Characterization of the WNT/\(\beta\)-catenin signaling pathway in the development of mouse ovarian surface epithelium (mOSE) and follicular ovulatory capability

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Abstract

Wnts are secreted extracellular signaling molecules that act locally to control diverse developmental processes such as cell fate specification, cell proliferation, and cell differentiation. Three WNT signaling pathways have been identified. The best characterized is the canonical or WNT/β-catenin signaling pathway. Recently, the WNT signaling pathway has been implicated in ovarian development and differentiation. However, little is known about the expression or role of β-catenin/Tcf-signaling activity within ovarian compartments. In order to aid in the ongoing pursuit of elucidating the mechanisms that govern ovarian differentiation and development, I explored the possible roles of the canonical WNT signaling pathway in the development of the ovarian surface epithelium (OSE) and follicular ovulatory capability. To examine canonical Wnt-signaling within the ovary, I utilized the Tcf-lacZ-reporter mice.

In Manuscript I, I present a detailed spatio-temporal pattern of β-catenin/Tcf mediated expression in the OSE throughout development. Cells covering the indifferent gonad at E11.5 were β-catenin/Tcf signaling (lacZ-positive). With further development and sexual differentiation, lacZ staining was lost over the testis but maintained on embryonic ovaries. This staining became dispersed and the proportion of lacZ-positive OSE cells decreased to relative constancy when female mice reached maturity. FACS analyses revealed the lacZ-positive cell population exhibits cytoprotective mechanisms as indicated by enrichment within a side population. The results indicate that the mouse OSE is heterogeneous and may contain a population of progenitor cells.
In Manuscript II, I investigated the molecular connection between β-catenin and Tcf-mediated lacZ activity and assessed whether β-catenin stabilization regulates β-catenin/Tcf-mediated gene expression and OSE proliferation. β-catenin was detected on the lateral membranes of ovarian epithelium. I demonstrated that treatment of OSE cells with Wnt agonist stabilized β-catenin but failed to induce β-catenin/Tcf-lacZ expression. Furthermore, E-cadherin expression was down-regulated and the proliferative potency of OSE cells increased. Of four ovarian cancers cell lines screened, only the HEY cell line demonstrated induction of luciferase reporter expression upon canonical WNT stimulation. These studies indicate that nuclear localization of β-catenin is insufficient for β-catenin/Tcf mediated gene expression in OSE cells, suggesting GSK-3β inhibition as a result of altered WNT signaling is of major importance in the control of epithelial-mesenchymal transition and cell proliferation leading to tumorigenesis.

In Manuscript III, I investigated the role of the canonical WNT signaling pathway in the development of follicular ovulatory capability. Oocytes in primordial and primary follicles did not show active WNT signaling. β-catenin/Tcf-signaling was activated at the secondary follicle stage and the proportion of β-catenin/Tcf-signaling (lacZ-positive) follicles increased with follicular maturation. In contrast, the majority (>90%) of oocytes recovered from the oviducts at estrus and following hormone stimulation were lacZ-negative. The results indicate that the canonical WNT signaling pathway is active in growing oocytes and suggest that canonical WNT signaling may be involved in the development of follicular ovulatory capability and identifies non-ovulatory follicles.
Résumé

Les Wnts sont des molécules de signalisation sécrétées dans l’espace extracellulaire pour réagir localement et contrôler divers processus du développement comme la spécification, la prolifération et la différenciation des cellules. Trois voies de signalisation WNTS ont été identifiées. La plus connue est la voie canonique appelée aussi la voie WNT/β-catenin.

Récemment des études ont montré que la voie de signalisation WNT serait impliquée dans le développement et la différenciation ovarienne. Malgré ces études, peu est connu sur l’expression et la fonction de l’activité β-catenin/Tcf dans les différents compartiments d’ovaire. Pour clarifier les mécanismes moléculaires responsables dans le développement d’ovaire, j’ai étudié le rôle de la signalisation WNT/β-catenin durant la formation d’épithélium superficiel ovarien et dans la capacité ovulatoire des follicules. Pour visualiser l’implication de la voie canonique dans l’activation ovarienne, nous avons utilisé des souris transgéniques qui expriment la protéine β-galactosidase (lacZ) en réponse des protéines β-catenin/Tcf.

Le premier article que je présente (Manuscrit I) décrit l’activation spatiotemporal de β-catenin/Tcf dans l’épithélium superficiel ovarien au cours du développement de la souris. Au jour E11.5, la gonade non-différenciée est marquée positivement pour LacZ indiquant que β-catenin/Tcf est activée. Au cours de la différenciation sexuelle de la souris, l’expression de LacZ disparaît dans les testicules. Par contre, dans l’ovaire embryonnaire l’expression de LacZ est maintenue. À la maturation sexuelle de la souris femelle, l’expression de LacZ est devenue plus
diffuse et la quantité de cellules qui expriment LacZ a diminué. Une analyse de cytométrie en flux a indiqué que la population de cellules positive pour LacZ démontre des mécanismes cytoprotecteurs. En conclusion, les résultats suggèrent que l’épithélium superficiel ovarien est constitué d’une population de cellules hétérogène et mais aussi de cellules progénitrices.

Le deuxième article que je présente (Manuscrit II) étudie la stabilisation de β-catenin, les gènes induit par le complexe de β-catenin/Tcf et la prolifération d’épithélium superficiel ovarien. β-catenin est localisée dans les membranes latérales d’épithélium ovarien. J’ai démontré que la stimulation des cellules d’épithélium superficiel ovarien avec une agoniste Wnt stabilise β-catenin mais n’induit pas l’expression de lacZ dans les souris transgéniques. En plus, le niveau d’expression du gène E-cadherin a baissé et la prolifération des cellules d’épithélium superficiel ovarien a augmenté. Des quatre lignées cellulaires de cellules ovariennes cancéreuses qu’on a examinées, seulement la lignée HEY avait une réponse transcriptionnelle à la stimulation de protéines WNT canoniques. Nos études révèlent que la localisation nucléaire de β-catenin n’est pas suffisante pour induire l’expression de ces gènes cibles dans l’épithélium superficiel ovarien. Dans les cellules cancéreuses la voie de signalisation WNT est altérée de sorte que l’inhibition de GSK-3β est augmentée et β-catenin est activée ce que contrôle la prolifération et la transition d’épithélium à mésonchyme durant la tumorigénèse.

Le troisième article que je présente (Manuscrit III) examine le rôle de la voie canonique durant le développement folliculaire. Les ovocytes des follicules primordiaux et primaires ne montraient...
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Dedication

This thesis is dedicated to the memory of my late father, Paa Usongo Isaac Akuta. I miss him every day, but I am glad to know he initiated this process, offering support as well as encouragement to make it possible. Thanks to mom, Ogwe Ruth Usongo, for dealing with me being worlds away and for making so many things possible.
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<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNase</td>
<td>Deoxyribonuclease</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FDG</td>
<td>Fluorescein-di-β-D-galactopyranoside</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>LacZ</td>
<td>β-galactosidase enzyme</td>
</tr>
<tr>
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<td>Lithium chloride</td>
</tr>
<tr>
<td>M</td>
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<td>mg</td>
<td>Milligram</td>
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<td>ml</td>
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<tr>
<td>M-MLV</td>
<td>Moloney Murine Leukemia Virus</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<tr>
<td>ng</td>
<td>Nanogram</td>
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<tr>
<td>NHS-biotin</td>
<td>N-Hydroxysuccinimidobiotin</td>
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<tr>
<td>Term</td>
<td>Definition</td>
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<tr>
<td>---------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
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<td>Ribonucleic acid</td>
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<tr>
<td>RNase</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute Media</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>s.e.m.</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>TAE</td>
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<td>TBS</td>
<td>Tris-buffered saline</td>
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<tr>
<td>Tcf</td>
<td>T-cell factor</td>
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<tr>
<td>TJ</td>
<td>Tight junctions</td>
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<tr>
<td>μg</td>
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<td>Micromolar</td>
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<td>5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside</td>
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Publications

1. **Macalister Usongo** and Riaz Farookhi. β-catenin/Tcf-signaling appears to establish the murine ovarian surface epithelium (OSE) and remains active in selected postnatal OSE cells. *BMC Developmental Biology* 2012, 12:17 [Manuscript I]

2. **Macalister Usongo**, Xinfang Li, and Riaz Farookhi. Activation of the canonical WNT signaling pathway promotes ovarian surface epithelial proliferation without inducing β-catenin/Tcf-mediated reporter expression. *Developmental Dynamics* 2012 (accepted: DVDY-12-0189.R1) [Manuscript II]

**Contribution of authors**

1. **Macalister Usongo** and Riaz Farookhi. *BMC Developmental Biology* 2012, 12:17

   *Contribution:* The candidate, Macalister U., performed all experiments presented in the publication, analyzed the data and wrote the manuscript. RF designed the research and provided guidance.

2. **Macalister Usongo**, Xinfang Li, and Riaz Farookhi. *Developmental Dynamics* 2012 (accepted: DVDY-12-0189.R1)

   *Contribution:* The candidate, Macalister U., performed all experiments presented in the publication with the exception listed below. Macalister U. designed the research, performed data analysis and wrote the manuscript. Xinfang Li performed the transfection experiments and assisted in Western blot analysis. RF supervised the research.


   *Contribution:* The candidate, Macalister U., performed all experiments presented in the publication with the exception listed below. Macalister U. designed experiments and methods, carried out the laboratory experiments, analyzed data, interpreted results and wrote the paper. Aida Rizk assessed the proportion of lacZ-positive follicles in prepubertal mice following hormone stimulation. RF provided guidance.
CHAPTER 1

Background
1.0. Introduction

The process of sexual development requires a network of endocrine and paracrine signalling pathways, hormones, and transcription factors during a critical window in embryogenesis. In mammals, the reproductive systems of the male and female embryos consist of an indifferent gonad indistinguishable by morphological criteria. The ovary and testis develop from a single primordium. For this reason, the gonadal primordium is called “the bipotential gonad”. Thus the process of gonadal development begins with the formation of the indifferent gonad, followed by the development of a testis or an ovary. Although the switch that initiates divergent development of the bipotential gonad is diverse among species, the underlying mechanisms that lead to sexual differentiation are likely conserved (Kim and Capel, 2006). Gonadal development is divided into four main phases; germ cell specification and migration, formation of the bipotential gonad, sex determination, and sexual differentiation.

1.1. Germ cell specification and migration

Primordial germ cells (PGCs) are established early during embryonic development. In mammals, gametogenesis begins during gastrulation when the PGCs are first determined from epiblast cells and then specified in the extraembryonic mesoderm at the basis of allantois (Ginsburg et al., 1990; Lawson
and Hage, 1994). PGCs are derived from part of the population of epiblast cells that will give rise mainly to the extraembryonic mesoderm (Lawson and Hage, 1994). Recent studies in mice have shown that bone morphogenetic proteins (BMP4 and BMP8b) emitted from the extraembryonic ectoderm are essential for specification of PGCs (Lawson et al., 1999; Ying et al., 2000). Both BMP4 and BMP8b are expressed in the extraembryonic ectoderm before and during gastrulation. The PGCs begin as a cluster of about 25-30 cells at E7.0 at the base of the allantois (Fig. 1) and are destined to give rise to the entire population of germ cells in the adult animal (Ginsburg et al., 1990; Lawson and Hage, 1994; Saitou, 2009).
Figure 1. Schematic illustration of the emergence of PGCs. PGCs originates from the proximal epiblast as a cluster of alkaline-phosphatase (AP)-positive cells in the extraembryonic mesoderm (ExM) at around embryonic day (E) 7. AVE: anterior visceral endoderm; EM: embryonic mesoderm; DE: definitive endoderm; PGCs: primordial germ cells. [Modified from (Saitou, 2009)]

Initially, epiblast cells are known to express a number of markers such as SSEA-1, AP, Oct4. These markers gradually disappear from areas of epiblast not involved in germ line formation, possibly signifying loss of totipotency (Tam and Zhou, 1996). Characteristically, PGCs stain strongly for alkaline phosphatase (AP) activity and continue to express AP during their proliferation in the developing hindgut and migration into the genital ridges (Buehr, 1997).

Generally, PGCs are formed at some distance from the prospective gonad and
have to migrate through different tissues before finally reaching the developing
gonad by E11.5. PGCs begin their migration into the embryonic mesoderm by
E8.5, continue to migrate through the hindgut endoderm at E9.5, along the
developing mesentery of the hind-gut and dorsal body wall mesenchyme by
E10.5, until they finally colonize the gonadal ridges by E11.5 (Molyneaux et al.,
2001). Studies in mice have shown that colonization of the gonadal anlagen is
caused by active movement of PGCs (Donovan et al., 1986). It has been
demonstrated that two factors control PGC migration in mouse embryos: the
intrinsic capacity of PGCs to spread and move, and extrinsic factors released from
the target tissues controlling the direction of migration and supporting the PGC
population (Donovan et al., 1986; Godin et al., 1990). Once in the gonadal ridge,
PGCs lose their motile behaviour. During their migration, PGCs proliferate and
increase in number. In mouse, PGCs increase from approximately 100 cells at
E8.5 to 4000 by E12.5, eventually resulting in approximately 25,000 PGCs at
E13.5 (Godin et al., 1990; Tam and Snow, 1981). Several genes have been shown
to be important in PGC development in the mouse embryo. Mutations in the
receptor tyrosine kinase c-kit (Buehr et al., 1993), Steel, encoding the c-kit ligand
(Runyan et al., 2006), and the POU domain transcription factor Oct4 (Kehler et
al., 2004), all result in drastic reduction of germ cells.

Once in the gonad, germ cells undergo several rounds of division before they
differentiate. The behaviour of germ cells is similar in the male and female
embryo. However, after entering the genital ridges, their fates diverge (McLaren, 1998). In males at about E13.5, germ cells cease dividing and remain in G0/G1 as gonocytes until after birth. Proliferation then resumes, with the first germ cells not entering meiosis until about a week later (McLaren, 1995). In the ovary, germ cells undergo one final mitosis at 13.5 dpc and then enter meiosis (McLaren and Southee, 1997). If XX or XY germ cells migrate into ectopic tissue by error, they will go into meiotic arrest (McLaren and Southee, 1997). Germ cells are known to play a more active role in the development of the ovary than they do in testis development. They are not required for testis cord formation, whereas they are needed for the organization of the ovary into follicles and its maintenance thereafter (McLaren, 1991).

1.2. Formation of the bipotential gonad

In mammals, the indifferent or bipotential gonad arises as paired structures within the intermediate mesoderm. The intermediate mesoderm in mice can first be recognised at E9, forming two bilateral longitudinal ridges on the posterior body wall known as the urogenital ridges. In mice, the gonads form around E10.5 on the developing urogenital ridge. The urogenital ridge comprises 3 overlapping kidney units: (1) the pronephros, including the adrenal primordium near its caudal end; (2) the mesonephros, the central region from which the gonad arises; and (3) the metanephros, the most posterior region from which the definitive kidney
arises (Capel, 2000). The presumptive gonads emerge as swellings of the
coelemic epithelium overlaying the medio-lateral surface of the mesonephros by
E10. Subsequently, cell proliferation within the thickening coelomic epithelium
gives rise to the somatic cell component of the gonad (Karl and Capel, 1998b).
It is also possible that surrounding tissues contribute to the somatic cell
component of the gonad. The somatic cell types in the bipotential gonad have
sexually dimorphic fates, and in response to appropriate signals, will follow either
a male or female developmental pathway.

Primordia for the male and female ductal system are present within the urogenital
ridge. The mesonephric Wolffian ducts are the progenitors of the male ductal
system. Closely associated with the Wolffian ducts are the paramesonephric
Müllerian ducts, the progenitors of the female ductal system. The Müllerian duct
differentiates into the oviduct, uterus, cervix and upper part of the vagina, while
the Wolffian duct differentiates into the epididymis, vas deferens and seminal
vesicle (Behringer et al., 1994). By E14.5, the Müllerian duct becomes female-
specific and the Wolffian duct male-specific, with subsequent, sex specific
degeneration of Wolffian or Müllerian ducts in females and males, respectively
(Fig. 2)
Figure 2. Differentiation of the gonad and its associated sex ducts. Originally, both Müllerian and Wolffian ducts are present at the bipotential stage. In males, the Müllerian ducts degenerate, whereas the Wolffian ducts differentiate into epididymides, vasa deferentia, and seminal vesicles under the control of androgens. In females, the Wolffian duct regresses and the Müllerian duct differentiates into oviduct, uterus, and upper vagina. Adapted from [(Wilhelm et al., 2007)].

1.3. Genes involved in the formation of the indifferent gonad

There are several gene mutations identified by mutational analysis in mice and humans that cause abnormal gonad development. Four genes, mostly transcription factors, are known to be involved in the differentiation of the intermediate
mesoderm as a whole. Knock outs of these genes in mice typically results in
gonadal agenesis. These genes have been identified as Sf-1, Wt-1, Lhx9 and
Emx2.

Steroidogenic factor 1 (Sf-1; also called nuclear receptor subfamily 5, group A,
member 1 and designated NR5A1) is a key regulator of steroidogenic enzymes
and is expressed in all primary steroidogenic tissues including the adrenal cortex,
testicular Leydig cells, theca and granulosa cells of the ovary and the corpus
luteum (Ikeda et al., 1993). Sf-1 is a transcription factor belonging to the
subfamily of nuclear receptors, the orphan receptors, for which no clear activating
ligand is known (Parker et al., 1999). Sf-1 plays a role in steroid biosynthesis by
regulating steroid hydroxylases. It is expressed in the urogenital ridge by E9.0 and
is responsible for differentiation of the adrenal glands and for normal sexual
development of the gonads (Ikeda et al., 1994; Luo et al., 1994). Sf-1 null mice
show complete gonadal agenesis, thus establishing its role in sexual
differentiation and primary steroidogenic tissue formation.

Wilms tumor suppressor (Wt1), a proline rich zinc finger transcription factor, is
critical for kidney and gonad formation. It is expressed during early development
of the urogenital ridge, with expression becoming restricted to Sertoli and
granulosa cells after sex determination (Pelletier et al., 1991; Sharma et al., 1992).
Wt1 mutations are associated with Wilm’s tumor, a childhood malignancy arising
from persistent renal stem cells (Armstrong et al., 1993). \textit{Wt1} is first expressed in the intermediate mesenchyme by E9.0 and is suggested to play a role in mediating mesenchymal to epithelial transition during embryonic development (Armstrong et al., 1993). \textit{Wt1} functions as a transcriptional activator as well as repressor. \textit{Wt1} encodes several protein isoforms, the best known being the +KTS and –KTS isoforms, which differ only by the presence or absence of three amino acids (Hammes et al., 2001). Reduction of +KTS isoforms, as found in Frasier patients, causes male-to-female sex reversal, male pseudohermaphroditism (the external genitalia have a female appearance despite an XY genotype) and gonadal dysgenesis. Denys-Drash syndrome (DDS) involves reduction in the –KTS isoform leading to urogenital abnormalities and degenerated gonads. The different phenotypes are due to the different roles the isoforms play during gonadal development. However, \textit{Wt1} deficient mice die \textit{in utero} with a complete absence of kidneys, gonads and adrenals (Kreidberg et al., 1993). Gonadal development is initiated in the mutant mice, but is then arrested by E12.5, indicating a role for \textit{Wt-1} in the establishment of the genital ridge. All available evidence points to normal development of the gonads in \textit{Wt1}\textsuperscript{+/-} animals whereas humans heterozygous for \textit{Wt1} experience cryptorchidism (Kreidberg et al., 1993).

\textit{Lhx9} belongs to the LIM homeobox gene family with roles in early gonadal development. The LIM homeodomain proteins are transcription factors characterized by the presence of two NH2-terminal LIM domains involved in
protein-protein interactions, followed by a DNA-binding homeobox domain (Hobert and Westphal, 2000). *Lhx9* is expressed on the medial surface of E9.5 urogenital ridge, with increased expression detected in the mesothelial lining, subjacent mesenchyme, and lower levels in deeper layers of the gonadal ridge by E11.5 (Birk et al., 2000). *Lhx9* mutants lack gonads by E13.5. The urogenital ridge first forms in a similar manner to that in wild-type, but no discrete gonad evolves, suggesting that signals controlled by Lhx9 are responsible for gonadal growth and the proliferation and invasion of the epithelium into the mesenchyme, an event essential for gonadal formation (Birk et al., 2000). No histological evidence of apoptosis was detected and the massive cell proliferation normally seen in the genital ridge at E11.5 was markedly attenuated in the mutant embryos. However, it was observed that expression of *Sf-1* in these mutants was reduced to minimal levels in the urogenital ridge, indicating that *Lhx9* lies upstream of *Sf-1* in the developmental cascade.

*Emx2*, a mouse homeobox-containing gene homologous to the *Drosophila empty spiracles (ems)* gene, is expressed in the early primordia of organs forming the excretory and reproductive systems (Pellegrini et al., 1997). Specifically, *Emx2* is expressed in the epithelial components of the pronephros and mesonephros, Wolffian and Müllerian ducts, ureteric buds, and the “bipotential” gonads. *Emx2* mutants lack gonads and genital tracts (Miyamoto et al., 1997). In *Emx2* mutants, thickening of the coelomic epithelium marking marks the first stage of gonadal
development is not prominent, and degeneration of the Wolffian duct and mesonephric tubules is abnormally accelerated without the formation of the Müllerian duct.

1.4. **Sex determination**

Sex determination is defined by the combination of sex chromosomes determining the sex of an offspring. In many cases, sex chromosomal genes determine the sex of an organism (genotypic sex determination). Genotypic sex determination (GSD) entails any mechanism of sex determination in which a genetic factor is the primary sex-determining signal. GSD occurs by any of the following two mechanisms; 1) genetic action, when at least one specific gene is considered to be the central regulator in a cascade of events leading to the determination of sexual phenotype; 2) the presence of distinct sex chromosomes (Manolakou et al., 2006).

