Université de Montréal

IDENTIFICATION AND CHARACTERIZATION OF THE TRANSCRIPTIONAL TARGETS OF THE WNT/β-CATENIN SIGNALING PATHWAY IN GRANULOSA CELLS

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Mémoire présenté à la Faculté de médecine vétérinaire

en vue de l'obtention du grade de
maître ès science (M.Sc)
en science vétérinaires
option reproduction

Juillet, 2009

Université de Montréal

Faculté des études supérieures et postdoctorales

Ce mémoire intitulé

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présenté par

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a été évalué par un jury composé des personnes suivantes :

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RÉSUMÉ

Les Wnts représentent une famille de glycoprotéines de signalisation qui sont connues pour les nombreux rôles qu'ils jouent durant le développement embryonnaire et dans la cancerogénèse. Plusieurs Wnts, leurs récepteurs (*Fzd*) et d'autres composants des voies de signalisation des Wnt sont exprimés dans l'ovaire postnatal, et il a été démontré que l'expression de certains de ces gènes est régulée pendant le développement et l'ovulation/luteinization folliculaires. Toutefois, leurs rôles physiologiques dans l'ovaire demeurent mal définis. Pour étudier le rôle de WNT4 dans le développement folliculaire, nous avons entrepris d'identifier ses cibles transcriptionnels dans les cellules de la granulosa. Pour ce faire, nous avons employé la souris *Catnb*$^{flox(ex3)/flox(ex3)}$, chez laquelle une activation constitutive de la voie de Wnt/β-catenin a lieu suite à l'action de la recombinare Cre. Des cellules de la granulosa de ces souris ont été mises en culture et infectées avec un adenovirus pour causer la surexpression de WNT4 ou l'expression de Cre. L'ARN a alors été extrait de ces cellules et analysé par micro-puce. Les résultats ont démontré qu'une forte proportion des gènes induits par WNT4 étaient des gènes impliqués dans la réponse cellulaire au stress. Presque tous gènes induits par WNT4 ont également été induits par Cre, indiquant que WNT4 signale via la voie Wnt/β-catenin dans ces cellules. Nos résultats suggèrent donc que WNT4 favorise la survie des follicules par l'induction de gènes de réponse au stress dans les cellules de la granulosa, augmentant ainsi la résistance cellulaire à l'apoptose.

Mots-clés : Développement folliculaire, Cellules de la granulosa, WNT4, β-catenin, Micro-puce.

ABSTRACT

The Wnts comprise a large family of local-acting, secreted glycoprotein signaling molecules that are known mostly for the numerous roles they play in embryonic development and cancer. Several Wnts, their cognate receptors of the Frizzled (*Fzd*) family and other components of the Wnt signaling pathways are expressed in the postnatal ovary, and several have been shown to exhibit specific patterns of regulation in response to gonadotropin stimulation. Nonetheless, their role(s) in ovarian physiology remain poorly defined. To study the role of WNT4 in follicle development, we endeavoured to identify its transcriptional targets in granulosa cells. To this end, we used the *Catnb*$^{flox(ex3)/flox(ex3)}$ mouse model, in which constitutive activation of the Wnt/β-catenin pathway is obtained following Cre-mediated genetic recombination. Cultured granulosa cells from these mice were infected with adenoviruses to either overexpress WNT4 or to express Cre. RNA from these cells was then extracted and subjected to microarray analysis. Results revealed that a large proportion of the genes induced by WNT4 were genes previously shown to mediate cellular stress responses. Nearly all genes that were up-regulated by WNT4 were also induced by the Cre, indicating that WNT4 signals via the Wnt/β-catenin pathway in these cells. Our findings suggest that WNT4 mediates ovarian follicle survival by inducing a stress response in granulosa cells, thereby increasing their resistance to apoptosis.

Key Words: Follicle development, Granulosa cells, WNT4, β-catenin, Microarray

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LIST OF ABBREVIATIONS

AMH	anti mullerian hormone	Car8	carbonic anhydrase 8
APC	adenomatous polyposis coli	DSH	dishevelled
Amh	anti mullerian receptor	DWnt2	*Drosophila* Wnt gene 2
Ad	adino viruses	ES	embryonic stem cells
BMP15	bone morphogenetic protein15	EREG	EGF-like growth factor epiregulin
BTC	betacellulin	EGF	epidermal growth factor
CL	corpus luteum	eGFP	enhanced Green fluorescent proteins
CC	cumulus cells		
COC	cumulus-oocyte complex	GTP	guanosine triphosphate
cAMP	cyclic adenosine monophosphate	GDF9	growth differentiation factor-9
cDNA	complementary deoxyribonucleic acid	GnRH	gonadotropin-realising
		GSK-3	glycogen synthase kinase-3
CamKII	calcium/calmodulin regulated kinase II	FSH	follicle stimulating hormone
Cyp11a1	cholesterol side-chain cleavage enzyme	FST	follistatin
CRD	cysteine-rich domain	FOXO	forkhead box O
Catnb1	mouse beta-catenin1	Fzd	frizzled
CTNNB1	human beta-catenin1	hCG	human chrionic gonadotropin
Cre	cyclic REcombinase		

JNK	c-jun N-terminal kinase	qRT-PCR	quantitative real time polymerase chain reaction
KitL	kit ligand		
KO	knock out		
KGF	keratinocyte growth factor	RT-PCR	reverse transcription polymerase chain reaction
LH	luteinizing hormone		
ROS	reactive oxygen species		
LHR	luteinizing hormone receptor	RNA	ribonucleic acid
		SCF	stem cell factor
LRP	low density lipoprotein	SFRP	soluble frizzled related protein
LEF	lymphoid enhancer factor		
LIF	leukemia inhibitory factor	TGF-β	transforming growth factor-β
LoxP	locus of X-over P1		
MMTV	mouse mammary tumor	TCF	t-cell factor
MGC	mural granulosa cells	TSH	thyroid stimulating
MIS	müllerian inhibitory substance hormone	20-α-HSD	20-α-hydroxysteroid
		3β-HSD	3β-hydroxysteroid
NR5A1	orphan nuclear receptor steroidogenic factor-1	XFzd7	Xenopus Frizzled 7
		Wg	wingless
PRL	prolactin	Wnt4	wingless-type MMTV integration site family member 4
PRL-R	prolactin receptor		
PKC	protein kinase C		
PCP	planar cell polarity	Wnt2	wingless-type MMTV integration site family member 2
PR	progesterone receptor		
Pthlh	parathyroid hormome-peptide		

Acknowledgements

I would like to express profound gratitude to my director, Dr Derek Boerboom, for his invaluable support, encouragement, supervision and useful suggestions throughout this research work. His support, patience and continuous guidance enabled me to complete my work successfully. I appreciate his vast knowledge and skill in many areas.

I am highly thankful to my lab members Alexandre Boyer, Evelyne Lapointe, Marie-Noëlle Lague, Céline Forget and Zheng Xiaofeng for their help and friendship over the one and a half years, and to all the members of CRRA, especially Dr Bruce Murphy, Dr Christopher A. Price, Micheline Sicotte and Micheline St-Germain.

I sincerely thank the members of the jury, Dr Christopher A. Price and Dr Younès Chorfi, for their valuable suggestions throughout this study.

I would also like to thank my father and my family for the support they provided me throughout my entire life.

I. Introduction

Ovarian folliculogenesis is regulated by both endocrine and intraovarian mechanisms that coordinate the processes of oocyte growth and somatic cell proliferation and differentiation. Within the follicle, surrounding granulosa cells are critical for normal follicle development and function. This project focuses on the role of secreted glycoprotein signaling molecules Wnts that are known mostly for the numerous roles they play in embryonic development and cancer (Logan and Nusse 2004, Lustig and Behrens 2003). Particular emphasis is given to study the role of WNT4 in follicle development in granulose cells. The first indication of the importance of Wnt signaling in ovarian processes came from a study by Vainio et al (1999), which reported that inactivation of the *Wnt4* gene in mice results in the loss of a large fraction of the reserve of oocytes in the days prior to birth.

A recent descriptive study has shown Wnt4 to be expressed postnatally in follicular granulosa cells and corpora lutea (Hsieh et al 2002). Furthermore, constitutive activation of the Wnt/b-catenin pathway in granulosa cells *in vivo* results in differentiation defects and the formation of granulosa cell tumors (Boerboom 2005, Boerboom, 2006). These observations suggested to us that Wnt4 could be a somatic cell factor involved in some aspect of follicle development. Unfortunately, proof of this could not be obtained from the Wnt4 knockout mouse, which dies shortly after birth due to kidney defects. We aimed to determine the role of Wnt4 in ovarian follicular development by conditional gene targeting in granulosa cells, and to define the mechanism of Wnt4 action by identifying its downstream regulatory targets using an *in vitro* microarray-based approach. Our results demonstrated that Wnt4 regulates the expression of a series of genes associated with the cellular stress response. We therefore propose that Wnt4 acts to induce an adaptive response that is critical for granulosa cell survival during the later stages of follicle growth.

II. Literature Review

1. Ovarian follicular development

The ovary is a functional unit involved in the production of mature eggs and endocrine homeostasis, and the ovarian follicle plays a central role in female reproductive physiology. In recent years, considerable progress has been made towards elucidating the complex intraovarian control mechanism that, in concert with systemic signals, coordinate the recruitment, selection and growth of follicles from the primordial stage through to ovulation and corpus luteum formation.

1.1 Functional anatomy of the ovary

1.1.1 The ovarian follicle

The ovarian follicle is composed of an external layer of theca cells in continuity with the ovarian stroma. Separated from the theca layer by a basal lamina, mural granulosa cells are the major epithelial component of the follicle. During follicular growth, oocytes assume a central position in this structure and are surrounded by, and in close contact with, replicating granulosa cells. With the development of the antral cavity, the somatic granulosa cells surrounding the oocyte, termed cumulus cells, detach from the mural layer and become phenotypically distinct (1).

From the morphologic point of view, ovarian follicles may be classified in three major groups**:** 1) Primordial follicles, 2) Growing follicles, and 3) Graafian follicles (Figure 1).

Primordial follicles consist of 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. Primordial follicles lack a distinct vasculature. A discrete follicular capillary bed develops later in the growing follicles. The number of primordial follicles that undergo folliculogenesis to reach the mature, graafian stage from puberty on is only a small

fraction of the number of follicles available in the pool of primordial follicles. Most follicles will either undergo a process of regression called atresia at some stage of folliculogenesis or remain as primordial follicles with no sign of growth (1,53). Growing follicles are follicles that have left the resting stage as primordial follicles and have begun growth, but have not yet developed a theca layer or antrum (cavity). A growing follicle is characterized as developing two or more layers of granulosa cells surrounding the oocyte. With continued growth, additional layers of granulosa cells appear to surround the oocyte. A zona pellucida surrounding the oocyte may be seen at this stage. Graafian follicles are follicles in which an antrum is clearly visible. By the end of the follicular phase the ruptured follicle will develop into the corpus luteum (1,54).

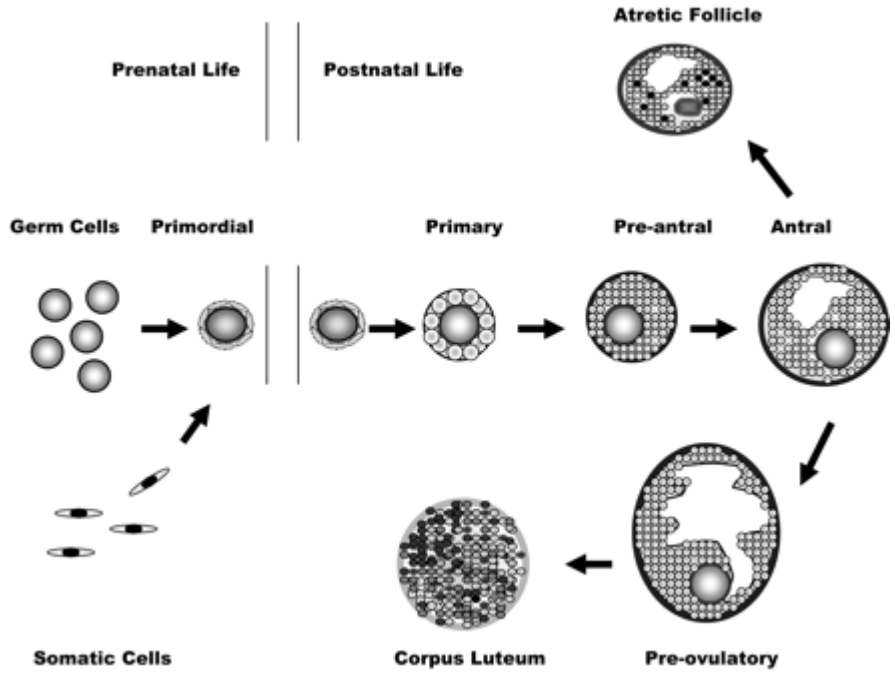

Figure 1. Ovarian follicle development (Mouse). Folliculogenesis begins with the establishment of a finite pool of primordial follicles. Primordial follicles must grow to the primary, pre-antral and antral stages before they reach the pre-ovulatory stage and are capable of releasing an oocyte for fertilization. After ovulation, the remaining granulosa and thecal cells differentiate into a structure known as the corpus luteum (CL). At the antral stage, however, 99.9% of antral follicles will undergo atresia (follicle death). (Reproduced from reference 2)

1.1.1.1 The oocyte

The oocyte, the female gamete, develops within the confines of the ovarian follicle, which provides a chemical and physical microenvironment for its growth and maturation. The oocyte is in a quiescent stage of meiosis in the primordial follicle, arrested at the diplotene stage or dictyate stage. In the Graafian follicle, it is in the stage of a primary oocyte, and is surrounded by a glycoprotein layer, the zona striata, or zona pellucida. The nucleus of such an oocyte is called a germinal vesicle. During pre-ovulatory phase, meiosis I is completed, and the oocyte is now called a secondary oocyte (1,53).

Within the follicle, paracrine communications between the oocyte and surrounding granulosa cells are critical for normal cell development and function. Recent studies have revealed the particular role of the granulosa cell-derived KitL, and its interactions with the oocyte-specific growth differentiation factor-9 (GDF9) and bone morphogenetic protein-15 (BMP15/GDF9b) (Figure 2) (3). In rodents, both GDF9 and BMP15 have been shown to promote proliferation of granulosa cells from small antral follicles, and BMP15 has been reported to inhibit FSH-stimulated progesterone production, and is an inhibitor of luteinization. Evidence from studies with *Gdf9*$^{-/-}$ mice and granulosa cell cultures indicate that GDF9 suppresses expression of both KitL-1 and KitL-2. In contrast, BMP15 promotes KitL expression in monolayers of granulosa cells from rat early antral follicles and enhances the expression of both KitL-1 and KitL-2 mRNA in mouse oocyte-granulosa cell complexes grown *in vitro*. FSH is known to regulate BMP15 expression in a dose-dependent manner via Kit signaling, and Kit signaling promotes both oocyte growth and cell survival (3).

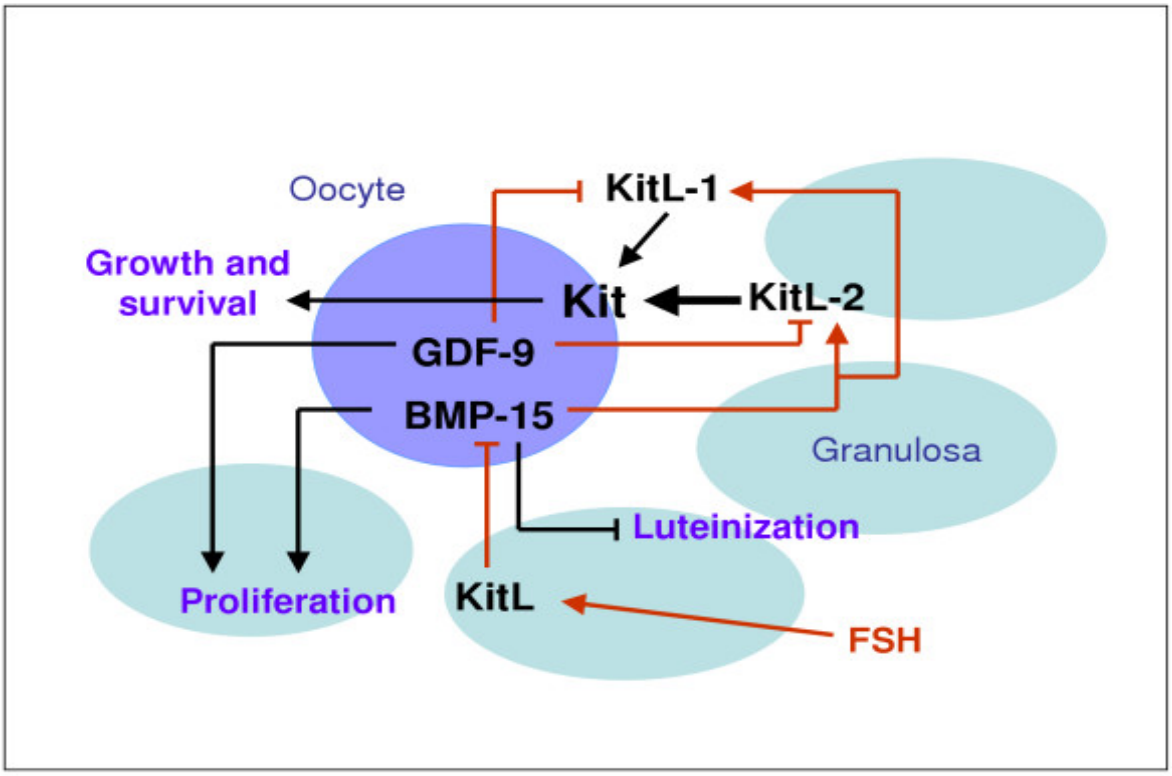

Figure 2. GDF9, BMP15 and KitL interactions during rodents oocyte and follicular development. Black lines indicate actions; red lines indicate effects on mRNA expression. The thick black arrow denotes the relative importance of KitL-2 in activating Kit receptors to promote oocyte growth. (Reproduced from reference 3)

1.1.1.2 Theca interna

Two layers of theca cells, theca interna and theca externa, are discernible, and together with the granulosa cells, form the wall of the follicle. The theca interna is formed by ovarian fibrocytes and stromal cells as the follicle matures. These cells undergo differentiation into epithelioid cells, rich in granules and cytoplasmic organelles. Theca cells express receptors for luteinizing hormone (LH). LH initiates the production of androgens by the theca cells, most notably androstendione, which are aromatized by granulosa cells to produce estrogens, primarily estradiol (1). Two growth factors of the TGF-β family, bone morphogenetic proteins -4 and -7, are expressed by ovarian theca cells and have recently been implicated as positive regulators of the primordial-to-primary follicle transition (4).

