

Published in final edited form as:

*Dev Biol.* 2013 November 15; 383(2): 295–306. doi:10.1016/j.ydbio.2013.08.026.

## Disruption of mitotic arrest precedes precocious differentiation and transdifferentiation of pregranulosa cells in the perinatal *Wnt4* mutant ovary

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### Abstract

Mammalian sex determination is controlled by antagonistic pathways that are initially co-expressed in the bipotential gonad and subsequently become male- or female-specific. In XY gonads, testis development is initiated by upregulation of *Sox9* by SRY in pre-Sertoli cells. Disruption of either gene leads to complete male-to-female sex reversal. Ovarian development is dependent on canonical Wnt signaling through *Wnt4*, *Rspo1* and  $\beta$ -catenin. However, only a partial female-to-male sex reversal results from disruption of these ovary-promoting genes. In *Wnt4* and *Rspo1* mutants, there is evidence of pregranulosa cell-to-Sertoli cell transdifferentiation near birth, following a severe decline in germ cells. It is currently unclear why primary sex reversal does not occur at the sex-determining stage, but instead occurs near birth in these mutants. Here we show that *Wnt4*-null and *Rspo1*-null pregranulosa cells transition through a differentiated granulosa cell state prior to transdifferentiating towards a Sertoli cell fate. This transition is preceded by a wave of germ cell death that is closely associated with the disruption of pregranulosa cell quiescence. Our results suggest that maintenance of mitotic arrest in pregranulosa cells may preclude upregulation of *Sox9* in cases where female sex-determining genes are disrupted. This may explain the lack of complete sex reversal in such mutants at the sex-determining stage.

### Keywords

sex determination; ovary; *Wnt4*; *Rspo1*; *Sox9*; meiotic germ cells

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## Introduction

Mammalian sex determination is controlled by opposing pathways that guide differentiation of the embryonic gonad towards a testis or ovary fate. The gonad is initially identical in XX and XY embryos and has the potential to follow either a male or female developmental pathway. Primary sex determination within the gonad dictates subsequent sexual differentiation outside of the gonad (secondary sex determination) through hormone production.

In the XY mouse gonad, male fate is determined by the expression of *Sry* from the Y chromosome between 10.5 and 12.5 days post coitum (dpc). *Sry* expression establishes Sertoli cell fate in the supporting cell lineage, shifting the bipotential gonad towards the testis fate (Hacker et al., 1995; Bullejos et al., 2001) by upregulating *Sox9*, a critical component of the male pathway (Sekido et al., 2004; Sekido et al., 2008). Inappropriate upregulation of *Sox9/SOX9* in the XX gonad overrides female differentiation and leads to female-to-male sex reversal (Vidal et al., 2001; Qin et al., 2005), while mutation of murine (Chaboissier et al., 2004; Barrionuevo et al., 2006) or human (Foster et al., 1994; Wagner et al., 1994) *Sox9/SOX9* in XY individuals leads to male-to-female sex reversal. The establishment of *Sox9* expression is followed by a rapid morphological reorganization of the XY gonad: mesonephric endothelial cells migrate into the gonad to form a male-specific coelomic vessel (Martineau et al., 1997; Cool et al., 2008; Coveney et al., 2008; Combes et al., 2009), cells at the coelomic surface undergo rapid proliferation (Schmahl et al., 2000), and *Sox9*-expressing supporting cells cluster around germ cells forming testis cords.

In the absence of a signal to upregulate *Sox9* expression, XX supporting cells upregulate several genes that establish the ovarian fate and differentiate as pregranulosa cells (reviewed in Liu et al., 2010a). Early XX gonads do not undergo obvious morphological changes. Between 14.5 dpc and birth, germ cells, surrounded by mitotically arrested pregranulosa cells, are arranged in clusters termed ovigerous cords. Throughout female reproductive life, pregranulosa cells in quiescent follicles are induced to resume proliferation as small cohorts of follicles are recruited for maturation.

