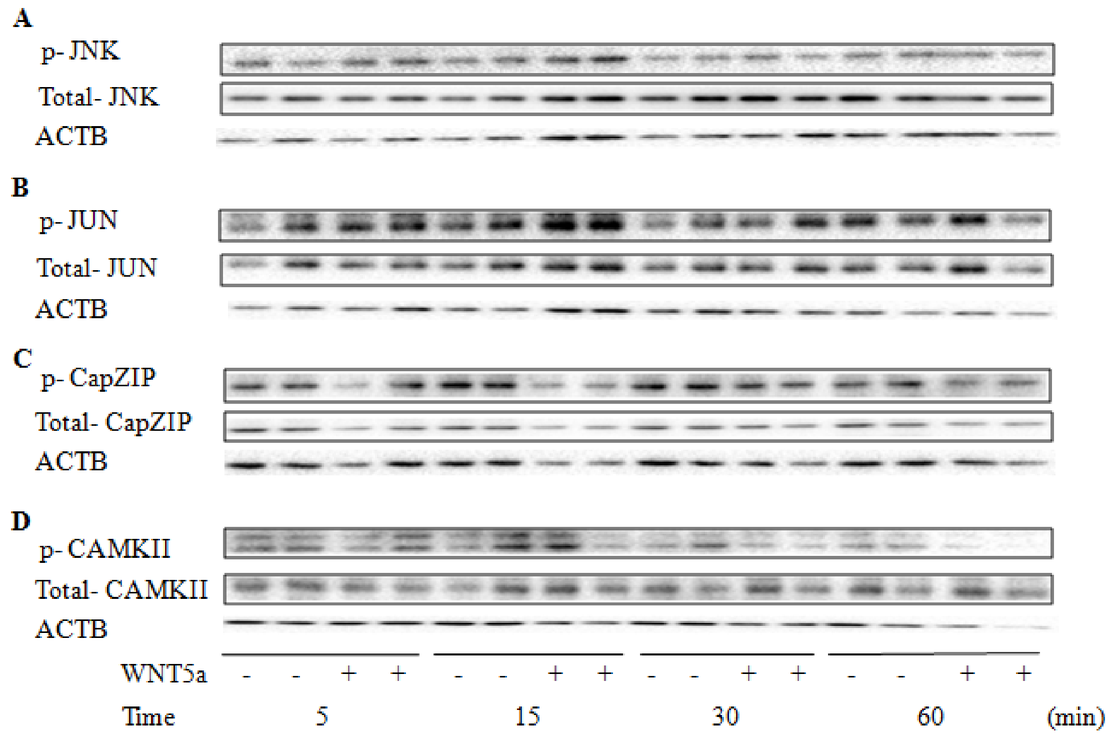


Figure S2. Expression of WNT5a target genes in *Wnt11*$^{flox/-}$;*Amhr2*$^{cre/+}$ and *Wnt*5a$^{flox/-}$;*Wnt11*$^{flox/-}$;*Amhr2*$^{cre/+}$mice.

RT-qPCR analysis of the indicated genes was done on granulosa cell samples collected from immature mice 48h after eCG treatment (n=4 animals/genotype). All data were normalized to the housekeeping gene *Rpl19*. Control = corresponding genotype lacking the *Amhr2*^{cre} allele. Columns = means, error bars = SEM, asterisks indicate a statistically significant (*: $P<0.05$, **: $P<0.01$) difference vs control.

Figure S3.