1.1.1.3 Granulosa and cumulus cells

As follicles grow and an antrum is formed, granulosa cells separate into two sub-types: the cumulus granulosa cells (CC), those surrounding and in intimate metabolic contact with the oocyte; and the mural granulosa cells (MGC), the cells lining the follicle wall forming a stratified epithelium with the basal lamina. The granulosa cells produce the sex steroids, as well as the growth factors such as Inhibin, Activin, BMP-2, BMP-5, BMP-6 and AMH (antimullerian hormone) that are thought to interact with the oocyte and theca cells during its development. Also, FSH stimulates granulosa cells to convert androgens (coming from the thecal cells) to estradiol by aromatase. After ovulation the luteinizing granulosa cells produce progesterone (5).

The highly specialized cumulus cells have trans-zonal cytoplasmic processes, which penetrate through the zona pellucida and abut the oocyte membrane, forming the cumulus-oocyte complex (COC) which allows the transfer of molecules that are necessary for oocyte

growth and development (5). Cumulus cells have a high rate of proliferation, low steroidogenic capacity, and low LHR expression, but have the capacity to secrete hyaluronic acid and undergo expansion. Cumulus expansion is induced as a consequence of the LH surge and is dependent on the induction of specific genes as cyclooxygenase-2 (5).

1.1.2 The ovarian stroma

The bulk of the ovary, both cortex and medulla, consists of stroma in which the follicles are interspersed. Ovarian stroma consists of peculiar spindle-shaped stromal cells similar to fibroblasts and arranged into a characteristic whorled texture. Unlike ordinary connective tissue, ovarian stroma is highly cellular while supporting fibers (both reticular fibers and collagen) are inconspicuous. The outside (peritoneal) surface of the ovary is covered not by a standard serosa with simple squamous mesothelium but rather by a peculiar simple cuboidal-to-columnar mesothelium. This mesothelium is sometimes unhelpfully called "germinal epithelium" although it does not contain germ cells (1).

1.2 The ovarian cycle

The ovarian life cycle begins with the formation of a primordial germ cell that becomes an egg, is fertilized, and then becomes a new female organism in which the events of the ovary life cycle are repeated (Figure 3). The life of the ovary has four major development phases: embryogenesis, folliculogenesis, ovulation, and luteogenesis (6).

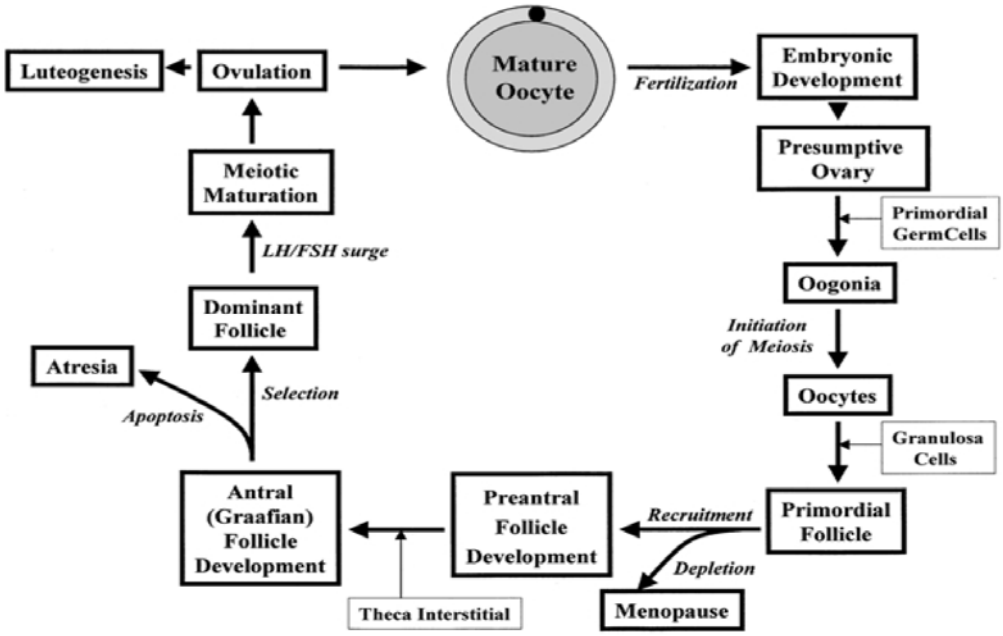

Figure 3. The ovarian life cycle in mammals. Embryogenesis is the phase whereby populations of primordial germ cells and somatic cells become an ovary containing oocytes and granulosa cells located within primordial follicles. This is followed by folliculogenesis in which oogenesis, granulosogenesis, and thecogenesis occur as a recruited primordial follicle grows and develops to preovulatory stage or it dies by atresia. Next, ovulation is the phase whereby the oocyte transforms into a mature egg, which is secreted into the oviduct to await fertilization. Finally, during luteogenesis, the follicle, lacking an oocyte, luteinizes into an endocrine structure, the corpus luteum, which if implantation does not occur, dies by a process termed luteolysis. (Reproduced from reference 6)

1.2.1 The follicular reserve

In most mammals, the ovarian endowment of primordial follicles (ovarian reserve) is fully established before or slightly after birth. The perinatal ovary is endowed with several million primordial follicles that are in a growth-arrested state. Only a fraction of these survive and are recruited into the growing follicle population. The precise mechanisms that 're-awaken' these growth-arrested follicles remain to be elucidated, although it has been recognized that the rate at which primordial follicles join the growing pool is positively related to the size of the ovarian reserve. This observation is compatible with the notion of intraovarian signalling between growing follicles, resting follicles and ovarian stroma. Certainly, there is compelling evidence that bi-directional communication between the oocyte and surrounding granulosa cells (epithelial-derived), and granulosa cells and thecal-interstitial cells (stromal/mesenchymal-derived) is obligatory for normal follicle recruitment and development (4). Growth of primordial follicles is generally thought to be FSH-independent, although a recent study found that FSH increases primordial follicle reserve in hypogonadal mice.This findings revealed that in the absence of endogenous gonadotrophins, promotes an increase in primordial follicle reserve despite also stimulating follicular growth in mature females (50). The assembly of the primordial follicles and transition of the primordial follicle to the primary follicle are critical processes in ovary. These processes directly affect the number of oocytes and inappropriate coordination of these processes contributes to premature ovarian failure (POF) and infertility. The pre-granulosa cells that surround the oocyte in primordial follicles express a number of peptide factors, including kit ligand (KL, also known as stem cell factor, SCF) and leukemia inhibitory factor (LIF), bone morphogenic proteins (BMP's), keratinocyte growth

factor (KGF) and basic fibroblast growth factor (bFGF), that have been shown *in vitro* to promote the transition of primordial follicles into primary follicles, stimulate oocyte growth and the recruitment and proliferation of theca cells from the surrounding stromal tissue. Endocrine factors such as progesterone have also been identified that influence follicular assembly. Müllerian inhibitory substance (MIS) can inhibit the primordial to primary follicle transition (51). The receptor for KL (c-kit) is expressed by oocyte and thecal-interstitial cells, enabling them to respond to this growth factor.

1.2.2 Recruitment and growth

The term recruitment used by different investigators to describe two distinct decision points during follicle development. The dormant primordial follicles are *recruited* into the growing follicle pool in a continuous manner, whereas increases in circulating FSH during each reproductive cycle *recruit* a cohort of antral follicles. During initial recruitment, intraovarian and/or other unknown factors stimulate some primordial follicles to initiate growth, whereas the rest of the follicles remain quiescent for months or years. Initial recruitment is a continuous process that starts just after follicle formation, long before pubertal onset. In contrast, cyclic recruitment starts after pubertal onset and is the result of the increase in circulating FSH during each reproductive cycle that rescues a cohort of antral follicles from atresia. LH levels may reduce the number of resting follicles. In transgenic mice overexpressing a long-acting LH, primordial follicles are lost from the resting pool more rapidly than controls (7,52). Follicle progression through the antral stage of development is associated with continued proliferation of granulosa and theca cells, increased thecal vascularisation, further oocyte enlargement and a relatively rapid increase in diameter and volume. The increasing size and histotypic complexity of the follicle

imposes limits on the diffusion-dependent transfer of secreted signalling molecules between cells in different intrafollicular compartments (4). Primordial follicles undergo initial recruitment to enter the growing pool of primary follicles (Figure 4). The duration required for this step is unknown. In the human ovary, during cyclic recruitment, increases in circulating FSH allow a cohort of antral follicles to escape apoptotic demise. Among this cohort, a leading follicle emerges as dominant by secreting high levels of estrogens and inhibins to suppress pituitary FSH release. The result is a negative selection of the remaining cohort, leading to its ultimate demise.

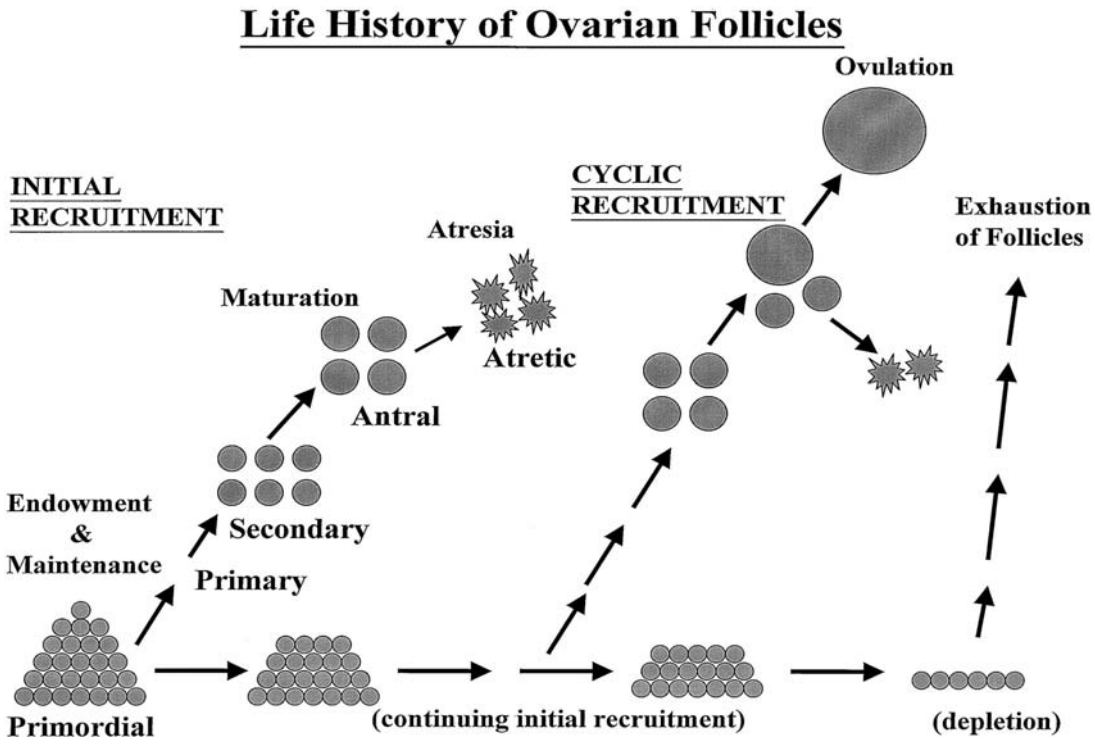

Figure 4. Life history of ovarian follicles in mammals: endowment and maintenance, initial recruitment, maturation, atresia or cyclic recruitment, ovulation, and exhaustion. A fixed number of primordial follicles are endowed during early life, and most of them are maintained in a resting state. Growth of some of these dormant follicles is initiated before and throughout reproductive life (initial recruitment). Follicles develop through primordial, primary, and secondary stages before acquiring an antral cavity. At the antral stage most follicles undergo atresia; under optimal gonadotropin stimulation that occurs after puberty, a few of them are rescued (cyclic recruitment) to reach the preovulatory stage. Eventually, depletion of the pool of resting follicles leads to ovarian follicle exhaustion and senescence. (Reproduced from reference 7)

Concomitantly, increases in local growth factors and vasculature allow a positive selection of the dominant follicle, thus ensuring its final growth and eventual ovulation. In the rodent, the duration of follicle development is thought to be much shorter than in women. The time required between the initial recruitment of a primordial follicle and its growth to the secondary stage is more than 30 days, whereas the time for a secondary follicle to reach the early antral stage is about 28 days in the rat (7).

1.2.3 Ovulation

Ovulation is the stage in the ovarian cycle in which the oocyte breaks free from the Graafian follicle by causing a self inflicted wound and floating out of the ovary. The production of LH and FSH by the anterior pituitary allows for the secondary follicle to grow. The primary oocyte is arrested in prophase I. When development of the secondary follicle is complete, an increase in LH causes the primary oocyte to complete meiosis I. Meiosis II starts in the oocyte in the graafian follicle, and metaphase II occurs before ovulation. In the pre-ovulatory phase, the ovarian follicle will undergo a series of transformations called cumulus expansion; this is stimulated by the secretion of FSH. After this is done, a hole called the stigma will form in the follicle, and the ovum will leave the follicle through this hole. LH increases prostaglandin levels causing local smooth muscular contractions at the base of follicle. This allows for the oocyte to be pushed out of the follicle and the follicular contents are released (1,8).

1.2.4 Luteinization

Luteinization is essential to the success of early gestation. It is the process by which elements of the ovarian follicle, usually including both theca interna and granulosa cells, are provoked by the ovulatory stimulus to develop into the corpus luteum. Although there are

significant species differences in luteinization, some elements pervade, including the morphological and functional differentiation to produce and secrete progesterone. Following ovulation, the granulosa cells remaining in the collapsed follicle and thecal cells carried from the theca interna by invading capillaries begin to hypertrophy, take on lipid material, and become the lutein or luteal cells of mature corpora lutea. The corpus luteum is a temporary endocrine organ which, for most of the domestic species, functions for only a few days in the cycling nonpregnant animal. During the diestrual phase of the cycle, the corpus luteum produces maximal amounts of progesterone. The corpus luteum is followed by regression to the corpus albicans by the end of diestrus in domestic species (1). In rodents, PRL is known to be required for the normal function of the CL throughout pregnancy. In PRL or PRL-R knock-out mice, ovulation and fertilization occur normally, the CL forms, but the mice are sterile because uterine decidualization and embryo implantation fail to take place PRL enhances progesterone production via the suppression of 20α-hydroxysteroid dehydrogenase (20α-HSD) expression (55). In cultured rat luteal cells, both PRL and TGF-ß suppressed 20α-HSD activity. The suppressive effect of PRL was attenuated by a TGF-ß antibody, indicating that the luteotrophic action of prolactin is at least in part mediated by TGF-ß. Furthermore, human granulosa lutein cells treated with TGF-ß1 showed a significant reduction in apoptosis. Therefore, it appears that TGF-ß isoforms in concert with prolactin support the CL by suppressing apoptosis (4,55).

1.3 Hormonal regulation of follicular development

FSH is an essential survival hormone for the prevention of the programmed demise of early antral follicles in rodents. FSH and LH are unlikely to exert direct actions on primordial follicles because functional gonadotropin receptors have not yet developed in them. FSH is

also important in the final differentiation of granulosa cells in antral and pre-ovulatory follicles to allow the biosynthesis of estrogens and to prepare the pre-ovulatory follicles for ovulation (8). FSH receptors exist primarily on the granulosa cell to stimulate follicular growth. FSH stimulates LH receptor expression in granulosa cells, and activates the aromatase and 3β-hydroxysteroid dehydrogenase (3β-HSD) enzymes by increasing cAMP (cyclic adenosine monophosphate) (Figure 5). LH acts on the theca to produce androgens (androstenedione and testosterone), which are then transported to the granulosa cells, where they are aromatized to estrogens (estrone and estradiol). Concomitant with increased estrogen production, mitosis is stimulated in granulosa cells, augmenting cell number. The rising concentration of estradiol exerts a negative feedback effect on FSH release from the pituitary, which halts the development of all the other follicles so that they become atretic (8). In granulosa cells primed by exposure to large amounts of estradiol and FSH, LH acts synergistically with FSH to increase LH receptors and induces luteinization of the follicle, thereby increasing progesterone production. LH also stimulates prostaglandin synthesis by intracellular production of cAMP (8). Prostaglandins play a role in follicle rupture, since the prostaglandin content of pre-ovulatory follicles increases at the time of the gonadotropin surge. Progesterone augments the activity of proteolytic enzymes, which act together with prostaglandins to promote follicular degradation and rupture.