GSD occurs in different constitutions: single-locus complementary sex determination (slCSD), as in the insect order of Hymenoptera, the ratio of X chromosomes to autosomes and the sex switching gene, sxl as in *Drosophila melanogaster*; XX/XY sex determining system in mammals, and the ZZ/ZW pattern of sex chromosomes as in some reptiles, amphibians, fish (Manolakou et al., 2006). However, in some reptiles, sex is not controlled genetically but by a temperature dependent sex determination (TDS) mechanism (Harvey and Slatkin, 1982).
In the mammalian XX/XY sex determining system, sex determination depends on
the presence or absence of a signal from a male sex determining gene on the Y
chromosome. Testis development is associated with a single Y-linked gene locus,
the testis determining factor (TDF) in humans, or its homolog Tdy in mice. If Tdy
is expressed, testes are formed, and most male secondary sexual characters
develop under the influence of hormones secreted by the testes. In the absence of
Tdy, the embryo develops as a female, and its gonads differentiate as ovaries.

1.5. Sexual differentiation

Most organisms have two sexes. Sexual differentiation is a broad term used to
describe the process through which an individual becomes male or female. It is a
consequence of the action of hormones produced following gonadal
determination. In addition to germ cells and coelomic epithelial cells, other
somatic cell components are present within the indifferent gonad. The founding
cell populations in the bipotential gonad have the capacity to differentiate into
either ovarian or testicular cell types. The somatic cells will differentiate into
follicular cells in the ovary or Sertoli cells in the testis, supporting the growth and
maturation of germ cells. Sex hormones produced by steroidogenic cells, theca
and granulosa cells in females and Leydig cells in males, are then required for the
development of secondary sex characteristics. One crucial event that tilts the
balance of sex differentiation towards male development is the expression of a gene on the Y chromosome, called the testis determining region of the Y (Sry).

1.5.1. Testis differentiation

The earliest morphological landmark of testis organogenesis is organization of the testis cords at E12.5. Testis development is associated with a single Y-linked gene locus located on the distal part of the short arm of Y chromosome. The search for the testis determining gene led to the identification of the male sex determining gene, Sry (Gubbay et al., 1990; Sinclair et al., 1990). In mammals, Sry expression determines the sex of the organism by initiating the development of a testis instead of an ovary from the bipotential primordium (Koopman et al., 1991). Sry is expressed in the XY gonad between 10.5 and 12.5 d.p.c and does not require the presence of germ cells (Bullejos and Koopman, 2001; Koopman et al., 1990). Sry expression initiates Sertoli cell differentiation, marking the initiation of testis specific development and providing a starting point for cell fate decisions in the gonad. Evidence from adult XX-XY chimeric mouse testis indicates that the Sry gene is required only in sertoli cells in the developing testis, suggesting that a critical threshold number of Sry-expressing cells (more than 30%) is needed to recruit the gonad to the testis pathway (Palmer and Burgoyne, 1991). Sry is expressed from the centre of the genital ridges, or simultaneously from the central and anterior regions, and then moves posteriorly. Sry extinction appears to follow a similar pattern. Sry encodes a DNA binding protein of the HMG-box family that
recognizes both chromatin structure and a DNA binding sequence (Stros et al., 2007). The presence of the DNA binding domain suggests the protein could function as a transcription factor. Sry binds to the minor groove of DNA through the HMG-box and induces a sharp bend. The DNA binding domain of mammalian Sry proteins is highly conserved, indicating the usefulness of the domain in the function of the protein. Interestingly, mutations in Sry that cause male to female sex reversal are located within the HMG box (Cameron and Sinclair, 1997). The regulation of Sry expression has to be tightly controlled, as mice with weak Sry allele or expressing Sry too late show ovotestis formation (Wilhelm et al., 2007). If Sry is deleted from the Y chromosome in an XY mouse, the gonad differentiates as an ovary (Wilhelm et al., 2007). It is possible for XX individual with no Y chromosomal sequences that include SRY to have a completely normal male phenotype indicating that Sry is not the only sex-determining gene (Vilain and McCabe, 1998).

Following Sry expression, all the known major autosomal genes involved in the sex determination pathway begin to diverge. One of the best candidate genes downstream of Sry known to show male-specific upregulation is Sox9, a member of the Sry-related homeobox (SOX) family of transcription factors. Sox9 plays a critical role in male sexual differentiation as over-expression of Sox9 in XX mice leads to testis development (Vidal et al., 2001) whereas disruption of Sox9 expression in the XY gonad causes male–to-female sex reversal (Chaboissier et
al., 2004; Wagner et al., 1994). These effects are similar to the results obtained by over-expressing or deleting Sry in the developing gonad. This suggests that the establishment of Sox9 expression is a critical step downstream of Sry in testis differentiation. Sox9 is expressed in the bipotential gonad and upregulated immediately after Sry expression. It is expressed at low levels in both male and female gonads, upregulated by E11.5 in XY gonads and down-regulated in XX gonads, and specifically expressed in Sertoli cells once testis cords are formed (Capel, 2000). Sox9 co-localizes with Sry in the nucleus of pre-sertoli cells as early as E11.5 and is important for the induction of anti-Müllerian hormone (Amh; also known as Müllerian inhibitory substance, MIS) gene expression (Brennan and Capel, 2004). Sox8, a close homologue of Sox9, also activates the Mis promoter, and has been shown to reinforce Sox9 action in testis formation (Chaboissier et al., 2004).

The fate of the testis during differentiation is established in somatic precursor cells by the collective functions of transcription factors and signaling molecules. One of these factors is Fgf9. Fgf9 is a member of the Fgf gene family, which comprises 22 functionally diverse polypeptides in humans and mice regulating a broad range of cellular activities, including proliferation, survival, migration, and differentiation in many organs during embryonic development (Itoh and Ornitz, 2008). Fgf9 is expressed in the bipotential gonad and later resolves to an XY-specific pattern by E12.5 (Capel et al., 2006). Mice mutants for Fgf9 display male-to-female sex reversal caused by disruption of all testis-specific cellular
events (Colvin et al., 2001). The reproductive system phenotype of Fgf9-mutants varies from testicular hypoplasia to complete male-to-female sex reversal.

1.5.2. Ovary differentiation

Ovarian development is initiated during fetal life and involves key events, such as gonadal sex differentiation, germ cell mitosis, atresia and entry into meiosis. Early ovarian differentiation is marked by the presence of mesonephric tissue located toward the central part of the gonad, which serves to concentrate PGCs toward the periphery of the future cortical region (Matthew, 2009). As early as tail somite 15 stage (approximately 11.2 dpc.), coelomic epithelial cells invade the subjacent mesenchyme tissue and gradually surrounds the PGCs (Karl and Capel, 1998a). The first recognizable step in the process of ovarian differentiation involves organization of germ cells (or oogonia) and epithelial (pregranulosa) cells into epithelial structures called ovigerous cords, which are continuous with the ovarian surface epithelium (Guigon and Magre, 2006a). Ovigerous cords are delimited by a basement membrane and are loosely surrounded by somatic cells. Ovigerous cords break down to form primordial follicles consisting of an immature oocyte arrested early in meiosis surrounded by a flattened epithelium which will eventually become the granulosa cells (Richards and Pangas, 2010). The primordial follicles represent the entire stock of follicles with which the ovaries are endowed. Once growth is initiated, the primordial follicle is transformed into a primary follicle and the oocyte grows. The supporting cell lineage will become
granulosa cells and the steroidogenic cell lineage becomes theca cells. Granulosa cells support germ cells inside the developing follicle. Theca cells, located in the ovarian interstitium outside the basement membrane produce androgens, which are ultimately converted to estradiol by granulosa cells (Hirshfield, 1991). The ovary is covered by the surface epithelium, which is a modified mesothelium, also called coelomic epithelium. Throughout gestation, ovaries are generally smaller than the testis and consistently have a more elongated appearance.

1.5.2.1. Ovarian surface epithelium (OSE)

The ovarian surface epithelium (OSE) has long been known as the ‘germinal epithelium’ due to the belief that it gave rise to germ cells. The origin of germ cells in the mouse is clearly established; germ cells migrate from the base of the allantois via the gut mesentery and populate the gonad between 10.5 and 11.5 dpc (Ginsburg et al., 1990; Gomperts et al., 1994). The coelomic epithelium covering the primitive gonad, however, contributes to the supporting cell lineage of the developing ovary (Karl and Capel, 1998b). OSE cells migrate into the mesenchyme and become associated with germ cells to form sex cords. These cords later break up as pregranulosa cells to encapsulate oogonia and form primordial follicles.
1.5.2.2. Embryonic development of the OSE

It is well-known that sex organs first appear in the embryo as elongated swollen ridges of peritoneum on the ventro-median surfaces of the mesonephros. The future OSE forms part of the coelomic epithelium, which is the mesodermally-derived epithelial lining of the intraembryonic coelom. This intraembryonic coelomic epithelium is the precursor of the pleura, peritoneum, pericardium and Müllerian duct-derived epithelium, i.e. the oviduct, uterus, and proximal one-third of the vagina are derived from the peritoneal mesothelium (Byskov, 1986; Naora, 2007). The OSE is a continuation of the peritoneal mesothelium and overlies the ovary (Auersperg et al., 2001). The OSE overlies the presumptive gonadal area and, by proliferation and differentiation, it gives rise to part of the gonadal blastema (Auersperg et al., 2001). This embryonic process that is closely related to epithelial-to-mesenchymal interaction somehow indicates an intimate biological relationship between the epithelium and mesenchyme in mesodermal tissues (Fig 3). During the course of development, the OSE changes from a flat-to-cuboidal simple epithelium with a fragmentary basement membrane to one with a well defined basement membrane by term. OSE differs from the rest of the extraovarian mesothelium during fetal development in that it does not express cancer antigen 125 (CA125), a cell surface glycoprotein expressed by epithelial ovarian tumours as well as by other tissues of Müllerian origin (Jacobs and Bast, 1989). This difference could be evidence of divergent differentiation between OSE and other mesothelium. Thus part of the coelomic epithelium that gives rise
to the OSE does not reach the stage of differentiation where CA125 is expressed as in other coelomic epithelial derivatives. This interpretation is in keeping with the concept that OSE is developmentally less mature than other mesothelium and that its development is arrested at an early stage.

Figure 3. Sequential changes in the gonadal ridge, which is covered by modified coelomic epithelium (shaded). The coelomic epithelium proliferates and forms cords that penetrate into the ovarian cortex, giving rise to granulosa cells in the primordial follicles. The Mullerian ducts (Mul. duct) develop as invaginations of the celomic epithelium dorsolaterally from the gonadal ridges. Adapted from [(Auersperg et al., 2001)].
1.5.2.3. Structure of the OSE

The OSE is an inconspicuous monolayer of squamous-to-cuboidal cells covering the mammalian ovary. It is characterized by expression of cytokeratin 8, with some stromal features such as vimentin (Auersperg et al., 2001). It has been suggested that squamous and cuboidal forms of OSE cells represent cell groups that respectively have or have not undergone postovulatory proliferation (Gillett et al., 1991). In addition to these two cell forms, OSE cells tend to assume columnar shapes, especially within clefts and ovarian inclusion cysts. It is not known whether changes in OSE cell shape are the result of crowding or whether they reflect genetically determined metaplastic changes. The importance of surface invaginations and inclusion cysts lies in the propensity of the OSE to undergo metaplastic changes (Maines-Bandiera and Auersperg, 1997; van Niekerk et al., 1991). The OSE is separated from the ovarian stroma by a basement membrane (basal lamina) and differs from all other epithelia by its tenuous attachment to the basement membrane from which it is easily detached by mechanical means (Auersperg et al., 2001). Underneath the OSE is a dense collagenous connective tissue layer, the tunica albuginea. Epithelial tightness is maintained by formation of specialized structures known as tight junctions (TJ). Intercellular contact and epithelial integrity of OSE are maintained by simple desmosomes, tight junctions (Stevenson and Keon, 1998), integrins (Kruk et al., 1994), and cadherins (Davies et al., 1998).
1.5.2.4. OSE Function

Apart from restricting the movement of molecules and ions across the epithelial sheet, TJs also have a role in the maintenance of the apical/basolateral polarity of epithelial cells (Stevenson and Keon, 1998). Functionally, OSE is implicated in the ovulatory process and is responsible for repair and re-epithelialization of the ovulatory wound (Murdoch, 1994; Szotek et al., 2008). In the adult, the OSE is believed to actively participate in the ovulatory process. It has been suggested that proteolytic enzymes released from cytoplasmic granules of epithelial cells degrade the tunica albuginea and underlying apical follicular wall, thereby weakening the ovarian surface to the point of rupture (Murdoch and McDonnel, 2002). OSE cells located directly over the point of ovulatory rupture undergo apoptosis and are shed from the ovarian surface before ovulation (Murdoch, 1995). Thus the wound created at the ovarian surface is repaired by rapid proliferation of OSE cells from the perimeter of the ruptured follicle (Osterholzer et al., 1985). During postovulatory repair, the OSE undergoes epithelial-mesenchymal conversion as a homeostatic wound healing mechanism, as well as to accommodate OSE cells that become trapped within the ovary at ovulation (Auersperg et al., 2001). The OSE at the ovulation sites acquire a flat squamous-like appearance, which is thought to be a metaplastic process in response to injury at ovulation. A repeat of the wounding and re-epithelialization process provides an opportunity for the accumulation of mutations that may contribute to carcinogenesis. The OSE is exposed to hormones and growth factors produced by
The concentration of these factors is high at certain periods and this occurs in a cyclic manner. Thus, the OSE is more prone to be influenced than other types of mesothelium within the abdomen. OSE cells express receptors for estrogens, androgens, progestins, GnRH, FSH, LH (Leung and Choi, 2007), and growth factor receptors such as those for EGF and TGFα (Auersperg et al., 2001). The effects of these agents on the physiology and pathology of OSE are incompletely defined and/or controversially discussed.

1.5.2.5. Granulosa cells

Ovarian somatic development has remained poorly understood. However, a dual origin of somatic cells of the ovary has been demonstrated (Wartenberg, 1982). The gonadal blastema is formed by two types of somatic cells: cells segregating from the mesonephros and cells of the proliferating coelomic epithelium (Wartenberg, 1982). Mesonephric cells invade the growing cortex through the rete blastema and interact with cells derived from the coelomic epithelium thereby forming the primary gonadal blastema. The differentiation of the female gonad manifests itself in an unequal distribution of somatic cells: light cells concentrated in the ovarian cortex whereas dark cells dominate in the central core or medullary region. In addition, the coelomic epithelium has been shown to be a source of Sertoli cells in mice (Karl and Capel, 1998a). Therefore, as the counterpart cell type of Sertoli cell in the ovary, coelomic epithelial cells in XX gonads give rise
to granulosa cells. Thus, the OSE is believed to be part of the progenitor cells of ovarian somatic cells.

Granulosa cell differentiation requires proper expression of forkhead-domain transcription factor L2, Foxl2. Foxl2 is a transcription factor expressed specifically in pre-granulosa cells. Foxl2 is necessary for gonadal differentiation and for female gonadal specification (Uda et al., 2004). Homozygous Foxl2 mutants are infertile due to failure of the granulosa cells to progress from squamous to cuboidal form, thereby preventing the formation of primary follicles (Schmidt et al., 2004). This indicates that Foxl2 plays an important role in regulating the development of granulosa cells.

1.5.2.6. Theca cells

Theca cells are steroidogenic cells positioned outside the basement membrane of the ovarian follicle (Erickson et al., 1985). These cells are a constant feature of all antral follicles and are responsible for producing androgens. Their exact origin remain unknown but scattered evidence indicates that they continuously arise from a population of unspecialized mesenchymal cells (Erickson et al., 1985). Because theca cells are only present and adjacent to growing ovarian follicles, it seems their differentiation is closely associated with granulosa cells (Magoffin, 2005). Theca cells are thought to differentiate from stromal tissue. There are two
thecal layers- an inner theca interna and an outer theca externa. The theca interna, a highly vascularized layer of cells, first appears in the secondary stage of follicle development when the oocyte is fully grown and the follicle has acquired two to three layers of granulosa cells. The theca externa constitutes a loosely organized band of non-steroidogenic cells between the theca interna and the interfollicular stroma (Erickson et al., 1985). In the preovulatory follicle, the theca interna is the predominant site of androgen synthesis. Because theca cells differentiate adjacent to developing follicles, it is hypothesized that the growing follicles secrete a signal that stimulates thecal differentiation. Magarelli and Magoffin have demonstrated that small-molecular-weight proteins secreted by granulosa cells, named thecal differentiating factors (TDFs), stimulate thecal differentiation (Magoffin and Magarelli, 1995). Thus the differentiation of theca cells requires granulosa cells. Both theca and granulosa cells types are required for ovarian follicles to produce estrogen.

1.5.2.7. Follicular development

The follicle is the functional unit of the ovary, serving to protect and nourish the oocyte. Most follicles present in the mammalian gonad at birth will undergo atresia at some point along their developmental pathway. Ovarian folliculogenesis is characterized by two opposing processes: follicular growth and atresia. Ovarian follicles are committed to either development or atresia once a third layer of granulosa cells is formed (Oakberg, 1979). During fetal development, ovigerous
cords breakdown into follicular units comprised of an oocyte arrested at the diplotene stage of meiotic prophase, delimited by a basement membrane (Guigon and Magre, 2006a). Prior to primordial follicular formation, massive germ cell degeneration takes place, regulating the number of follicles endowed within the ovary (Kaipia and Hsueh, 1997). In contrast to male germ cells, proliferation of female oogonia occurs prenatally in mice. Throughout life, follicles are recruited from the primordial follicle pool for further growth and development in a continuous manner. Folliculogenesis begins with germ cell recruitment into a pool of primordial follicles, which will later progress through the primary, secondary, antral and preovulatory follicle stages (Guigon and Magre, 2006a). By puberty (6 weeks old in mice), increases in circulating FSH during each reproductive cycle recruit a cohort of antral follicles (McGee and Hsueh, 2000). This cyclic recruitment of follicles in rodents (estrous cycle) is characterized by hormonal and physiological changes (Byers et al., 2012). In the absence of gonadotropin stimulation, the follicles (and oocyte) degenerate.

As the primordial follicle is transformed into a primary follicle, the oocyte grows and produces a mucopolysaccharide coating called the zona pellucida that separates the oocyte from the surrounding granulosa cells. Oocyte growth is accompanied by an increase in the size of the oocyte and granulosa cell proliferation. During this process, the oocyte acquires a normal morphology, the
ability to resume and complete meiosis, and to initiate and sustain embryonic development (Krisher, 2004). Only a subset of oocytes are capable of supporting meiosis, fertilization and early embryo development to the blastocyst stage, and the proportion of these competent oocytes increases with follicular size (Mermillod et al., 2008). The developmental competence of oocytes increases in parallel with follicular size (Marchal et al., 2002). In the mouse, oocytes in primordial follicles appear not to require any stimulation to initiate growth (Miyano, 2003). Although meiosis and early development may be completed successfully, there are a variety of other processes occurring within the oocyte that are required for complete developmental competence (Krisher, 2004).

Follicular maturation is controlled by complex regulatory mechanisms orchestrated by several factors including the pituitary gonadotropins FSH and LH (Richards, 1994), intraovarian growth-regulatory factors such as IGFs, EGF, TGF-α, TGFβ, bFGF (Gougeon, 1996), and steroids such as estradiol (Edson et al., 2009). Additional locally-produced factors belonging to the TGFβ superfamily such as activins, inhibins, follistatin (Knight and Glister, 2001), as well as oocyte-derived factors including GDF9 and BMP-15 (Matzuk, 2000), are required at different stages of follicular development. Thus, it is evident that folliculogenesis in mice is controlled at two levels through intragonadal factors and extragonadal factors.
1.6. Differentiation of the sex ducts and external genitalia

During embryonic development, the embryo has both sex ducts. However, normally only one of these systems will develop any further. Sex differentiation is complete when common sex ducts develop into either a male-typical or a female-typical pattern. This process is under hormonal control. In the male, the Müllerian duct regresses under the influence of AMH, and testosterone promotes Wolffian duct differentiation into the epididymis and vas deferens (Rey, 2005). The result of these hormonal actions is a fetus with male (Wolffian) ducts but no female (Mullerian) ducts. In females, in the absence of AMH, the Müllerian ductal system differentiates further into the oviduct, uterus and upper part of the vagina, while the Wolffian ductal system regresses. In the anlagen of the external genitalia, testosterone is converted by 5α-reductase into dihydrotestosterone (DHT), a more potent androgen that binds the androgen receptor to induce external virilisation (Rey, 2005). The external genitalia develop into the female phenotype in the absence of the Y chromosome, while differentiation occurring in males is dependent solely on androgen production by the testes, with DHT being specifically required for full virilisation (Wiener et al., 1997)

1.7. Hormonal control of sex determination

After sex determination, further differentiation of the gonad is almost totally hormone-dependent. *Sry* expression induces the bipotential gonad to differentiate
into a testis that produces two hormones, anti-Müllerian hormone (AMH) or Müllerian-inhibiting substance (MIS), and testosterone. Androgen synthesis within the mitochondria and secretion by Leydig cells is essential for masculinization of the fetus. Testosterone, produced by the Leydig cells, is responsible for the development of the epididymis, vas deferens, seminal vesicles. AMH, in turn, causes regression of the Müllerian ducts, which would otherwise give rise to specific female structures (Cai, 2009). AMH belongs to the transforming growth factor β (TGF β) family. AMH signals via the membrane-bound serine/threonine kinase type II receptor, requiring recruitment and phosphorylation of a type I receptor.