Two transcriptional regulators are critical for maintaining ovarian cell fate:  $\beta$ -catenin and FOXL2.  $\beta$ -catenin activity is controlled by the canonical Wnt signaling pathway, which has been investigated from multiple perspectives including *Wnt4*-deficiency (Vainio et al., 1999), *Rspo1*-deficiency (Chassot et al., 2008b; Tomizuka et al., 2008) and conditional loss of  $\beta$ -catenin specifically in a subset of gonadal somatic cells (Manuylov et al., 2008; Liu et al., 2009). Loss of function of these Wnt pathway components in XX gonads leads to a partial female-to-male sex reversal characterized by ectopic coelomic vessel formation, upregulation of steroidogenic enzymes, and reduced germ cell viability. While  $\beta$ -catenin can directly antagonize SOX9 expression in XY gonads (Maatouk et al., 2008), a loss of canonical Wnt signaling in XX gonadal somatic cells is not sufficient to cause stable upregulation of SOX9 during primary sex determination. Near birth, ovaries lacking either *Rspo1* or *Wnt4* exhibit limited sex reversal marked by the appearance of cord-like structures and the expression of AMH (Vainio et al., 1999; Chassot et al., 2008b) and SOX9 (Chassot et al., 2008b). Curiously, although  $\beta$ -catenin is downstream of *Rspo1* and *Wnt4*, there is no

evidence of sex reversal near birth in ovaries where  $\beta$ -catenin is conditionally disrupted in the majority of somatic cells (Manuylov et al., 2008).

*Foxl2*, another regulator of ovarian differentiation, is expressed in the supporting cells of the ovary upon commitment to the female pathway. Unlike Wnt pathway mutations, loss of *Foxl2* in mice does not have an embryonic phenotype in the gonad; however, one week after birth, granulosa cells begin to transdifferentiate to Sertoli-like cells and upregulate SOX9 (Schmidt et al., 2004; Ottolenghi et al., 2005; Uhlenhaut et al., 2009). This suggests that, similar to  $\beta$ -catenin, *Foxl2* is also critical for maintaining ovarian cell fate by repressing SOX9; however, its dominant role is after birth.

Here we describe a transformation of pregranulosa cell identity that results from a *Wnt/Rspo* deficiency. Building off previous work demonstrating a requirement for *Wnt4* in germ cell survival (Vainio et al., 1999; Yao et al., 2004), we find that the loss of germ cells is non-random and occurs in an anterior-posterior wave across the ovary, mimicking the pattern of meiotic entry observed for ovarian germ cells. This germ cell loss is accompanied by the exit of pregranulosa cells from their normal quiescent state followed by the expression of markers normally associated with proliferative granulosa cells found in growing follicles of the postnatal ovary. Near birth, these cells show evidence of transdifferentiation to a male fate with the onset of SOX9 expression in a subset of cells. Additionally, we show that *Rspo1* mutants undergo a similar stepwise transformation of the pregranulosa lineage and speculate as to why these alterations are not observed in  $\beta$ -catenin mutants or at earlier stages of fetal ovarian development.