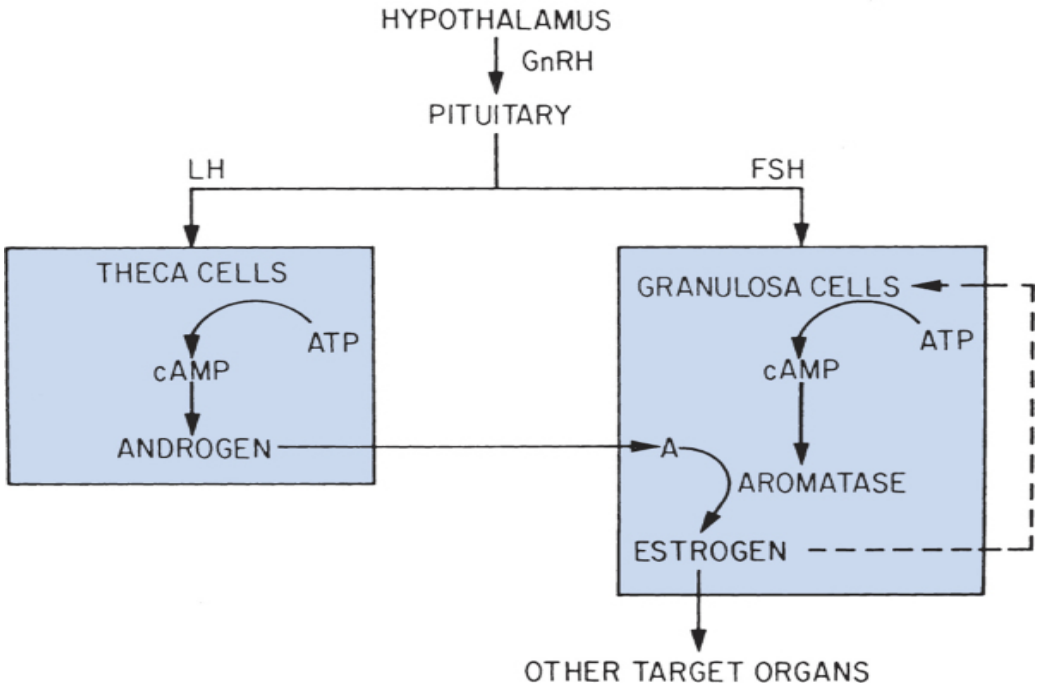

Figure 5. Action of gonadotropins on ovary: LH stimulates theca cells to synthesize androgen by cyclic-AMP (cAMP)-mediated action. FSH stimulates granulosa cells to activate aromatase via cAMP-mediated action. Aromatase in granulosa cells converts androgen to estrogen, which is then utilized by target organs. Estrogen also stimulates granulosa cell proliferation. (Reproduced from reference 9)

1.3.1 The gonadotropins

The hypothalamic releasing and release-inhibiting hormones are substances that either stimulate or inhibit the release of adenohypophyseal hormones. These factors pass via the hypophyseal portal system to influence the release of hormones of the adenohypophysis. The vertebrate gonadotropin-releasing hormone (GnRH) is a decapeptide involved in regulating reproduction. The release of GnRH from the hypothalamus regulates the production of gonadotropins in the pituitary and these gonadotropins are responsible for gonadal development and growth in vertebrates. Two hormones from the adenohypophysis affect the gonads: follicle-stimulating hormone and luteinizing hormone (1,9).

1.3.1.1 Follicle stimulating hormone

Follicle-stimulating hormone (FSH) is a glycoprotein hormone synthesized and secreted by gonadotropes in the anterior pituitary gland. Its structure is similar to those of LH, TSH (thyroid stimulating hormone), and hCG (human chorionic gonadotrophin). FSH has a beta subunit, which confers its specific biologic action and is responsible for interaction with the FSH-receptor. The sugar part of the hormone is composed of fucose, galactose, mannose, galactosamine, glucosamine, and sialic acid, the latter being critical for its biologic half-life. FSH regulates development, growth, pubertal maturation, and reproductive processes. In both males and females, FSH stimulates the maturation of germ cells. In females, FSH initiates follicular growth, specifically affecting granulosa cells. With the concomitant rise in inhibin B, FSH levels decline in the late follicular phase. This is to be critical in selecting only the most advanced follicle to proceed to ovulation in domestic species. At the end of the luteal phase, there is a slight rise in FSH that is important to start the next ovulatory cycle.

FSH release at the pituitary gland is controlled by pulses of gonadotropin-releasing hormone (GnRH) (Figure 6). Those pulses, in turn, are subject to estrogen feed-back from the gonads (1,9). During the estrous cycle before ovulation small amounts of estrogene (E2) are secreted from the ovary. Estrogen stimulates the release of GnRH from the hypothalamus and luteinisation hormone (LH) from the anterior pituitary. GnRH also stimulates release of LH from the anterior pituitary. GnRH, is released and transported to the anterior pituitary in a pulsatile manner, where it binds to specific high-affinity receptors and regulates gonadotropin biosynthesis and secretion. The stimulation of gonadotropin biosynthesis and secretion by Gn RH is dependent on the pulsatile nature of GnRH delivery to the anterior pituitary. At higher GnRH pulse frequencies, LH secretion increases more than FSH secretion (56). The GnRH pulses frequencies shown to regulates the expression of the gonadotropine subunit genes LHß *in vivo* by increased the messenger RNA (mRNA) levels (57).

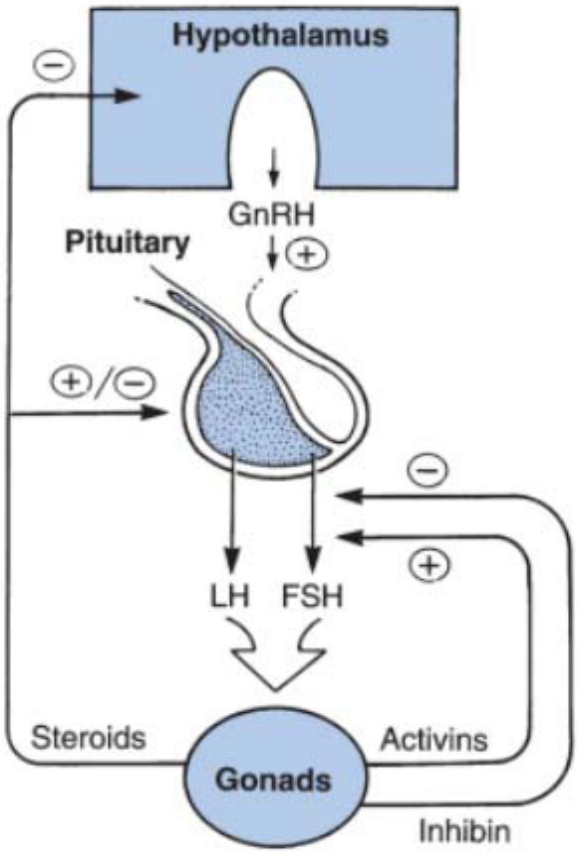

Figure 6. Regulation of gonadotropins. The release of GnRH from the hypothalamus regulates the production of gonadotropins in the pituitary. Activins stimulate FSH and LH release in opposition to Inhibin. These gonadotropins in turn are subject to steroids feedback from the gonads. (Reproduced from reference 9)

1.3.1.2 LH

Luteinizing hormone is a hormone synthesized and secreted by gonadotropes in the anterior lobe of the pituitary gland. In the female, an acute rise of LH – the LH surge – triggers ovulation. LH has a beta subunit that is responsible for interaction with the LH receptor. The biologic half-life of LH is 20 minutes, shorter than that of FSH (3-4 hours) or hCG (24 hours). In both males and females, LH is essential for reproduction (1). LH receptors are expressed on the maturing follicle that produces an increasing amount of estradiol with the final maturation of the follicle. The estrogen rise leads via the hypothalamic interface to the "positive feed-back" effect, a release of LH over a 24-48 hour period. The LH surge triggers ovulation and the conversion of the residual follicle into a corpus luteum that, in turn, produces progesterone to prepare the endometrium for a possible implantation. LH also supports thecal cells in the ovary that provide androgens and hormonal precursors for estradiol production (1).

1.3.2 Autocrine and paracrine regulators

A plethora of growth factors, many belonging to the transforming growth factor-ß (TGF-ß) superfamily, are expressed by ovarian somatic cells and oocytes in a developmental, stage-related manner and function as intraovarian regulators of folliculogenesis (Figure 7). In addition to the above-mentioned BMP4, BMP7, BMP15 and GDF9, the granulosa cell-expressed anti-Mullerian hormone (AMH, also known as Mullerian-inhibiting substance) promotes the growth of pre-antral follicles while inhibiting follicle cell differentiation (4). Studies on later stages of follicle development indicate positive roles for granulosa cell-derived 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 prevention of premature luteinization and/or atresia (4). Concomitantly, activin, TGF-ß and several BMPs may exert paracrine actions on theca cells to attenuate LH-dependent androgen production in small to medium-size antral follicles. Dominant follicle selection in monovular species may depend on differential FSH sensitivity amongst a growing cohort of small antral follicles. Changes in intrafollicular activins, GDF-9, AMH and several BMPs may contribute to this selection process by modulating both FSH- and IGF-dependent signalling pathways in granulosa cells. Activin may also play a positive role in oocyte maturation and acquisition of developmental competence. LH-induced androgen secretion is required to sustain a high level of estradiol secretion during the pre-ovulatory phase (4). LH stimulation of ovarian follicles involves activation of a local epidermal growth factor (EGF) network (10). LH activation of mural granulosa cells stimulates cAMP signalling, which, in turn, induces the expression of the EGF-like growth factors epiregulin (EREG), amphiregulin (AREG), and betacellulin (BTC). These growth factors function by activating EGF receptors in either an autocrine or juxtacrine fashion within the mural layer, or they diffuse to act on cumulus cells. Activation of EGF receptor signalling in cumulus cells, together with cAMP priming, triggers oocyte nuclear maturation and acquisition of developmental competence as well as cumulus expansion (10). The complex intraovarian control mechanisms, in concert with systemic signals, coordinate the recruitment and progression of primordial follicles through to ovulation and CL formation.

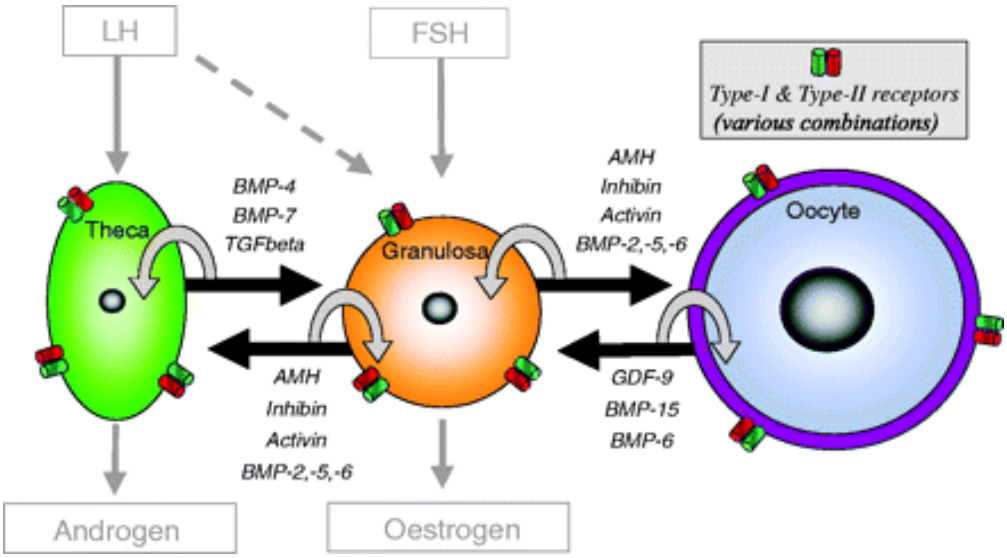

Figure 7. TGF-ß superfamily members implicated in the bi-directional communication between theca and granulosa cells, and granulosa cells and oocyte. Both autocrine (thick grey arrows) and paracrine (thick black arrows) signalling events are likely, depending on the expression of combinations of type-I and type-II receptors on the cell surface. (Reproduced from reference 4)

2. Wnts and Wnt signaling

Wnts comprise a large family of secreted, local-acting signalling glycoproteins that play critical roles in embryonic development. Following embryogenesis, Wnts and Wnt signaling pathway components continue to be expressed in many tissues, and play additional roles in adult tissue homeostasis (11). Wnt signaling has also received considerable attention from cancer researchers in recent years because many of its components play important roles in tumor formation (12,41,42). The name Wnt was coined as a combination of Wg (wingless) and Int and can be pronounced as "wint" (13). The wingless gene had originally been identified as a segment polarity gene in Drosophila melanogaster that functions during embryogenesis and also during adult limb formation during metamorphosis. The Int-1 gene and the wingless gene were found to be homologous, with a common evolutionary origin evidenced by similar amino acid sequences of their encoded proteins (14). The Int genes were originally identified as vertebrate genes near several integration sites of mouse mammary tumor virus (MMTV) (15).

2.1 The Wnt family

2.1.1 Phylogeny and structure

Wnt genes have been identified in animals from hydra to humans. In humans, 19 Wnt proteins have been identified that share 27% to 83% amino-acid sequence identity and a conserved pattern of 23 or 24 cysteine residues (Figure 8). Several human Wnt genes are located very close to each other in the genome, including Wnt6 and Wnt10a which are located on chromosome 2, and Wnt1 and Wnt10b located on chromosome 12 (16). Several

additional pairs of Wnt genes are also clustered within the human genome, including Wnt2 and Wnt16, Wnt3a and Wnt14, and Wnt3 and Wnt15. In the mouse, there are at least 18 Wnt genes (Wnt1, 2, 3, 3A, 4, 5A, 5B, 6, 7A, 7B, 8A, 8B, 10A, 10B, 11, 15). The mouse Wnt1/Wnt10b, Wnt6/Wnt10a, and Wnt3/Wnt15 gene pairs are located on the same chromosomes, and in the Wnt1/Wnt10b and Wnt6/Wnt10a pairs, the close proximity of these genes has been conserved from mouse to human. In the Drosophila genome, the paralogous genes Wg, DWnt6 and DWnt10, are located adjacent to one another on the second chromosome and are all transcribed in the same orientation (16). The majority of human Wnt genes contain four coding exons, with exon 1 containing the initiation codon. Wnt genes that differ from this pattern include Wnt14, with three exons, Wnt2, Wnt5b, and Wnt11, with five exons, and Wnt8b with six exons. Several Wnts-Wnt2b/13, Wnt8a/d, and Wnt16 - have alternative amino or carboxyl terminal, which result from the use of alternative 5' or 3' exons (16).

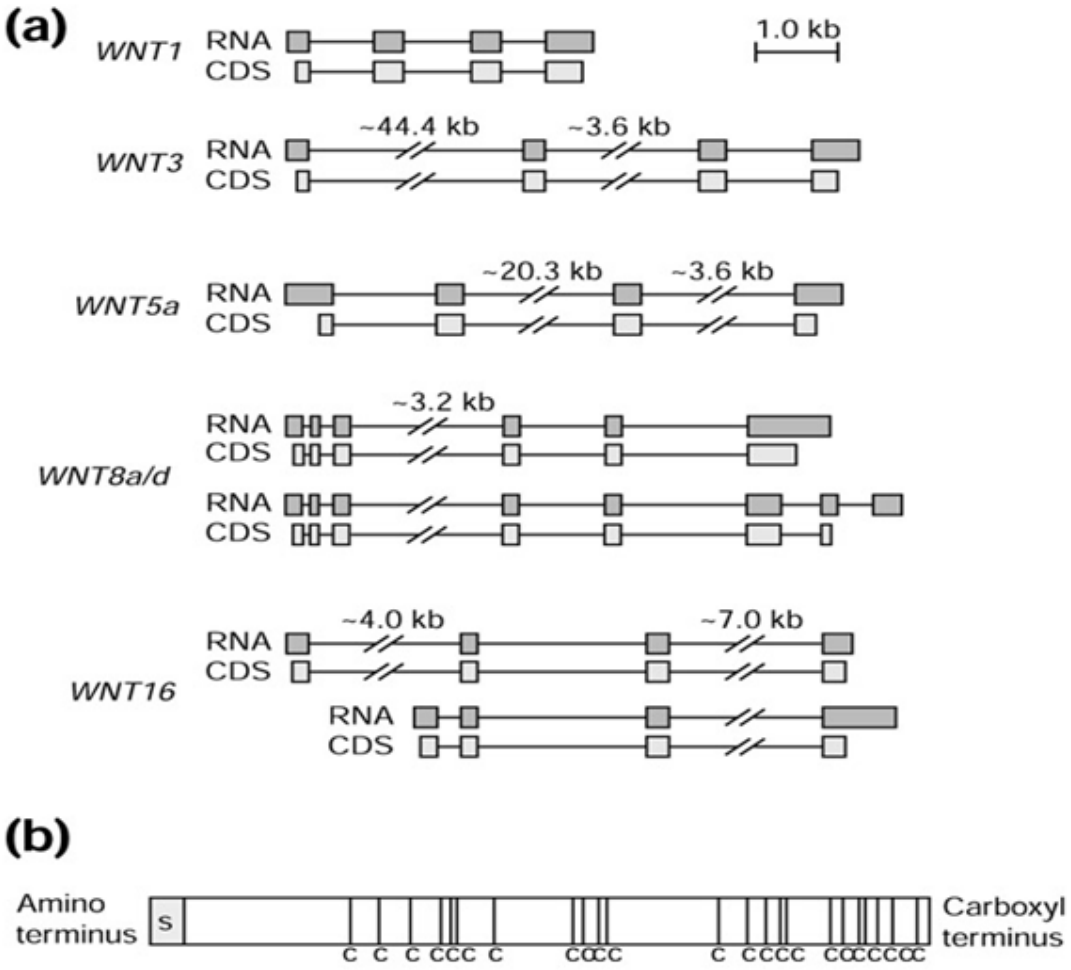

Figure 8. (a) Structures of selected members of the human Wnt gene family. Exons are shown as boxes and introns as lines. For each gene, RNA represents the portion of the gene that is transcribed and CDS represents the portion that encodes protein. Wnt8a/d is an example of a gene with 3' alternative splicing and *WNT16* is an example of a gene with alternatively used 5' exons. **(b) Structural features of the Wnt protein**. The amino terminus contains a signal sequence (S). All Wnts contain 23 or 24 conserved cysteine residues (C) with similar spacing, suggesting that the folding of Wnt proteins depends on the formation of multiple intramolecular disulfide bonds**.** (Reproduced from reference 16)

Members of the Wnt gene family are defined by their sequence similarity to mouse Wnt1 and Wingless in Drosophila. In vertebrates, the orthologs in different species are highly similar in sequence. For example, human Wnt1 and mouse Wnt1 are 98% identical and human WNT3a and Xenopus Wnt5a are 84% identical at the amino-acid level. Phylogenetic analyses of vertebrate and invertebrate Wnts demonstrate orthologous relationships between several human and Drosophila Wnts. The evolutionary relationship between the five C. elegans Wnt genes and human Wnt genes is less apparent, making it difficult to determine which C. elegans Wnt genes may have orthologs in the human genome (16).