Insulin-like factor 3 (Ins3; also known as Leydig insulin-like hormone, Ley I-L), a member of the insulin-like hormone superfamily, has recently been identified, and mutations in this gene cause abnormal development of the reproductive organs. Ins3 is expressed early in fetal mouse Leydig cells. Ins3-/- male mice are bilaterally cryptorchid - the gubernacular bulbs fail to develop and resemble the normal female gubernacular structure (Zimmermann et al., 1999). Histological analyses of the testes of Ins3-/- mice revealed defects in spermatogenesis. Double-mutant male mice for Ins3 and Ar (androgen receptor) genes showed testes positioned adjacent to the kidneys and steadied in the abdomen by the cranial suspensory ligament, demonstrating that the testicular factors androgen and Ins3
are essential for establishment of the sexual dimorphic position of the gonad (Zimmermann et al., 1999).

A role for estrogens in testicular function remained elusive, until the generation of mouse mutants for the aromatase gene or the estrogen receptors, α and β. Estrogen action is mediated through two receptors: estrogen receptor α (ERα) and estrogen receptor β (ERβ). Ovaries of mice lacking the aromatase gene (ArKO) develop cells possessing structural and functional characteristics of testicular interstitial cells and of seminiferous tubule-like structures lined with Sertoli cells (Britt et al., 2002). Estrogen replacement can prevent this transdifferentiation, thus establishing a role for estrogen in maintaining female somatic interstitial and granulosa cells. ERα deficient female mice (αERKO) have hypoplastic uteri and hyperaemic ovaries without any corpora lutea and are infertile. Male αERKO mice are also infertile, demonstrating that a functional receptor is essential for normal fertility in both sexes (Walker and Korach, 2004). αERKO male mice are sterile due to pleiotropic defects, including a reduction in the number (80%) and motility (5%) of epididymal sperm. In contrast, ERβ mutants (βERKO) develop normally and are indistinguishable histologically from their wild-type controls. In addition, double mutants lacking both receptors (αβERKO) show partial gonadal female-to-male sex reversal, which is not detectable at birth but appears later in adulthood (Couse et al., 1999). Both sexes are infertile, with the male mutant phenotype resembling that of αERKO. The ovaries of αβERKO female mice
contain structures that resemble seminiferous tubules, including Sertoli-like cells, and expression of Müllerian inhibiting substance and Sox9.

1.8. Genes involved in gonadal development

The expression pattern of some genes suggests a role in development and differentiation of the gonad. *GATA-4, Fog2, insulin-like growth factor (Igf)*, and *Dmrt1* are expressed in the developing gonad at the time of sex determination.

*Gata4* belongs to the GATA family of zinc finger proteins, which are transcription factors that play critical roles in various developmental processes. At E11.5, *Gata4* is expressed in somatic cells of both XX and XY genital ridges (Viger et al., 1998). By E13.5, *Gata4* expression becomes sexually dimorphic. In XY gonads expression is upregulated in Sertoli cells and downregulated in interstitial cells, whereas in XX gonads, expression is downregulated in all cells. The early function of *Gata4* in differentiation of the gonad is not known, as *Gata4*-deficient mice die in utero by E9.5 due to cardiac defects (Kuo et al., 1997). *In vivo*, the function of GATA factors requires physical interaction with multitype zinc-finger proteins of the FOG (Friend of GATA) family. Mouse homozygous for the null allele of *Fog2* or homozygous for a conditional knock-out of *Gata4* abrogating the interaction of *Gata4* with *Fog*, extending embryo survival to E13.5, have been generated (Tevosian et al., 2002). Analysis of the gonads established an essential role for *Fog2* and *Gata4* in gonad differentiation, indicating that testis development is blocked through interference with their direct
physical interaction. Testis cord development was absent in XY mutant gonads. The results also establish the need for Fog2 in normal development of ovaries and testes, as both XY and XX mutant gonads looked alike and did not resemble normal XY or XX gonads.

The insulin family signalling pathway is essential for normal development in mice. Triple knock outs of three insulin-receptor genes, \( Ir, Igf1r, \) and \( Irr \) results in complete male to female sex reversal (Nef et al., 2003). Triple mutants show complete sex reversal at both the histological and molecular levels. XY gonads were reduced in size and later expressed female markers. Based on the fact that \( Sry \) expression and \( Sox9 \) was reduced, and later male markers were absent in triple mutants, the study demonstrated that insulin signalling is required for establishment of the male pathway.

Doublesex-related gene (\( Dmrt1 \)) is the mammalian homologue of the \( C. \ elegans \) sex-determining gene \( Mab-3 \) and the Drosophila sex-determining gene \( doublesex (dsx) \). The sexual dimorphic pattern of \( Dmrt1 \) expression in mouse gonads suggests a role in gonadal differentiation (De Grandi et al., 2000). \( Dmrt1 \) is expressed in the genital ridges of both XY and XX and the signal appears to be identical in both sexes. At E12.5 in the male, it appears to mark the newly forming testis cords, while in the female it has a punctuate pattern throughout the ovary. By E14.5, the expression becomes clearly sexually dimorphic. In males,
expression is still strong in the cords, while in the female it drops to an undetectable level. $Dmrt1^-$ mutation causes severe defects in adult testis, and homozygous $Dmrt1^{+/+}$ mutants have severely hypoplastic testes (Raymond et al., 2000). However, $Dmrt1$ is not required for ovary development in the mouse.

The X-linked orphan nuclear receptor DAX1 (dosage-sensitive sex reversal, adrenal hypoplasia congenita, critical region on the X chromosome, gene 1) has reported functions in testis development. Dax-1-deficient females are fertile although some models of sex determination still portray $Dax1$ as an anti-testis gene (Goodfellow and Camerino, 1999). $Dax1$ has been demonstrated to play a crucial role in testis differentiation through regulation of the development of peritubular myoid cells and formation of intact testis cords (Meeks et al., 2003). The gonad is histologically indifferent at E11.5, and there were no apparent differences between wild-type male, female or $Dax1$-deficient gonad at 11.5 dpc. Thus, $Dax1$ is not required for gonadal development from the urogenital ridge. Fetal Leydig cell development is arrested in $Dax1$ mutants, and sertoli cells appear disorganized. DAX1 acts in part by repressing the transcription of other nuclear receptors e.g. $Sf1$ (Ito et al., 1997). Other regulatory peptides that have been shown to impact gonadal differentiation and development are members of the WNT family.
1.9. The WNT signaling pathway

Wnts are secreted cysteine rich glycoprotein molecules (Miller, 2002) involved in a number of developmental processes including the establishment of cell fate, proliferation and differentiation (Cadigan, 2008; Logan and Nusse, 2004b; Wodarz and Nusse, 1998). The name Wnt was derived from the Drosophila gene, wingless (Wg), and the related mammalian oncogene, Int-1. Members of this family of proteins are highly conserved with paracrine and autocrine functions. In humans, 19 Wnt genes have been identified and chromosomally located, and all Wnt proteins are similar in size (Miller, 2002)). Although their structure is unknown, as they are insoluble, all have 23 or 24 cysteine residues, the spacing of which is highly conserved. This suggests that Wnt protein folding may depend on the formation of multiple intramolecular disulfide bonds. Wnt proteins associate with glycosaminoglycans in the extracellular matrix, and remain close to the cell surface (Miller, 2002). Recent data indicates that Wnts can function as concentration-dependent, long-range morphogenic signals that act on distant neighbors (Logan and Nusse, 2004b). This suggests that they are secreted proteins. Mutation of porcupine in Drosophila leads to a lack of Wnt activity due to retention of Wg protein in the endoplasmic reticulum (van den Heuvel et al., 1993). In these mutants, the Wnt protein was retained in intracellular compartments suggesting that porcupine provides an accessory function for Wg protein secretion or transport. In mice, deletion of Porcn blocks
Wnt ligand secretion but Porcn-deficient cells remain responsive to exogenous Wnts (Barrott et al., 2011).

To elicit their functions, Wnt proteins bind to their specific receptors. The frizzled (FZD) receptors are a family of seven transmembrane proteins, 10 of which are encoded in humans known to serve as receptors for WNT signaling proteins (Bhanot et al., 1996). The specificity of Wnt/Fzd interactions remains unresolved due to the large number of Wnt and Fzd genes, the many possibilities for Wnt/Fzd binding, and functional redundancies (Kikuchi et al., 2007). Wnts transduce their signal through one of three intracellular signaling pathways: 1) the canonical 'WNT/β-catenin' pathway, 2) the 'WNT/Ca^{2+}' pathway, and 3) the 'WNT/polarity' pathway (also called the 'planar cell polarity' pathway). Distinct sets of Wnt molecules can activate each of these pathways, leading to unique cellular responses (Miller, 2002).

1.9.1. The canonical 'WNT/β-catenin' pathway

The WNT/β-catenin pathway leads to an increase in intracellular β-catenin, which acts as a co-transcription factor with members of the T-cell factor/lymphoid enhancer (Tcf/Lef) family of transcription factors to drive target gene expression (Behrens et al., 1996). WNT signaling is regulated at various levels, such as
through the presence or absence of multiple Wnt proteins, co-receptors, intracellular signaling molecules, and transcription factors (Krishnan et al., 2006). WNT signaling is also tightly regulated by a series of inhibitors including members of the Dickkopf (Dkk), secreted frizzled-related protein (sFRP) family as well as Wnt inhibitory factor (WIF) (Logan and Nusse, 2004a).

In the absence of a Wnt ligand, β-catenin is tightly regulated through phosphorylation by a multi-protein destruction complex including casein kinase 1α, glycogen synthase kinase 3-β (GSK3-β), the tumor suppressor adenomatous polyposis coli (APC), and axin. Sequential phosphorylation of β-catenin – first by casein kinase 1α at Ser-45, and then at Ser-33, 37 and Thr-41 by GSK3-β – targets it for proteosomal destruction through the ubiquitin pathway (Willert and Jones, 2006). Under these circumstances, β-catenin is stable at the plasma membrane in cell adherens junctions, where it acts as an adapter protein between the cytoplasmic tail of E-cadherin receptors and α-catenin, a cytoskeleton binding protein. In the canonical pathway, Wnts bind Fzd receptors in association with the co-receptors LDL-receptor related protein, LRP,-5/6 (He et al., 2004). Wnt binding to the Fzd receptor and LRP co-receptor induces phosphorylation of the LRP intracellular domain, inhibition of the destruction complex, and the stabilization and accumulation of β-catenin (Willert and Jones, 2006). The stabilized β-catenin then translocates to the nucleus to form a complex with Tcf/Lef proteins, resulting in transcriptional regulation of downstream Wnt target
genes (Giles et al., 2003). A simple version of the canonical WNT signaling pathway is shown below (Fig 4).
Figure 4. A simplified version of the canonical WNT/β-catenin pathway in mammals. A) In the absence of Wnt ligands, β-catenin binds to a destruction complex containing APC, Axin, GSK3 kinase, and is marked for proteasomal degradation following phosphorylation (P). Consequently, β-catenin cannot reach the nucleus, and cannot co-activate TCF-responsive genes. Groucho, a corepressor, also prevents the activation of Tcf/Lef-responsive genes in the absence of β-catenin, resulting in no transcription. B) Wnt proteins bind to the Frizzled/LRP receptor complex at the cell surface. Signaling promotes hyperphosphorylation of LRP5/6 and enhances Dishevelled (Dsh) phosphorylation, which jointly recruit Axin to the receptor complex at the plasma membrane. Unphosphorylated β-catenin is no longer rapidly degraded, accumulates within the cytoplasm and subsequently enters the nucleus, where it acts as a co-activator for Tcf/Lef-responsive genes [Adapted from (Nusse, 2005)]
1.9.1.1 β-catenin

β-catenin is a member of the armadillo (ARM) repeat protein family and is a central component of cadherin cell adhesion complex, as well as playing an essential role in the Wingless/WNT signaling pathway (Heuberger and Birchmeier, 2010). β-catenin turnover in the cell is tightly regulated through a ubiquitin-dependent proteolysis system to ensure steady state levels (Aberle et al., 1997). It is a protein with a molecular weight of about 86 kD. In the canonical WNT signalling pathway, β-catenin is a key mediator, serving as a transcriptional co-activator through binding to DNA-binding transcription factors. Although several proteins are known to recruit β-catenin to the nucleus, the most well-known transcription factors that interact with β-catenin are the T-cell factor (Tcf)/lymphoid enhancer factor (Lef). In the absence of Wnt, cytoplasmic β-catenin levels stay low, and the transcription factors Lef and Tcf interact with Groucho in the nucleus to repress Wnt-specific target genes (Heuberger and Birchmeier, 2010). When β-catenin is stabilized, it translocates to the nucleus and converts Tcf from a transcriptional repressor to an activator to trigger the expression of target genes.

1.9.2. The WNT/Ca\textsuperscript{2+} pathway

The WNT/Ca\textsuperscript{2+} pathway can be activated by Wnt4, Wnt5a or Wnt11, leading to intracellular release of calcium ions (Kuhl et al., 2000). This is sufficient to
activate calcium sensitive enzymes like protein kinase C (PKC), calcium-calmodulin dependent kinase II (CamKII) or calcineurin (Kuhl, 2004). The WNT/calcium pathway plays important roles in dorso-ventral patterning of the embryo, regulating cell migration and has inhibitory effects on the WNT/β-catenin pathway.

1.9.3. The Planar Cell Polarity (PCP) Pathway

Activation of the PCP pathway functions through apparent activation of small GTPases, the heterotrimeric G proteins, and, in some cases, C-Jun N-terminal kinase, and does not lead to either a stabilization of β-catenin or an influx of calcium (Montcouquiol et al., 2006). The WNT/PCP signaling pathway controls cell polarity through regulation of cytoskeletal organization and is responsible for the regulation of coordinated orientation of structures within the plane of various epithelia.

1.9.4. The role of Wnts in ovarian differentiation and development

Unlike in males, differentiation of the female gonad was thought to be the default pathway. However, recent evidence suggests this is not the case. In mouse, Wnt4 has been associated with female sexual development (Vainio et al., 1999). Wnt4 is expressed in both sexes prior to 11.5 days post-coitum (dpc) in the developing
gonad. Indeed, Wnt4 is required during embryonic development of female gonad to suppress formation of male-specific coelomic vessel, and prevent endothelial and steroidogenic cell migration into the developing ovary (Heikkila et al., 2002; Jeays-Ward et al., 2003). After sex determination, expression of Wnt4 persists in the female gonad, but is downregulated in the male gonad. The fact that Wnt4 expression is maintained in female gonad suggests it plays a role in ovarian development and differentiation. It has been demonstrated that the outcome of mammalian sex determination hinges on the balance between Fgf9 and Wnt4 (Kim et al., 2006). In addition to Wnt4, sex-specific expression has been observed for other Wnt genes, such as Wnt5a, Wnt6 and Wnt9a in the ovary (Cederroth et al., 2007). The overlapping expression of multiple Wnts in the gonads suggests functional redundancy, or that these could act synergistically through a common signaling pathway such as the WNT/β-catenin pathway. Recent findings implicate an additional gene in female sex-determination. R-spondin (Rspo), plays an essential role in ovarian development by regulating β-catenin (Parma et al., 2006; Tomizuka et al., 2008). Expression of Rspo proteins overlaps with Wnt expression during development (Kazanskaya et al., 2004), indicating close linkage between Rspo proteins and the WNT signaling pathway.

The normal function of Wnt4 is to repress certain aspects of the male pathway, through blocking migration of endothelial cells into XX gonads, as well as inhibiting the formation of the coelomic vessel. Wnt4 appears to do this by regulating the expression of follistatin (Fst). Follistatin is a secreted protein
expressed by numerous cells, capable of binding to and antagonizing the function of members of the TGFβ superfamily, including the activins and certain BMPs (Patel, 1998). Follistatin appears to play regulatory roles in the postnatal ovaries and testes (Knight and Glister, 2001). However, it was not previously appreciated that follistatin expression is sexually dimorphic. Fst and Bmp2 are expressed downstream of Wnt4 during ovary development, as neither Bmp2 nor Fst is expressed at any stage between 12.5 and 14.5 dpc in Wnt4−/− XX gonads (Yao et al., 2004). Fst mutants develop similar phenotype to Wnt4 mutants. In the XX gonad, inactivation of Follistatin leads to formation of the coelomic vessel and germ cells loss, similar to that observed in Wnt 4 mutants (Yao et al., 2004).

1.10. Rationale and objectives

Whereas WNT signaling plays key roles in embryonic development of the ovary (Ottolenghi et al., 2007; Vainio et al., 1999; Yao et al., 2004), several studies have described the expression of WNT signaling components in adult mouse ovary (Harwood et al., 2008; Hsieh et al., 2005; Hsieh et al., 2002; Hsieh et al., 2003; Kimura et al., 2006; Ricken et al., 2002). Thus, WNT signaling may provide potential local regulatory systems on which ovarian cell types may be dependent. However, little is known about the expression or role of β-catenin/Tcf-signaling activity within the OSE, follicle or oocyte during development.
The overall aim of this study was to characterize the developmental expression, localization, and modulation of \( \beta \)-catenin/Tcf signaling activity within the OSE and oocytes to further understand the possible roles of the canonical WNT signaling pathway in ovarian development. The specific goals for the studies described in this thesis are to:

1. Characterize WNT/\( \beta \)-catenin signaling in mouse OSE cells during ovary development

2. Determine whether \( \beta \)-catenin expression correlates with \( \beta \)-catenin/Tcf-mediated activity in OSE cells and whether \( \beta \)-catenin stabilization can regulate \( \beta \)-catenin/Tcf-mediated gene expression and OSE proliferation.

3. Assess \( \beta \)-catenin/Tcf-mediated expression in oocytes during follicular growth and differentiation in order to determine the role of the canonical WNT signaling pathway in the development of follicular ovulatory capability.
β-catenin/Tcf-signaling appears to establish the murine ovarian surface epithelium (OSE) and remains active in selected postnatal OSE cells

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Preface

The ovarian surface epithelium (OSE), squamous/cuboidal cells encompassing the mammalian ovary, is implicated in ovulation and responsible for repair and re-epithelialization of the ovulatory wound. The majority of epithelial ovarian cancers are thought to arise from the OSE. Emerging evidence implicates WNT signaling in ovarian endometrioid adenocarcinomas whereas some ovarian tumors are Wnt-inactive. Wnts are secreted extracellular signaling molecules that act locally to control diverse developmental processes such as cell fate specification, cell proliferation, and cell differentiation including embryonic development of the ovary. Using a β-catenin/Tcf-responsive β-galactosidase (lacZ) reporter mouse (Tcf-LacZ mouse), we detected β-catenin/Tcf signaling in adult OSE. In order to determine the ontogeny of these cells, we assessed β-catenin/Tcf activation of lacZ expression during mouse ovary development.
Abstract

We investigated the spatio-temporal pattern of β-catenin/Tcf expression in the ovarian surface epithelium (OSE) using responsive transgenic (Tcf-LacZ) mice. The generated β-galactosidase response (lacZ+) identified the cell population that overlies the medio-lateral surface of the indifferent gonad at embryonic day (E) 11.5. From E12.5 onwards, lacZ expression disappeared in cells covering the testis but remained with ovary development. LacZ+ OSE cells were present throughout embryonic and postnatal ovarian development but demonstrated an age-dependent decrease to a small proportion when animals were weaned and remained at this proportion with aging. Flow cytometric (FACS) and ovarian section analyses showed lacZ+ cells constitute approximately 20% of OSE in postnatal (day 1) mice which fell to 8% in 5 day-old animals while in prepubertal and adult mice this accounted for only 0.2% of OSE. Apoptosis was undetected in OSE of neonates and β-catenin/Tcf-signaling cells were proliferative in neonatal mice indicating that neither cell death nor proliferation failure was responsible for the proportion alteration. It appeared that lacZ+ cells give rise to lacZ− cells and this was confirmed in cell cultures. The DNA-binding dye DyeCycle Violet was used to set up the side population (SP) assay aimed at identifying subpopulations of OSE cells with chemoresistance phenotype associated with ABCG2 transporter activity. FACS analysis revealed lacZ+ cells exhibit cytoprotective mechanisms as indicated by enrichment within the SP. The study raises the possibility that
WNT/β-catenin-signaling cells constitute a progenitor cell population and could underlie the pronounced histopathology observed for human ovarian cancer.
Introduction

Wnts are secreted cysteine rich glycoprotein ligands that transduce their signal through at least three distinct pathways (Miller, 2002). The WNT/β-catenin pathway (termed canonical WNT-signaling) is the best studied and arises from an increase in non-phosphorylated intracellular β-catenin content, transport to the cell nucleus and association with members of the Tcf/Lef transcription factor family to drive target gene expression (Behrens et al., 1996). Wnts are involved in a number of developmental processes including the establishment of cell fate, proliferation, and differentiation (Cadigan, 2008; Logan and Nusse, 2004b; Wodarz and Nusse, 1998). In mice, Wnt4 is associated with female sexual differentiation (Vainio et al., 1999) and is required during emergence of the female gonad to prevent formation of the male-specific coelomic blood vessel and steroidogenic cell migration (Heikkila et al., 2002; Jeays-Ward et al., 2003). In addition to Wnt4, sex-specific expression has been found for Wnt5a, Wnt6 and Wnt9a within the gonad (Cederroth et al., 2007). Recent findings also implicate a family of secreted ligands (R-spondin) in female sex-determination (Parma et al., 2006; Tomizuka et al., 2008). The R-spondins (Rspo) play an essential role in ovarian development through stabilization of cytoplasmic β-catenin. Mutation of RSPO1 is associated with human sex reversal (Parma et al., 2006). Expression of Rspo proteins overlaps with expression of Wnts during development indicating a link between Rspo and the WNT signaling pathway (Kazanskaya et al., 2004).
While Wnts play a key role in embryonic development of the ovary (Ottolenghi et al., 2007; Vainio et al., 1999; Yao et al., 2004), several studies describe the expression of Wnts and WNT signaling components in adult rodent ovaries (Harwood et al., 2008; Hsieh et al., 2005; Hsieh et al., 2002; Hsieh et al., 2003; Kimura et al., 2006; Ricken et al., 2002). Some of these, including $Wnt4$ (Lyons et al., 2004) and $Wnt2b$ (Katoh et al., 2001), are associated with activation of canonical WNT signaling. Interestingly, $Wnt2b$ is expressed in the ovarian surface epithelium (OSE) (Ricken et al., 2002). OSE is a simple epithelium of squamous or cuboidal cells and it, as well as Mullerian duct derivatives (oviduct, uterus, and proximal one-third of vagina), are derived from the peritoneal mesothelium (Byskov, 1986; Naora, 2007). Functionally, OSE is implicated in the ovulatory process and is responsible for repair of the ovulatory wound (Murdoch, 1994; Szotek et al., 2008). Deregulation of WNT-signaling in OSE has been implicated in ovarian tumorigenesis (Gatcliffe et al., 2008).