## Materials and Methods

### Mouse Strains and Genotyping

*Oct4-Gfp* (Yoshimizu et al., 1999), *Wnt4* (Vainio et al., 1999), *Wnt4<sup>GC/+</sup>* (Maatouk et al., 2012), *Bax* (Baxtm1Sjk/J; (Knudson et al., 1995), *Kit<sup>Wv</sup>* (Little et al., 1937),  *$\beta$ -cat<sup>flox/flox</sup>* (B6.129-Ctnnb1tm2Kem/KnwJ; (Brault et al., 2001) and *Sfl-Cre* (Bingham et al., 2006) mice were maintained on a C57BL/6 genetic background. *Rspo1* mice (Chassot et al., 2008b) were maintained on a mixed 129/C57BL6/J background (4 to 5 back-crosses to C57BL6/J background). The *BRE-LacZ* (*BRE-Hspa1a-lacZ*) transgenic line, in which *LacZ* expression is driven by a series of Smad1/5/8 binding sites from the *Id1* promoter (Blank et al., 2008), was maintained on an outbred CD-1/ICR genetic background. Genotyping was performed on tail samples using previously published primers and standard PCR methods (see references above). For the *Kit<sup>Wv</sup>* mutation, a TaqMan SNP Genotyping Assay (Applied Biosystems) was developed and run on a StepOnePlus thermal cycler (Applied Biosystems) following the supplier's protocol. Primer and probe sequences (5'-3') are as follows: Forward primer GCTACCTGGGCAATCACATGAATAT, Reverse primer TGAGTCTCGAGTTGCCATCTCT, FAM-conjugated probe for the *Kit<sup>Wv</sup>* allele CATGCATGGTGGGAG and VIC-conjugated probe for the *Kit<sup>+</sup>* allele CATGCACGGTGGGAGG.

## Matings and busulfan treatment

*Wnt4* homozygous mutant embryos were generated by timed matings of *Wnt4*<sup>+/-</sup> or *Wnt4*<sup>GC/+</sup> males and females. *Rspo1*<sup>-/-</sup> males are viable and fertile; therefore, *Rspo1*<sup>-/-</sup> embryos were generated by mating homozygous mutant males to *Rspo1*<sup>+/-</sup> females. Attempts to genetically ablate germ cells from *Wnt4* mutant ovaries were unsuccessful (3 of 40 XY embryos were *Wnt4*<sup>-/-</sup>; *Kit*<sup>Wv/Wv</sup> and 0 of 58 XX embryos were *Wnt4*<sup>-/-</sup>; *Kit*<sup>Wv/Wv</sup>, Mendelian inheritance would predict ~3-4 (6.3%) of the XX *Wnt4*<sup>-/-</sup>; *Kit*<sup>Wv/Wv</sup> embryos to be double mutants.); therefore, germ cells were depleted by treatment with busulfan. Timed pregnant *Rspo1*<sup>+/-</sup> and *Wnt4*<sup>+/-</sup> females were intraperitoneally injected with 10 mg of busulfan (Sigma) dissolved in 50% DMSO at 11.5 dpc.

To block germ cell death in *Wnt4* mutant ovaries, *Wnt4*<sup>+/-</sup>, *Bax*<sup>+/-</sup>, and *Oct4-Gfp* mice were bred to generate *Wnt4*<sup>+/-</sup>; *Bax*<sup>+/-</sup>; *Oct4-Gfp* males and females. These offspring were intercrossed, resulting in double homozygous *Wnt4*<sup>-/-</sup>; *Bax*<sup>-/-</sup>; *Oct4-Gfp* embryos.

## Immunocytochemistry and BrdU Assay

Immunostaining and BrdU assays were performed as previously described (Mork et al., 2012). Briefly, following timed matings, gonads were dissected from embryos and fixed for several hours or overnight at 4°C in 4% paraformaldehyde. Samples were embedded in OCT and cryosectioned (18 µm) or whole-mount immunostained with antibodies listed in Table S1. For the BrdU incorporation assay, timed pregnant females were injected intraperitoneally with 1.5 mg BrdU (Sigma) at 18.5 dpc, and embryos collected 4-6 hours later.

## Results

### Germ cell death in *Wnt4* mutant embryos occurs in an anterior-posterior wave

At 13.5 dpc, influenced by signals from the surrounding somatic cells, proliferative germ cells within the testis enter mitotic arrest while those in the ovary initiate meiosis. Progression into meiosis is non-uniform with germ cells at the anterior end of the ovary entering meiosis first, followed by a wave of meiotic entry in the posterior direction (Menke et al., 2003; Yao et al., 2003; Bullejos et al., 2004). In the ovary, meiotic germ cells undergo several waves of apoptosis that are considered a normal part of ovarian development (Rucker et al., 2000; McClellan et al., 2003). However, germ cell apoptosis is increased in ovaries homozygous for a null allele of *Wnt4*, where at 16.5 dpc, apoptotic germ cells are found throughout the ovary, leaving approximately 10% of the original germ cell population remaining at birth (Vainio et al., 1999; Yao et al., 2004).