Wnt genes have been identified in vertebrates and invertebrates, but appear to be absent from plants, unicellular eukaryotes such as Saccharomyces cerevisiae and from prokaryotes. To date, in vertebrates, 16 Wnt genes have been identified in Xenopus, 11 in chick, and 12 in zebrafish; in invertebrates, Drosophila has seven Wnt genes, Caenorhabditis elegans five and Hydra at least one (16). The deduced evolutionary relationships between 18 of the 19 known human Wnt genes and between selected human and non-human WNTs are shown in Figure 9.

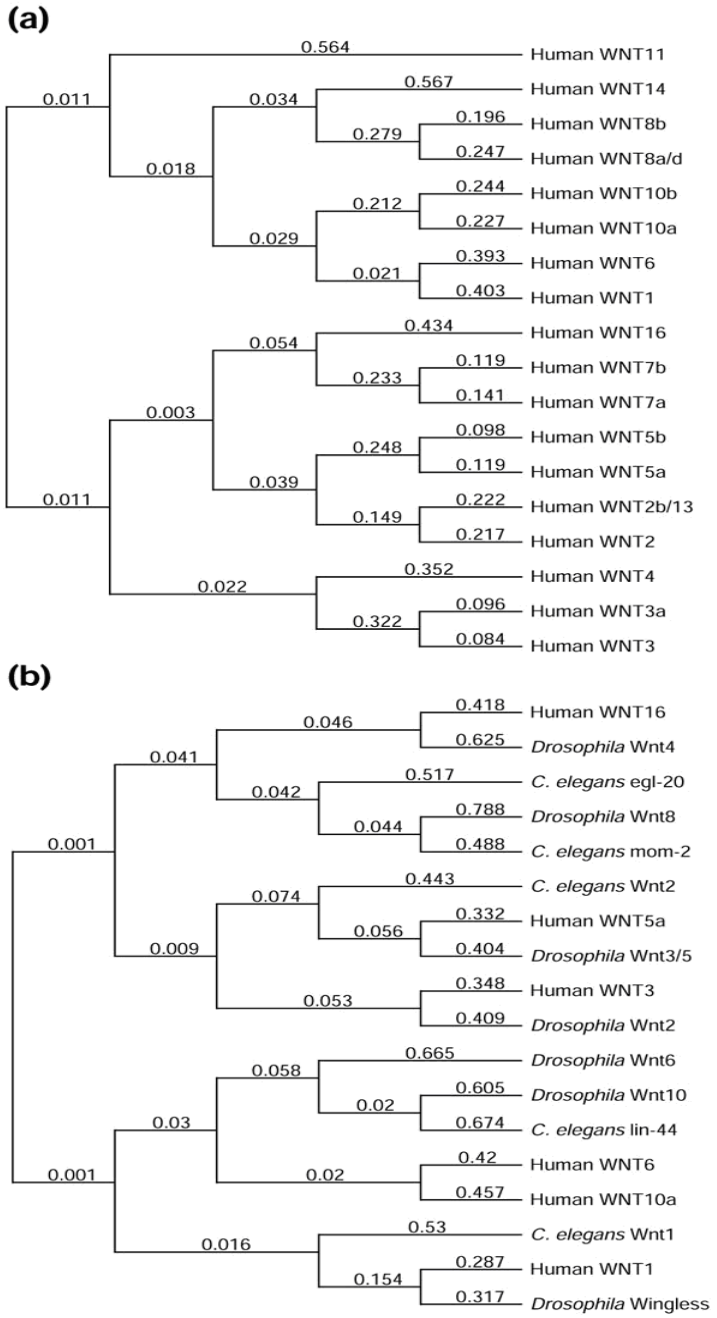

Figure 9. Predicted evolutionary relationships between members of the Wnt gene family. (a) Predicted relationships between 18 of the 19 known human WNT protein sequences. **(b)** Predicted evolutionary relationships between selected human WNT proteins and WNT proteins from mouse, Xenopus, Drosophila, and C. elegans. (Reproduced from reference 16)

2.1.2 Physiological roles of Wnts

Wnts have diverse roles in governing cell fate, proliferation, migration, polarity, and death. In adults, Wnts function in homeostasis, and inappropriate activation of Wnt signaling is implicated in a variety of cancers (12).

Loss-of-function mutations in 9 of the 18 mouse Wnt genes have been generated, and the phenotypes of mutant embryos demonstrate the diverse functions of Wnt genes during embryogenesis. For example, knocking out Wnt1 results in a dramatic loss of a portion of the midbrain and deletion of the rostral cerebellum (17). A targeted knockout of Wnt3 in mice results in defects in axis formation and gastrulation, suggesting a conserved role for Wnts in regulating the establishment of the dorsal-ventral axis in vertebrates (18). Inactivation of Wnt4 results in the absence of kidneys (19), masculinization of mutant females (absence of the Müllerian duct and continued development of the Wolffian duct) and oocyte loss due to defects in the early steps of follicle organization (20). Targeted knockout of Wnt7a also has pleiotropic effects, including ventralization of the limbs (21), female infertility due to failure of Müllerian duct regression (22), and a delay in the morphological maturation of glomerular rosettes in the cerebellum (23). Overexpression and antisense 'knockdown' analyses in Xenopus have shown that XFzd7 is important for establishing dorsal cell fates (24). In flies, Wnt signaling has a variety of functions during development. The *wg* gene is required for cell-fate choices in the ventral epidermis during embryogenesis, as well as for many other functions, and DWnt2 is required for testis and adult muscle development (25). In C. elegans, genetic analyses have defined a number of roles for Wnts, including establishment of polarity and endodermal cell fates in the early embryo and regulation of cell migration, among many others (26).

2.2 Wnt signal transduction

Wnts can signal through at least three different pathways. In the canonical pathway, Wnt-mediated signaling proceeds initially through binding to cell surface receptors of the frizzled family; the signal is subsequently transduced through several cytoplasmic components to β-catenin, which enters the nucleus and activates transcription (27). Non-canonical Wnt signaling pathways act independently of β-catenin, and include the Wnt/Ca^{2+} pathway and the Wnt/polarity pathway (also called the "planar polarity" pathway) (28).

2.2.1 The canonical pathway

The intracellular signaling pathway activated by Wnts was originally identified as a ß-catenin-dependent signaling pathway that is highly conserved among various species. In this "canonical" pathway, Wnt binds to its cell-surface receptor, which consists of Frizzled (Fzd) and low-density-lipoprotein receptor-related protein 5/6 (LRP5/6) (Figure 10).

Dishevelled (DSH), a key component of a membrane-associated Wnt receptor complex, then inhibits a second complex of proteins that includes axin, GSK-3 (glycogen synthase kinase 3), and the protein APC (adenomatous polyposis coli). The axin/GSK-3/APC complex normally promotes the proteolytic degradation of the β-catenin in resting cells. Wnt signaling antagonizes this "β-catenin destruction complex", a pool of cytoplasmic β-catenin then stabilizes, and some β-catenin is able to enter the nucleus and interact with TCF/LEF family transcription factors to promote specific gene expression. Tcf/β-catenin complexes directly regulate the expression of numerous known target genes, many of which, such as cyclinD1 and c-myc, are implicated in cellular proliferation (29).

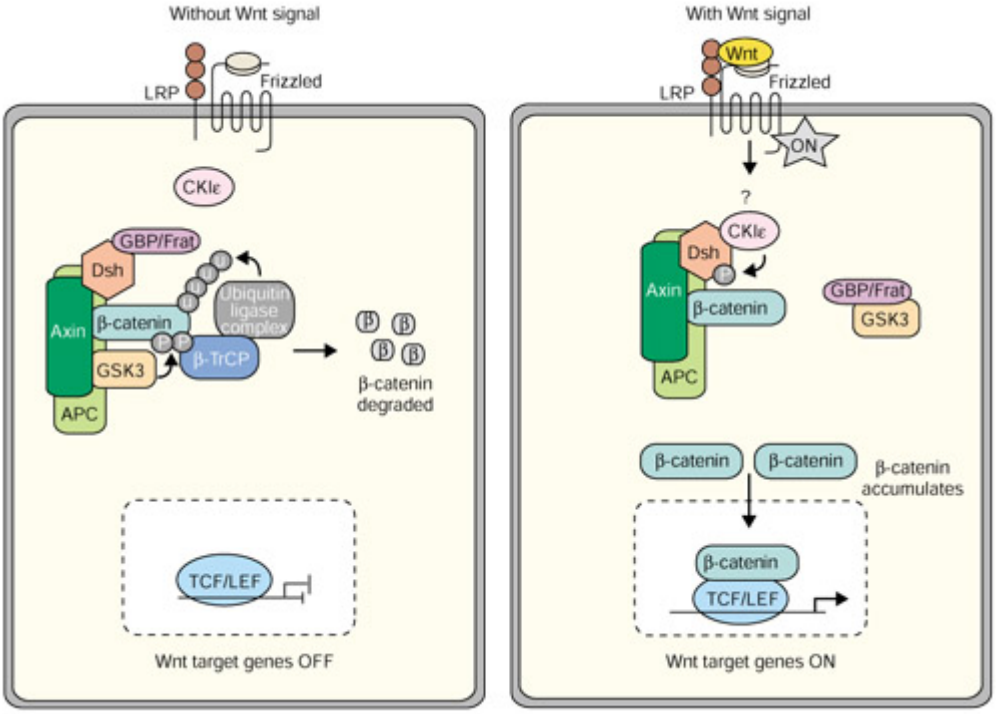

Figure 10. The canonical Wnt/β-catenin pathway. In the absence of Wnt signal (left), a multi-protein destruction complex that includes the APC and a member of the Axin family facilitates the phosphorylation of β-catenin by GSK3 promotes degradation of the β-catenin. When a cell is exposed to a Wnt (right), the Wnt interacts with its coreceptors Frizzled and LRP. Activation of Frizzled and LRP leads to the phosphorylation of Dsh, Dsh then functions through its interaction with Axin to antagonize GSK3, preventing the phosphorylation and ubiquitination of β-catenin. Unphosphorylated β-catenin escapes degradation, accumulates in the cell, and enters the nucleus, where it interacts with members of the TCF/LEF family of HMG-domain transcription factors to stimulate expression of target genes. (Reproduced from reference 30)

2.2.2 The Calcium pathway

The Ca^{2+} pathway, which is mediated by specific Wnts and Fzds, can increase the intracellular Ca^{2+} concentration, probably through trimeric GTP-binding proteins, calcium/calmodulin-regulated kinase II (CamKII) and the activation of protein kinase C (PKC) (Figure 11). The downstream targets of CamKII and PKC are currently unknown, but it has been shown that activation of the Wnt/Ca^{2+} pathway can antagonize the Wnt/β-catenin pathway in Xenopus, although it is unclear at what level this interaction occurs (31)

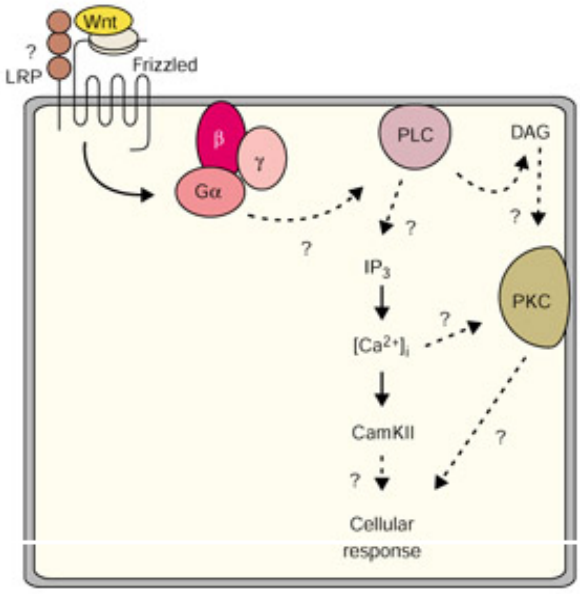

Figure 11. The Wnt/Ca^{2+} signaling pathway. The pathway involve activation of the two pertussis-toxin-sensitive G proteins, $G_{\alpha o}$ and $G_{.\alpha t}$, in combination with $G_{\beta 2}$. G-protein activation then leads to an increase in intracellular Ca^{2+} and the stimulation of Ca^{2+}/calmodulin-dependent kinase II (CamKII). Activation of the Wnt/Ca^{2+} pathway results in stimulation of PKC activity in the form of the translocation of PKC to the plasma membrane. (Reproduced from reference 30)

2.2.3 The planar cell polarity pathway

The planar cell polarity (PCP) pathway, which was originally identified in Drosophila, is mediated by small G proteins (including Rac and Rho), c-Jun *N*-terminal kinase (JNK) and Rho-associated kinase (Rho-kinase) (Figure 12). Although it is conceivable that the pathway is involved in the regulation of tissue polarity, cell migration and cytoskeleton arrangement based on observations on fly genetics, its exact roles in mammals are not clear (32).

Some of the proteins involved in planar cell patterning of the Drosophila wing are also involved in the regulation of cell movements during gastrulation and neurulation in vertebrates. In drosophila, Wnt/polarity signaling is required for the orientation of trichomes - or hairs - of the adult wing and for chirality of ommatidia in the eye, and may regulate asymmetric cell divisions of certain neuroblasts (33).

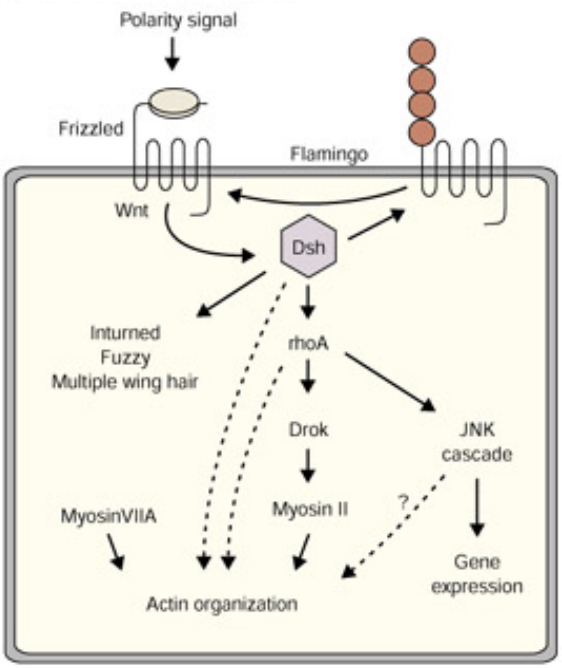

Figure 12. The Wnt/polarity pathway. The planar cell polarity (PCP) pathway is mediated by some Fzd, activates small G proteins, c-Jun *N*-terminal kinase (JNK) and Rho-associated kinase (Rho-kinase) to regulate cytoskeletal organization. (Reproduced from reference 30)

2.3 Wnt signalling in the ovary

Recent descriptive studies have demonstrated the regulation of several Wnts and Wnt signalling pathway components in the ovary. Interestingly, several of these have been shown to exhibit specific patterns of regulation in response to gonadotrophin stimulation, suggesting that they may function during specific phases of follicular development (34, 36, 39, 40).

2.3.1 Wnt4

The first indication of the importance of Wnt signalling in ovarian processes came from a study by Vainio et al (19), which reported that inactivation of the Wnt4 gene in mice results in the loss of a large fraction of the reserve of oocyte in the days prior to birth. Subsequent studies have shown that Wnt4 expression is required during embryonic female gonadal development to repress the formation of the male–specific coelomic blood vessel (35). It remains unclear how this altered vascular development relates to the observed perinatal oocyte depletion in Wnt4 knockout mice. A recent descriptive study has shown that Wnt4 mRNA is expressed in granulosa cells of small follicles in the postnatal ovary and that this expression is increased in pre-ovulatory follicles as well as in association with ovulation and luteinization. Unfortunately, Wnt4 KO mice die shortly after birth due to developmental kidney defects, precluding further analysis of Wnt4 function in the mature rodent ovary (36).

2.3.2 Fzd4

Expression of Fzd4 in granulosa cells is stimulated by LH-R activation. Although the role of both Wnt4 and Fzd4 in luteinization is not yet clear, the results obtained to date suggest that Fzd4 a potential receptor for Wnt4 and that Wnt4/Fzd4 signaling is important for the

regulation of luteal cell formation and function. The recent deletion of the Fzd4 gene has revealed that the null mice fail to form functional CLs despite normal follicular development and ovulation of fertilizable oocytes. Analyses at defined stages of reproductive function indicate that immature Fzd4 knockout mouse ovaries contain follicles at many stages of development and respond to exogenous hormone treatments in a manner similar to their wild-type littermates, indicating that the processes controlling follicular development and follicular cell responses to gonadotropins are intact. Adult Fzd4$^{-/-}$ mice also exhibit normal mating behavior and ovulate, indicating that endocrine events controlling these processes occur. However, *Fzd4*$^{-/-}$ mice fail to become pregnant and do not produce offspring. Histological and functional analyses of ovaries from timed mating pairs at Days 1.5–7.5 postcoitus (p.c) indicate that the corpora lutea of the Fzd4$^{-/-}$ mice do not develop normally. Fzd4 null ovaries exhibit CL of altered appearance and reduced expression of genes such as Lhcgr, Prlr, Cyp11a1 and Sfrp4 known to be associated with luteinization, luteal cell morphology is altered, and markers of angiogenesis and vascular formation are reduced. Thus, Fzd4 appears to impact the formation of the corpus luteum by mechanisms that more closely phenocopy Prlr null mice (36).