The spatio-temporal pattern of $\beta$-catenin/Tcf-signaling activity within murine OSE (mOSE) was investigated using a specific transgenic reporter mice (Tcf-LacZ) strain (Mohamed et al., 2004b). We show that $\beta$-catenin/Tcf activation identifies a cell population in the mesothelium that overlies the indifferent gonad. By embryonic day (E) 12.5, the majority of cells in the overlying epithelium of the ovary retain $\beta$-catenin/Tcf-signaling cells while they disappear in cells covering the testis. At parturition the proportion of signaling to non-signaling
mOSE cells decrease and fall to a small but sustained proportion in adult females. The \(\beta\)-catenin/Tcf-signaling population in adult females is enriched for a side population which is believed to be a characteristic of stem cells (Goodell et al., 1996). These observations might explain why ovarian cancers show oviduct/uterine histopathology as OSE share an origin with Mullerian duct derived reproductive tissues (Kurman and Shih Ie, 2010).
Results

\textit{β-Catenin/Tcf-activated lacZ expression is seen on cells covering the indifferent mouse gonad}

Prominent lacZ\(^+\) stained cells overlie the entire medio-lateral surface of the indifferent gonad on embryonic day 11.5 (E11.5; Fig. 1A). Coelomic epithelial cells extending beyond the genital ridge towards either the rostral or caudal end of the mesonephros (Fig. 1A, arrowhead), did not stain indicating that only the mesothelium directly covering the gonad has β-catenin/Tcf-mediated signaling. To identify the cell types in which β-catenin/Tcf-signaling was activated, paraffin-embedded sections of E11.5 gonads were examined. Somatic (surface and sub-surface) as well as Mullerian duct cells of E11.5 gonads showed lacZ expression (Fig. 1B; dark arrows and arrow head, respectively); germ cells, distinguished by their large round nuclei, were not stained (Fig. 1B, white arrowheads).

\textit{β-Catenin/Tcf-mediated lacZ expression shows sexual dimorphism in the cells covering the developing gonad}

Gonadal β-catenin/Tcf-mediated lacZ-expression was examined at later stages of embryonic development. Sexual dimorphic patterns of lacZ staining were observed at E12.5 (Fig 2, upper and lower left panels). In E12.5 females,
pronounced staining was observed over the entire gonadal surface while E12.5 males displayed less extensive staining compared to either the E11.5 indifferent gonad (Fig. 1A) or the E12.5 ovary (Fig. 2, upper left panel).

The ovarian surface at E13.5 maintained extensive lacZ expression with a bias towards the cranial pole (Fig 2, black arrows). In contrast, staining in the E13.5 testis had almost disappeared with some staining retention at the caudal pole (Fig 2, arrowhead). Staining in E14.5 ovaries was similar to that at E13.5 with an anterior bias towards the cranial suspensory ligament. E14.5 testes were devoid of lacZ+ cells except for the region near the mesonephric tubules and faint staining at the caudal pole. In E15.5 ovaries, lacZ staining remained prominent while testes were devoid of lacZ+ cells with the exception of the mesonephric tubule attachment (right panels, Fig. 2). In both sexes, lacZ staining was present in Wolffian (WD) and Mullerian (MD) ducts as well as mesonephric tubules (MT). The sex-specific degeneration of the MD in the male by E15.5 (Fig. 2, lower right panel) is apparent with retention of WD. The female (Fig. 2 upper right panel) loses her WD at a later age, generally at E16-5 – E17.5 (not shown).

The disappearance of β-catenin/Tcf-mediated lacZ expression on the surface of developing testes raised the possibility of cell loss and coverage with a tough
capsule i.e. establishment of the testicular tunica albuginea. In order to assess this possibility, basement membranes were highlighted by Periodic acid-Schiff (PAS) staining of lacZ-stained E15.5 gonad sections. A thin basement membrane (arrow; Fig. 3 left panel) was observed below OSE and the lacZ⁺ and lacZ⁻ epithelial cells (arrowhead; Fig. 3 left panel). Germ and pregranulosa cells, organized into structures termed ovigerous cords in developing ovaries (Guigon and Magre, 2006b), were delimited by a thin basement membrane. Gonadal surface cells of E15.5 testis did not stain for lacZ expression and appeared more elongated than E15.5 OSE cells (arrowhead; Fig. 3 right panel). A basement membrane was observed underneath and between the epithelial cells of E15.5 testis and appeared to thicken at this stage (arrow; Fig. 3 right panel). Testis cords consisting of germ and Sertoli cells (Karl and Capel, 1998b) were surrounded by a basement membrane. These observations indicate that the testicular loss of β-catenin/Tcf-mediated lacZ expression is not due to surface cell elimination.

**β-catenin/Tcf-mediated lacZ expression in mOSE is heterogeneous during postnatal development**

Whole-mount lacZ staining of ovaries derived from postnatal mice (P1 – P180) revealed staining and demonstrated an age-dependent decrease in the relative proportion of stained cells (Fig 4a). Histological examination of sections derived from P1 to P21 mice showed staining in the OSE (Fig 4b) and an age-dependent
decrease in lacZ⁺ OSE cells. No distribution pattern for lacZ⁺ cells within the OSE was detected at these ages.

In order to quantify changes in the proportion of lacZ⁺ to total OSE with animal age, cells were isolated from the ovarian surface using an enzymatic procedure and analyzed by flow cytometry. This approach was tested to ensure that OSE cells only were released. Ovary-encompassing OSE was selectively labeled using the water-soluble reagent NHS-biotin. Exposure of an intact ovary to the reagent for a brief time interval followed by quenching with excess glycine provides specific OSE labeling. Tight junctions between OSE cells and the presence of a basement membrane prevent the reagent from entering into the ovarian parenchyma (Aravindakshan et al., 2006). Indeed, OSE cells are labeled specifically with NHS-biotin with minimal penetration into the parenchyma as visualized by the binding of FITC-conjugated avidin to NHS-biotin stained P21 ovary (Arrow head; Fig 5a panel A).

The efficiency of OSE isolation was assessed after subjecting NHS-biotin labeled ovaries to enzymatic treatment. The majority (> 90%) of the labeled cells were removed and the residual ovary remained relatively intact (Fig 5a panel B). FACS analysis of the isolated cells showed that approximately 86 ± 2.3% (mean ± sem; n=3) of the cells were labeled with avidin-FITC (Fig 5b) after enzyme treatment. The proportion of viable cells, assessed by concurrent analysis of cell viability (7-AAD labeling), corresponded with those of unlabeled OSE from control ovaries.
After sorting NHS-biotin-labeled cells through FACS, cytokeratin 8 (CK8) detection with Cy3-labeled goat anti-mouse antibody was conducted (Fig. 5c). Ovarian section from P21 mice was used as a control for the specificity of CK8 antibody to OSE cells (Fig. 5d).

Fig 6a illustrates the percentage of lacZ-positive mOSE cells as a function of animal age. The proportion of lacZ+ cells within OSE decreased from approximately 8% at P5 to 0.2% by P21 and it remained at this level with continued aging (Fig 6a). FACS results were confirmed by counting lacZ-positive and negative cells in serial sections of P1, P5, and P10 ovaries (Fig 6b). The percentage of lacZ-positive OSE cells at P5 and P10 by serial counting was similar to the same measurement through FACS analysis at these ages. Sections from older animals were difficult to evaluate owing to the relative rarity of lacZ-positive OSE cells and the larger number of sections generated.

Since the proportion of OSE cells expressing lacZ decreased with age, it is possible that β-catenin/Tcf expression drives cellular apoptosis. Accumulating evidence suggests that β-catenin is involved in cell cycle arrest (Mao et al., 2001) and apoptosis (Kim et al., 2000a). To investigate this possibility, the occurrence of apoptosis in P1 and P5 ovaries was analyzed by TUNEL. The positive control (DNase-treated ovary section) displayed staining in all cells (Fig. 7, bottom panel). There was no evidence of DNA fragmentation in P1 and P5 ovaries (Fig.
7, top and middle panel), suggesting absence of apoptosis even though significant changes in the proportion of lacZ+ cells were seen at these ages (Fig. 6).

It was possible that the age-dependent decrease in the proportion of lacZ+ cells arose because they were non-replicating. Cell proliferation was assessed immunohistochemically after incorporation of the thymidine analog, bromodeoxyuridine (BrdU), into cellular DNA. To enumerate proliferating lacZ+ cells, ovaries were collected 48 hr post BrdU injection and stained for lacZ and BrdU (Fig 8). At P4, approximately 25% of lacZ+ cells are BrdU-labeled but by P9 this falls to 8% (Table 1). This indicates lacZ+ cell replication decreases with age. The observation that there is an age-dependent decrease in the number and rate of replication of lacZ+ cells raises the possibility that proliferating lacZ+ cells give rise to non-signaling cells. To assess this possibility, OSE cells from P5-P9 were harvested and cultured. In four days, a 2-fold increase in lacZ+ cells and a three-fold increase in the number of lacZ- cells were obtained (Fig. 9). There was, however, no significant difference in proliferation rate between lacZ+ and lacZ- cells. Prolonged culture for 8 days led to a significant decrease in lacZ+ cells whereas the number of lacZ- cells increased. The latter observation suggests non-signaling cells arose from the previously signaling ones. To further confirm that lacZ- arose from their positive counterparts, lacZ+ cells were sorted from lacZ- cells and cultured separately. After 8-days of culture, the majority of lacZ+ cells did not stain for lacZ (data not shown). LacZ- cells, however, did not stain for lacZ following prolonged culture.
**Wnt and fzd expression in OSE were unchanged with age**

Table 2 is a summary of Wnt and fzd transcripts assessed for animals of different ages. Interestingly, with the exception of fzd6 these particular Wnts and fzds were either expressed or not expressed regardless of animal age. The cause for non-expression of fzd6 in adult mice is not known nor which particular Wnt signals through this receptor. Fzd6 is an essential component of the hair patterning pathway (Guo et al., 2004) and mutation of fzd6 is known to cause autosomal-recessive nail dysplasia (Frojmark et al., 2011). Since the proportion of lacZ\(^+\) OSE cells in weaned (P21) and adult mice are similar, it is unlikely that the loss of fzd6 transcription is affecting this aspect of WNT-signaling in OSE cells.

**LacZ\(^+\) OSE cells are enriched with a potential stem cell population**

The observation that there is an age-dependent decrease to relative constancy of \(\beta\)-catenin/Tcf-signaling OSE cells prompted an examination of whether these cells exhibit a stem cell characteristic termed side population (SP). SP is detected by dual wavelength flow cytometry of the cellular efflux of the DNA-binding dyes Hoechst 33342 or dyeocyte violet (DCV). SP cells appeared as a characteristic tail in the flow cytometry of isolated OSE (Fig 10). The SP cells were small round cells of approximately six \(\mu\)m in diameter (Fig 11). The dependence of the SP phenotype on expression of ABCG2 transporter pump was demonstrated by pre-incubation with the ABCG2 inhibitor verapamil. The results
indicate that while ~ 0.6% OSE of prepubertal mice is SP (Table 3), ~ 23.9 ± 2%
(mean ± SE, n=3) of lacZ-positive cells are SP positive. This also indicates that
the lacZ+ cell population is heterogeneous with potential stem and non-stem cell
components.
Discussion

Transgenic reporter (Tcf-LacZ) mice indicate activation of β-catenin/Tcf signaling within OSE. This dominates the epithelium covering the indifferent gonad and is followed by its maintenance in a sex-specific manner. The majority, most likely all of the cells overlying the differentiating ovary, retain β-catenin/Tcf activity, which begins to disappear with testis formation. Further ovarian development shows an age-dependent decrease in the proportion of β-catenin/Tcf-responsive cells to relative constancy in the mature ovary. RT-PCR analysis detected the expression of multiple Wnts and fzds within the OSE. Side population analysis indicates enrichment in β-catenin/Tcf-signaling cells. These findings suggest that β-catenin/Tcf signaling cells form the definitive OSE and raise the possibility that these signaling cells constitute a putative stem cell population.

β-catenin/Tcf-signaling plays critical role in embryonic patterning and cell fate determination in a variety of tissues (van Noort and Clevers, 2002). Wilms’ tumor suppressor 1 (Kreidberg et al., 1993a) and empty-spiracles homeobox gene 2 (Miyamoto et al., 1997) expressed within the thickening coelomic epithelium of the developing gonad are required for genital ridge development. The presence of a lacZ+ cell population overlying the E11.5 gonad suggests that the mouse gonad may be formed from a β-catenin/Tcf-signaling cell population. Coelomic
epithelial cells migrate into XX and XY gonad and contribute to supporting cell lineage of the gonad (Karl and Capel, 1998b). The localization of lacZ+ cells within E11.5 gonads raises the possibility that β-catenin/Tcf-mediated expression may be involved in differentiation of somatic cells of the developing gonad.

β-catenin is dispensable for testis formation and maintenance whereas it is required for maintenance of ovarian characteristics (Liu et al., 2009). Several genes known to play a role in sexual development are transcribed in a sexually dimorphic fashion (Capel, 2000; Greenfield, 1998). We observed sexual dimorphic β-catenin/Tcf-mediated expression that is distinct by E12.5. It is established that the presence of germ cells in female gonads is required for proper development of the ovary (McLaren, 2000) and that sexual differentiation and meiotic entry of germ cells in embryonic XX gonads progress in an anterior-to-posterior pattern (Menke et al., 2003). Our analysis demonstrated that lacZ expression in ovaries show the opposite wave pattern: an anterior bias observed by E13.5 and extends to the early postnatal period. This staining pattern wave is similar in its spatial distribution to that of Adams19 a marker of ovarian somatic differentiation (Menke et al., 2003; Menke and Page, 2002) and raises the possibility that OSE differentiation begins posteriorly and moves anteriorly. This pattern of β-catenin/Tcf-mediated expression in the XX gonad may be due to a gradient of meiosis promoting substances produced by a fixed source in or near the
anterior portion of the ovary. There is some evidence that the ovary in germ cell 
deficient mice is covered by OSE cells (Sakata et al., 2003). Thus, the observed 
avarian lacZ expression pattern is unlikely due to a substance produced by germ 
cells but could be reflective of local environmental cues that are present in the 
embryonic female gonad.

*Sry* expression blocks β-catenin-mediated transcription (Bernard et al., 2008b). 
The observation that loss of lacZ expression begins at the anterior portion of the 
developing testis mimicking *Sry* expression suggests that the anterior-posterior 
loss of lacZ on the surface of embryonic testis may be due to *Sry* expression. 
Because *Sry* is not expressed in the coelomic epithelium of a differentiating testis 
(Albrecht and Eicher, 2001), it is unlikely that the loss of lacZ is due to *Sry* 
expression. Alternatively, non-cell autonomous paracrine signals such as *Sox9*, 
*Mis*, and *Dhh* emanating from Sertoli cells (Tevosian et al., 2002) may be 
responsible for blocking β-catenin/Tcf expression. It is possible that active 
steroidogenesis present in embryonic testis but absent in fetal ovary (Greco and 
Payne, 1994) may be preventing the β-catenin/Tcf-pathway from being efficiently 
expressed in the developing testis.

Several methods have been described for isolating OSE cells (Clark-Knowles et 
al., 2007; Nicosia et al., 1984; Quinn et al., 2009; Symonds et al., 2005). We have
established a simple procedure for isolating a relatively pure population of OSE cells. This procedure owes its success to the fact that the NHS-biotin label is retained following enzymatic digestion. Additionally, biotinylation did not affect OSE cell viability. Sorted NHS-labeled cells stained for CK8 indicating that they were derived from the OSE.

Some studies support a positive role of β-catenin in cell mitosis (Olmeda et al., 2003), whereas others suggest a potential involvement in cell cycle arrest (Mao et al., 2001) or a direct involvement in apoptosis (Kim et al., 2000a). β-catenin accumulation within the cell nucleus is however involved in cell fate decision (Lluis et al., 2010). We have shown that age-dependent decrease in β-catenin/Tcf-signaling cells is not due to the absence of cell proliferation or the result of selective apoptosis. Similar studies in day-2 and day-4 neonatal mouse ovaries reported the absence of apoptosis in all ovarian cell types (Dharma et al., 2009). We suspect that lacZ+ cells were quiescent with continued aging and prepared for differentiation. It is likely that upon mitosis, a lacZ+ cell looses its ability to signal through the β-catenin/Tcf pathway and becomes non-signaling. This was reflected by the loss in proliferative capacity of lacZ+ cells from P4 to P9. In vitro, lacZ+ cells showed a transient increase in cell number followed by appearance of lacZ− cells suggesting that loss of lacZ expression is due to cellular differentiation. The transient increase in lacZ+ cells may originate from symmetric division of lacZ+
cells or insufficient downregulation of β-catenin/Tcf-LacZ expression in vitro following mitosis. The observation that sorted lacZ⁺ cells cultured separately from their non-signaling counterparts gave rise to lacZ⁻ cells provides the supporting evidence that loss of lacZ expression is due to OSE differentiation.

Knockout studies of various Wnt molecules led to the discovery that Wnts perform critical functions during early development of reproductive tissues (Parr and McMahon, 1998; Vainio et al., 1999). Our studies show expression of various Wnt ligands and fzd receptors transcripts within OSE. These are similar to previous studies assessing Wnt/fzd expression within the ovary (Harwood et al., 2008; Hsieh et al., 2002). Among the Wnts found to be expressed in the OSE are Wnt4 and Wnt2, which have been shown to impact ovary development (Vainio et al., 1999; Wang et al., 2010a). The overlapping expression of multiple Wnts within the OSE is suggestive of functional redundancy.

Gene expression analysis supports the hypothesis that human OSE cells are multipotent (Bowen et al., 2009). This hypothesis has recently been validated by a study that described putative stem cells within the OSE as cells expressing markers of pluripotency (Virant-Klun et al., 2011). A side population-enriched and label-retaining cell population in the coelomic epithelium of adult mouse ovary has been identified as possible stem/progenitor cells (Szotek et al., 2008).
We obtained a distinguishable SP within mouse OSE. The observation that the SP is enriched for β-catenin/Tcf-signaling cells raises the possibility that lacZ-positive cells constitute OSE progenitors. The fact that cancer stem cells are a subset defined by increased WNT/β-catenin activity (Vermeulen et al., 2010) and that Wnt/β-catenin is essential for maintenance of intestinal stem cells (Fevr et al., 2007) supports our hypothesis. There is accumulating evidence suggesting that somatic stem cells may undergo mutagenic transformation into cancer stem cells (Rossi et al., 2008). Because many of the properties that define somatic stem cells also define cancer stem cells, our identification of a β-catenin/Tcf-signaling cell population in OSE raises the possibility that endometrioid adenocarcinomas may arise as a result of transformation of the lacZ+ cells. The common origin of the β-catenin/Tcf-signaling cells with oviduct/uterus may explain the suggestion that OSE cancers show uterine/oviductal characteristics (Kurman and Shih Ie, 2010).
**Conclusion**

Our findings indicate that β-catenin/Tcf-signaling cells are present early in OSE development. The maintenance of a constant number of β-catenin/Tcf-signaling cells accompanied by the increase in appearance of non-signaling cells raises the possibility that the original β-catenin/Tcf-signaling cells give rise to a replacement as well as an expanding population of non-signaling progeny. Taken together, our results indicate that the mouse OSE is heterogeneous and may contain a population of progenitor cells. The physiological necessity for restoring OSE after ovulations may reflect the need for establishing and maintaining the progenitor cells. It also raises the possibility, specifically in primates, that transformation of OSE progenitor cells can generate the varied histopathology observed in ovarian cancer.
Materials and Methods

Reagents and chemicals

Deoxyribonuclease I, collagenase (Type IV), and 5-bromo-2'-deoxyuridine (BrdU) were purchased from Sigma Aldrich (St Louis, MO). Glycine, N, N-dimethyl formamide, and other general chemicals were of tissue culture grade and purchased from Fisher Scientific (Nepean, ON). Dulbecco's Modified Eagle Medium (DMEM), Minimum Essential Medium (MEM) alpha, 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-gal), dithiothreitol (DTT), propidium iodide (PI), 4’,6-diamidino-2-phenylindole (DAPI), 7-aminoactinomycin D (7-AAD), and 5-chloromethylfluorescein di-b-D-galactopyranoside (CMFDG) kit were purchased from Invitrogen (Burlington, ON). Bovine serum albumin (BSA) and fetal bovine serum (FBS) were purchased from Wisent (St-Bruno, QC), Hoechst 33342 was purchased from Roche (Laval, QC), Tween-20 from Bio Basic Inc (Markham, ON), mowiol from Calbiochem (La Jolla, CA), and N–Hydroxysulfosuccinimide (Sulfo-NHS)-biotin from Pierce Thermo Fisher (Nepean, ON). Streapavidin phycoerythrin-Cy5 (PE-Cy5) was obtained from Biolegend (San Diego, CA). The mouse monoclonal antibodies against cytokeratin-8 (TROMA-1) and BrdU (G3G4) were obtained from Developmental Studies Hybridoma Bank (DSHB; Iowa City, IA), Cy3-labeled goat anti-mouse IgG antibody (Jackson Immuno Research, West Grove, PA) and avidin-FITC (EY Laboratories, San Mateo, CA) were obtained through Cedarlane Laboratories.
Animals

All animal procedures followed the guidelines established by the Canadian Council of Animal Care and approved by the Animal Care Committee of the Royal Victoria Hospital, McGill University. CD1 mice bearing the β-catenin/Tcf-responsive lacZ reporter gene (Tcf-LacZ mice) have been described (Mohamed et al., 2004b). Dr. Daniel Dufort (Department of Obstetrics and Gynecology, McGill University, Montreal, Canada) provided us with female and male CD1 mice, homozygous for the transgene, for colony establishment. Dr Makoto C. Nagano (Department of Obstetrics and Gynecology, McGill University, Montreal, Canada) provided us with wild type CD1 mice. Female mice were examined daily for vaginal plugs. The day of plug detection was considered day 0.5 of gestation and the day after birth designated postnatal day 1 (P1). Gonads were isolated from a minimum of three mice for analysis and all experiments replicated at least thrice.