Intriguingly, we found that the loss of germ cells that occurs in response to disruption of *Wnt4* progressed from the anterior to the posterior end of the ovary. At 15.5 dpc, the number of germ cells at the anterior end of *Wnt4*<sup>-/-</sup> ovaries was reduced compared to the anterior end of control gonads (Fig. 1A, D). Within one day, the area occupied by germ cells encompassed barely half of the ovary (Fig. 1B, E) and near birth only a small population of germ cells survived, clustered at the posterior end of the ovary (Fig. 1C, F). This orderly wave of germ cell loss was consistently observed using three independent markers of germ

cells; the cell surface markers PECAM1 and CDH1, expressed by both XX and XY germ cells, and a marker of the meiotic synaptonemal complex, SCP3 (Fig. 1 and Fig. S1).

Because the decision to enter meiosis versus mitotic arrest depends on the somatic environment, we investigated whether a subset of germ cells in XX *Wnt4* mutants (exhibiting partial sex reversal) differentiated as male germ cells and entered mitotic arrest. However, we found no evidence of germ cell sex reversal. Co-labeling of germ cells with PECAM1 and SCP3 did not reveal a population of SCP3-negative germ cells, and DNMT3L, a marker of mitotically arrested male germ cells, was not expressed in *Wnt4*-null ovaries (Fig. S1). These findings uncovered an interesting pattern of germ cell loss that leaves a small cohort of surviving meiotic germ cells at the posterior end of the ovary at birth.

### **Pregranulosa cell quiescence is disrupted specifically in agametic regions of *Wnt4*-null ovaries**

Contrary to the Sertoli cell lineage, which proliferates throughout fetal development, the pregranulosa cell lineage is mitotically arrested and remains so until after birth when primordial follicles are selected for maturation and granulosa cells differentiate and resume proliferation (Hirshfield, 1992; Mork et al., 2012). We previously showed that mitotically arrested pregranulosa cells express an inhibitor of the cell cycle, p27 (*Cdkn1b*), at all fetal stages (Hirshfield, 1992; Mork et al., 2012). Loss of *Wnt4* had no effect on the initial entry of pregranulosa cells into mitotic arrest; however, coincident with the loss of germ cells beginning at 15.5 dpc, we observed downregulation of p27 in a small population of FOXL2-expressing pregranulosa cells at the anterior end of the ovary (Fig. 2A, B). Similar to the wave of germ cell loss, loss of p27 expression progressed along the anterior-posterior axis (Fig. 2C, D). Near birth, only pregranulosa cells residing adjacent to surviving meiotic germ cells retained expression of the mitotic arrest marker.

It was previously reported that near birth, *Wnt4*-null ovaries begin showing evidence of sex reversal, expressing markers of Sertoli cells such as AMH and showing morphological structures reminiscent of testis cords (Vainio et al., 1999). To localize cells that might be sex-reversing with respect to the anterior-posterior disruption of the ovarian program, we co-labeled 17.5 dpc ovaries with FOXL2 and AMH. As previously observed, AMH was ectopically expressed in *Wnt4* mutant ovaries, but surprisingly, was restricted to the anterior half of the ovary which had undergone a concomitant loss of both germ cells and somatic expression of p27 (Fig. 2E, F). AMH-expressing cells were not observed at the posterior end of the ovary, where surviving meiotic germ cells resided (Fig. 2G, H).