2.3.3 Fzd1

Fzd-1 mRNA is expressed at low levels in mouse pre-ovulatory follicles, but increases markedly after an ovulatory surge of hCG. Fzd1 transcripts increase first in the theca cells and then in the granulosa cells of ovulating follicles, but are low in corpora lutea. Induction of Fzd1 in the hours preceding ovulation suggests that Fzd1 gene could be involved in the transcriptional regulation of factors known to impact ovulation, such as PR, Ptgs2, collagenases and Adamts1 (34). Recently, rat Fzd1 has been shown to activate the ß-catenin

pathway via G protein signalling (37). Therefore, in the ovulating follicle, Fzd1 may control granulosa cell movement or differentiation or regulate genes that impact follicle rupture or luteinization.

2.3.4 SFRP 4

SFRP4 (Secreted frizzled-related protein 4) is a member of the SFRP family of Wnt signaling antagonists. It contains a cysteine-rich domain (CRD) that is homologous to the putative Wnt-binding site of Frizzled proteins and that is thought to mediate its interaction with Wnts (38). In the mouse ovary, expression of SFRP-4 mRNA was upregulated in granulosa cells of large antral follicles after hCG administration, and was also elevated in corpora lutea. In hypophysectomised rat ovaries, sFRP-4 expression was similarly induced by hCG and further upregulated by prolactin (PRL). PRL also stimulated secretion of sFRP-4 protein from luteinized rat granulosa cells in culture. Therefore, regulation of sFRP-4 by LH and PRL may be important for modulating Fzd1 known to be expressed in periovulatory follicles, and Wnt4/Fzd4 expressed in corpora lutea (39).

2.3.5 Wnt-2

This gene is a member of the WNT gene family. The WNT gene family consists of structurally related genes which encode secreted signaling proteins which are involved in the Wnt signaling pathway. Very recent study shows that Wnt2 overexpressed in rat hepatic sinusoidal endothelial cells (HSECs) and shown to be activated the canonical Wnt signalling pathway (58). The function of Wnt-2 in reproduction is not known yet. Wnt-2 is expressed in the developing pericardium, lung, and placenta. Targeted disruption of the Wnt-2 gene in mice results in placentation defects (59). In the rat ovary, granulosa cells express Wnt-2 mRNA and all growing follicles were positive for Wnt-2. Primordial

follicles were observed in a few sections and cells in these follicles also appeared positive (40). These observations indicate that Wnt-2 is expressed in adult reproductive tissues and may displays hormonal regulation.

2.3.6 Wnt2b

This gene encodes a member of the wingless-type MMTV integration site (WNT) family of highly conserved, secreted signaling factors. Wnt-2b is expressed in several types of human cancer, such as basal cell carcinoma, gastric cancer, breast cancer, cervical cancer and leukemia. Wnt-2b is one of canonical WNTs transducing signals through Frizzled (FZD) and LRP5/LRP6 receptors to β-catenin-TCF/LEF signaling cascade (60). The expression of a number of wnt genes (e.g. Wnt-2, Wnt-4) has been associated with the development and cellular differentiation of tissues and organs of the male and female reproductive systems (19,34). As well, the Wnt-2b mRNA is detected in rat ovarian surface epithelium (OSE) and human ovarian cancer cells (40,60). The presence of Wnt-2b in these tissues suggests that this Wnt may be involved of the ovarian development and function.

Hypothesis and Objective

The overall objective of the present work was to identify the physiological roles of Wnt4 in the ovary. Given the pattern of Wnt4 expression throughout late follicle development, we hypothesized that Wnt4 may be required for some aspect of late follicle development, survival or ovulation/luteinization. To address this hypothesis, we endeavoured to identify the transcriptional targets of Wnt4 in granulosa cells, with the presumption that the nature of these targets would provide insight into its physiological function(s).

WNT4 acts as an ovarian follicle survival factor and regulates stress response genes in granulosa cells

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Running title: WNT4 is required for follicle survival

Key words: Follicle, Granulosa cell, Conditional gene targeting, Ctnnb1, Wnt4, Microarray, Stress response

Grant support: Canadian Institutes of Health Research operating grant and the Canada Research chair in Ovarian Molecular Biology and Functional Genomics (D. Boerboom); NIH grants HD16272 (J.S. Richards), and HD07495 (J.S. Richards).

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ABSTRACT

To study the physiological role of WNT4 in the postnatal ovary, a mouse strain bearing a floxed *Wnt4* allele was created and mated to the *Amhr2*$^{tm3(cre)Bhr}$ strain to target deletion of *Wnt4* to granulosa cells. *Wnt4*$^{flox/-}$;*Amhr2*$^{tm3(cre)Bhr/+}$ mice had significantly reduced ovary weights and produced smaller litters ($P<0.05$). Serial follicle counting demonstrated that, while *Wnt4*$^{flox/-}$;*Amhr2*$^{tm3(cre)Bhr/+}$ mice were born with a normal ovarian reserve and maintained normal numbers of small follicles until puberty, they had only 25.2% of the normal number of healthy antral follicles due to an increased rate of atresia. Some *Wnt4*$^{flox/-}$;*Amhr2*$^{tm3(cre)Bhr/+}$ mice had no antral follicles or corpora lutea and underwent premature follicular depletion. To determine the mechanism of WNT4 action, cultured granulosa cells were caused to overexpress WNT4 or dominant-stable CTNNB1 and subsequently analyzed by microarray for alterations in gene expression. Results showed that WNT4 induced a series of genes associated with the cellular stress response via the WNT/CTNNB1 signaling pathway. Together, these results indicate that WNT4 mediates follicle survival by regulating genes that increase granulosa cell resistance to stress and prevent their apoptosis.

INTRODUCTION

The WNTs comprise a large family of local-acting, secreted glycoprotein signaling molecules that are known mostly for the numerous roles they play in embryonic development and cancer (Logan 2004, Lustig 2003). Several WNTs, their cognate receptors of the Frizzled (*Fzd*) family and other components of the WNT signaling pathways are expressed in the postnatal ovary, but their role(s) in ovarian physiology remain poorly defined (Hsieh 2002, Rickens 2002, Hsieh 2003, Hsieh 2005). The first indication of the importance of WNT signaling in the ovary came from a study by Vainio et al (1999), which reported that inactivation of the *Wnt4* gene in mice

results in the loss of a large fraction of the reserve of oocytes in the days prior to birth. Subsequent studies have shown that WNT4 expression is required during embryonic female gonadal development to suppress the formation of the male-specific coelomic blood vessel, and that follistatin (FST) acts downstream of WNT4 to mediate this effect (Jeays-Ward 2003, Yao 2004). In addition, WNT4 is required to prevent the migration of a steroidogenic cell population from the mesonephros into the female gonad (Heikkela 2002, Jeays-Ward 2003). It remains unclear, however, how (or if) altered vascular development and the presence of ectopic steroidogenic cells in WNT4 knockout ovaries relate to the perinatal oocyte depletion phenotype. WNT4 expression in the mouse embryonic gonad is restricted to the somatic cells (Vainio 1999), and a descriptive study has shown postnatal WNT4 expression selectively in follicular granulosa cells and corpora lutea (Hsieh 2002). Interestingly, WNT4 expression increased markedly in response to an ovulatory dose of hCG in rat and mouse granulosa cells, suggesting that it could be important for some aspect of late follicle development, ovulation and/or luteinization (Hsieh 2002). Unfortunately, proof of this could not be obtained from the *Wnt4* knockout mouse, which dies shortly after birth due to kidney defects (Stark 1994), precluding analyses of postnatal ovarian follicular development.

Some insight into the role(s) of *Wnt4* in granulosa cells may have been provided by a recent series of studies that examined the role of dysregulated WNT signaling in the pathogenesis of granulosa cell tumors (Boerboom 2005, Boerboom 2006, Lague 2008). In these studies, a WNT signaling pathway known as the canonical or WNT/CTNNB1 pathway was constitutively activated in mouse granulosa cells by conditional gene targeting. The resulting *Ctnnb1*$^{tm1Mmt/+}$;*Amhr2*$^{tm3(cre)Bhr/+}$ mice developed unusual, follicle-like structures by 5-6 weeks of age that consisted of nests of disorganized,

pleiomorphic granulosa cells that often formed a central, antrum-like cavity (Boerboom 2005). However, the structures did not develop and regress in a manner similar to follicles, as they grew no larger than the size of antral follicles and subsequently persisted for the rest of the life of the animal. These structures were subsequently defined as premalignant lesions, as they were found develop into granulosa cell tumors in older mice, or following the inactivation of the anti-oncogene *Pten* (Boerboom 2005, Lague 2008). Based on these observations and the fact that WNT4 can signal via the WNT/CTNNB1 pathway in certain contexts (Lyons 2004, Terada 2003, Biason-Lauber 2004), we hypothesized that WNT4 may regulate follicle development by controlling some aspect(s) of granulosa cell proliferation, differentiation and/or survival.

MATERIALS AND METHODS

Gene targeting. The *Wnt4*^{flox} allele was created using standard gene targeting techniques. Briefly, a targeting construct was built using the pKOII vector as a backbone (Bardeesy 2002), into which was inserted a 478bp chromosomal DNA fragment flanked by *loxP* sites and encompassing the third and fourth exons and third intron of the *Wnt4* gene, along with parts of the second and fourth introns (Fig. 1Ab). Homologous chromosomal sequences consisting of the 7000bp upstream and 1518bp downstream of the floxed region were then inserted into the vector so as to generate the construct illustrated in Fig. 1Ab. A BglII restriction site for use in Southern screening was also inserted before the upstream *loxP* site. All genomic sequences were isolated from R1 embryonic stem (ES) cell genomic DNA by PCR using the Expand long template PCR system (Roche Molecular Biochemicals, Laval, Canada), and LoxP and restriction sites were inserted within the oligonucleotide primers used to isolate the genomic DNA

fragments. Following linearization with Not1, targeting construct DNA was introduced into the R1 ES cell line (Nagy 1993) by electroporation, and recombinant colonies were selected by addition of 400µg/ml Geneticin (Invitrogen, Burlington, Ontario) to the culture medium for 8-9 days. Selected colonies were cultured in triplicate, and duplicate sets were analyzed by Southern blotting to screen for proper homologous recombinants as previously described (Sambrook 1989). Two of 384 colonies screened showed a second band consistent with the expected size for the targeted allele (Fig. 1B). Both cell lines were subsequently microinjected into blastocysts and transferred to pseudopregnant recipients according to standard protocols. Chimeric males derived from both cell lines sired pups heterozygous for the targeted allele (Fig 1Ac).

Animals models. To eliminate the possibility of the neo cassette interfering with WNT4 expression from the targeted allele, mice bearing the targeted allele were mated to the 129S4/SvJaeSor-*Gt(ROSA)26Sor*$^{tm1(FLP1)Dym}$/J strain (The Jackson Laboratory, Bar Harbor, ME) to target Flp1-mediated excision of the frt-flanked neo cassette (Fig. 1Ab) to the germline. The excision of the neo cassette (Fig. 1Ad) was verified by PCR from tail biopsies from *Wnt4*$^{flox/+}$*;Gt(ROSA)26Sor*$^{tm1(FLP1)Dym/+}$ mice, and the Flp1 transgene was eliminated the following generation by mating to wild-type mice. To generate a null *Wnt4* allele (*Wnt4*-, Fig. 1Ae), *Wnt4*$^{flox/flox}$ mice were mated to the B6.C-Tg (CMV- cre) 1Cgn/J strain (The Jackson Laboratory) which expresses Cre in all tissues including the germline. Following PCR verification of the *Wnt4*$^{-}$ allele (Fig. 1C), the Cre transgene was eliminated from *Wnt4*$^{+/-}$;*Gt(ROSA)26Sor*$^{tm1Sor/+}$ mice the following generation by mating to wild-type mice. Complex genotypes were then obtained by selective breeding of the *Wnt4*$^{-}$, *Wnt4*^{flox} and previously-described *Amhr2*$^{tm3(cre)Bhr}$ parental strains (Jamin 2002). Genotyping analyses for the *Amhr2*$^{tm3(cre)Bhr}$ allele were performed by PCR on DNA

obtained from tail biopsies as previously described (Jorgez 2004). Genotyping analyses for the *Wnt4* alleles was performed using the oligonucleotide primers 5'-GCCAGGCTGTCTGCTGGCTCA -3' (Fig 1Ca), 5'GCATATGAGGCCTGCTGAATGCT -3' (Fig 1Cb) and 5'- TAGGAACTTCAATTCCCCGCAAGA -3' (Fig. 1Cc) and using the same reagents and cycling parameters used for *Amhr2*$^{tm3(cre)Bhr}$ genotyping. This protocol results in PCR products of approximately 250bp for the wild-type *Wnt4* allele, 210bp for the floxed allele, and 110bp for the knockout (i.e., Cre-recombined) allele (Fig. 1C). All animal procedures were approved by the Institutional Animal Care and Use Committee and were conform to the USPHS Policy on Humane Care and Use of Laboratory Animals.

Semi-quantitative and real-time RT-PCR. Semi-quantitative RT-PCR to measure relative expression of *Wnt4* and Ribosomal protein L19 (*Rpl19*, control gene) was performed on 1µg ovarian RNA samples from mice of the genotypes indicated in Fig. 1D and purified with the RNeasy mini kit (Qiagen). RT-PCR was done using the Superscript one-step RT-PCR kit (Invitrogen) as directed by the manufacturer. Oligonucleotides primers used for *Wnt4* and *Rpl19* are indicated in Table 1. Cycling conditions were 94°C for 1 minute followed by 25 cycles (*Rpl19*) or 35 cycles (*Wnt4*) of 94°C for 55°C for 1 minute, 72°C for 1 minute. Preliminary experiments done for *Wnt4* and *Rpl19* ensured that the cycle numbers selected fell within the linear range of PCR amplification (data not shown). PCR products were separated by electrophoresis on 1.8% TAE-agarose gels containing ethidium bromide and photographed under UV light. Expression of selected genes identified by microarray analysis was verified by real-time RTPCR using a set of RNA samples derived as described below, and distinct

from those used in the original array analyses. Reactions were formulated using the Superscript III Platinum two-step qRT-PCR kit with SYBR green (Invitrogen)

according to the manufacturer's instructions and using the oligonucleotide primer pairs listed in Table 1. Thermal cycling and data capture were performed using a Rotor-Gene RG-3000 apparatus (Corbett Research, Mortlake NSW 2137, Australia) and using to the manufacturer's recommended conditions. Relative gene expression was calculated using Rotor-Gene 6.0 software (Corbett Research) by comparing amplification curves to a series of standard curves obtained by amplification of serial dilutions of an *Rpl19* cDNA fragment. All data were subsequently normalized by dividing expression levels of individual genes by corresponding *Rpl19* values.

Cell culture, adenoviral infection and microarray analyses. Granulosa cells were obtained for culture and microarray analyses from 20-26 day-old *Ctnnb1*$^{tm1Mmt/tm1Mmt}$ (Harada 1999) animals that had been given eCG (Folligon, Intervet, Whitby, Canada, 5 IU, i.p.) 48h prior to sacrifice, and cells were isolated using the needle puncture method as previously described (Zeleznik 1974). Cells were suspended in DMEM/F-12 medium (Invitrogen) with 1% fetal bovine serum and allowed to attach overnight at a final density of ~50%. Cells were then infected with adenoviruses to express eGFP or Cre, or overexpress WNT4 (Chen 2006) for 24h in serum-free medium, and subsequently harvested for RNA extraction as described above. The Ad-WNT4 virus was a kind gift from Dr Peter Hornsby (University of Texas), the Ad-Cre and Ad-eGFP viruses were obtained from the Baylor College of Medicine Vector Development Laboratory (Houston, TX). Preliminary experiments demonstrated that an infection efficiency of >80% could be obtained at an MOI of ~50 (as determined by analysis of fluorescent signal in Ad-eGFP-infected cells) and that the Ad-Cre and Ad-WNT4 viruses produced efficient recombination

of the floxed *Ctnnb1* alleles and robust WNT4 overexpression, respectively (not shown). Triplicate RNA samples from each treatment were analyzed using mouse expression set 430 microarrays (Affymetrix, Santa Clara, CA). All steps of RNA quality control, probe synthesis, hybridization, washing, array scanning, and statistical analyses were done by the Microarray Core Facility of the Baylor College of Medicine (Houston, TX). To determine fold-induction values for probe sets caused by WNT4 overexpression or by Cre expression, signal strengths obtained on Ad-WNT4 or Ad-Cre arrays were normalized by dividing by corresponding values obtained on Ad- eGFP arrays. Probe sets called present on all three Ad-WNT4 arrays, that were induced > 5-fold on average by Ad-WNT4 treatment, and that corresponded to recognized coding genes were selected for further analyses. Using this method, no genes were found to be repressed > 5-fold following Ad-WNT4 treatment.

Follicle counting. Left ovaries from 5d and 42d-old $Wnt4^{flox}$ and $Wnt4^{flox}$;$Amhr2^{tm3(cre)Bhr}$ mice were collected, stored in Bouin's fixative for 24h, and rinsed in 70% ethanol prior to paraffin embedding. Ovaries were then serially sectioned at a thickness of 6mm, and every fifth section was stained with hematoxylin and eosin. Stained sections were photographed at 400X magnification so as to obtain image sets representing the entire section. All follicles with a visible nucleus were subsequently classified by stage of development and scored as healthy or atretic by systematic analysis of the image sets. The follicle classification system was based on Pedersen's system (Pedersen 1968): Oocyte surrounded by a layer of flat granulosa cells = primordial, Pedersen class 3 = primary, Pedersen classes 4-5 = secondary, Pedersen classes 6-8 = antral. Atresia was determined for each follicle based on histological criteria and using a weighted scoring system (based on Byskov 1974, Devine 2000, Peluso 1980). Primary criteria of atresia scoring were the presence and degree of 1) pycnosis (condensed granulosa cells or cells with condensed nuclei), 2) loss of

granulosa cell attachment to oocytes, or loss of cumulus cells, 3) presence of polymorphonuclear neutrophils or lymphocytes, and 4) pycnotic oocytes (primary follicles only). Secondary criteria were granulosa cell vacuolation, sparse or missing granulosa cells, and integrity of the basement membrane.