Tissue Processing

Mice were sacrificed by cervical dislocation. Gonads were isolated, washed in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 2
mM KH$_2$PO$_4$, pH 7.4) and fixed for 5-15 min in freshly prepared 4%
paraformaldehyde (PFA) in PBS. After rinsing in wash buffer (PBS containing 2
mM MgCl$_2$, 0.1% Triton, 0.05% sodium deoxycholate), gonads were stained in
the dark overnight at 37°C in wash buffer supplemented with 1 mg/ml X-gal,
0.04% N, N-dimethyl formamide, 5 mM potassium ferricyanide and 5 mM
potassium ferrocyanide to disclose β-galactosidase activity (Iglesias et al., 2007).
Following staining, gonads were washed in PBS and photographed or processed
for histology.

**Histology**

X-gal-stained ovaries were post-fixed in 4% PFA overnight at room temperature
(RT), rinsed in PBS, and embedded in paraffin. Sections were cut 6 µm thick,
mounted on glass slides and counterstained with hematoxylin and eosin. In studies
where OSE cell numbers were estimated, sections were stained with periodic acid
Schiff (PAS) to define the basement membrane and counterstained with Hoechst
33342 to identify cell nuclei. The total number of OSE cells was estimated by
applying the nucleator and fractionator principle described by Gundersen
(Gundersen, 2002). Only cells with large visible nuclei were counted. Every
fourth ovary section was evaluated and an estimate of the total number of OSE
cells per ovary determined by multiplying the cell counts by four. LacZ-positive
OSE cells were determined by evaluating every section of the ovary since these
cells were not uniformly distributed.

**OSE labeling and isolation**

OSE cells were isolated as follows: ovaries (2 ovaries /0.5 ml DMEM) were placed in a 1.5 ml capped tube and incubated for 60 min at 37°C in DMEM containing 1 mg/ml Type IV collagenase, 1 mg/ml deoxyribonuclease I, and 0.53 mM EDTA. Ovaries were agitated every 10 min by swirling the tube for a few seconds. Released cells were transferred to a fresh tube. The ovaries were rinsed in fresh DMEM and additional released cells combined with the previously isolated cell suspension. The cell suspension was vortexted and cells pelleted by centrifugation at 500 g for 5 min. The cell pellet was washed with PBS and resuspended in PBS.

We needed to confirm that our isolation procedure yielded primarily OSE cells with minimal contamination by other ovarian cells. Additionally, we wanted to assess the efficiency of the OSE isolation procedure. This was accomplished by labeling OSE cells *in situ*. We took advantage of a water-soluble and membrane impermeable biotinylation reagent (Sulfo NHS-biotin) that reacts chemically with exposed amine groups of cell surface proteins. Intact ovaries were incubated in 1 mg/ml Sulfo NHS-biotin in PBS for 1 min at 4°C. The reaction was quenched by incubating the ovary in ice-cold PBS containing 0.1M glycine for 1 min. Selective labeling of OSE cells was confirmed by preparing sections of NHS-biotin-labeled
ovaries followed by incubation with avidin-conjugated FITC (1:200 in PBS).
Sections of enzymatically-treated ovaries were also examined for the extent of
OSE removed by avidin-FITC staining.

**Immunofluorescence**

Cytokeratin 8 (CK8) staining was performed on paraffin-embedded sections and
isolated OSE cells. Six μm thick paraffin tissue sections were deparaffinized with
xylene and rehydrated in graded ethanol. Antigen retrieval was performed by
boiling the sections in 10 mM sodium citrate buffer, pH 6.0, for 25 min. After
rinsing in PBST (PBS + 0.5% Tween), nonspecific binding was blocked for 30
min in blocking solution (5% BSA in PBST). Sections were incubated with
primary antibody (TROMA-1) in blocking solution at 4°C overnight. The primary
antibody was omitted for negative control slides. Slides were subsequently rinsed
in PBST, incubated with Cy3-labeled goat anti-mouse antibody diluted 1:200 in
blocking solution for 60 min in the dark, counterstained with DAPI, and mounted
in mowiol. Isolated OSE cells (50,000 cells/ml) were cytospun onto slides, fixed
in 4% PFA for 5 min, and processed for CK8 staining as described above. Cell
counts were made in five microscopic fields and approximately 100 cells were
counted per field.
Fluorescence-activated cell sorting (FACS) analysis

LacZ expression in isolated OSE cells was detected using the DetectaGene Green CMFDG LacZ gene expression kit as outlined by the supplier (Invitrogen). OSE cells were washed in PBS and incubated with pre-warmed 0.1M CMFDG in PBS at 37 °C for 15 min. PI was added to label dead cells and FACS analysis performed on a Becton-Dickinson FACScan. Wild type CD1 OSE cells were used as controls. Dual parametric analysis of forward versus side scatter was the primary gate for identification of cells in the appropriate size range and to eliminate cell debris.

TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) staining

Six μm thick paraffin-embedded lacZ-stained ovary sections from each age under investigation were examined for the presence of fragmented DNA indicating apoptosis using the In Situ Cell Death Detection Kit, Fluorescein, according to the manufacturer's instructions (Roche). Sections were counterstained with PI to label cell nuclei and mounted in mowiol.
**BrdU labeling**

A solution of 1 mg 5'-bromo-2'-deoxyuridine (BrdU)/100 µl PBS was freshly prepared and filter sterilized (0.45µm filter). Mice received BrdU by injection (i.p.) of 100 µl of sterile preparation. Ovaries were collected 48 hrs later and stained for X-gal, post fixed in 4% PFA overnight and embedded in paraffin. To detect proliferating cells, 6 µm thick paraffin sections were analyzed using a monoclonal mouse antibody (DSHB) specific for BrdU. Sections were dewaxed, washed in PBST (PBS + 0.5% Tween 20) and incubated with 2N HCl at RT for 1hr. Subsequently, slides were rinsed 3x5 mins in PBST and blocked in a blocking solution (3% BSA in PBST) for 30 mins. Sections were then incubated with anti-BrdU antibody diluted 1:100 in blocking solution at 4°C overnight. Following primary antibody incubation, slides were rinsed 3x5 mins in PBST, incubated with an FITC-labeled goat anti-mouse secondary antibody diluted 1:100 in blocking solution overnight at 4°C, counterstained with DAPI, and mounted in mowiol.

**RT-PCR**

Total RNA was isolated from FACS-sorted OSE cells using the miRNeasy Mini kit (Qiagen, Toronto, ON) and incorporating on-column RNase-free DNase digestion. Quantity and quality of mRNA samples were assured by analysis with the Thermo Scientific NanoDrop 2000 (Thermo Scientific, Wilmington, DE). To
survey the expression of WNT signaling components, total RNA (100 ng) was
reverse transcribed using M-MLV reverse transcriptase (Invitrogen). Polymerase
chain reactions (PCRs) were conducted as described previously (van den Berg et
al., 1999). Wnts for RT-PCR analysis were selected based on previous studies
(Harwood et al., 2008; Hsieh et al., 2002). A PCR reaction for a known
housekeeping gene, GAPDH, was generated as an internal control. The annealing
temperature for each primer pair was optimized using positive control tissues to
generate single bands corresponding to correct product sizes. Information
regarding positive control tissue in which the genes of interest are expressed was
obtained from: (i) Mouse Genome Database (MGD)
(http://www.informatics.jax.org 09/2009), RT-PCR database and included brain
(for Wnt2, Wnt4, Wnt7a, Wnt8a, Wnt 11), eye (Wnt5a), testis (Wnt3a); and (ii) a
previous study (Zeng et al., 2007). The RT-PCR protocol was performed on two
separate RNA preparations. For each sample, a RT-minus control was included to
provide for a negative control for subsequent PCR. All minus RT controls were
negative. PCR products were visualized on a UV transilluminator after
electrophoresis on a 1% agarose gel in TAE buffer (40mM Tris, 1mM EDTA and
20mM acetic acid) and SafeView nucleic acid staining.
Side population (SP) analysis

OSE cells were labeled with DyeCycle Violet (DCV) according to a modified protocol (Telford, 2010). Briefly, OSE cells were suspended in the appropriate medium (DMEM containing 2% FBS and 2mM HEPES) at 1 x 10⁶ cells/ml. Before DCV incubation, cells were pre-incubated for 15 mins with or without 50 μM verapamil. DCV was added to the cell suspension at a final concentration of 5 μM and the mixture incubated for 30 minutes at 37°C in the dark. Propidium iodide was added to a final concentration of 1 μg/mL to identify dead cells. FACS analysis and sorting were performed on a dual laser flow cytometer (Becton Dickinson). The SP was defined as described previously (Goodell et al., 2005).

Cell culture

OSE cells isolated from postnatal day 5 (P5) to P9 mice were grown in MEM alpha (Walsh et al., 2003) supplemented with 4% FBS and 20 U/ml PenStrep at a density of ~3000 cells/well in 96-well plates. The cells were grown in a humidified incubator at 37°C and 5% CO₂.
**Statistical analysis**

Data were analyzed using SYSTAT 10.2 statistical software (SYSTAT Software, Richmond, CA). Analysis of variance (ANOVA) was used with Tukey's test in the post hoc analysis for cell counts to ascertain group mean differences. Data for the percentage of lacZ-positive OSE cells obtained following CMFDG labeling was subjected to ANOVA after arcsine transformation (McDonald, 2009). Data are presented as mean ± standard error of the mean. \( P \leq 0.05 \) was considered significant.

**Authors’ contributions**

R.F. designed research; U.M. performed research and analyzed data; R.F and U.M. wrote the paper.

**Acknowledgements**

We are grateful to Ken McDonald (Life Science Complex, McGill University) for his technical assistance in flow cytometry. The authors thank Makoto C. Nagano for discussions. This study was funded by grants from the Canadian Institutes of Health Research (CIHR) and the Natural Sciences and Engineering Research Council of Canada (NSERC).
Tables

Table 1. Proliferating lacZ\(^+\) cells as fraction of total lacZ\(^+\) OSE cells. Data are presented as mean ± standard error of the mean (n=3).

<table>
<thead>
<tr>
<th>Animal age (days)</th>
<th>LacZ(^+) BrdU/ LacZ(^+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>23.6±1.67</td>
</tr>
<tr>
<td>9</td>
<td>8.0±0.48</td>
</tr>
</tbody>
</table>
Table 2. Expression of wnts and frizzleds in OSE cells harvested from different animal ages and primer sequences for RT-PCR analysis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>P5</th>
<th>P10</th>
<th>P21</th>
<th>Adult (&gt;6 wks old)</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wnt2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>CGACTGGGGTGCTGAGTG</td>
<td>AGGGGAGCTTGCTTGAGTC</td>
</tr>
<tr>
<td>Wnt2b</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>CATGATCAACAGAGGAGTT</td>
<td>CAGCCTTGCTCAAAGACAGT</td>
</tr>
<tr>
<td>Wnt3a</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>CAAGCCCGCGATGCTCCT</td>
<td>ACTCCCCGGGGCGCTTCAGTC</td>
</tr>
<tr>
<td>Wnt4</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>GGGAGCTCATGCTCTCAG</td>
<td>GCCGCTCAACGCTTTAGATG</td>
</tr>
<tr>
<td>Wnt5a</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>AGGGAGCTCATGCTCTCAG</td>
<td>GTCGACTCGCTTCTGAGTCT</td>
</tr>
<tr>
<td>Wnt7a</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>CAGGCCGAGTACGACAGCC</td>
<td>CAGCCTCCCCGACTCCACT</td>
</tr>
<tr>
<td>Wnt8a</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>TGCCGCTCATGCTCTCAG</td>
<td>GTCGACTCAGTTGAGTCT</td>
</tr>
<tr>
<td>Wnt11</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>CGGCTCAGCTATGCTCAAGT</td>
<td>AGCTCGAGGAGGAGACT</td>
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<tr>
<td>GAPDH</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ACAACCTTGGCATGAGTGA</td>
<td>GATCGAGGAGGATGGCTCTG</td>
</tr>
<tr>
<td>Fzd 1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>CCGAGCTCAAGTCTCCTCG</td>
<td>GGGAACTCTCGCAGTCTG</td>
</tr>
<tr>
<td>Fzd 2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>TTCTCCAGTGCCACCACT</td>
<td>GACCTCGAGTTCTGACTCA</td>
</tr>
<tr>
<td>Fzd 3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>CCAAGAACTGCTGTTGCT</td>
<td>GACCTCGAGTTCTGACTCA</td>
</tr>
<tr>
<td>Fzd 4</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>TCCGACTCATGTCCCTCAG</td>
<td>GACCTCGAGTTCTGACTCA</td>
</tr>
<tr>
<td>Fzd 5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>GACGCGAGTTGCTTGGTA</td>
<td>TCGTTCAATGTCAATGGAA</td>
</tr>
<tr>
<td>Fzd 6</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>CACAAATCATGCGCCCTCAG</td>
<td>GGTGTTCTACGAGAATCTG</td>
</tr>
<tr>
<td>Fzd 7</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>GCTCACAACCCACACTCT</td>
<td>GCACGCAGTTGCTGACATAG</td>
</tr>
<tr>
<td>Fzd 8</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>CTTCACTGCTCTCTCCAG</td>
<td>ACGTGAAGCACTCGAGTT</td>
</tr>
<tr>
<td>Fzd 9</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>CCAAGTACGACTGAGAAGAG</td>
<td>GAAGGGTAACCGTGAGAAGG</td>
</tr>
<tr>
<td>Fzd 10</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>GACGCAAGGCTGTACCTAG</td>
<td>ATGAAAGGAAAGGCTGAC</td>
</tr>
</tbody>
</table>

+/- indicates presence or absence of RT-PCR product
Table 3. Percentage of side population (SP) cells relative to total viable OSE cells and β-catenin/Tcf-signaling cells as a fraction of total lacZ⁺ OSE cells in SP and main population (MP).

<table>
<thead>
<tr>
<th>Animal age (days)</th>
<th>% SP/total viable cells</th>
<th>% lacZ⁺ cells in SP</th>
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<td>35</td>
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Figures

Figure 1. Coelomic epithelium overlying the indifferent gonad displays β-catenin/Tcf-mediated lacZ expression. Panel A: Whole-mount X-gal staining of E11.5 urogenital ridge. LacZ positive cells overlie the medio-lateral surface of the indifferent gonad (dotted line demarcates the gonad). The mesonephric duct (MD) and mesonephric tubules (MT) also stain for lacZ. Arrowhead indicates coelomic epithelium extending beyond the gonad is not stained. Scale bar = 10 µm. Panel B: Section of an X-gal stained E11.5 urogenital ridge. LacZ staining is present in the gonadal surface epithelium (arrows), cells beneath the surface and the mesonephric ducts (black arrow head). Germ cells (white arrow head) do not stain for lacZ. Scale bar = 50 µm.
Figure 2. β-catenin/Tcf-mediated lacZ expression is maintained in the embryonic female gonad. Time course of β-catenin/Tcf-mediated transcription in female (upper panels) and male (lower panels) embryonic gonads. Whole-mount X-gal staining demonstrates β-catenin/Tcf expression is sexually dimorphic from E12.5 onwards. Blue (lacZ) staining reflecting β-catenin/Tcf-mediated transcription is observed in the mesonephric tubules (MT), mesonephric duct (MD), Mullerian duct (MU), Wolffian duct (WD), ovaries (o) and testis (t). Black arrows indicate the anterior region of the female gonad. Arrowhead indicates the ventral surface and posterior tip of the male gonad. All gonads are positioned with the anterior region at the top of each panel. Scale bar = 10 µm.
Figure 3. Loss of β-catenin/Tcf-mediated lacZ expression in the testis is not due to gonadal surface cell elimination. E15.5 ovary (XX; left panel) and testis (XY; right panel) after Periodic acid-Schiff staining. The basement membrane (arrow) is deposited underneath the epithelial layer (arrow head) of gonads and surrounds ovigerous cords (OC) in XX and testis cords (TC) in XY gonads. Scale bar = 50 µm.
Figure 4. Heterogeneity of β-catenin/Tcf-mediated lacZ expression in the ovarian surface epithelium (OSE). (a) β-catenin/Tcf-mediated lacZ expression during ovary development from postnatal day 1 (P1) to P180 indicating an age-dependent decrease in the proportion of stained cells. Scale bar = 100 µm. (b) X-gal staining of OSE cells during postnatal development from P1 to P21. LacZ+ cells are present within the OSE (arrow heads) and indicate an age-dependent decrease in the proportion of stained OSE cells. Scale bar = 50 µm
Figure 5. NHS-biotin labels OSE cells. (a) Panel A: A representative P21 NHS-biotin-labeled ovary section stained with avidin-FITC (green) and counterstained with Hoechst 33342 (blue). NHS-biotin labels OSE cells with little penetrance of the reagent into ovarian parenchyma. Panel B: Collagenase/DNase I treatment results in isolation of a significant proportion of the OSE leaving remainder of the ovary relatively intact. Dotted line demarcates the OSE location. Arrowheads indicate labeled OSE cells. Scale bar = 100 µm. (b) FACS analysis of non-NHS labeled (PBS) and NHS-biotin-labeled P21 OSE cells stained with avidin-FITC. Histogram showing that the majority of the cells stained for avidin-FITC indicating that they retained the NHS-biotin label following enzymatic treatment. A minimum of 10,000 cells were analyzed. (c) Cytokeratin 8 (CK8) staining of OSE cells. OSE cells were isolated following enzymatic digestion of NHS-biotin labeled ovary in collagenase/DNase I in DMEM. Released cells were stained with avidin-FITC and FITC-positive cells sorted, stained for CK8 (red) and counterstained with DAPI (blue). Scale bar = 50 µm. (d) CK8 staining of P21 ovary. Immunostaining for CK8 labels OSE specifically (red). Scale bar = 50 µm.
Figure 6. Age-dependent decrease in β-catenin/Tcf-signaling OSE cells. (a) The percentage of lacZ-positive OSE cells as a function of animal age estimated by FACS analysis. Error bars represent one SD from the mean and at least three replicates were used. *, denotes $P \leq 0.05$ between group means. A minimum of 10,000 cells were analyzed. (b) Serial count analysis of percentage of lacZ-positive OSE cells as a function of age. The percentage of lacZ-positive OSE cells at P5 and P10 is quite similar to that obtained at these ages using FACS. Error bars represent one SD from the mean and at least three ovaries were counted. *, denotes $P \leq 0.05$ between means.
Figure 7. Apoptosis is not detected in neonatal OSE cells. Apoptosis was assessed by TUNEL on X-gal-stained P1 and P5 ovaries. TUNEL positivity (green) was not observed in the ovary. DNase-treated ovary served as a positive control. Slides were counterstained with PI (red). Scale bar = 50 µm.
Figure 8. LacZ$^+$ and lacZ$^-$ neonatal OSE cells are proliferative. P2 mice were injected with BrdU 48 h prior to analysis. Ovaries were collected, X-gal stained, probed with anti-BrdU antibody (green) and counterstained with PI (red) to label cell nuclei. BrdU-positive cells were observed within somatic cells of the ovary (left panel) suggesting that mitosis is not restricted to the OSE. White arrow heads show BrdU$^+$ and black arrow heads lacZ$^+$ cells. Scale bar = 50 µm.
Figure 9. Decrease in β-catenin/Tcf-signaling OSE cells in vitro. OSE cells isolated from P5-P9 mice were cultured in alpha MEM supplemented with 4% FBS. The number of lacZ+ cells was estimated after incubation with the fluorescent substrate CMFDG. LacZ+ cell numbers doubled within first four days of culture and decreased subsequently with prolong culture. In contrast, lacZ-negative cells increased steadily. These results suggest that lacZ+ cells lose their ability to signal through the β-catenin/Tcf-signaling pathway and become non-signaling.
Figure 10. OSE cells show a side population (SP). Dye cycle violet (DCV) SP analysis of OSE cells. OSE cells harvested from prepubertal mice were stained with DCV in the presence or absence of the ABCG2 inhibitor verapamil. Addition of verapamil resulted in reduction in SP. The ratio of SP cells to total viable cells is indicated as a percentage in the scatter plot. Boxed cells (labeled MP) represent the main population.
Figure 11. SP cells from mouse OSE. Isolated OSE cells were stained with DCV and SP cells sorted into 3% paraformaldehyde.
CHAPTER 3

[Manuscript II]

Activation of the canonical WNT signaling pathway promotes ovarian surface epithelial proliferation without inducing β-catenin/Tcf-mediated reporter expression