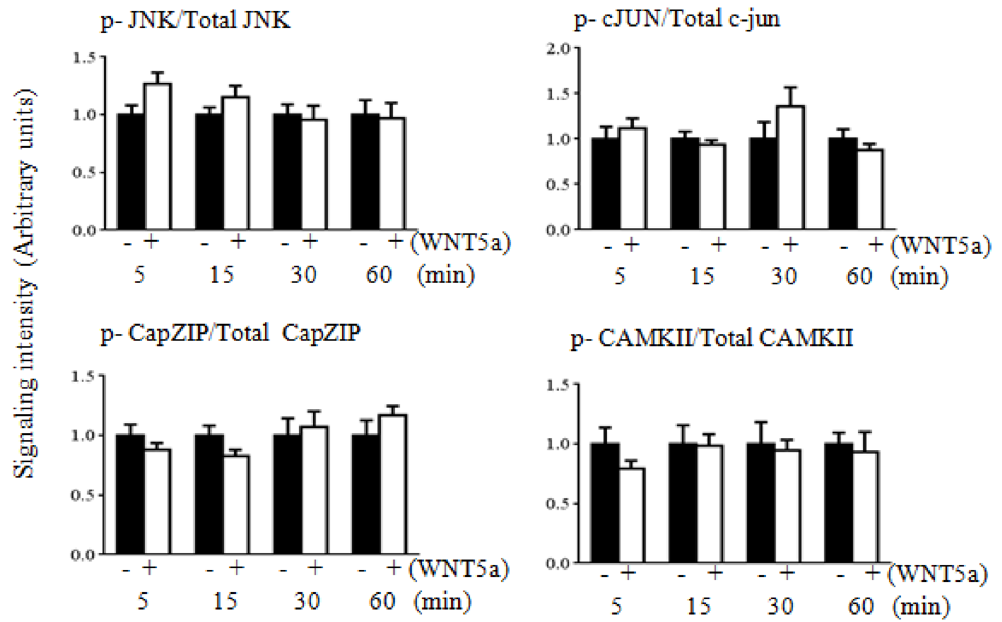


Figure S3. WNT5a does not regulate non-canonical signaling effectors in granulosa cells. Primary cultured granulosa cells were treated or not with WNT5a (3.5 µg/ml) for the indicated times, and the expression of the indicated proteins and phosphoproteins was studied by immunoblotting. Representative immunoblots showing 2 samples/treatment and time are shown in the upper panels, quantitative analyses of 4 samples/treatment and time are shown in the graphs. Columns = means, error bars = SEM, values for the WNT5a treated groups are expressed relative to their corresponding controls at each time point.

Figure S4.