In vitro and in vivo experiments showed that exposure of fetal ovaries to AMH leads to a loss of germ cells (Vigier et al., 1987; Behringer et al., 1990) followed by transdifferentiation of granulosa cells into Sertoli-like cells at postnatal stages (Behringer et al., 1990). To determine whether the wave of germ cell death was caused by the ectopic expression of AMH, we examined the onset of AMH expression. In 15.5 dpc *Wnt4*<sup>-/-</sup> ovaries, when germ cells are first lost from the anterior end of the gonad, we detected no AMH expression (Fig. S1C-D) excluding AMH as the cause of germ cell death.

The appearance of AMH/FOXL2 double-positive cells overlapped the domain where the mitotic arrest marker, p27, was lost. This suggested that the AMH/FOXL2 double-positive cells may resume proliferation. To test this, pregnant females were injected with the synthetic thymidine analog BrdU several hours before embryo collection to identify cells undergoing active DNA replication. Co-labeling for BrdU and AMH revealed BrdU/AMH double-positive cells, confirming that proliferation had resumed in a subset of pregranulosa cells (Fig. 2H, inset). Curiously, the AMH-expressing cells continued expressing the pregranulosa cell marker FOXL2 (Fig. 2F, inset), challenging the prior assertion that AMH expression in the perinatal *Wnt4*-null ovary is an indicator of sex reversal (Vainio et al., 1999).

### **Wnt4-null pregranulosa cells resemble differentiating granulosa cells rather than Sertoli cells**

AMH is expressed throughout the embryonic stages of testis development but is not expressed in Sertoli cells of adult testes (reviewed in Behringer et al., 1994). In females, AMH is not expressed during embryonic stages of ovarian development; however, after birth, AMH is expressed in differentiating granulosa cells of growing follicles from the primary to early antral stages (Ueno et al., 1989a; Ueno et al., 1989b). In *Wnt4*-null ovaries, pregranulosa cells continue to express FOXL2, suggesting that their female fate is maintained (Fig. 2F). The aberrant proliferation that occurs concomitantly with the expression of AMH could indicate that in the absence of *Wnt4*, pregranulosa cells are precociously differentiating as granulosa cells of growing follicles, rather than transdifferentiating to Sertoli-like cells. To test this possibility, we investigated additional markers of granulosa and Sertoli cells.

The transcription factor DMRT1 is transiently expressed in male and female gonads around the sex-determining stage, but is downregulated in females shortly thereafter and remains on in Sertoli cells (Raymond et al., 1999; Raymond et al., 2000). In the 18.5 dpc testis, DMRT1 and AMH were co-expressed in Sertoli cells; however, in *Wnt4*-null ovaries, AMH-expressing cells were negative for DMRT1 (Fig. 3A, B).

Bmp signaling plays an important role during multiple stages of ovarian development (reviewed in Knight et al., 2006). In the postnatal ovary, *Bmp15* in germ cells and *Bmp7* in theca cells regulate follicle progression. Earlier in development, around the stage of sex determination, *Bmp2* is specifically expressed immediately under the coelomic surface of the ovary, and its expression is dependent upon *Wnt4* (Yao et al., 2004). Consistent with the expression of *Bmp2* in the early gonad, we found that a *LacZ* reporter for SMAD1/5/8-dependent BMP signaling (*BRE-LacZ*; (Blank et al., 2008) was expressed in somatic cells of the ovary, including FOXL2-positive pregranulosa cells, and was restricted to granulosa cells of all follicle stages after birth (Fig. S2 and S3). In the absence of *Wnt4*, at 18.5 dpc we detected *BRE-LacZ* expression in germ cell-depleted regions at the anterior end of the ovary, specifically in AMH-expressing cells (Fig. 3D).

Based on these additional markers, *Wnt4*-null pregranulosa cells undergo a transformation that resembles the transition from quiescent to differentiating granulosa cells in postnatal growing follicles. This precocious differentiation involves escape from mitotic arrest,



resumption of proliferation, and ectopic expression of AMH. Considering the continued expression of granulosa cell markers, *FOXL2* and *BRE-LacZ*, the lack of expression of the Sertoli marker *DMRT1*, and the rare occurrence of *SOX9*-expressing cells (discussed below), our results suggest that the loss of *Wnt4* leads to the precocious differentiation of pregranulosa cells.