Developmental dynamics (accepted: DVDY-12-0189.R1)
Preface

In chapter 2, we demonstrated that β-catenin/Tcf-signaling (lacZ⁺) OSE cells decreased to a small but constant proportion when mice were weaned. If β-catenin is required for Tcf-mediated lacZ expression, we can expect that ectopic activation of the WNT signaling pathway will induce Tcf-mediated lacZ activity and OSE proliferation. Here, we assessed whether β-catenin expression correlates with Tcf-mediated lacZ activity in OSE cells. Using LiCl and Wnt3a-conditioned media as a tool to activate the canonical WNT signaling pathway, we further investigated whether β-catenin stabilization can regulate β-catenin/Tcf-mediated gene expression and OSE proliferation.
Activation of the canonical WNT signaling pathway promotes ovarian surface epithelial proliferation without inducing β-catenin/Tcf-mediated reporter expression

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Running Title: Modulation of β-catenin/Tcf-signaling activity in the ovarian epithelium

- β-catenin is localized on OSE cell membrane during postnatal development
- Stimulation of the canonical WNT pathway promotes OSE cell proliferation without inducing Tcf/Lef-mediated reporter expression
- Canonical WNT pathway stimulation increases Tcf/Lef-mediated reporter gene activation in a cancer cell line-specific manner

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Keywords: Ovarian surface epithelium, Wnts, β-catenin/Tcf-signaling, lacZ, ovarian cancer cell lines
Abstract

**Background:** In response to canonical WNT signaling, β-catenin cooperates with Lef/Tcf (lymphoid enhancer factor/T-cell factor) transcription factors to activate target genes. Canonical WNT signaling is involved in development, wound repair, and tumorigenesis. Studies examining the involvement of canonical WNT signaling in development of ovarian surface epithelium (OSE) and ovarian carcinogenesis, however, have recently begun to emerge. In this study, we investigated the modulation of β-catenin and β-catenin/Tcf-signaling activity within the OSE using responsive transgenic mice and examined response of primary OSE cells and ovarian cancer cell lines to activation of canonical WNT signaling. **Results:** β-catenin was localized on the lateral membrane of the ovarian epithelium. Stimulation of primary OSE cells *in vitro* with LiCl or Wnt3a led to GSK-3β inhibition and stabilization of β-catenin but failed to induce β-catenin/Tcf-LacZ expression. Furthermore, E-cadherin expression was down-regulated and the proliferative potency of OSE cells increased. Of four ovarian cancers cell lines screened, only the HEY cell line demonstrated induction of luciferase reporter upon canonical WNT stimulation. **Conclusions:** These observations suggest that in ovarian adenocarcinoma, dysregulated WNT signaling may not always be indicative of β-catenin/Tcf-mediated transcriptional activity.
Introduction

Wnts are secreted cysteine-rich glycoprotein molecules involved in a number of developmental processes including establishment of cell fate, proliferation and differentiation (Cadigan, 2008; Logan and Nusse, 2004b; Miller, 2002; Wodarz and Nusse, 1998). The WNT signaling pathway has been implicated in ovarian development (Boyer et al., 2010). In female gonad, expression of Wnt4 acts antagonistically to male pathway and the outcome of mammalian sex determination hinges on the balance between Fgf9 and Wnt4 (Kim et al., 2006). In addition to Wnt4, sex-specific expression has been observed for other wnt genes such as Wnt5a, Wnt6 and Wnt9a in the ovary (Cederroth et al., 2007). Whereas WNT signaling plays key roles in embryonic development of the ovary (Ottolenghi et al., 2007; Vainio et al., 1999; Yao et al., 2004), several studies have described the expression of WNT signaling components in adult mouse ovary (Harwood et al., 2008; Hsieh et al., 2005; Hsieh et al., 2002; Hsieh et al., 2003; Kimura et al., 2006; Ricken et al., 2002). Some of these, including Wnt4 (Lyons et al., 2004) and Wnt2b (Katoh et al., 2001) are associated with activation of the canonical WNT signaling pathway. Recent findings also implicate a family of secreted ligands (R-spondin) in female sex-determination (Parma et al., 2006; Tomizuka et al., 2008). The R-spondins (Rspo) play an essential role in ovarian development through stabilization of cytoplasmic β-catenin. Mutation of Rspo1 is associated with human sex reversal (Parma et al., 2006). In the ovary, Wnts are primarily restricted to the ovarian surface epithelium or stroma and the expression
pattern changes at specific stages of ovarian development (Hsieh et al., 2002; Ricken et al., 2002). Thus, Wnts may provide potentially local regulatory systems on which ovarian cells may be dependent.

Canonical WNT signaling leads to increased intracellular β-catenin which acts as a co-transcription factor with members of T-cell factor/lymphoid enhancer (Tcf/Lef) family of transcription factors to drive target gene expression (Behrens et al., 1996). One of the target genes, cyclin D1, serves as a central regulator of cell cycle and cell proliferation (Shtutman et al., 1999). Additionally, β-catenin binds to E-cadherin to form adherens junctions essential for cell-cell adhesion (Wheelock and Johnson, 2003). Loss of E-cadherin leads to impairment of cadherin-catenin complex at cell junction contributing to increased free β-catenin and Tcf-mediated gene expression (Gottardi et al., 2001).

In a previous study, we demonstrated that β-catenin/Tcf-signaling (lacZ+) OSE cells overlie the differentiating ovary but decreased to a small proportion when mice were weaned (Usongo and Farookhi, 2012). Here, we show that β-catenin is localized on the membrane of ovarian epithelium during postnatal development. Stimulation of primary OSE cells with Wnt agonists promotes stabilization of β-catenin and proliferation without inducing β-catenin/Tcf-mediated lacZ activity. These observations suggest that stabilization of β-catenin is not always associated with β-catenin/Tcf-related transcriptional activity in OSE cells.
Results

**β-catenin is localized on OSE cell membrane**

Because N-terminally dephosphorylated (active) represents a better marker for canonical WNT signaling than total β-catenin, we examined subcellular localization of active β-catenin in OSE cells during postnatal development by immunofluorescence. β-catenin was localized at the cell membrane of the OSE at P5 (Fig 1, white arrow). By P35, the plasma membrane was still the most common location of β-catenin. This indicates that during postnatal development, β-catenin is predominantly localized at the membrane of the OSE.

**Activation of the canonical WNT signaling pathway stabilizes β-catenin without inducing β-catenin/Tcf-mediated reporter expression in OSE cells**

Activation of β-catenin/Tcf-signaling results from nuclear accumulation of β-catenin (Rubinfeld et al., 1996). We investigated the possibility that stabilization of β-catenin drives β-catenin/Tcf-mediated reporter expression in OSE cells. We sought to analyze this by ectopically activating the β-catenin/Tcf signaling cascade. To this end, we employed two strategies. Firstly, we activated β-catenin by challenging OSE cells with exogenous Wnt-Wnt3a. Stable expression of several *Wnt* genes, including *Wnt3a*, stabilizes β-catenin in mammalian cells thereby mimicking the canonical WNT signaling pathway (Shimizu et al., 1997).
Secondly, we used LiCl, an agonist that mimics the WNT signaling pathway by inhibiting glycogen synthase kinase 3-β (GSK-3β) to stabilize β-catenin protein (Stambolic et al., 1996).

In order to establish a cell culture model in which the effect of β-catenin modulation could be investigated, primary OSE cells were stimulated with conditioned media (CM) from Wnt3a-expressing L cells (Shibamoto et al., 1998). To determine at what point the canonical WNT signaling pathway becomes activated, OSE cells were exposed to conditioned media from Wnt3a expressing (Wnt3a-CM), parental L cells (LCM) or LiCl and β-catenin levels measured at three different time points over a 24-hour period. Treatment of OSE cells with Wnt3a-CM or LiCl resulted in a time-dependent increase in β-catenin protein expression, with the highest increase in β-catenin protein occurring after 24 hours of stimulation compared to controls (Fig 2a). Consequently, the effect of OSE cell stimulation was investigated after 24 hours.

Immunofluorescent analysis revealed that β-catenin was mostly localized at the cell membrane in non-stimulated OSE cells (Fig 2b). Treatment with Wnt3a-CM or LiCl increased β-catenin expression and nuclear localization in OSE cells. Wnt3a-CM induced a change in cell morphology; manifested by elongated cell shape, a characteristic observed in Wnt-stimulated epithelial cells (Wong et al., 1994). Unexpectedly, activation of Tcf/Lef transcription determined by β-galactosidase staining indicated absence of β-galactosidase activity (Fig 2c).
Furthermore, LiCl-dependent stabilization of \( \beta \)-catenin did not activate the lacZ reporter. The Fallopian tube served as positive control for \( \beta \)-galactosidase staining (Fig 2d). Collectively, the data demonstrates that activation of the canonical WNT signaling pathway is insufficient for induction of \( \beta \)-catenin/Tcf-mediated lacZ expression in OSE cells.

**LiCl and Wnt3a activate the canonical WNT signaling pathway by inhibiting GSK-3\( \beta \)**

A prior study suggests LiCl mimics canonical WNT/\( \beta \)-catenin activation by inhibiting GSK-3\( \beta \) through phosphorylation at Ser-9 (Hedgepeth et al., 1997). Although the target of lithium responsible for this indirect regulation has not been identified, we investigated the effect of LiCl or Wnt3a-CM on GSK-3\( \beta \) phosphorylation. The inhibition of GSK-3\( \beta \) was assessed by immunoblot analysis using a phospho-Ser-9-specific antibody following 24 hr stimulation. LiCl or Wnt3a-CM stimulation increased phosphorylated GSK-3\( \beta \) (Fig 3). This demonstrates that LiCl and Wnt3a-CM activate the canonical WNT signaling pathway in OSE cells through GSK-3\( \beta \) inhibition.
**E-cadherin is expressed in OSE cells and decreased following canonical wnt stimulation**

The ability of cadherins to sequester β-catenin from the nucleus could come from direct binding to β-catenin or indirectly from increased cell adhesion. The extracellular domain of E-cadherin is involved in calcium dependent cell adhesion, while its intracellular domain binds β-catenin. β-catenin in turn binds α-catenin, which connects adherens junctions with the cytoskeleton (Nelson and Nusse, 2004). Thus canonical WNT signaling may destabilize adherens junction by both E-cadherin downregulation and β-catenin nuclear translocation.

Immunohistochemical expression of E-cadherin was observed along the cytoplasmic membrane of OSE cells and decreased following canonical WNT stimulation (Fig 4a). Immunoblot analyses showed a two-fold decrease in E-cadherin protein levels upon stimulation (Fig 4b), indicating both LiCl and Wnt3a-CM decreased E-cadherin expression in OSE cells.

To eliminate cadherin-mediated adhesion as a confounding factor in β-catenin/Tcf-mediated expression, cells were incubated with EDTA. Incubation of cells with EDTA greatly facilitates calcium removal and it is one of the most effective ways of blocking cadherin-mediated cell adhesion (Takeichi and Nakagawa, 2001). This process may further increase cytosolic/nuclear accumulation of free β-catenin. The morphology of OSE cells cultured for 24 hrs with EDTA is shown in Fig 4c. Inclusion of EDTA in the culture media appeared
to diminish cell compactness. Although cell spreading appeared reduced, the expression of β-catenin/Tcf-mediated lacZ reporter was not observed even when cells were stimulated with Wnt3a-CM.

Canonical WNT stimulation promotes OSE cell proliferation in culture

Excess or dysregulated β-catenin signaling activity drives tumorigenesis (Behrens and Lustig, 2004). To address whether β-catenin stabilization induces OSE proliferation, we performed immunocytochemistry using an antibody against PCNA. PCNA is an auxiliary protein of DNA polymerase δ, which is essential for DNA replication and is expressed during the cell-cycle transition from the G0 to G1 phase and further increased during the S phase (Celis et al., 1987). Subconfluent and asynchronous primary OSE cells were incubated with 20 mM LiCl and NaCl as control or Wnt3a and LCM as control for 24 hrs. Immunofluorescent labeling with antibodies against PCNA showed that both control and stimulated cells displayed PCNA staining, indicative of chromatin-bound protein engaged in DNA replication with no significant differences in the percentages of PCNA-positive cells between treatments and their controls (>90% cells stained for PCNA). Very few control cells, however, presented an intense PCNA staining which could correlate with the very few proliferating cells in this condition (Fig 5a). Thus it seemed likely that inhibition of GSK-3β led to enhancement of proliferative potency. To assess DNA synthesis, we examined the
rate of G1 to S phase transition using the BrdU incorporation assay. BrdU-positive cells significantly increased with Wnt3a (Fig 5b) or LiCl (Fig 5c) treatment compared to controls. Taken together, the data demonstrate that stimulation of the canonical WNT signaling pathway in OSE induced G1 to S phase cell cycle progression.

*LiCl and Wnt3a increase Tcf/Lef-mediated reporter gene activation in a cell line-specific manner*

The overwhelming majority of epithelial ovarian cancers are thought to arise from the surface epithelium and emerging evidence implicates WNT signaling in ovarian endometrioid adenocarcinomas (Gatcliffe et al., 2008). For this reason, we examined whether or not activation of the canonical WNT signaling pathway in human ovarian cancers leads to β-catenin/Tcf-dependent expression. We used LiCl (or NaCl as control) together with TOPFLASH-luciferase, a positive control reporter plasmid containing Tcf/Lef-1–binding sites to screen ovarian cancer cell lines (HEY, OVCAR3, SKOV3 and SW626) for responsiveness to canonical Wnt stimulation. Of the four cell lines, only HEY (human ovarian carcinoma) showed activation of luciferase reporter upon stimulation. OVCAR3, SKOV3 and SW626 did not show induction of reporter activity. LiCl or Wnt3a induced more than 30-fold Lef-1–dependent transactivation over NaCl or LCM control, respectively (Fig 6). These observations indicate that canonical WNT stimulation is capable of
driving Tcf-dependent reporter activity in some but not all ovarian cancer cell lines.
Discussion

β-catenin is a key effector of the WNT pathway and an important component of the cadherin/catenin intercellular adhesion complexes (Kikuchi, 2000). Accumulating evidence suggests only the non-cadherin bound β-catenin is involved in signaling (Fagotto et al., 1996). Others, as well as results of this study revealed that β-catenin is localized at the cell membrane of normal OSE (Davies et al., 1998; Fan et al., 2010; Rask et al., 2003). The localization of β-catenin at the cell membrane suggests it is associating with E-cadherin to mediate cell adhesion. In the embryonic mouse gonad, β-catenin is localized on OSE cell membrane at E12.5 (Bernard et al., 2008a). This developmental stage corresponds to the stage when the female gonad is overlaid by lacZ+ cells (Usongo and Farookhi, 2012). To reconcile this observation, we speculate that the high membranous staining of β-catenin may mask nuclear detection. It is also possible that nuclear accumulation of β-catenin may be a dynamic process and as such difficult to localize. The expression of non canonical Wnts within the OSE (Usongo and Farookhi, 2012) may rapidly redirect any nuclear β-catenin to the cell membrane, as has been the case with Wnt 4 expression (Bernard et al., 2008a).

The morphological alterations in response to canonical WNT stimulation were probably accompanied by rearrangement of actin cytoskeleton. Cytoskeletal
alterations in response to Wnt3a have been noted in epithelial cells (Shibamoto et al., 1998). Since β-catenin is important for both cadherin-mediated cell–cell adhesion and WNT-dependent signal transduction, it represents a putative link between these signaling pathways. Despite β-catenin stabilization, Tcf-mediated lacZ expression was not induced. Similar observations have been made with primary bovine aortic endothelial cells (Mao et al., 2001) and human T lymphocytes (Prieve and Waterman, 1999). Staining of Lef-1, a member of the HMG-box transcription factors was confined to cytoplasm and cell borders of human OSE cells and epithelial-derived tumour cells suggesting that a complex of β-catenin/Lef for transcriptional regulation is less likely to be formed in OSE (Rask et al., 2003). Even when Lef and β-catenin are abundantly present in the nucleus, β-catenin/Lef-1 complexes do not always activate reporter gene expression (Prieve and Waterman, 1999). Therefore, it is conceivable that additional modifications of the WNT pathway must occur to get activity. This suggests that deregulation of β-catenin as in some cancers might not always lead to β-catenin/Tcf-mediated transcriptional activity. Another interpretation is that Tcf/Lef transcription may be one of many downstream events in the WNT signal transduction pathway and that β-catenin activation alone is insufficient to drive Tcf/Lef gene transcription.

GSK-3β is known to regulate the function and subcellular localization of Snail (Zhou et al., 2004). Snail upregulation due to GSK-3β inhibition likely served as a
mechanism for E-cadherin downregulation. Although β-catenin/Tcf-mediated lacZ expression was not induced, it is possible that loss of cell-cell adhesion triggers early and temporary changes in gene expression. Thus it can be assumed that loss of cadherin-mediated cell adhesion will result in a multitude of secondary effects following disruption of adherens junctions.

Accumulating evidence suggests that canonical WNT signaling induces cell proliferation (Behrens and Lustig, 2004; Logan and Nusse, 2004b; Wodarz and Nusse, 1998). In ovarian cancer, Wnt7a is known to regulate tumor growth and progression through the WNT/β-catenin pathway (Yoshioka et al., 2012). As described for other cellular systems (Rao et al., 2005; Welshons et al., 1995), inactivation of GSK-3β induced OSE cell proliferation. Induction of cell proliferation by GSK-3β inactivation is known to occur through the canonical β-catenin/Tcf pathway (Rao et al., 2005), non-canonical GSK-3β-NF-AT signaling (Qu et al., 2011), or by inhibition of GSK-3β-dependent cyclin D1 proteolysis (Diehl et al., 1998). Also, the ERK pathway is involved in Wnt3a-induced fibroblast proliferation with the degree of cell proliferation significantly more reduced by co-treatment with ERK1 and ERK2 siRNAs than with β-catenin siRNA treatment (Yun et al., 2005). Since lacZ expression was not induced following stimulation, we therefore suggest that OSE cell proliferation occurred via pathways other than the β-catenin/Tcf-signaling pathway. Consistent with this
hypothesis, it has been shown that LiCl stimulates proliferation of neural precursor cells without stabilizing β-catenin (Ohteki et al., 2000; Qu et al., 2011). It is possible that β-catenin stabilization is a consequence of GSK-3β inhibition and may be involved in destabilizing cell adhesion. While investigations were performed in primary OSE cells, β-catenin/Tcf-mediated expression was assessed in human ovarian cancers. β-catenin/Tcf-mediated reporter expression in ovarian cancer cell lines raised the possibility that other components of the WNT pathway in addition to β-catenin are necessary for Tcf-mediated expression. These may be cell type specific. Thus, the phenotype of cells in response to canonical WNT signaling is a result of GSK-3β inhibition, increased levels/or activities of β-catenin, or both. This may alter gene expression and/or modulate cell adhesion, mechanisms that are instrumental in regulating cellular patterning, tissue architecture and cancer progression.
Conclusion

The results make it clear that β-catenin; the central player of the canonical WNT signaling pathway, is localized at the membrane of OSE cells. Stimulation of the canonical WNT signaling pathway with LiCl or Wnt3a led to stabilization of β-catenin and induction of cell proliferation but was insufficient to drive β-catenin/Tcf-mediated expression. In epithelial ovarian cancers, only one of four cell lines demonstrated β-catenin/Tcf reporter activity following activation of the canonical WNT signaling pathway. Collectively, our data suggests that GSK-3β inhibition as a result of altered WNT signaling is of major importance in the control of epithelial-mesenchymal transition and cell proliferation leading to tumorigenesis.
Experimental Procedures

Reagents

Deoxyribonuclease I, collagenase (Type IV), and 5'-bromo-2'-deoxyuridine were purchased from Sigma Aldrich (St Louis, MO). Dulbecco's Modified Eagle Medium (DMEM) and propidium iodide (PI) were purchased from Invitrogen (Burlington, ON), fluorescein di-beta galactopyranoside (FDG) was obtained from Molecular Probes (Eugene, Oregon), Bovine serum albumin (BSA) was from Wisent (St-Bruno, QC), Fugene HD transfection reagent, Hoechst 33342 from Roche (Laval, QC), Tween-20 from Bio Basic Inc (Markham, ON), and mowiol from Calbiochem (La Jolla, CA). The mouse monoclonal anti-BrdU antibody was obtained from Developmental Studies Hybridoma Bank (Iowa City, IA), anti-E-cadherin antibody from BD (Mississauga, ON), anti-PCNA antibody from Santa Cruz Biotechnology (Santa Cruz, CA), anti-β-catenin and GSK3 antibody from ABM (Richmond, BC), and anti-α tubulin antibody from Abcam (Cambridge, MA). Cy3-labeled goat anti-rabbit IgG and FITC-labeled goat anti-mouse IgG antibody (Jackson Immuno Research, West Grove, PA) were obtained through Cedarlane Laboratories (Burlington, ON).

Animals

All animal procedures followed the guidelines established by the Canadian Council of Animal Care and were approved by the Animal Care Committee of the
Royal Victoria Hospital, McGill University. CD1 mice bearing the β-catenin/Tcf-responsive lacZ reporter gene (TopGal mice) have been described previously (Mohamed et al., 2004b). Female and male CD1 mice homozygous for the transgene were provided for colony establishment by Dr. Daniel Dufort (Department of Obstetrics and Gynecology, McGill University, Montreal, Canada). The day after birth was designated postnatal day 1 (P1). Gonads were isolated from a minimum of three mice and all experiments performed in triplicates.

**OSE isolation**

Isolated ovaries were washed in PBS and OSE cells isolated. OSE cells were isolated as follows: ovaries (2 ovaries /0.5 ml DMEM) were incubated for 60 min at 37°C in DMEM containing 1 mg/ml Type IV collagenase, 1 mg/ml deoxyribonuclease I, and 0.53 mM EDTA. Ovaries were agitated every 10 min by swirling the tube for a few seconds. After the 60 min incubation, the released cells were transferred to a fresh tube. The ovaries were rinsed in fresh DMEM and additional released cells combined with previously isolated cell suspension. The cell suspension was vortexed, centrifuged at 500 g for 5 min at RT and resuspended in alpha MEM + 4% FBS.
**Cell lines and culture**

All of the ovarian cancer lines, with the exception of OVCAR3, were cultured in MEM supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 0.1 mM non-essential amino acids (Life Technologies Inc., Burlington, ON) and 1% Penstrep. OVCAR3 cells were maintained in RPMI 1640 containing 20% FBS, 2 mM L-glutamine and 0.1 mM non-essential amino acids (Life Technologies Inc.). Primary OSE cells were grown in alpha MEM + 4% FBS and 20 U/ml PenStrep.