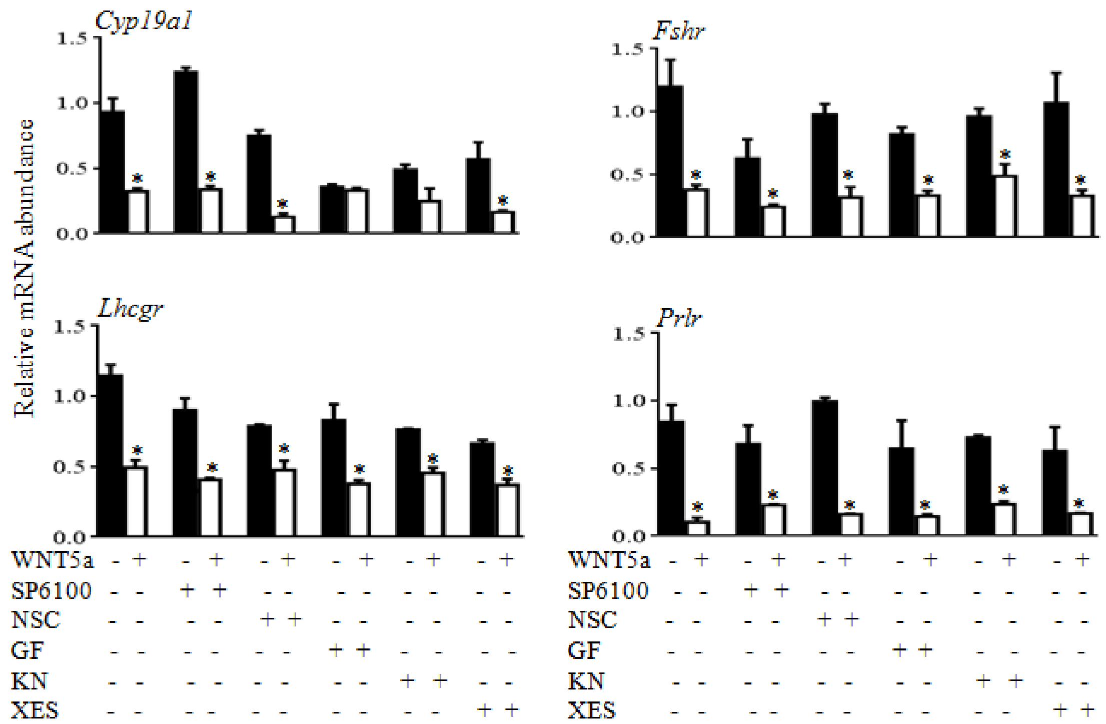


Figure S4. Inhibition of noncanonical signaling does not prevent WNT5a-mediated suppression of its target genes.

Primary cultured granulosa cells were treated with WNT5a (3.5 µg/ml) for 15 minutes, with or without pretreatment with the inhibitors SP600125 (SP6), NSC23766 (NSC), GF109203X (GF), KN-93 (KN) or Xestrospongin C (Xes), and the mRNA levels of the indicated WNT5a target genes were studied by RT-qPCR. Expression of each transcript was normalized to the housekeeping gene *Rpl19*, n = 4 samples/treatment. Columns = means, error bars = SEM, asterisks (*) indicate a statistically significant difference vs control, $P < 0.05$.

4.11 Tables

Table 1. Mating trials

Variable	*Amhr2*$^{cre/+}$	*Wnt5a*$^{flox/-}$	*Wnt5a*$^{flox/-}$;*Amhr2*$^{cre/+}$
Female	6	6	6
Total litters	38	34	24
Total pups	278	282	96
Litter size	7.32±0.30^{a}	8.29±0.32^{a}	4.00±0.38^{b}

Values are means ± SEM.

Values without common superscripts are significantly different (P<0.05).

Table 2. Ovary weights

Variable	*Wnt5a*$^{flox/-}$	*Wnt5a*$^{flox/-}$;*Amhr2*$^{cre/+}$
Immature +eCG		
N	12	12
Weight (mg)	5.53±0.22^{a}	3.83±0.24^{b}
Adult		
N	12	14
Weight (mg)	5.38±0.22^{a}	4.18±0.22^{b}

Values are means ± SEM.

Values without common superscripts are significantly different (P<0.05).

Table 3. Ovulatory rates

Variable	*Wnt5a*$^{flox/-}$	*Wnt5a*$^{flox/-}$;*Amhr2*$^{cre/+}$
Number	4	4
Total COCs	37	15
Litter size	9.25±0.62[a]	3.75±1.93[b]

COCs: Cumulus oocyte complexes

Values are means ± SEM.

Values without common superscripts are significantly different ($P<0.05$).

Table 4. Hormone assays

Variable	*Wnt5a*$^{flox/-}$	*Wnt5a*$^{flox/-}$;*Amhr2*$^{cre/+}$
Immature +eCG (number)		
N	4	4
E2 (pg/ml)	76.08±18.32[a]	197.20±40.79[b]
P4 (ng/ml)	9.55±2.14[a]	1.83±0.25[b]

Values are means ± SEM.

Values without common superscripts are significantly different ($P<0.05$).