Immunohistochemistry. Immunohistochemical analysis of activated caspase-3 was done on formalin-fixed, paraffin-embedded, 7-μm ovary sections using the VectaStain Elite avidin-biotin complex method kit (Vector Labs, Burlingame, CA) as directed by the manufacturer. Sections were probed with a primary antibody against activated caspase-3 (Cell Signaling Technology, Inc., Danvers, MA, catalog #9661) and staining was done using the 3,3'-diaminobenzidine peroxidase substrate kit (Vector Labs). Sections were briefly counterstained with hematoxylin prior to mounting.

Statistical analyses. Effects of genotype on litter sizes, ovary weights and follicle numbers were analyzed by unpaired, two-tailed t-tests. P-values lower than 0.05 were considered statistically significant. Analyses were done using Prism 4.0a software (GraphPad Software, Inc., San Diego, CA).

RESULTS

Generation of a floxed Wnt4 allele. To study the role of WNT4 in granulosa cells *in vivo*, a floxed *Wnt4* allele was created so as to permit conditional targeting using the *Cre-Lox* system. A targeting construct designed to insert *LoxP* sites upstream of the third exon and downstream of the fourth exon of *Wnt4* was built (Fig 1A), and two properly targeted embryonic stem cell lines were obtained following transfection and antibiotic selection (Fig 1B). Male chimeric mice obtained by injection of the targeted ES cells into blastocysts gave germline transmission of the floxed allele upon mating to wild-

type C57BL/6 females. Female *Wnt4*$^{flox/flox}$ mice were found to have morphologically normal genitourinary tracts at birth (Fig 1E), developed normally through adulthood and demonstrated fertility indistinguishable from C57BL/6 wild-type mice (data not shown), indicating an absence of functional hypomorphism of the *Wnt4*^{flox} allele. Genetic recombination of the *Wnt4*^{flox} allele was then obtained by mating *Wnt4*$^{flox/flox}$ mice to the B6.C-Tg(CMV-cre)1Cgn "Cre-deleter" strain, resulting in offspring bearing a presumptive null (*Wnt4*$^{-}$) allele (Fig 1C). Upon backcross, the resulting *Wnt4*$^{-/-}$ mice were found to die within 48h after birth, and this was associated with severe developmental anomalies of the kidneys, reproductive tract and gonads (Fig 1E) that were essentially identical to those previously described in *Wnt4*$^{-}$ null mice (Vainio 1999, Stark 1994). These findings demonstrated the functionality of the *Wnt4*^{flox} allele, and that its recombination results in complete ablation of WNT4 activity.

Conditional targeting of Wnt4 in granulosa cells results in infertility and decreased ovary size. To target deletion of *Wnt4* to granulosa cells, mice bearing the *Wnt4*^{flox} allele were mated to the *Amhr2*$^{tm3(cre)Bhr}$ strain, which features the *Cre* transgene knocked-in to the *Amhr2* locus (Jamin 2002). The *Amhr2*$^{tm3(cre)Bhr}$ allele was chosen due to its ability to drive *Cre* expression perinatally in granulosa cell precursors (Lague 2008), and therefore to target *Wnt4* from the early stages of follicle development. The *Wnt4*$^{-}$ locus, created as described above, was also used so as to generate mice with the *Wnt4*$^{flox/-}$*;Amhr2*$^{tm3(cre)Bhr/+}$ genotype, an experimental design meant to maximize the efficiency of *Wnt4* ablation in granulosa cells. Semi-quantitative RT-PCR performed on RNA extracted from adult ovaries demonstrated a substantial reduction in *Wnt4* mRNA in *Wnt4*$^{flox/-}$*;Amhr2*$^{tm3(cre)Bhr/+}$ animals compared to littermate controls bearing a wild-type *Wnt4* allele or lacking the *Amhr2*$^{tm3(cre)Bhr}$ allele (Fig 1D). Real-time RT-PCR analyses showed *Wnt4* mRNA to be

62% less abundant on average in adult *Wnt4*$^{flox/-}$*;Amhr2*$^{tm3(cre)Bhr/+}$ ovaries relative to *Wnt4*$^{flox/-}$ controls (n = 14 animals/genotype). To study the effects of WNT4 loss on fertility, four *Wnt4*$^{flox/-}$*;Amhr2*$^{tm3(cre)Bhr/+}$ and four control females were placed with wild-type males for 6 months. While similar numbers of litters were obtained from both groups, the average litter size of *Wnt4*$^{flox/-}$*;Amhr2*$^{tm3(cre)Bhr/+}$ females was only 54% that of the control group (Fig 2A).

Histopathologic examination of the gonads and reproductive tracts of adult *Wnt4*$^{flox/-}$*;Amhr2*$^{tm3(cre)Bhr/+}$ females revealed no obvious anomalies, except that the ovaries appeared somewhat smaller in most animals examined (Fig 2B). Analysis of ovarian weights confirmed the histological observations, showing that *Wnt4*$^{flox/-}$*;Amhr2*$^{tm3(cre)Bhr/+}$ ovaries were only 49% the size of those of littermate controls (Fig 2C). Despite reduced size, all stages of follicular development and corpora lutea could be identified in most *Wnt4*$^{flox/-}$*;Amhr2*$^{tm3(cre)Bhr/+}$ ovaries, but large antral follicles and corpora lutea appeared less abundant.

Wnt4*$^{flox/-}$*;Amhr2*$^{tm3(cre)Bhr/+}$ *mice have defects in follicular development and survival.

To further study ovarian follicle development in *Wnt4*$^{flox/-}$;*Amhr2*$^{tm3(cre)Bhr/+}$ mice, serial sections were prepared from ovaries of 5d and 42d-old animals, and follicles were counted and categorized by phase of development and viability (ie, healthy or atretic). Results from 5d-old mice showed no significant differences ($P > 0.05$) in numbers of primordial or primary follicles between *Wnt4*$^{flox/-}$*;Amhr2*$^{tm3(cre)Bhr/+}$ mice and *Wnt4*$^{flox/-}$ controls (Fig. 3A), indicating that *Wnt4*$^{flox/-}$*;Amhr2*$^{tm3(cre)Bhr/+}$ mice are born with normal numbers of oocytes, and that early folliculogenesis occurs normally. Both primordial and primary follicle numbers in 42-old *Wnt4*$^{flox/}$

;Amhr2$^{tm3(cre)Bhr/+}$ ovaries appeared somewhat lower than controls, however statistical testing failed to show that these differences were significant ($P > 0.05$), and total numbers of secondary follicles were essentially identical in both groups (Fig. 3B). However, *Wnt4*$^{flox/-}$;*Amhr2*$^{tm3(cre)Bhr/+}$ ovaries had far fewer healthy antral follicles, with only 25.2% the number found in *Wnt4*$^{flox/-}$ mice. The loss of follicles between the secondary and antral stages was attributed to increased follicular atresia in *Wnt4*$^{flox/-}$*;Amhr2*$^{tm3(cre)Bhr/+}$ ovaries. Although comparable numbers of atretic antral follicles were found in *Wnt4*$^{flox/-}$*;Amhr2*$^{tm3(cre)Bhr/+}$and *Wnt4*$^{flox/-}$ mice, fully 46.2% of all antral follicles were classified as atretic in *Wnt4*$^{flox/-}$*;Amhr2*$^{tm3(cre)Bhr/+}$ ovaries, versus 16.0% in *Wnt4*$^{flox/-}$ controls. A more modest increase in the proportion of atretic follicles was also noted at the secondary stage, as more than half of all secondary follicles in *Wnt4*$^{flox/-}$*;Amhr2*$^{tm3(cre)Bhr/+}$ ovaries were atretic, versus fewer than a third in controls (Fig. 3B). These data therefore indicated that the decreased ovary size and fertility observed in *Wnt4*$^{flox/-}$*;Amhr2*$^{tm3(cre)Bhr/+}$ mice were due to an increased rate of follicular atresia, resulting in a partial depletion of antral follicles. A more severe phenotype was observed in a small subset (~5%) of *Wnt4*$^{flox/-}$*;Amhr2*$^{tm3(cre)Bhr/+}$ mice. These mice had very small ovaries (< 1 mg) that were devoid of antral follicles and corpora lutea at 8-12wks of age (Fig 4A). Many of the larger secondary follicles in these ovaries were atretic, as determined by histologic analyses and activated caspase-3 immunohistochemistry (Fig. 4B). However, the rarity of these mice precluded quantitative analyses of follicle numbers and rates of atresia. The severe ovarian phenotypes were always accompanied by variable degrees of growth retardation, poor body condition, a disheveled appearance, and frequently premature death. This was attributed to the presence of kidney defects (Fig. 4C) presumably related to those previously reported in *Wnt4*$^{-}$ null mice (Sark 1994). A single *Wnt4*$^{flox/-}$;*Amhr2*$^{tm3(cre)Bhr/+}$ mouse with the severe

ovarian/kidney phenotype survived to the age of 8 months, and showed a virtually complete depletion of its follicular reserves (Fig. 4D).

WNT4 regulates the transcription of cellular stress response genes via the WNT/CTNNB1 pathway. To determine the mechanism by which WNT4 mediates granulosa cell survival, a strategy was devised to identify its downstream transcriptional targets. Granulosa cells from immature, eCG-treated 20-26 day-old *Ctnnb1*$^{tm1Mmt/tm1Mmt}$ mice were placed in culture and treated with adenoviruses to express either eGFP (Ad-eGFP, control) or WNT4 (Ad-Wnt4), and genes specifically induced by Ad-Wnt4 treatment were identified by microarray analysis. Interestingly, 16 of 45 (35.6%) of transcripts induced > 5-fold by Ad-Wnt4 corresponded either to genes with known roles in mediating cellular stress responses, or to genes known to be responsive to stress (Fig. 5)(Gouraud 2007, Pradervand 2004, Ohaka 2005, Liu 2006, Dokladny 2008, Raff 2003, Colgan 2006, Fang 2008, Guyton 1996, Khurana 2006, Chen 2006, Espey 2002, May 2005, Morimoto 2008, Kokame 2000, Zhang 2008). Additional analyses of the array data using less stringent parsing methods revealed the marked (> 10-fold) induction of a set of 6 additional known stress response genes by Ad-Wnt4 (Fig. 5) (Fang 2008, Neufeld 1999, Froyland 2006, Martin 2005, Sarkar 2007, Bruder 2008). The induction of select genes identified by microarray was confirmed by real-time RT-PCR using RNA samples derived independently from those used for the microarray analyses (Fig. 5). Although *Fst* and *Bmp2* were previously identified as downstream targets of WNT4 signaling in the embryonic ovary (Yao 2004, Coveney 2008), neither *Fst* nor *Bmp2* was strongly regulated by Ad-Wnt4 in cultured granulosa cells as determined by microarray (not shown) or by qRT-PCR (fold induction by Ad-Wnt4, *Fst*: 0.407 ± 0.296, *Bmp2*: 2.153 ± 1.073). As WNT4 appears to be able to signal via both canonical and non-canonical WNT signaling pathways depending on

the cell type and physiological context (Bernard 2007, Tevosian 2008), an additional microarray experiment was conducted to determine if WNT4 signals via the WNT/CTNNB1 pathway in granulosa cells. Cultured *Ctnnb1*$^{tm1Mmt/tm1Mmt}$ granulosa cells were infected with an adenovirus to express Cre (Ad- Cre), which acts to stabilize CTNNB1 and therefore constitutively activates the WNT/CTNNB1 pathway in the *Ctnnb1*^{tm1Mmt} model (Harada 1999). These samples were subjected to microarray analysis, and results showed that all genes found to be up-regulated >5-fold on the Ad-WNT4 arrays were also induced on the Ad-Cre arrays, with 45 of 51 (88.2%) induced >2-fold (Fig.5). This suggests a substantial overlap between the transcriptional targets of WNT4 and those of the WNT/CTNNB1 pathway, and therefore that WNT4 signals via this pathway in granulosa cells.

DISCUSSION

Whereas the roles of WNTs in embryonic development and cancer are well established, their roles in adult tissues have only recently become an area of intense investigation. WNT4 in particular has well-established roles in sex determination and embryonic gonadal development (Vainio 1999, Jeays-Ward 2003, Yao 2004), but is also expressed postnatally in ovarian follicles and corpora lutea (Hsieh 2002). The fact that WNT4 expression increases in response to gonadotropin during late follicle development (Hsieh 2002) was particularly suggestive to us of its importance for ovarian function. In this study we report for the first time that WNT4 expression in the adult ovary is required for normal female fertility. Our analyses of *Wnt4*$^{flox/-}$;*Amhr2*$^{tm3(cre)Bhr/+}$ ovaries revealed that these animals are born with a normal complement of ovarian follicles and that these follicles subsequently develop normally through the secondary stage, but the number of healthy

antral follicles present at puberty was substantially decreased. This observation could be attributed either to a blockage of post-secondary follicular development, or to an increased rate of antral follicle atresia, or both. Our findings that the proportion of antral follicles undergoing atresia in *Wnt4*$^{flox/-}$;*Amhr2*$^{tm3(cre)Bhr/+}$ ovaries is markedly increased and that WNT4 overexpression increases the expression of genes associated with resistance to apoptosis *in vitro* argue that WNT4 acts as an ovarian follicle survival factor, but do not exclude the possibility that it is required for additional aspect(s) of follicle development. We were not able to conclusively determine why a subset of *Wnt4*$^{flox/-}$;*Amhr2*$^{tm3(cre)Bhr/+}$ mice failed to develop any antral follicles. This phenotype was presumed to be a more severe version of the developmental blockage/follicular atresia phenotype observed in most mice, and appears to lead to premature follicular depletion, perhaps due to accelerated recruitment from the follicular reserve to offset the loss of antral follicles to atresia. A considerable inter-animal variability was observed in the degree of knockdown of WNT4 expression in *Wnt4*$^{flox/-}$;*Amhr2*$^{tm3(cre)Bhr/+}$ ovaries, presumably due to inefficient and inconsistent recombination of the floxed *Wnt4* allele, as has been observed in several studies using the *Amhr2*$^{tm3(cre)Bhr/+}$ allele to target granulosa cells (Boerboom 2005, Li 2008, Pangas 2008, Pangas 2007). We speculate that the severe ovarian phenotype observed in some *Wnt4*$^{flox/-}$;*Amhr2*$^{tm3(cre)Bhr/+}$ mice was the result of complete or near-complete abrogation of WNT4 expression. The reason why this was rarely observed may relate to the kidney anomalies that invariably accompanied it. Indeed, these animals appeared sickly and grew poorly, likely as a result of inadequate kidney function. *Wnt4*$^{flox/-}$;*Amhr2*$^{tm3(cre)Bhr/+}$ mice were weaned at a frequency ~20% lower than the predicted Mendelian ratio (data not shown), indicating that a significant proportion died perinatally. This would suggest that the majority of the analyses presented in this report were in fact conducted with the

subpopulation of *Wnt4*$^{flox/-}$;*Amhr2*$^{tm3(cre)Bhr/+}$ mice in which ovarian recombination of the floxed *Wnt4* allele was the least efficient. The reason why kidney defects arose in *Wnt4*$^{flox/-}$;*Amhr2*$^{tm3(cre)Bhr/+}$ mice with the severe ovarian phenotype was not examined, but was almost certainly the result of unintended targeting of cell population(s) involved in the development of the kidney (Stark 1994).

Interestingly, an oocyte depletion phenotype was previously reported in *Wnt4*$^{-}$ null mice, resulting in the loss of most of the oocyte reserve in the days before birth (Vainio 1999). In light of our findings, this could suggest that WNT4 acts as a survival factor even at the earliest stages of folliculogenesis, and that loss of WNT4 expression in somatic cells results in the loss of oocytes even prior to germ cell cyst breakdown and follicle formation (Pepling 2006). However, several problems exist with the latter theory. Notably, no perinatal depletion of the oocyte reserve or increased atresia of primordial or primary follicles was detected in *Wnt4*$^{flox/-}$;*Amhr2*$^{tm3(cre)Bhr/+}$ mice despite that fact that Cre expression in the ovary from the *Amhr2*$^{tm3(cre)Bhr}$ allele occurs prior to birth (Lague 2008). Furthermore, *Fst, Bmp2* and other WNT4 target genes that have been identified in embryonic ovaries (Coveney 2008, Yao 2004) are not similarly regulated by WNT4 in cultured granulosa cells. Indeed, we report herein a set of adult granulosa cell WNT4-responsive genes that appears entirely distinct from its embryonic counterpart. The prenatal oocyte depletion phenotype observed in *Wnt4*$^{-}$ null mice may thus be unrelated to the follicle survival function of WNT4, and could simply be secondary to the severe developmental defects present in the *Wnt4*$^{-}$ null female gonad, as has been previously suggested by Yao et al (2004). We therefore propose that the physiological roles of WNT4 in the adult ovary are entirely distinct from its roles during the embryonic development of the gonad. Our microarray analysis of WNT4 downstream target genes has revealed that

the mediation of a stress response is a likely mechanism of WNT4 action in granulosa cells. The main role of the cellular stress response is to favor cell survival by inducing the expression of genes required for processes such as DNA repair, metabolic adaptations and anti-apoptotic signaling (Das 2008, Sedding 2008, Kultz 2005). The growing antral follicle represents a potentially hostile microenvironment, as the granulosa cell layer is avascular and must rely on diffusion through intercellular junctions for the exchange of nutrients and metabolic waste products. Intrafollicular hypoxic and oxidative stresses are thought to be much greater during the later phases of follicle development, as the thickness of the granulosa cell layer increases in parallel with the metabolic demands of activities such as proliferation and steroidogenesis (Fischer 1992, Banini 2004, Tsai-Turton 2006, Agarwal 2003). We therefore propose that WNT4 promotes follicle survival by permitting the adaptation of granulosa cells to the stressful microenvironment of the growing antral follicle, and thereby increasing their resistance to apoptosis. The involvement of WNT/CTNNB1 signaling in the response to oxidative stress has also recently been demonstrated in osteoblasts (Manolagas 2007, Jin 2008), and WNT4 can decrease oxidative stress-induced apoptosis in mesangial cells caused by high glucose treatment (Lin 2006, Lin 2008). The mediation of stress responses may therefore be a widespread role of WNT signaling in adult tissues. We have previously reported that sustained WNT/CTNNB1 signaling in the granulosa cells of transgenic mice causes the formation of follicle-like premalignant lesions that develop in a manner similar to normal follicles, but fail to differentiate properly or to regress (Boerboom 2005,Boerboom 2006). These lesions degenerate into granulosa cell tumors in a stochastic manner late in life, or following the loss of the anti-oncogene *Pten* (Boerboom 2005, Lague 2008). In light of the present study, we now propose that the anti-apoptotic activity of the WNT/CTNNB1 signaling pathway in

granulosa cells represents a likely mechanism by which it contributes to carcinogenesis. WNTs can signal via at least three distinct pathways, often referred to as the canonical (WNT/CTNNB1), WNT/calcium and planar cell polarity pathways

(Tevosian 2008). WNT4 seems capable of functioning alternatively as a canonical or as a non-canonical WNT, depending on the exact cell type and conditions (Bernard 2007, Tevosian 2008). In this report, we offer the first incontrovertible evidence that WNT4 can signal via the WNT/CTNNB1 pathway in granulosa cells. While our data do not exclude the possibility that WNT4 can also signal via non-canonical mechanisms, the fact that stabilization of CTNNB1 in granulosa cells induced essentially all of the genes that were induced by WNT4 overexpression argues that the WNT/CTNNB1 pathway is likely the major transducer of the WNT4 signal. The mechanisms of WNT4 signaling during sex determination and the embryonic development of the ovary have not yet been completely resolved, and could involve CTNNB1 as well as non-canonical effectors (Tevosian 2008).