Wnt3a-expressing L cells were provided by Dr. Daniel Dufort (McGill University, Montreal, QC, Canada) and were cultured in DMEM containing 10% FBS and antibiotics. Conditioned media (CM) were derived from these cells (Wnt3a-CM) and from control L cells (LCM) by seeding the cells at approximately 30% confluence and collecting the media after four days of culture. The conditioned media were cleared of cellular debris by centrifugation (300xg for 10 min) and stored at 4°C after filtration through a 0.2 µm filter (Millipore, Mississauga, ON, Canada). Cells were cultured in appropriate media at 37°C with 5% CO₂ humidified air and grown to 70-90% confluence. Cells were subsequently incubated with 20mM NaCl or 20mM LiCl and LCM or Wnt3a-CM for an additional 24 h.
**β-galactosidase assay**

OSE cells were assayed for β-galactosidase using a fluorescence assay with fluorescein di-beta galactopyranoside as a substrate. Conditions for staining were as per manufacturer’s recommendations.

**Tissue processing and X-gal staining**

Mice were sacrificed by cervical dislocation. Fallopian tubes were isolated, washed in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8 mM Na$_2$HPO$_4$, 2 mM KH$_2$PO$_4$, pH 7.4) and fixed for 5–15 min in freshly prepared 4% paraformaldehyde (PFA) in PBS. After rinsing in wash buffer (PBS containing 2 mM MgCl$_2$, 0.1% Triton, 0.05% sodium deoxycholate), the tissue was stained in the dark overnight at 37°C in wash buffer supplemented with 1 mg/ml X-gal, 0.04% N, N-dimethyl formamide, 5 mM potassium ferricyanide and 5 mM potassium ferrocyanide to disclose β-galactosidase activity (Iglesias et al., 2007). Following staining, the Fallopian tubes were washed in PBS and processed for histology.

**Immunofluorescence**

Cells grown on glass cover-slips were fixed with ice cold methanol at - 20°C and blocked with 1% BSA/PBST for 60 min at RT. Cells were subsequently incubated overnight at 4°C with one of the following antibodies; E-cadherin (1/50 dilution),
PCNA (1/100 dilution) and β-catenin (1/100 dilution). The primary antibody was omitted for negative control slides. After washing 3x5 min with PBST, the secondary antibody was added and incubated in the dark for 2 h at RT. Cells were rinsed with PBS, counterstained and mounted using mowiol.

**BrdU labeling and immunohistochemistry**

To detect proliferating cells, cultures were incubated with BrdU at a final concentration of 10μM for 24h. Cells were fixed with 4% para-formaldehyde for 5 min at RT. The fixed cells were treated with 2 M HCl for 1 hr at RT, followed by 3x5 min wash with PBST. 3% BSA in PBST was applied for 30 minutes to block non-specific binding of antibodies. Cells were then incubated overnight at 4°C with anti-BrdU antibody. Following primary antibody incubation, slides were washed 3x5 min in PBST, stained with an FITC-labeled goat anti-mouse secondary antibody diluted 1:100 in blocking solution overnight at 4°C in the dark, counterstained with DAPI, and mounted in mowiol.

**Transfection and luciferase assay**

Cells were transfected with the use of Fugene HD transfection reagent. Cells were plated in 24-well plate two days before transfection so that they were ~80% confluent on the day of transfection. Cells were cotransfected with plasmid DNA
containing TOPFLASH reporter construct (van de Wetering et al., 1991) and β-galactosidase using a 5:2 ratio of Fugene HD transfection reagent (µl) to DNA (µg). To do this, 0.5 µg total DNA [0.15 µg TOPFLASH + 0.05 µg expression vector coding for β-galactosidase gene (internal control for transfection efficiency) + 0.3 µg empty vector] was diluted in 25 µl Opti-MEM 1 Reduced Serum Medium, and 1.25 µl of Fugene HD transfection reagent was added to it. The DNA-Fugene HD complex was incubated for 15 mins at room temperature. The DNA-Fugene HD transfection reagent complexes were applied to the cells and incubated for 24 hrs at 37°C at 5% CO2. Following transfection, cells were stimulated with 20mm NaCl, 20mM LiCl, LCM or Wnt3a-CM for 24 hrs. Cells were harvested with 100ul extraction buffer (1% Triton X-100, 15 mM magnesium sulphate, 4 mM EGTA, 1 mM dithiothreitol and 25 mM glycyglycine) on ice. Luciferase activity was measured in an EG&G Berthold Luminometer (EG&G Optoelectronics, Vaudreuil, QC, Canada) using 45 µl cell lysate and normalized to β-galactosidase activity (Valderrama-Carvajal et al., 2002).

**SDS-Polyacrylamide gel electrophoresis and Western blotting**

Western blot analyses were performed as previously described (Harlow and Lane, 1988). Subconfluent cultures were rinsed twice with cold PBS and total cell lysates prepared in radioimmunoprecipitation assay (RIPA) buffer (150 mM
NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 7.5) containing aprotinin (30 μg/ml), phenylmethylsulfonyl fluoride (PMSF; 10 μg/ml) and sodium orthovanadate (1 mM). The samples were sonicated for 10 seconds and centrifuged at 4ºC for 10 minutes at 10,000 x g. The supernatants were recovered and total cell lysates containing equivalent amounts of protein were diluted 1:6 with 6X Laemmli sample buffer and boiled for 5 min. Aliquots (10-20 μg protein) were resolved by SDS-PAGE and transferred to PVDF membranes (Bio/Can Scientific Inc., Mississauga, ON). Membranes were washed with TBST (TBS containing 0.1% Tween-20) and blocked in TBST containing 5% fat free instant milk. Membranes were incubated with primary antibodies (α-tubulin 1:10000, β-catenin 1:1000 and E-cadherin 1:500) in blocking solution overnight at 4ºC. The membranes were washed 3x15 min in TBST and incubated for one hour at RT with a 1:10000 dilution of alkaline phosphatase-conjugated goat anti-rabbit and 1:6000 anti goat antibody. Immunoreactive bands were detected by chemiluminescence using CSPD (Roche Molecular Biochemical, Laval, QC, Canada). Image J densitometry software (NIH, Bethesda, MD) was used for gel band quantitative densitometric analysis. Selected bands were quantified based on relative intensities.

**Cell counting**

BrdU-labeled cells and PI-stained nuclei were counted from at least 3 fields (×10 magnification). Nuclei were quantified using Image J nucleus counter software
The percentages of proliferating cells were calculated as the number of positive BrdU labeled nuclei divided by number of PI-stained nuclei in all fields.

**Statistical analysis**

Data were analyzed using SYSTAT 10.2 statistical software (SYSTAT Software, Richmond, CA). Analysis of variance (ANOVA) was used with Tukey's test in the post hoc analysis for cell counts to ascertain group mean differences. Data was subjected to ANOVA after arcsine transformation (McDonald, 2009). Data are presented as mean ± standard error of the mean. A p value of \( \leq 0.05 \) was considered significant.

**Acknowledgements**

This study was funded by grants from the Canadian Institutes of Health Research (CIHR) and the Natural Sciences and Engineering Research Council of Canada (NSERC).
Figure 1. **β-catenin is localized on OSE membrane.** β-catenin staining of paraffin-embedded sections of P5 and P35 ovaries. β-catenin (red) was detected predominantly in the plasma membrane (white arrow) of OSE cells. Hoechst 33342 was utilized for counterstaining. Scale bar represents 50 microns.
Figure 2. Activation of canonical WNT signaling stabilized β-catennin protein in OSE cells. (a). β-catenin immunoblot analysis of OSE whole-cell lysates. Subconfluent OSE cells grown in alpha MEM supplemented with 4% FBS were treated for up to 24 h. Expression of β-catenin was evaluated by immunoblotting using specific antibodies at indicated times. Image J software was used to quantify band intensity. Data represents mean ± standard error of at least 2 independent experiments. Each sample was plotted on Y-axis as ratio of relative density normalized to α-tubulin and compared to control.
Figure 2b. Immunofluorescence staining of primary OSE cells for β-catenin.
OSE cells grown in alpha MEM supplemented with 4% FBS were stimulated with Wnt3a-CM, 20 mM LiCl or 20 mM NaCl (control) for 24 hours and stained with an antibody specific for active β-catenin. Hoechst 33342 was used as a nuclear marker.
**Figure 2c. β-catenin stabilization fails to induce β-catenin/Tcf-mediated lacZ activity.** Sub-confluent OSE cells were grown in Wnt3a-conditioned media (Wnt3a-CM) or L-cell media (LCM) for 24 hrs and analyzed for lacZ expression. β-galactosidase activity was not observed following incubation with the fluorescent substrate FDG. Wnt3a-CM, however, induced changes in OSE cell morphology.
Figure 2d. Fallopian tube β-galactosidase staining served as positive control.
Scale bar represents 50 microns.
Figure 3. Canonical WNT stimulation inhibits GSK-3β in OSE cells. Sub-confluent OSE cultures were grown in media containing 20 mM LiCl (NaCl as control) or Wnt3a-CM (LCM as control) for 24 hrs, followed by western blotting analysis on phosphorylated (Ser 9) GSK-3β. Results were analyzed using Image J software. Each sample was plotted on Y-axis as ratio of relative density normalized to α-tubulin and compared to control.
Figure 4. Activation of canonical WNT signaling decreases E-cadherin expression in OSE cells (a). Subcellular localization of E-cadherin in OSE cells. E-cadherin is expressed along the cell membrane. Sub-confluent OSE cell cultures were stimulated with either 20 mM NaCl (control), 20 mM LiCl or Wnt3a-CM for 24 hrs and analyzed immunohistochemically with an antibody specific for E-cadherin. Both LiCl and Wnt3a-CM decreased E-cadherin expression. Propidium iodide was used as nuclear marker.
Figure 4b. E-cadherin immunoblot analysis of OSE whole-cell lysates. Image analysis indicates differential E-cadherin expression. Each sample was plotted on Y-axis as ratio of relative density normalized to α-tubulin and compared to control.
Figure 4c. Morphology of OSE cells cultured with media (left panel) or media + 1mM EDTA (right panel) for 24 hrs. Compactness of OSE cells was diminished in the presence of EDTA (compare left to right panel)
Figure 5. Activation of canonical WNT signaling increases the number of proliferating BrdU⁺ OSE cells in culture. (a) Immunofluorescence staining of PCNA in OSE cells. Two-color fluorescence of cells stained for PCNA (red) and counterstained for DNA with DAPI (blue). No significant difference in the percentage of PCNA-positive cells was found between treatments and controls.
Figure 5b. Wnt3a stimulation increases cell proliferation. Sub-confluent OSE cells were treated with LCM (control) or Wnt3a-CM in the presence of 10μM BrdU for 24h, probed with anti-BrdU antibody, and counterstained with propidium iodide. The proportion of proliferating cells was analyzed using Image J software. Data represent mean ± standard error, and significance was determined with Tukey post hoc analysis following ANOVA.
**Figure 5c.** LiCl stimulation increases OSE cell proliferation. Data represent mean ± standard error, and significance was determined with Tukey post hoc analysis following ANOVA.
Figure 6. LiCl and Wnt3a-CM induced TOPFLASH luciferase reporter containing Tcf/Lef-1 binding motifs in HEY cell line. HEY cells were transfected with TOPFLASH plasmids and treated for 24 hr with NaCl, LiCl, LCM or Wnt3a after which, luciferase activity was measured. Wnt3a or LiCl treatment led to increased luciferase activity only in HEY cells. The data represent mean ± standard error, and significance was determined with Tukey post hoc analysis following ANOVA.
CHAPTER 4

[Manuscript III]

β-catenin/Tcf-signaling in murine oocytes identifies non-ovulatory follicles

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Preface

We have assessed canonical WNT signaling in the murine ovary using transgenic mice harboring a reporter construct that activates β-galactosidase (lacZ) expression in response to β-catenin/Tcf binding (β-catenin/Tcf mice). Analysis of serial sections revealed β-catenin/Tcf expression in adult ovaries, as indicated by X-gal staining, was only observed in the germinal vesicle of some oocytes and in a small proportion of cells of the ovarian surface epithelium. After ovulation, most oocytes collected from the oviduct did not stain. Based on this observation, we hypothesized that lacZ-positive oocytes identifies non-ovulatory follicles. Here we have focused our attention on β-catenin/Tcf-signaling in oocytes with the objective of determining if this signaling 1) is associated with particular stages of follicular development; 2) is affected by gonadotropin stimulation and 3) if its presence or absence distinguishes follicles selected for ovulation.
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Abstract

Wnts are secreted glycoproteins molecules that signal through one of three signaling pathways. The best characterized pathway involves stabilization of the multifunctional protein β-catenin which in concert with members of the T-cell factor (Tcf) family activates specific gene transcription. We have examined putative WNT/β-catenin signaling in the murine ovary using transgenic mice harboring a reporter construct that activates β-galactosidase (lacZ) expression in response to β-catenin/Tcf binding (Tcf-LacZ mice). Primordial and primary follicles did not stain for lacZ and the proportion of β-catenin/Tcf signaling oocytes was lower than that of non-signaling oocytes throughout estrous cycle. β-catenin/Tcf signaling oocytes were observed in follicles from the secondary stage of development and their proportion increased with follicular maturation (secondary follicles: 20%; early antral and antral 70%). In contrast, the majority (>90%) of ovulated oocytes did not stain for lacZ. Since the oocyte possesses components for WNT signal transduction, our data suggest that β-catenin/Tcf-signaling is involved in the development of follicular ovulatory capability and identifies non-ovulatory follicles.
Introduction

The follicle is the functional unit of the ovary serving to protect and nourish the oocyte. Ovarian follicles and their contained oocytes are committed to either development or atresia once a third layer of granulosa cells is formed (Oakberg, 1979). Folliculogenesis begins with recruitment of germ cells into a pool of primordial follicles which will later progress through the primary, secondary, antral and then preovulatory follicle stages (Guigon and Magre, 2006a).

Follicular maturation is controlled by regulatory mechanisms orchestrated by several factors including the pituitary gonadotropins FSH and LH (Richards, 1994), intraovarian growth-regulatory factors such as IGFs, EGF, TGF-α, TGFβ, bFGF (Gougeon, 1996), and steroids such as estradiol (Edson et al., 2009). Additional locally produced factors belonging to the TGFβ superfamily such as activins, inhibins, follistatin (Knight and Gister, 2001) as well as oocyte-derived factors including GDF9 and BMP-15 (Matzuk, 2000), are also required at different stages of follicular development. Throughout life, follicles are recruited from the primordial follicle pool for further growth and development in a continuous manner. By puberty (6 weeks old in mice), increases in circulating FSH during each reproductive cycle recruit a cohort of antral follicles (McGee and Hsueh, 2000). This cyclic recruitment of follicles (estrous cycle) in rodents can divided into 4 stages (proestrus, estrus, metestrus, and diestrus) characterized
by hormonal and physiological changes (Byers et al., 2012). In the absence of
gonadotropin stimulation, the follicles (and oocyte) degenerate.

During oogenesis and folliculogenesis, the oocyte acquires a normal morphology,
an ability to resume and complete meiosis, initiate and sustain embryonic
development (Krisher, 2004). Only a subset of oocytes are capable of supporting
meiosis, fertilization and early embryo development to the blastocyst stage and
the proportion of these competent oocytes increases with follicular size
(Mermillod et al., 2008). The developmental competence of oocytes increases in
parallel with follicular size (Marchal et al., 2002). Although meiosis and early
development may be completed successfully, there are a variety of other
processes occurring within the oocyte that are required for complete
developmental competence (Krisher, 2004).

The Wnts comprise a large family of secreted cysteine rich glycoprotein
molecules (Miller, 2002). Wnts transduce their signal through one of three
signaling pathways, of which the best characterized, the WNT/β-catenin pathway,
leads to an increase in intracellular β-catenin protein which acts as a co-
transcription factor with members of T-cell factor/lymphoid enhancer (Tcf/Lef)
family of transcription factors to drive target gene expression (Behrens et al.,
1996). Wnts are involved in a number of developmental processes including
establishment of cell fate, proliferation and differentiation (Cadigan, 2008; Logan
and Nusse, 2004b; Wodarz and Nusse, 1998). The WNT signaling pathway has
been implicated in ovarian development. Ovaries of Wnt4 knockout mice are abnormal, containing structures similar to testicular tubules and exhibiting dramatically reduced oocyte numbers (Boyer et al., 2010; Vainio et al., 1999). Early in embryonic development, Wnt4/5a signaling coordinates germ cell entry into meiosis (Naillat et al., 2010). Multiple WNT signaling pathway genes are expressed within the oocyte (Wang et al., 2004; Zheng et al., 2006). In addition to Wnt4, sex-specific expression within the gonad has been found for Wnt5a, Wnt6 and Wnt9a (Cederroth et al., 2007). While Wnts play a key role in embryonic development of the ovary (Ottolenghi et al., 2007; Vainio et al., 1999; Yao et al., 2004), several studies described the expression of WNT signaling components in adult rodent ovaries (Harwood et al., 2008; Hsieh et al., 2005; Hsieh et al., 2002; Hsieh et al., 2003; Kimura et al., 2006; Ricken et al., 2002). Furthermore, several molecules needed for activation of alternative WNT pathways are in place during oocyte growth (Harwood et al., 2008). These studies collectively suggest that the WNT signaling pathway may play a role in follicle (and oocyte) development.

Given the complex expression pattern of WNT signaling components in oocytes, it is important to assess whether β-catenin/Tcf-mediated expression can serve as an informative marker for oocyte quality and its ability to be ovulated. We have assessed canonical WNT signaling in the murine ovary using transgenic mice (Tcf-LacZ) harboring a reporter construct that activates β-galactosidase (lacZ) expression in response to β-catenin/Tcf binding (Mohamed et al., 2004a). We
provide evidence suggesting that β-catenin/Tcf signaling in oocytes identifies non-ovulatory follicles.
**Results**

**β-Catenin/Tcf-activated lacZ expression is seen in oocytes of growing follicles**

To begin to explore β-catenin/Tcf-signaling activity in oocytes, we processed ovaries from transgenic mice during postnatal development for X-gal staining. Histological examination of sections at postnatal day 1 (P1) (Fig 1 A), P12 (Fig 1 B), P21 (Fig 1 C) and in cycling (Fig 1D) mice demonstrated β-galactosidase (lacZ) staining in oocytes (Fig 1 B/C/D, black arrowhead) and granulosa cells of some growing follicles (Fig 1 E). LacZ-positive cells were also observed within corpus luteum of cycling mice (Fig 1 F). The Fallopian tube/uterus served as positive control for β-galactosidase staining (Fig 1 G). Although lacZ staining levels were not uniform, a follicle was designated lacZ-positive only when partial or full staining of the oocyte was observed. Primordial and primary follicles did not stain and no distribution pattern was observed for lacZ-positive follicles within the ovary.

*The proportion of lacZ-positive follicles is lower than lacZ-negative follicles at all stages of estrous cycle*

Hormonal regulation of Wnt signaling pathway components has been detected in rodent ovaries. *Wnt4* expression is elevated in response to human chorionic gonadotropin (hCG) and highly expressed in corpora lutea (Hsieh et al., 2002). To
explore the possibility that hormones regulate β-catenin/Tcf signaling in oocytes, we examined lacZ expression in oocytes during the estrous cycle. The data set compiled for this study included follicles of one ovary from a pair of ovaries of cycling mice (n=4 for diestrus; n=5 for proestrus, estrus, and metestrus). We observed that the proportion of lacZ-positive was significantly lower than lacZ-negative follicles at all stages of estrous (Fig 2; p < 0.05).

**β-catenin/Tcf-signaling is increasingly activated in oocytes as the follicle matures**

Intraovarian factors that regulate early follicular development remain unknown. Once started, the initiation of follicular growth is continuous throughout life and occurs daily irrespective of hormonal status of the animal. We examined whether changes in follicular diameter due to growth and maturation are related to β-catenin/Tcf-mediated lacZ expression in oocytes. Measurements obtained for follicular diameter were stratified according to the phase of estrous cycle. LacZ-positive follicles were significantly larger than lacZ-negative follicles at all stages of estrous cycle (Fig 3a; p < 0.05).

Because follicular growth passes through distinct stages defined by certain structural characteristics, we examined if β-catenin/Tcf-mediated expression in oocytes is associated with specific stages of follicular development. The majority (> 70%) of secondary follicles did not stain for lacZ (Fig 3b; p<0.05). On the
contrary, we observed an increase in the proportion of stained to unstained follicles at the early antral stage. At least 70% of early antral follicles stained for lacZ (Fig 3c; p<0.05). The scarcity of antral follicles within the ovary prompted us to group antral follicles from individual ovaries of representative stages of estrous into a single group. Using this approach, we observed that approximately 60% of antral follicles stained for lacZ.

**β-catenin/Tcf-signaling oocytes are larger than non-signaling oocytes**

Oocytes in primordial follicles remain unchanged for shorter or longer periods of time in the ovary until there are recruited. We investigated the relationship between oocyte size and β-catenin/Tcf-signaling activity. Oocyte diameter was stratified according to stages of estrous cycle in the same manner as with follicular diameter. Statistical comparison revealed a significant difference between lacZ-positive and lacZ-negative oocytes. LacZ-positive were larger than lacZ-negative oocytes at all phases of estrous (Fig 4; p < 0.05).