4.12 Supplemental Tables

Table S1. Primer sequences

	Forward primer (5′- 3′)	Reverse primer (5′- 3′)
Areg	CTCGCAGCTATTGGCATCGGCA	TGGCATGCACAGTCCCGTTT
Axin2	CCACTTCAAGGAGCAGCTCAGCA	TACCCAGGCTCCTGGAGACTGA
Btc	CTTGCCCTGGGTCTTGCAAT	GAGGGAGTTTGCTCGTCCAC
Cyp11a1	GTGACCTTGCAGAGGTACACTGT	GTGACTCCAGCCTTCAGTTCACA
Cyp19a1	CTGAACATCGGAAGAATGCACAG	GAGTAGATAGGCCACACTTCTTC
Ereg	ACGTTGCGTTGACAGTGATTCTCAT	GGTCCCCTGAGGTCACTCTCTCAT
Esr1	CGTGTGCAATGACTATGCCTCT	TGGTGCATTGGTTTGTAGCTGG
Esr2	GTCAGGCACATCAGTAACAAGGG	ATTCAGCATCTCCAGCAGCAGGTC
Fos	AGCCAAGTGCCGGAATCGGA	AGGTCGGTGGGCTGCCAAAA
Fshr	TGGATGTCATCACTGGCTGTGTCA	ATTCTGGAAGGCCTCAGGGTTGAT
Grem1	CTGGAGACCCAGAGTACCGT	TGCGATTCATTCTGTCACTTCC
Il1b	TAGGCTCATCTGGGATCCTCT	TCATTCTCATCACTGTCAAAAGGTG
Inhbb	AGATCATCAGCTTTGCAGAGACA	GCCTGCACCACGAATAGGTT
Jun	AACCGCATTGCCGCCTCCAA	TCCCTGAGCATGTTGGCCGT
Lhcgr	AGATGCACAGTGGCACCTTCCAG	ATGACGTGGCGATGAGCGTCT
Nr5a1	ACCTGGCGGTAGATGTGGTCCAA	TGTCGACTGGGCACGAAGGT
Prlr	ATACTGGAGTAGATGGGGCCAGGAG	CTTCCATGACCAGAGTCACTGTCAGGAT
Pten	GCCAAATTTAACTGCAGAGTTGC	ACAGTGAATTGCTGCAACATGA
Ptgs2	TCCCCTTCCTGCGAAGTTTA	TAGAAGAACCTTTTCCAGCACTTC
Star	GATTAAGGCACCAAGCTGTGCTG	CTGCTGGCTTTCCTTCTTCCAGC
WNT5a	GTCTTCCAAGTTCTTCCTAATGGCT	CCAGGTTGTTATAGAAGCTAACTTG
Wnt11	GAGGCTGTGCTCTTTGCC	CTGCCGGAGTCTTGGACAGT

Table S2. Mating trials

Variable	*Cyp19*	*Wnt5a*$^{flox/flox}$	*Wnt5a*$^{flox/flox}$;*Cyp19*-Cre
Female	5	6	6
Total litters	30	36	33
Total pups	219	257	189
Litter size	7.31±0.48^{a}	7.13±0.42^{a}	5.73±0.36^{b}

Values are means ± SEM.

Values without common superscripts are significantly different (P<0.05).

Table S3. Mating trials

Variable	*Wnt11*$^{flox/-}$	*Wnt11*$^{flox/-}$;*Amhr2*$^{cre/+}$
Female	5	6
Total litters	28	33
Total pups	199	247
Litter size	7.11±0.24	7.48±0.49

Values are means ± SEM.

Values without common superscript are significantly different (P<0.05).

Table S4. Mating trials

Variable	*Wnt11*$^{flox/flox}$	*Wnt11*$^{flox/flox}$*;Cyp19*-Cre
Female	6	6
Total litters	40	40
Total pups	289	306
Litter size	7.23±0.16	7.65±0.38

Values are means ± SEM.

Values without common superscript are significantly different (P<0.05).

Table S5. Mating trials

Variable	*Control*	*Wnt5a*$^{flox/-}$;*Wnt11*$^{flox/-}$; *Amhr2*$^{cre/+}$	*Wnt5a*$^{flox/flox}$;*Wnt11*$^{flox/flox}$;Cyp19-Cre
Female	5	7	6
Total litters	31	26	34
Total pups	240	127	241
Litter size	7.74±0.37[a]	4.88±0.82[b]	7.09±0.47[a]

Values are means ± SEM.

Values without common superscript are significantly different (P<0.05).

Chapter 5. General discussion and conclusion

5.1General discussion

Infertility is an important issue that affects many couples from all over the world. Beside human society and medicine, infertility is also a major concern for the agriculture industry, and is a source of major economic losses. Achieving a better understanding of basic molecular and cellular events that control follicle development should lead to improvements in infertility treatments, such as *in vitro* fertilization.