How WNTs such as WNT4 can signal via distinct pathways according to cell type or developmental stage remains unclear, although some evidence suggests that the precise composition of the cell surface receptor complex determines the downstream signaling mechanisms that a given WNT can activate (Tevosian 2008). The identity of the physiological receptor(s) for WNT4 remains unknown, but their differential expression during ovarian development and later follicle development could conceivably explain differences between the embryonic and adult functions of WNT4 in the ovary. In summary, we report for the first time that WNT4 is required for normal female fertility and ovarian follicle development. Our results demonstrate that WNT4 favors antral follicle survival by increasing granulosa cell resistance to apoptosis. Microarray analyses demonstrated that WNT acts by inducing the expression of stress response genes via the WNT/CTNNB1

signaling pathway. Taken together, these results reveal the essential role of a novel pathway in follicular development, and define the mechanism of WNT4 action in granulosa cells.

ACKNOWLEDGEMENTS

The authors would like to thank Lisa White, John Lydon, Céline Forget, Janet DeMayo and Aron Cory for expertise and technical assistance with microarray analyses, targeting vector design, embryonic stem cell procedures, mouse colony management, dissections and follicle counting. *Amhr2*$^{tm3(cre)Bhr}$ and *Ctnnb1*^{tm1Mmt} mice were kindly provided by Dr Richard R. Behringer (University of Texas, Houston) and Dr Makoto M.Taketo (Kyoto University, Kyoto), respectively. The R1 embryonic stem cell line was provided by Dr Andras Nagy, Reka Nagy, Dr Janet Rossant and Dr Wanda Abramow-Newerly (University of Toronto).

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FIGURE LEGENDS

Figure 1. Conditional targeting of Wnt4 in granulosa cells. A) Illustration of the strategies used to generate the *Wnt4*^{flox} and *Wnt4*$^{-}$ alleles, as detailed in Materials and Methods. *LoxP* sites were inserted upstream of the third (eIII) and downstream of the fourth (eIV) exons. The placement of the genomic DNA probe used for Southern blotting is indicated in (Aa). DTα = diphtheria toxin α-chain, neo = neomycin resistance cassette. B) Generation of embryonic stem cell lines heterozygous for the targeted *Wnt4* allele. The presence of the targeted allele was detected as a 10kb BglII restriction fragment by Southern blotting (clones AC1 and AE11). C) PCR genotyping analysis strategy (upper panel) and typical results from tail biopsies (lower panel). Oligonucleotides (a-c) were designed so as to generate PCR products of 250bp for the *Wnt4*^{flox} allele, 210bp for the *Wnt4*$^{+}$ allele and 110bp for the *Wnt4*$^{-}$ (i.e., Cre-recombined) allele. D) RTPCR analysis of *Wnt4* expression in ovaries from mice of different genotypes. Results demonstrate markedly decreased *Wnt4* expression in *Wnt4*$^{flox/}$;*Amhr2*$^{tm3(cre)Bhr/+}$ mice relative to controls. E) Urogenital tracts from newborn mice of the indicated genotypes. Wnt4$^{-/-}$ mice generated by recombination of the Wnt4flox allele suffer from developmental kidney defects identical to those previously reported in *Wnt4* knockout mice, indicating that recombination of the *Wnt4*^{flox} allele results in complete loss of function. O = ovary, A = adrenal, K = kidney.

Figure 2. *Wnt4*$^{flox/-}$;*Amhr2*$^{tm3(cre)Bhr/+}$ mice have small ovaries and reduced fertility. A) Results of 6-month mating experiment demonstrated that similar numbers of litters were produced by *Wnt4*$^{flox/-}$;*Amhr2*$^{tm3(cre)Bhr/+}$ female mice, but that these were of smaller size. Control animals consisted of two *Wnt4*$^{flox/-}$ mice, one *Wnt4*$^{flox/+}$;*Amhr2*$^{tm3(cre)Bhr/+}$ mouse and one *Wnt4*$^{flox/+}$ mouse, all of which had comparable fertility. Litter size is

expressed as mean ± SEM. ** = significantly different than control, $P < 0.05$. B) Sections from representative *Wnt4*$^{flox/-}$ (control) and *Wnt4*$^{flox/-}$;*Amhr2*$^{tm3(cre)Bhr/+}$ ovaries, demonstrating smaller ovary size in *Wnt4*$^{flox/-}$;*Amhr2*$^{tm3(cre)Bhr/+}$ animals. Animals were 8wk-old littermates. C) Ovary weights from adult (8-10 month) *Wnt4*$^{flox/-}$;*Amhr2*$^{tm3(cre)Bhr/+}$ and control mice. Control animals consisted of littermates of the experimental animals, genotypes were *Wnt4*$^{flox/-}$ (n = 7), *Wnt4*$^{flox/+}$;*Amhr2*$^{tm3(cre)Bhr/+}$ (n = 6) and *Wnt4*$^{flox/+}$ (n = 5), weights did not differ by genotype among the control animals ($P > 0.05$). Ovary weight is expressed as mean ± SEM. ** = significantly different than control, $P < 0.05$.

Figure 3. *Wnt4*$^{flox/-}$;*Amhr2*$^{tm3(cre)Bhr/+}$ mice have increased follicular atresia and fewer antral follicles. Ovaries from mice of the indicated ages and genotypes were serially sectioned, and all follicles from every fifth section were counted, categorized and scored as healthy or atretic. One ovary (left) from each animal was evaluated, n = 6 (both genotypes in panel A), n = 5 (*Wnt4*$^{flox/-}$;*Amhr2*$^{tm3(cre)Bhr/+}$ panel B) or n = 8 (*Wnt4*$^{flox/-}$ panel B). Data represent raw follicle count numbers, and were not adjusted to estimate the total ovarian follicle population. All data are expressed as mean (columns) ± SEM (error bars). * = significantly different than control, $P < 0.05$, ** = significantly different than control, $P < 0.01$.

Figure 4. Rare *Wnt4*$^{flox/-}$;*Amhr2*$^{tm3(cre)Bhr/+}$ mice have very small ovaries and no antral follicles. A) Ovary from a two month-old *Wnt4*$^{flox/-}$;*Amhr2*$^{tm3(cre)Bhr/+}$ mouse showing an absence of antral follicles or corpora lutea. B) Activated caspase-3 immunohistochemistry experiment showing an atretic secondary follicle from the ovary shown in A. C) Kidney from the animal shown in A, showing dramatic developmental anomalies and few glomeruli. D) Ovary from an 8 month-old *Wnt4*$^{flox/-}$;*Amhr2*$^{tm3(cre)Bhr/+}$ mouse showing complete depletion of follicles.

Figure 5. WNT4 and the WNT/CTNNB1 pathway regulate genes involved in the cellular response to stress. Granulosa cells isolated from immature, eCG-treated *Ctnnb1*tm1Mmt/tm1Mmt mice were placed in culture, and infected with adenoviruses to express eGFP (control), overexpress WNT4 ("Wnt4 array") or express Cre ("Cre array"). Triplicate RNA samples from each treatment were analyzed using Affymetrix mouse expression set 430 microarrays. Fold-induction of all probe sets by Ad-WNT4 or Ad-Cre treatment were calculated by dividing values by those obtained by Ad-eGFP treatment. Probe sets called present on all three Ad-WNT4 arrays were then sorted in decreasing order of fold-induction, and fold-induction values, values obtained for the probe sets on the Ad-Cre arrays, and gene names corresponding to the probe sets are listed. Induction of selected genes was also verified by real-time RTPCR using a distinct set of RNA samples ("Wnt4 qRTPCR" column). Genes associated with the cellular response to stress are highlighted in yellow. Another group of stress response genes that were strongly induced by Ad-Wnt4, but that did not meet the original array data parsing criteria, are listed in the second block. All values are listed as mean ± SEM (n = 3 for all array experiments, n = 4 for all RTPCR experiments). * = Although carbonic anhydrase activity is well known to increase in response to hypoxic stress, neither the Car8 nor Car13 isoforms have been previously implicated in the hypoxia response. ** = Expression of Car8 and Pthlh was only detected in the Ad-Wnt4-infected cells and not in the Ad-eGFP-infected cells, resulting in an infinite signal ratio.

Table 1

Primer name	Sequence (5 ‘ to 3’)
Bmp2-S	AGACGTCCTCAGCGAATTTGAGT
Bmp2-AS	TCTGGAAGTTCCTCCAGCGCTT
Car8-S	TTCGAAGGCTGAGGACACA
Car8-AS	GTAACTGAAGATAATGCAGGCTTGA
Car13-S	ACAATCCTCAACTGCAAAAGATCACT
Car13- AS	AAGAGGCTTCGGAATCTGGCCA
Ccl5-S	GCTGCCCTCACCATCATCCTCA
Ccl5-AS	CTAGCTCATCTCCAAATAGTTGATGT
Ccnc-S	TGCCTACATGTAGCCTGTGTCGT
Ccnc-AS	TGGCTGTAGCTAGAGTTCTGACT
Cp-S	CACAGGGGAGTATACAGTTCTGAT
Cp-AS	TGCTGCAGGCGAATGAGCTGTA
Ddit3-S	AACCTGGTCCACGTGCAGTCAT
Ddit3-AS	GGAATCTGGAGAGCGAGGGCTT
Fst-S	CAAAGTCCTGTGAAGATATCCAGT
Fst-AS	TAGGAAAGTTGTAGTCCTGGTCT
Herpud1-S	CACCACGTCGGGTGGTTTCCGT
Herpud1-AS	GCACAAGGGCCATCAGTTGGCT
Mt1-S	TGGACCCCAACTGCTCCTGCT
Mt1-AS	CAAACAGGCTTTTATTATTCACGTACT
Nt5e-S	AGTCCACCGGAGAGTTCCTGCA
Nt5e-AS	GTATTCAGAAACCACGCTGATATCT
Pth1h-S	GTATTCCTGCTCAGCTACTCCGT
Pth1h-AS	ACCTTGTTGGTTTCCTGAGTTAGGT
Rpl19-S	CTGAAGGTCAAAGGGAATGTG
Rpl19-AS	GGACACAGTCTTGATGATCTC
Snai2-S	GTGATGCCCAGTCTAGGAAATCGT
Snai2-AS	CCTATTGCAGTGAGGGCAAGAGA
Trib3-S	CCCAGGCGGTGCTGGCACCTT
Trib3-AS	CCAGGTTCTCCAGCACCAGCTT
Vegfa-S	TGATCAAGTTCATGGATGTCTACCA
Vegfa-AS	CTGCATTCACATCTGCTGTGCTGT
Wnt4-S	AGCTGTCATCGGTGGGCAGCAT
Wnt4-AS	ACTGTCCGGTCACAGCCACACT

Table 1. Sequences of oligonucleotide primers used for RT-PCR.

Figure 1

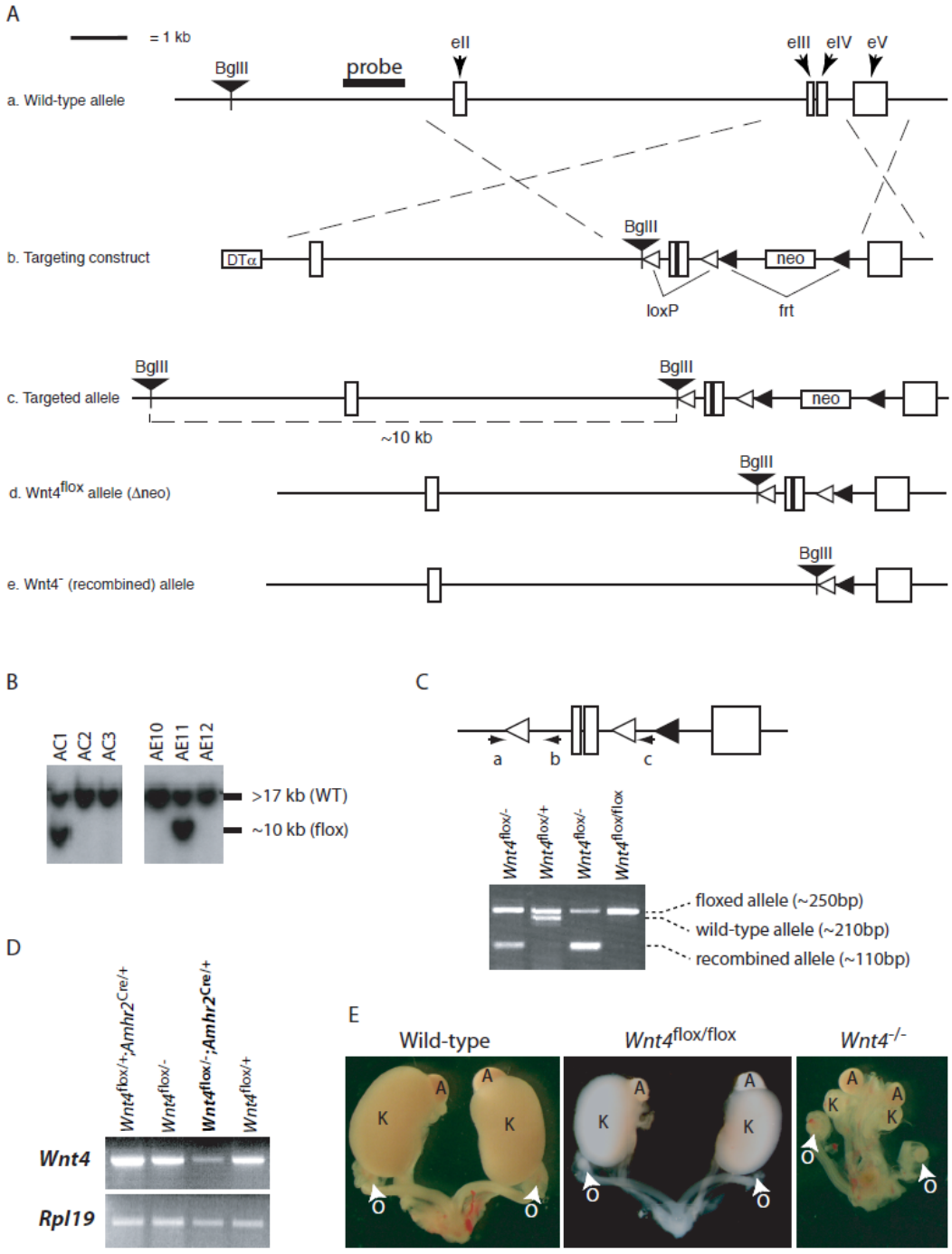

A
= 1 kb
BglII
probe
eII
eIII eIV eV
a. Wild-type allele
b. Targeting construct
DTα
neo
loxP
frt
c. Targeted allele
~10 kb
d. Wnt4flox allele (Δneo)
e. Wnt4- (recombined) allele
B
AC1 AC2 AC3
AE10 AE11 AE12
>17 kb (WT)
~10 kb (flox)
C
a b c
Wnt4flox/-
Wnt4flox/+
Wnt4flox/-
Wnt4flox/flox
floxed allele (~250bp)
wild-type allele (~210bp)
recombined allele (~110bp)
D
Wnt4flox/+;Amhr2Cre/+
Wnt4flox/-
Wnt4flox/-;Amhr2Cre/+
Wnt4flox/+
Wnt4
Rpl19
E
Wild-type
Wnt4flox/flox
Wnt4-/-
A
K
O

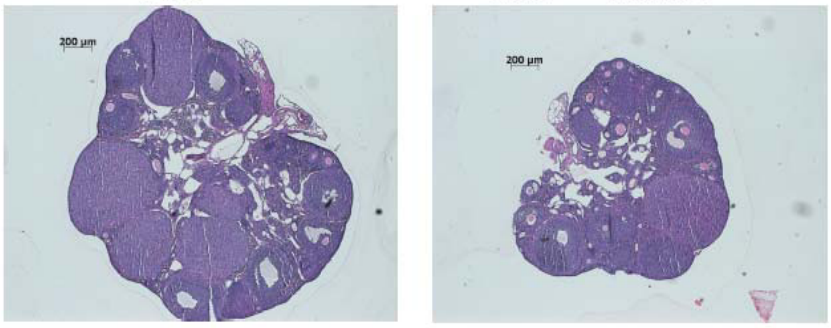
Figure 2

A. Mating experiment

	Control	*Wnt4*$^{flox/-}$;*Amhr2*$^{cre/+}$
n (females)	4	4
Total litters	25	21
Total pups	179	81
Litter size	7.19 ± 0.64	3.86 ± 0.38**

B.