**Gonadotropin stimulation increases the proportion of lacZ-positive oocytes in antral follicles of prepubertal mice**

To study the direct effects of FSH and hCG on β-catenin/Tcf mediated lacZ expression in oocytes, superovulation experiments using PMSG and hCG were
done in prepubertal mice. In non-stimulated mice, lacZ-positive oocytes were observed in approximately equal proportions of preantral and antral follicles (57% and 43%, respectively). β-catenin/Tcf-mediated lacZ staining in antral follicles increased 48h following PMSG treatment (preantral: 32%; antral: 68%), and this was further enhanced 24 h after hCG treatment (preantral: 20%; antral 80%). In contrast, the majority (>90%) of oocytes recovered from the oviducts did not stain for lacZ.

**Ovulated oocytes do not stain for lacZ**

To determine the reproductive consequence of β-catenin/Tcf-signaling activity, we examined ovulated oocytes for lacZ expression. For these experiments, a total of 110 oocytes were recovered from oviducts (n=14 mice) on the morning of estrus and examined for lacZ expression. The majority of oocytes recovered (91.8%) did not stain for lacZ and these were approximately 80.2 ± 0.55 µm in diameter (mean ± sem; n=11). These oocytes (lacZ-negative) had an intact cumulus mass (left panel, Fig 5a) representing fully-grown oocytes derived from Graafian follicles. Approximately 2% lacZ-negative ovulated oocytes were fragmented and 6% had reduced cumulus mass. On the contrary, cumulus-oocyte-complexes in which the oocyte stained for lacZ were smaller and contained few cells (right panel Fig 5a). Approximately 33% of these lacZ-positive oocytes were
fragmented (Fig 5b). A few (2 out of 9 total) ovulated lacZ-positive oocytes were similar in size and morphology to non-stained oocytes.
Discussion

The present study has revealed spatio-temporal β-catenin/Tcf-mediated expression in oocytes during follicular development. The proportion of lacZ-positive was lower than lacZ-negative oocytes throughout the estrous cycle. LacZ-positive were larger than lacZ-negative follicles and the proportion of stained to unstained follicles increased with follicular maturation. Despite a higher proportion of oocytes in antral follicles staining for lacZ, most ovulated oocytes did not show any indication of active Wnt signaling.

In the present study, we examined β-catenin/Tcf-mediated lacZ expression in oocytes as a read-out for canonical WNT signaling. Detection of β-galactosidase activity in oocytes from the secondary stage of follicular development suggests that canonical WNT signaling is inactive during primordial follicular formation. This is in agreement with a previous study indicating that β-catenin/Tcf signaling is transcriptionally inactive in developing germ cells (Chassot et al., 2008). In accordance with our results, Kimura et al have shown that canonical WNT molecules are expressed at low levels in primordial germ cells and over-expression of β-catenin impairs germ cell development (Kimura et al., 2006).

Recent evidence indicates that depleting β-catenin in granulosa cells of growing and pre-ovulatory follicles does not cause any overt effects in follicular...
development, ovulation or luteinization (Fan et al., 2010). The reason(s) why some granulosa and luteal cells, but not all, stain for lacZ remains to be elucidated. Even when β-catenin and its binding partner Lef are abundantly present in the cell nucleus, β-catenin/Lef-1 complexes do not always activate reporter gene expression (Prieve and Waterman, 1999). The lack of β-catenin/Tcf-mediated lacZ staining in all granulosa and luteal cells suggests that β-catenin/Tcf-signaling is not absolutely necessary in adult ovarian follicular cell growth and differentiation.

That majority of ovarian follicles do not show β-catenin/Tcf signaling during the estrous cycle was probably because most follicles were in the primary and secondary stages of follicular development and as such did not express lacZ. We noted that the proportion of lacZ-positive follicles increased with antrum formation (secondary follicles: ~20%; early antral and antral ~70%) suggesting β-catenin/Tcf–mediated gene expression in oocytes is initiated once a secondary follicle is formed and increases with follicular maturation. In addition, we observed a remarkable increase in the diameter of lacZ-positive follicles at proestrus. These observations may be explained by rapid follicular growth that is known to result from increased estradiol levels at this time. This event is likely the result of proestrus FSH surge (Hirshfield and Midgley, 1978). Since β-catenin/Tcf signaling activity is seen in oocytes of juvenile and prepubertal mice, our data suggests that activation of β-catenin/Tcf-signaling in
oocytes is independent of pituitary gonadotropins. It is possible that other factors associated with regulation of follicular growth, maturation, and differentiation activates β-catenin/Tcf-signaling in oocytes. The increased proportion of lacZ-positive antral follicles following hormone treatment suggests PMSG drives follicular maturation and β-catenin/Tcf–mediated lacZ activity in oocytes. Given that only lacZ-negative oocytes were ovulated, their expulsion from the ovary probably led to the observed increase in lacZ-positive follicles seen after hCG treatment. However, oocyte β-catenin/Tcf–mediated lacZ activity in hCG-treated mice is unlikely due to rising progesterone levels as progesterone is known to inhibit Wnt/β-catenin signaling by induction of DKK1 and FOXO1 (Wang et al., 2010b).

The diameter of an oocyte is often used as a marker for maturity or meiotic competence (Blanco et al., 2011). For this reason, one can infer that β-catenin/Tcf-signaling is activated in oocyte once it has reached a certain maturation state. The observation that ovulated oocytes did not stain for lacZ raises the possibility that a developmental defect exist within the follicle that precludes ovulation of lacZ-positive oocytes. This defect may be within the oocyte or follicle. It has been demonstrated that over-activation of β-catenin negatively effects LH-induced ovulation and luteinization (Fan et al., 2010). It is also known that disruption of pituitary LH synthesis, LH receptor binding or its downstream signaling pathways blocks ovulation and luteinization (Elvin and Matzuk, 1998). The failure of lacZ-positive oocytes to ovulate suggests impaired
responses to LH and reduced expression of genes required for ovulation and cumulus expansion. Work by Antosik et al. (2010) suggests that the morphology of cumulus oocyte complex is associated with oocyte quality and fertilization ability (Antosik et al., 2010). Interestingly, the fact that ovulated lacZ-positive oocytes showed anomalies in size and shape reinforces the idea that β-catenin/Tcf-signaling pathway identifies developmentally incompetent oocytes. There is accumulating evidence indicating that β-catenin is involved in apoptosis (Kim et al., 2000b) and that apoptosis is responsible for fragmentation of murine oocytes (Takase et al., 1995). Cytoplasmic fragmentation of ovulated lacZ-positive oocytes suggests that these oocytes were undergoing apoptosis.
Conclusion

In summary, our study indicates that the canonical WNT signaling pathway is active in oocytes from the secondary stage of follicular development. The proportion of β-catenin/Tcf-mediated lacZ-positive follicles increased with follicular maturation. Upon ovulation, >90% oocytes recovered from oviducts did not stain for lacZ. Since the oocyte and granulosa cells possess components for WNT signal transduction, our results suggests that canonical WNT signaling may be involved in the development of follicular ovulatory capability and identifies non-ovulatory follicles. Our findings may have clinical and animal biotechnological application related to assisted reproductive technologies. Identification of markers in follicles/oocytes that are correlated with developmental competence will increase efficiency of in vitro embryo production.
Materials and methods

Animals

All animal procedures followed guidelines established by the Canadian Council of Animal Care and approved by the Animal Care Committee of the Royal Victoria Hospital, McGill University. CD1 mice bearing the β-catenin/Tcf-responsive lacZ reporter gene (Tcf-LacZ mice) have been previously described (Mohamed et al., 2004a). Female and male CD1 mice homozygous for the transgene, were provided for colony establishment by Dr. Daniel Dufort (Department of Obstetrics and Gynecology, McGill University, Montreal, Canada).

Estrus cycle determination

The stage of estrus cycle was determined by cytologic evaluation of vaginal smears as described previously (Hong et al., 2010). Sterile PBS was gently flushed into the vagina using glass pipettes between 9 a.m. and 11 a.m. daily. The lavages were smeared on glass slides and examined microscopically in unstained wet preparations. The following criteria were used for identification of cycle stage: proestrus: nucleated cells; estrus: cornified cells; metestrus: leukocytes and cornified cells; diestrus: leukocytes.
**Collection and preparation of tissues**

Mice were sacrificed by cervical dislocation. Both left and right ovaries from each mouse were collected, washed in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4, 2 mM KH2PO4, pH 7.4) and fixed for 5-15 min in freshly prepared 4% paraformaldehyde (PFA) in PBS. After rinsing in wash buffer (PBS containing 2 mM MgCl2, 0.1% Triton, 0.05% sodium deoxycholate), gonads were stained in the dark overnight at 37°C in wash buffer supplemented with 1 mg/ml X-gal, 0.04% N, N-dimethyl formamide, 5 mM potassium ferricyanide and 5 mM potassium ferrocyanide to disclose β-galactosidase activity (Iglesias et al., 2007). After being stained, gonads were washed in PBS and processed for histology.

**Histology**

X-gal-stained ovaries were post-fixed in 4% PFA overnight at room temperature (RT), rinsed in PBS, embedded in paraffin for sectioning (7-μm thick) and standard counterstaining with hematoxylin and eosin (H & E).

**Quantification of follicles**

Follicles were classified according to the criteria as detailed in Myers et al. (2004)
into three main groups; secondary, early antral and antral follicles (Myers et al., 2004). The total number of follicles was estimated by applying the nucleator and fractionator principle described by Gundersen (Gundersen, 2002). Oocyte nuclear number was equated to follicular number. The number of follicles was estimated by counting oocytes with large visible nuclei. The percentage of lacZ-positive and lacZ-negative oocytes was estimated from the total number of follicles per ovary determined.

**Follicle and oocyte measurements**

Every sixth ovarian section was evaluated for the presence of oocytic follicles using a Leica DM IL microscope equipped with four, ten, twenty, and forty times magnification plan objectives (Leica Microsystems, Wetzlar, Germany). The microscope was fitted with a Hamamatsu ORCA ER digital camera (Hamamatsu photonics, Shizuoka, Japan) driven by MetaMorph software (Universal Imaging Corporation, Downingtown, PA). Captured images of follicles meeting the selection criteria described above for quantification of follicles were saved as tiff formatted images and transferred to Image J (NIH, Bethesda, MD). The accurate calculation of diameters was ensured by using the measuring tools in the Image J software. Additionally, when measuring diameters, two measurements were taken. The second measurement originated at a right angle from the midpoint of the first measurement. The two measurements were averaged and expressed as the
diameter of the structure. Data was collected in this manner to determine the diameters of the follicle and oocyte. Follicular diameters were measured from the basement membrane of the ovarian follicle. The diameter of the oocyte was measured excluding the zona pellucida.

Collection of oocytes

Sexually mature mice were examined daily for estrus. Oviductal tissue including oocytes were collected (n = 14 mice) after detection of estrus and processed for X-gal. Oocytes were collected by flushing the oviduct and examined for β-galactosidase activity.

Hormone stimulation and follicular estimation

Prepubertal (21-26 day-old) mice were injected intraperitoneally (i.p.) with PBS or 5 IU PMSG (pregnant mare's serum gonadotropin) followed 48h later with 5 IU hCG. PMSG and hCG were purchased from the National Hormone & Peptide Program, Torrance (CA 90509, U.S.A.). Ovaries were recovered 48h after treatment with PBS or PMSG and 24h after injection with hCG. In the case of hCG treatment, ovulated oocytes were recovered from the oviducts. Ovaries were fixed, sectioned (7µm), and processed for X-gal staining. Every fifth ovarian
section was evaluated and only follicles in which the oocyte germinal vesicle and nucleolus were visible were considered.

**Statistical analysis**

Data were analyzed using SYSTAT 10.2 statistical software (SYSTAT Software, Richmond, CA). Analysis of variance (ANOVA) was used with Tukey's test in the post hoc analysis for cell counts to ascertain group mean differences. Data are presented as mean ± standard error of the mean. P ≤ 0.05 was considered significant.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Figure 1. β-catenin/Tcf-mediated signaling is activated in growing oocytes during postnatal development. Ovaries were stained for β-galactosidase activity, sectioned and counterstained with H & E. At postnatal day 1 (P1), no β-galactosidase activity is seen in oocytes (A). Blue staining was detected specifically in oocytes from the secondary stage of follicular development at P12 (B), P21 (C) and in cycling mice (D) (black arrowhead). Primordial follicles did not stain for lacZ (white arrowhead, B/C). Only some growing follicles demonstrated staining of granulosa cells (white arrowhead, E). Within the corpus luteum, not all luteal cells stained for lacZ (white arrowhead, F). Fallopian tube β-galactosidase staining served as positive control (G). Scale bar = 50 μm.
Figure 2. The percentage of lacZ-positive is lower than lacZ-negative follicles at all stages of estrous cycle. Mean percentage of lacZ-positive follicles at different stages of estrous cycle. Data presented as means ± sem.
Figure 3. The proportion of lacZ-positive follicles increases with follicular growth and maturation. (a) Average diameter of lacZ-positive and lacZ-negative follicles at all stages of estrous cycle. LacZ-positive are larger in diameter than lacZ-negative follicles (p ≤ 0.05). Data presented as means ± sem. (n=4-5 ovaries).
Figure 3b. The percentage of stained and unstained secondary follicles at all stages of the estrous cycle. A higher proportion (>70%) of secondary follicles did not stain for lacZ throughout estrous. Values are presented as mean ± standard error.
Figure 3c. The percentage of lacZ-positive and lacZ-negative early antral follicles at all stages of the estrous cycle. Majority early antral follicles (>70%) stained for lacZ. Values are the mean ± standard error.
Figure 4. LacZ-positive are larger than lacZ-negative oocytes at all phases of estrus cycle \((p \leq 0.05)\). The average diameter of lacZ-positive and lacZ-negative oocytes at the four stages of estrous cycle. Data presented as means ± sem. \((n=4-5\) ovaries)
Figure 5. Ovulated oocytes do not show β-galactosidase activity and are morphologically normal. (a) Representative light microscopic image of lacZ-negative and lacZ-positive cumulus oocyte complex. LacZ-negative oocytes had an intact cumulus mass representing fully-grown and developmentally normal oocytes.
Figure 5b. Representative light microscopic image of fragmented lacZ-positive oocytes. Note the different levels of oocyte fragmentation.
CHAPTER 5

Conclusion and future direction
In summary, the present studies were conducted to characterize the developmental expression and modulation of \( \beta \)-catenin/Tcf signaling activity within the mouse ovary to further understand the possible roles of canonical WNT signaling in ovary development. Although WNT signaling has been shown to be involved in ovarian development, my research has provided the first evidence to indicate that \( \beta \)-catenin/Tcf signaling plays an important role in the development of the OSE and selection of follicles for ovulation. The following conclusions can be drawn from my studies:

1. **The mouse ovarian surface epithelium is heterogeneous and contains a population of \( \beta \)-catenin/Tcf signaling and non signaling cells.**

\( \beta \)-galactosidase expression (lacZ) identified a cell population that overlies the medio-lateral surface of the indifferent gonad and became sex specific by embryonic day 12.5. The proportion of \( \beta \)-galactosidase positive (presumptive WNT/\( \beta \)-catenin-signaling) OSE cells decreased with age during embryonic development. These results demonstrate that WNT/\( \beta \)-catenin-signaling cells are present early in OSE development. Evaluation of ovarian sections during postnatal growth showed \( \beta \)-galactosidase positive cells in the OSE. Approximately 20% of OSE in new-born ovaries, 8% in 5-day-old but <0.3% in animals 21 –days-old and older were \( \beta \)-galactosidase positive. These age-related changes in staining were confirmed by X-gal staining of intact ovaries and flow
cytometric analyses (FACS) of isolated OSE cells. Apoptosis was undetected in OSE of neonates and β-catenin/Tcf-signaling cells were proliferative in neonatal mice indicating that neither cell death nor proliferation failure was responsible for the proportion alteration. Based on the fraction of β-galactosidase positive cells and an estimate of the number of OSE cells at each of the ages examined, it appears that the WNT/β-catenin-signaling cells gave rise to a replacement, as well as an expanding, population of non-signaling progeny. This was confirmed in cell cultures. The maintenance of a constant number of WNT/β-catenin-signaling cells, accompanied by the increase in appearance of non-signaling cells, suggests that the WNT/β-catenin-signaling cells generate the adult OSE pattern by selective expansion of their non-signaling progeny. Additional FACS analyses revealed that the β-catenin/Tcf-signaling (lacZ+) cell population exhibits cytoprotective mechanisms as indicated by enrichment within a side population. Collectively, my results indicate that the mouse OSE is heterogeneous, and may contain a population of stem/progenitor cells necessary for the generation of the definitive OSE and to allow wound repair of this tissue following ovulation.

2. β-catenin/Tcf-mediated lacZ expression in OSE cells is inhibited downstream of nuclear localization of β-catenin.

Immunohistochemical staining for active β-catenin localized dephosphorylated β-catenin at the plasma membrane of OSE cells. Stimulation of OSE cells with LiCl
or Wnt3A, a Wnt signal activator through GSK-3β inhibition, led to stabilization and nuclear localization of active β-catenin. Unexpectedly, we could not detect reporter activity in OSE cells following treatment. The deprivation of Ca²⁺ failed to induce Tcf-mediated reporter expression even in the presence of Wnt3A. Nonetheless, activation of canonical WNT signaling increased OSE proliferation as measured by BrdU incorporation. Next, I examined the functionality of the canonical WNT pathway in ovarian cancer cell lines. My study showed that ovarian cancer cell lines exhibit distinct aberrations of the WNT signaling pathway which may affect cell proliferation and/or cell adhesion. Together, this study shows that active β-catenin accumulation and nuclear localization is not always indicative of β-catenin/Tcf transcriptional activity in OSE cells.

3. β-catenin/Tcf signaling in oocytes identifies non-ovulatory follicles.

LacZ expression in adult murine ovaries, as indicated by X-gal staining, was only observed in the germinal vesicle of some oocytes and in a small proportion of OSE cells. We focused our attention on β-catenin/Tcf-signaling in oocytes with the objective of determining if this signaling 1) is associated with particular stages of follicular development; 2) is affected by gonadotropin stimulation and 3) if its presence or absence distinguishes follicles selected for ovulation. Primordial and primary follicles did not stain for lacZ. β-catenin/Tcf signaling oocytes were observed in follicles from the secondary stage of development, and their
proportion increased with follicular maturation (secondary follicles: 20%; early antral and antral 70%). In prepubertal hormone-treated mice, lacZ-positive oocytes were observed in approximately equal proportions of large preantral and antral follicles (57 and 43%, respectively). After eCG treatment, more lacZ-positive oocytes were observed in antral follicles (preantral: 32%; antral: 68%) and this was further enhanced after hCG treatment (preantral: 20%; antral 80%). In contrast, the majority (>90%) of the oocytes recovered from the oviducts were lacZ negative. Since the oocyte possesses components for WNT signal transduction, we conclude that this signaling pathway is involved in oocyte maturation and the development of follicle ovulatory capability.

Our results provide new insights into the role of WNT/β-catenin signaling in ovarian development. Although our data suggests that WNT/β-catenin signaling is involved in the development of the OSE and follicular ovulatory capability, we have limited knowledge of the molecular mechanism regulating β-catenin/Tcf-mediated lacZ expression. The following proposed experiments will help in uncovering mechanisms that regulate β-catenin/Tcf-mediated lacZ expression in OSE cells and how the WNT signaling pathway is involved in selecting follicles for ovulation.
1. Elucidate the gene signature expression of the β-catenin/Tcf-signaling cell population by microarray analysis.

In chapter 2, I demonstrated that two distinct types of epithelial cells, β-catenin/Tcf signaling (lacZ+) and non-signaling (lacZ-) cells overlie the mouse ovary. The study also showed that the majority (most likely all) of the cells overlying the differentiating ovary retained lacZ activity and demonstrated an age-dependent decrease to relative constancy in the proportion of stained cells when animals reached maturity. In vitro, lacZ+ cells showed a transient increase in cell number followed by appearance of lacZ- cells suggesting that loss of lacZ expression is due to cellular differentiation. Collectively, the results suggested that lacZ+ OSE cells may be progenitor cells. In order to investigate the transcriptional events behind this stem cell-like state and determine a possible connection between lacZ+ and lacZ- cells, I propose a microarray study on these two cell populations. The goal is to determine whether lacZ+ cells constitute a progenitor cell population and thus provide a map of OSE differentiation. Total RNA will be isolated from lacZ+ cells and lacZ- cells at postnatal day 1 (P1), P10, and P21, and processed for microarray analysis. Significant changes in gene expression will be confirmed by Real Time PCR.
2. How is the β-catenin/Tcf-signaling pathway involved in the selection of follicles for ovulation?

In chapter 4, I showed that activation of the β-catenin/Tcf-signaling pathway occurs only in some oocytes and thereby identifies them as anovulatory. An interesting question is whether an oocyte switches from β-catenin/Tcf-signaling to non-signaling or vice versa before ovulation. To address this, follicles will be isolated from P12-P14 mice (these have a lot of primary/secondary follicles) and cultured in vitro. In order to determine whether an oocyte can switch from being lacZ+ to lacZ- or vise versa, β-catenin/Tcf-signaling activity within the oocyte will be determined on day 1 of culture and subsequently until the oocyte is ovulated. β-catenin/Tcf-signaling activity will be assessed using the fluorogenic substrates Fluorescein di-β-D-galactopyranoside (FDG) for detecting β-galactosidase expression in live cells.

To further address the physiological significance of β-catenin in ovulation, I propose use of the Cre/loxP system of recombination to permit tissue-specific deletion of β-catenin alleles in oocytes. One such method will involve the use of an oocyte specific gene such as Gdf9 or zona pellucida (Zp) genes. Gdf9 mRNA and its protein are not expressed in primordial follicles but are expressed beginning at the early primary follicle stage through ovulation. If oocyte secreted β-catenin regulates β-catenin/Tcf-signaling and follicular ovulatory capability, more oocytes will be ovulated in the knockout mice compared to controls.
CHAPTER 6

References and documentation
6.1. References


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