Recent descriptive studies have demonstrated the regulation of canonical WNTs and WNT signaling component in the adult ovary. Gonadotropins have been shown to regulate expression of several members of WNT signaling family, suggesting the importance of WNT members during follicle development (Hsieh et al., 2005, Hsieh et al., 2002, Hsieh et al., 2003, Ricken et al., 2002). However, little attention has been paid to the role of non-canonical WNTs in the ovary. Different animal models provide a comprehensive approach for determining the mechanisms underlying follicle development and fertility. For the first time, we investigated the function of WNT5a (a well known non-canonical WNT) in the granulosa cells of two different mammalian species, the mouse and the cow, taking advantage of the strengths and offsetting the weaknesses of each model. This research helps to increase basic knowledge about the roles of non-canonical WNT signaling, in the hopes of increasing our understanding of infertility issues in human and large animal species. Many aspects of follicle development in the cow render this species a good model for the study of ovarian function in women. Humans and cattle are both mono-ovular species with similar patterns of follicular wave emergence. In addition, wave numbers during the menstrual cycle and dominant follicle selection and ovulation processes appear to be similar (Baerwald et al., 2003). Pritpal et al. have shown that the cow is a valid model to study reproductive aging in women (Malhi et al., 2005).

Furthermore, milk production is a major agricultural industry in Canada, mainly in Ontario and Québec (www.dairyinfo.gc.ca). The dairy industry is presently suffering a major decline in fertility, which has been attributed to decreased follicle and oocyte quality due to increasing milk production over the last two decades (Buckley et al., 2014, Lucy, 2007b).

The development of transgenic mouse technology has expanded our understanding of ovarian physiology and follicle development, which can help to uncover possible causes of human fertility disorders. Using the mouse as an animal model has several advantages such as short generation time, a large number of pups per litter, immediate post partum estrus after giving birth, economy of mouse husbandry and the ability to manipulate the mouse genome with methods like transgenesis and knockouts (Jorgez et al., 2005).

WNT5a is a representative example of non-canonical WNT members, and has been extensively studied in different cell types. WNT5a is conserved in a wide variety of species from *C. elegans* to human and it is known to regulate many aspects of cellular functions including cell proliferation, differentiation, polarity, adhesion and migration. A loss of function of *WNT5a* (*Wnt5a*$^{-/-}$) resulted in perinatal lethality and a complex phenotype due to an inability to extend the embryonic anterior-posterior and proximal-distal axes in outgrowth tissues (Yamaguchi et al., 1999). Recent reports suggested that postnatal abnormalities in *Wnt5a* signaling could cause cancers, inflammatory diseases, and metabolic disorders (Kikuchi et al., 2012). In addition, meiosis is completely inhibited in the ovary of WNT4/5a double mutant embryos, indicating the importance of WNT5a in female germline development before birth (Kikuchi et al., 2012, Naillat et al., 2010).

In mammals, 19 different WNT proteins have been described (Hsieh et al., 2002, Ricken et al., 2002). Due to recent publications describing the roles of WNT4 and WNT2

in rodent granulosa cells, we expected these two WNTs to play important roles in the cow ovary as well. However, the expression level of *WNT4* and *2* transcripts was very low in bovine granulosa cells, in contrast with *WNT4* expression in human luteinized granulosa cells and rodent ovaries (Hsieh et al., 2003, Sanchez et al., 2014) and *WNT2* expression in bovine granulosa cells cultured under luteinizing conditions and rodent granulosa cells (Castanon et al., 2012c, Ricken et al., 2002). Differences in WNT4/2 expression between species may reflect differences in the role of WNTs and may also be due to differences in the stages of follicle development and differentiation between studies. In a previous bovine study (Castanon et al., 2012c), granulosa cells were cultured under luteinizing conditions, which was in contrast with the non-luteinized system used in the present study. Indeed, cells in this model can respond to physiological doses of FSH and IGF1 and produce estrogen, mimicking growing and early antral stage follicles (Gutierrez et al., 1997, Silva and Price, 2000). To assess *Wnt5a* mRNA levels in the mouse, we used an immature mouse model in which animals were treated with (FSH-mimetic) eCG for 48h, followed or not by (LH-mimetic) hCG. Our results showed that graded doses of FSH could decrease *WNT5a* mRNA and protein levels in cultured bovine granulosa cells; however, *Wnt5a* mRNA abundance increased in response to gonadotropins throughout follicle development in the mouse model. Beyond species differences, these contradictory results may also be due to the very different nature of the samples that were compared: the bovine granulosa cells were cultured from growing secondary and antral follicles over 6 days *in vitro*, whereas the mouse granulosa cells were freshly isolated from preovulatory follicles. The absence of oocytes in the bovine culture system may also have altered the *WNT5a* response to gonadotropins, due to the loss of interaction between germ cells and somatic cells.