Wnt4$^{flox/-}$ *Wnt4*$^{flox/-}$;*Amhr2*$^{cre/+}$



C. Ovary weights

	Control	*Wnt4*$^{flox/-}$;*Amhr2*$^{cre/+}$
n (ovaries)	36	18
Ovary weight (mg)	5.25 ± 0.31	2.56 ± 0.25**

Figure 3

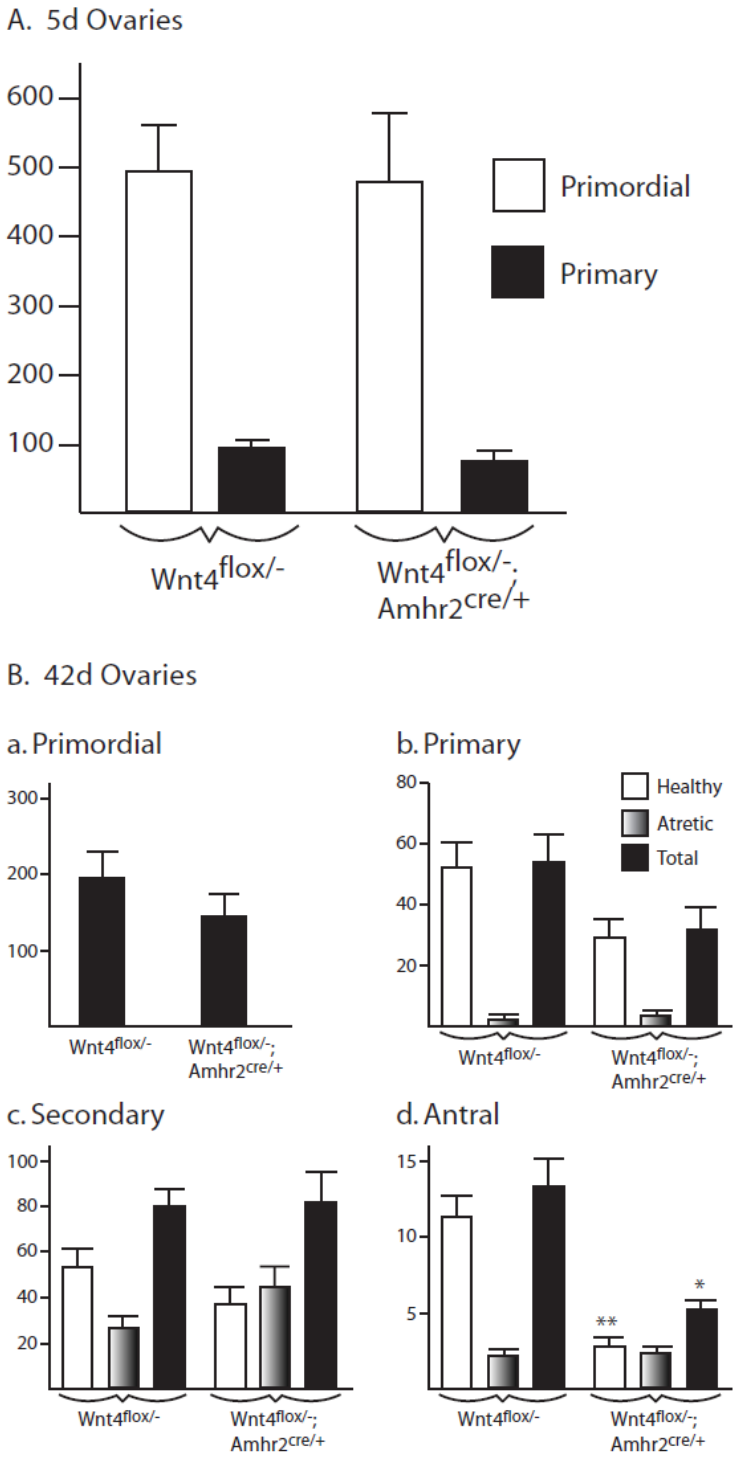

A. 5d Ovaries
600
500
400
300
200
100
Primordial
Primary
Wnt4flox/-
Wnt4flox/-;
Amhr2cre/+
B. 42d Ovaries
a. Primordial
300
200
100
Wnt4flox/-
Wnt4flox/-;
Amhr2cre/+
b. Primary
80
60
40
20
Healthy
Atretic
Total
Wnt4flox/-
Wnt4flox/-;
Amhr2cre/+
c. Secondary
100
80
60
40
20
Wnt4flox/-
Wnt4flox/-;
Amhr2cre/+
d. Antral
15
10
5
**
*
Wnt4flox/-
Wnt4flox/-;
Amhr2cre/+

Figure 4

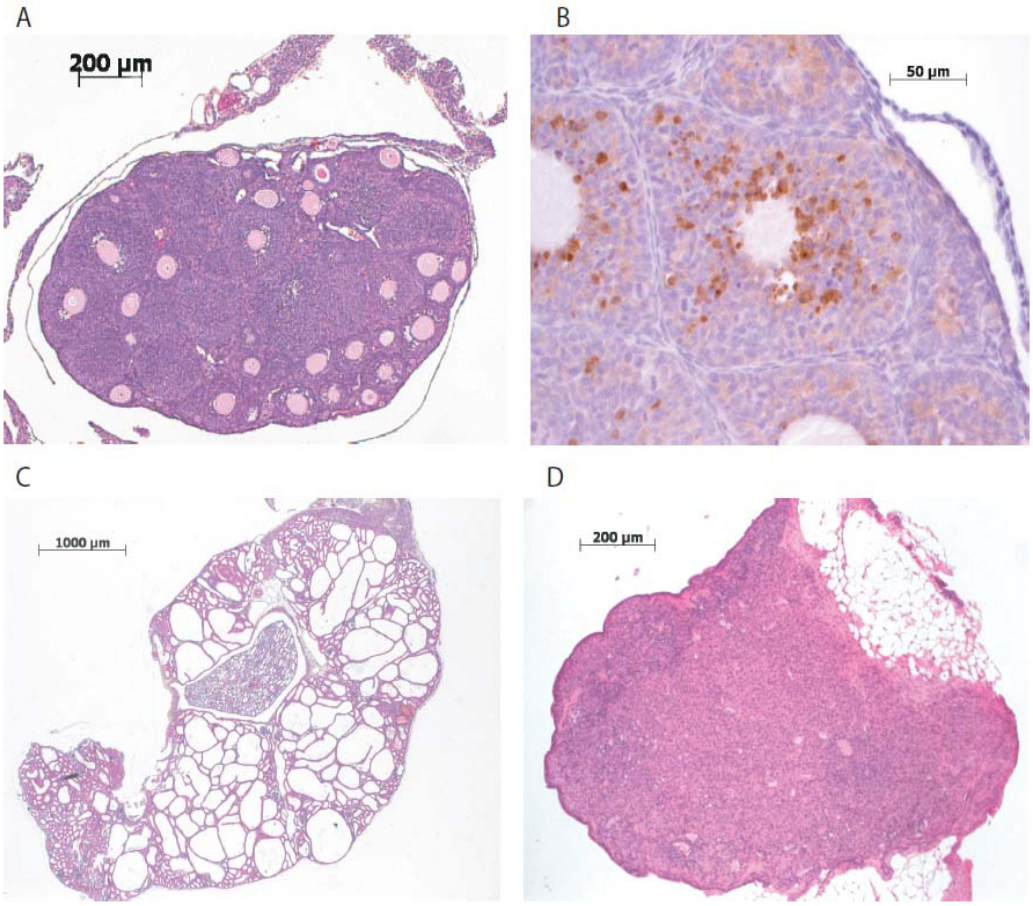


Figure 5

Wnt4 array fold induction	Wnt4 qRTPCR fold induction	Cre array fold induction	Gene symbol	Gene symbol and name
20,030 ± 19,285		8,821 ± 7,859	Ywhag	tyrosine 3-monooxygenase/tryptophan 5-mono-oxygenase activation protein, gamma polypeptide
19,102 ± 14,063		7,742 ± 4,057	Sprr1a	small proline-rich protein 1A
16,741 ± 8,782	17,68 ± 17,68	4,550 ± 2,376	Trib3	tribbles homolog 3 (Drosophila)
14,551 ± 13,515		9,917 ± 9,278	Lhx9	LIM homeobox protein 9
12,722 ± 11,827		7,524 ± 5,720	Mtmr7	myotubularin related protein 7
11,619 ± 7,797		7,220 ± 5,986	Saa3	serum amyloid A 3
11,414 ± 3,272		2,015 ± 0,860	Rsad2	radical S-adenosyl methionine domain containing 2
11,175 ± 7,415		2,200 ± 1,151	Usp18	ubiquitin specific peptidase 18
10,999 ± 9,002		3,763 ± 2,325	Mmel1	membrane metallo-endopeptidase-like 1
10,400 ± 8,652		4,423 ± 2,596	Klf4	Kruppel-like factor 4 (gut)
10,085 ± 4,482		2,279 ± 1,195	Rsad2	radical S-adenosyl methionine domain containing 2
9,107 ± 6,910		3,629 ± 2,494	Parp14	poly (ADP-ribose) polymerase family, member 14
8,489 ± 7,296		5,571 ± 4,521	Ocln	occludin
8,463 ± 1,072		4,145 ± 2,526	Tgtp	T-cell specific GTPase
8,445 ± 7,091		4,466 ± 3,287	Centg2	centaurin, gamma 2
8,253 ± 5,207		5,174 ± 2,260	Pkd2l2	polycystic kidney disease 2-like 2
7,761 ± 6,691		8,179 ± 7,219	Arhgap6	Rho GTPase activating protein 6
7,644 ± 2,333	7,183 ± 2,595	3,250 ± 0,762	Nt5e	5' nucleotidase, ecto
7,639 ± 1,848	**	2,527 ± 0,732	Car8*	carbonic anhydrase 8*
7,623 ± 4,875	5,748 ± 2,183	1,082 ± 0,240	Ddit3	DNA-damage inducible transcript 3
7,047 ± 5,225		7,899 ± 7,136	H2-Q7	histocompatibility 2, Q region locus 7
6,954 ± 5,755		1,955 ± 0,916	Timp4	tissue inhibitor of metalloproteinase 4
6,664 ± 3,032	2,783 ± 0,902	1,802 ± 0,575	Snai2	snail homolog 2 (Drosophila)
6,565 ± 4,599		3,235 ± 1,784	Ldb2	LIM domain binding 2
6,305 ± 4,396		3,629 ± 2,151	Siah2	seven in absentia 2
6,229 ± 4,138		3,944 ± 2,215	Ccl2	chemokine (C-C motif) ligand 2
6,116 ± 3,773		1,958 ± 0,515	Star	steroidogenic acute regulatory protein
6,057 ± 1,848		4,265 ± 2,013	Tnfrsf23	tumor necrosis factor receptor superfamily, member 23
5,975 ± 2,274	2,793 ± 0,820	16,940 ± 9,451	Mt1	metallothionein 1
5,695 ± 3,942		5,012 ± 4,102	Ndrg1	N-myc downstream regulated gene 1
5,639 ± 2,961		3,715 ± 2,393	Ero1l	ERO1-like (S. cerevisiae)
5,567 ± 4,081		2,304 ± 1,229	Nab1	Ngfi-A binding protein 1
5,476 ± 3,537		3,911 ± 2,491	Osmr	oncostatin M receptor
5,415 ± 4,498		2,444 ± 1,419	Abcg1	ATP-binding cassette, sub-family G (WHITE), member 1
5,412 ± 4,393		4,869 ± 3,510	Zfp53	zinc finger protein 53
5,359 ± 3,962		2,568 ± 0,626	Gbp2	guanylate nucleotide binding protein 2
5,324 ± 3,556		5,021 ± 3,050	Tph1	tryptophan hydroxylase 1
5,311 ± 0,523		2,491 ± 0,874	Gbp3	guanylate nucleotide binding protein 3
5,293 ± 3,169		1,197 ± 0,147	Dmn	desmuslin
5,195 ± 2,002		2,218 ± 1,021	Plcb4	phospholipase C, beta 4
5,165 ± 0,839	24,02 ± 15,34	1,064 ± 0,240	Herpud1	homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1
5,069 ± 1,198		3,339 ± 1,228	Ifitm3	interferon induced transmembrane protein 3
5,057 ± 4,380		4,945 ± 3,967	Ak3l1	adenylate kinase 3 alpha-like 1
5,026 ± 2,983		2,228 ± 1,195	Megf10	multiple EGF-like-domains 10
5,022 ± 1,835		2,190 ± 0,659	Irf7	interferon regulatory factor 7
52,518 ± 28,539	7,244 ± 3,295	12,746 ± 8,871	Car13*	carbonic anhydrase 13*
19,106 ± 11,154	9,813 ± 5,786	16,614 ± 15,409	Vegfa	vascular endothelial growth factor A
18,892 ± 13,515	28,26 ± 11,68	14,376 ± 11,913	Ccl5	chemokine (C-C motif) ligand 5
18,109 ± 12,406	32,67 ± 19,34	11,078 ± 8,672	Cp	ceruloplasmin
16,948 ± 8,402	10,42 ± 5,830	5,148 ± 3,301	Ccnc	cyclin C
12,122 ± 4,072	**	6,914 ± 1,347	Pthlh	parathyroid hormone-like peptide
	34,99 ± 15,32		Wnt4	wingless-related MMTV integration site 4

General discussion

The Wnts are a class of signalling molecules that are expressed in the adult ovary, but whose roles in ovarian physiology remain poorly defined. Recent descriptive studies have demonstrated the regulation of several Wnts and Wnt signalling pathway components in the ovary. Interestingly, several of these have been shown to exhibit specific patterns of regulation in response to gonadotropin stimulation, suggesting that they may function during specific phases of follicular development (34, 36, 39, 40).

The first indication of the importance of Wnt signalling in ovarian processes came from a study by Vainio et al (19), which reported that inactivation of the *Wnt4* gene in mice results in the loss of a large fraction of the reserve of oocyte in the days prior to birth. Subsequent studies have shown that *Wnt4* expression is required during embryonic female gonadal development to repress the formation of the male–specific coelomic blood vessel (35).

A recent descriptive study has shown that *Wnt4* mRNA is expressed in granulosa cells of small follicles in the postnatal ovary and that this expression is increased in pre-ovulatory follicles, as well as in association with ovulation and luteinization (34). Furthermore, constitutive activation of the Wnt/β-catenin pathway in granulosa cells *in vivo* results in differentiation defects and the formation of granulosa cell tumors (41,42). These observations suggested to us that WNT4 could be a somatic cell factor involved in some aspect of follicle development. Unfortunately, proof of this could not be obtained from the *Wnt4* knockout mouse, which dies shortly after birth due to kidney defects (20). Based on these studies, a primary objective of our work was to study the physiological role of WNT4 in the adult ovary by identifying its transcriptional targets in granulosa cells.

Stressors such as hypoxia and free radicals can damage proteins, lipids and DNA. To counteract the adverse effects of stress, cells possess intricate anti-stress defence mechanisms, which permit cellular adaptation and survival by mechanisms such as the repair or replacement of damaged molecules. When these fail, cells undergo apoptosis (43). Surprisingly, our microarray results showed that 35.6% of genes induced by WNT4 overexpression are associated with the cellular response to stress, and this was confirmed by quantitative real time RT-PCR. If WNT4 therefore acts to mediate a stress response, how is this relevant to follicular development? The granulosa cell layer is avascular and must rely on diffusion through intercellular junctions for the exchange of nutrients and metabolic waste products (47). The increase in follicular diameter during maturation is accompanied by an increase in vascular supply, but this is obviously not sufficient to bridge the growing diffusion distance between the capillary network surrounding the follicle and its center. The preovulatory follicle is therefore a stressful microenvironment, being both hypoxic and rich in genotoxic free radicals. We therefore propose that the WNT4-induced stress response permits the adaptation of granulosa cells to the microenvironment of the growing antral follicle, and thereby increasing their resistance to apoptosis. While the notion that WNT signalling promotes cellular survival in the ovary is novel, there is evidence that WNT proteins prevent apoptosis in other cell types. For example, uncommitted osteoblast progenitors and differentiated osteoblasts can be rescued from apoptosis by both β-catenin dependant and independent signalling cascades (44). Likewise, overexpression of the Wnt signalling co-receptor LRP5 mutation or deletion of the Wnt antagonist SFRP-1 in mice reduce osteoblast and osteocyte apoptosis (45,46).

The transcriptional mechanisms by which WNT4/β-catenin induces the expression of its stress response target genes remain to be elucidated. In many cell types, β-catenin mediates Wnt signalling by binding to and activating members of the T cell factor (TCF) transcription factor family. β-catenin has also been recently implicated as a pivotal molecule in defence against oxidative stress by serving as a co-factor of the forkhead box O (FOXO) transcription factors in the regulation of genes involved in bone, glucose, and lipid metabolism. With increasing age, increased reactive oxygen species production is thought to divert the limited pool of β-catenin from TCF/LEF to FOXO- mediated transcription (43). It is possible that a similar mechanism occurs in granulosa cells in response to oxidative stress. More recently, β-catenin was shown to interact with the orphan nuclear receptor steroidogenic factor-1 (NR5A1) to modulate the FSH/cAMP dependent expression of aromatase (CYP19A1) in rat ovarian granulosa cells (48). The enzyme CYP19A1 is present in the granulosa cells of healthy growing antral follicles, and converts androgens to estrogens. Estrogen is known to facilitate the differentiation of granulosa cells and to inhibit granulosa cell apoptosis (49). It is therefore tempting to speculate that WNT4/β-catenin signalling in granulosa cells is mediated through interactions with steroigenic factor-1, thereby regulating genes involved in estrogen synthesis, leading to increased estrogen levels and follicle survival. These hypothesis could be tested by in vitro gene regulation assays, measurement of steroid hormone production, NR5a1/β-catenin co-immunoprecipitation, and CYP19A1 chromatin immunoprecipitation following addition of WNT4.

Conclusion

In conclusion, we report that Wnt4 is required for normal follicle development and survival, particularly at the post-secondary stages. Wnt4 regulates the expression of a series of genes associated with the cellular stress response. We therefore propose that Wnt4 acts to induce an adaptive response that is critical for granulosa cell survival during the later stages of follicle growth.

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