To evaluate WNT5a function *in vivo*, we used a conditional knock out approach. We used the well established *Amhr2* and *Cyp19* Cre-*loxP* models to circumvent the embryonic lethality of *Wnt5a*$^{-/-}$ mice and eliminate WNT5a expression specifically in granulosa cells. *Amhr2-Cre* mice express Cre in granulosa cells of all stages of follicle development (Fan and Richards, 2010), whereas Cre expression in *Cyp19-cre* mice is limited to antral follicles (Stocco, 2008a). With the two granulosa cell-specific knockout models we could study the role of WNT5a in a follicle stage-dependent manner.

In the cow model we found that *WNT5a* mRNA abundance was reduced in dominant follicles relative to subordinate follicles, suggesting a link between WNT5a expression and follicle atresia. In addition, our results showed that estradiol secretion (a marker of healthy follicles) was significantly reduced when cultured cells were challenged with WNT5a. However, when we knocked out *Wnt5a* in mouse granulosa cells, we found that the proportion of antral follicles undergoing atresia increased considerably, as did serum estradiol levels. Our results obtained in cow model can be attributed to the reduction in *CYP19A1* and *FSHR* mRNA levels, which are responsive to FSH (Luo and Wiltbank, 2006, Silva and Price, 2000). Inhibition of the estrogenic pathways is a characteristic of atretic follicles (Grimes and Ireland, 1986, Rodgers and Irving-Rodgers, 2010), and therefore we expected WNT5a to play a role in atresia. Atresia of bovine follicles is associated with reduced proliferation and increased death of granulosa cells, which may occur through apoptotic or nonapoptotic pathways (Irving-Rodgers et al., 2001). However, in the cow model, WNT5a did not increase the rate of apoptosis or alter the cell cycle *in vitro*, suggesting that, by itself, WNT5a is not able to induce apoptosis in FSH-stimulated granulosa cells *in vitro*.

How to explain our finding that the knockout of *Wnt5a* in granulosa cells can cause apoptosis? One possibility is that the increase in estradiol synthesis with higher expression of *Cyp19a1* and *Fshr* can enhance secondary follicle development and early differentiation of granulosa cells, in a manner that is asynchronous with the development of the oocyte. Follicle development requires a coordination of growth and differentiation of the germ cells and somatic cells (Carabatsos et al., 2000). Loss of this coordination could result in the loss of gap junctions and connections between the somatic and germ cells, ultimately leading to follicle atresia. However, changes in gap junction components and follicular cell polarity caused by WNT5a loss were not studied in our model and remain to be elucidated. Our microarray data derived from cultured mouse granulosa cells indicated that the mRNA levels of many genes related to late stages of follicle development, oocyte maturation and cumulus expansion were up- or down-regulated in response to WNT5a, therefore a second possibility is that atresia in the *Wnt5a* conditional knockout is the result of oocyte development defects. Based on our results in the cow and in the mouse, loss of WNT5a appears to be a consequence of atresia rather than a cause.

As mentioned above, WNT5a activates the CTNNB1-independent pathways (Kikuchi et al., 2012, Zhu et al., 2014). Based on our results in article 1, WNT5a is able to activate the MAPK8 signaling pathway in bovine granulosa cells. However, in article 2, we were not able to find any significant alteration in the PCP and WNT/Ca^{2+} signaling cascades in the *Wnt5a* conditional knockout mouse model. These results showed that WNT5a can activate different signaling pathways according to species, cell type and probably stage of follicle growth. An important consideration is that the cow study was conducted using granulosa cells extracted from small antral follicles, whereas the mouse granulosa cells were isolated from immature mice treated with eCG, which promotes

follicle development to the preovulatory stage. Therefore, there is a possibility that the difference we observed in these two models arises from the different stages of follicle development that were studied. It seems likely that the results that we obtained in mice are mostly related to the role of WNT5a in ovulation, whereas in cow they relate to secondary and early antral stages of follicle development. Additional studies of the role of WNT5a in early stages of follicle development in the mouse model will be required to better clarify the potential role of WNT5a at the secondary to antral transition or even earlier in follicle development. Importantly, in addition to non-canonical signaling, non-canonical WNTs act to suppress canonical WNT signaling (Pourreyron et al., 2012, Sato et al., 2010). In both the bovine and murine primary cultured GC models, we were able to see a drastic reduction in CTNNB1 expression in response to WNT5a, indicating that this is a mechanism of WNT5a action that is conserved across species and throughout follicle development. Different mechanisms have been proposed to explain how WNT5a can suppress canonical signaling. FZDs are the receptors of both canonical and non-canonical WNTs, resulting in a competition between them to bind to these receptors (Bryja et al., 2009, Mikels and Nusse, 2006). WNT5a can increase SIAH ubiquitin ligase expression, resulting in the degradation of CTNNB1 (Topol et al., 2003). The other possible mechanism is that WNT5a can also activate retinoic acid-related orphan nuclear receptor α (RORα) via PKC, which inhibits the canonical pathway most likely by inhibiting coactivator recruitment to the promoters of target genes (Lee et al., 2010). If any or all of these mechanisms mediate the suppression of CTNNB1 in response to WNT5a in granulosa cells remains to be determined.

This basic research suggests potential applications for WNT5a for the treatment of ovarian disorders and cancer. Altered *Wnt5a* expression has been linked to ovarian cancer

(Ford et al., 2014, McDonald and Silver, 2009). It has been shown that activation of the WNT/β-catenin pathway causes granulosa cell tumors (GCTs) (Boerboom et al., 2005). As mentioned above, some studies had reported that WNT5a can also act as an antagonist of canonical WNTs (Blagodatski et al., 2014). Therefore, WNT5a may have a therapeutic use to suppress canonical WNT signaling in patients with GCT.

Premature ovarian failure (POF) is a condition which the ovarian reserve is depleted prematurely. POF has a spontaneous origin and a genetic etiology (Kuo et al., 2011). One of the major phenotypes of *Wnt5a*$^{flox/-}$;*Amhr2*$^{cre/+}$ mice was a depletion of the ovarian reserve that resembles POF. A recent study determined that failure in *Wnt5a* signaling inhibited meiosis in the ovary during embryonic development (Naillat et al., 2010). Together, these results showed the critical importance of *Wnt5a* not only during embryogenesis, but also after birth and in the adult. *Wnt5a* can control follicle development and survival, and seems to coordinate *Wnt5a* granulosa cell and oocyte growth in the developing follicles. Therefore, *Wnt5a* may play a role in POF, however we need to investigate its exact role.

The present study increases our knowledge of non-canonical WNT signaling in granulosa cells, however further studies are needed to determine if WNT5a coordinates granulosa cell and oocyte growth. Moreover, the potential role of WNT5a in processes such as ovulation, oocyte maturation, cumulus expansion, luteinization, steroidogenesis and proliferation has to be investigated.

An important area of future investigation will be to determine the relevance of our findings to human ovarian disorders. The role of WNT5a in ovarian cancer, POF and other dysfunctions has to be investigated. In addition, the potential of using WNT5a as a therapeutic agent to control canonical WNT signaling has to be determined.

5.2 Conclusion:

In conclusion, the present study provides novel insight into the role of WNT5a and its mechanisms of action in two different animal models. For the first time, our data demonstrated that WNT5a is essential for normal follicle development. We determined that WNT5a negatively regulates steroidogenesis by antagonizing canonical WNT signaling. Our results reveal the essential role of a non-canonical WNT during follicular development and identify a novel pathway that regulates gonadotropin action in granulosa cells.

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