### **Germ cell death is not a prerequisite for precocious differentiation of pregranulosa cells in *Wnt4* mutants**

In *Wnt4*-null mutants, *Wnt4* is never expressed, yet female somatic cells initially enter mitotic arrest and activate *Foxl2*, similar to wild type XX gonads. This suggests that *Wnt4* is not the primary or sole regulator of pregranulosa cell mitotic arrest. Near birth, we found that pregranulosa cells at the anterior end of the *Wnt4*-null ovary, which lacks germ cells, resemble differentiating granulosa cells, while those in the posterior end, adjacent to surviving meiotic germ cells, remain undifferentiated. The spatial correlation between regions of germ cell loss and disruption of pregranulosa cells quiescence led us to investigate whether the presence or absence of germ cells influenced the pregranulosa cell cycle state. To test this hypothesis, we produced *Wnt4* mutants in which germ cell death was rescued or accelerated.

Germ cell survival is regulated by a balance between *Bax* (pro-apoptotic) and *Bcl-x* (anti-apoptotic) activity (Rucker et al., 2000). Homozygous deletion of *Bax* results in an increased number of germ cells in wild type gonads and rescues germ cell death when germ cells migrate to ectopic locations (Stallock et al., 2003) or in mutants where germ cell survival is compromised (Suzuki et al., 2008; Cook et al., 2009). To rescue germ cells in the *Wnt4* mutant, we generated *Wnt4*<sup>-/-</sup>; *Bax*<sup>-/-</sup> double mutant embryos. In *Wnt4*<sup>-/-</sup>; *Bax*<sup>-/-</sup> double mutant gonads, germ cells were found throughout the ovary and were no longer limited to the posterior end, as in single *Wnt4*<sup>-/-</sup> mutants (Fig. 4). However, pregranulosa cells at the anterior end of the double mutant ovary still ectopically expressed AMH, similar to *Wnt4*<sup>-/-</sup> single mutants (Fig. 4C-D). Therefore, the presence of germ cells did not prevent aberrant differentiation of pregranulosa cells, suggesting that the loss of germ cells is not responsible for the precocious differentiation phenotype in *Wnt4* mutants.

To examine the consequence of ablating all germ cells from *Wnt4*-null ovaries, we exposed developing embryos to the chemotherapeutic drug busulfan, previously used to deplete germ cells from mid-gestation embryos (Merchant, 1975; Maatouk et al., 2012). Pregnant dams were treated with busulfan at 11.5 dpc to deplete germ cells shortly after they enter the gonads. Although germ cells were absent in busulfan-treated gonads, based on staining with the germ cell marker *CDH1* at 18.5 dpc (Fig. 4E-H), mitotic arrest of granulosa cells was not disrupted in control or *Wnt4*-mutant ovaries (data not shown and Fig. 6G). Furthermore, pregranulosa cells in *Wnt4*-null ovaries lacking germ cells did not activate AMH (Fig. 4E-H). The data suggests that the aberrant differentiation of pregranulosa cells in the *Wnt4* mutant depends upon prior contact with germ cells; in the absence of pre-meiotic germ cells, pregranulosa cells remain in their quiescent state.

### Pregranulosa cells lacking *Wnt4* transdifferentiate toward a Sertoli cell fate after passing through a transient differentiated granulosa cell state

There has been no case of complete female-to-male sex reversal upon mutation of any female gene at the sex-determining stage and the only mutations that disrupt initial female sex determination include members of the canonical Wnt signaling pathway. These mutations do not initially lead to de-repression of SOX9 in female supporting cells; however, near birth in *Rspo1*<sup>-/-</sup> single mutants as well as *Foxl2*<sup>-/-</sup>; *Wnt4*<sup>-/-</sup> double mutant ovaries, SOX9 expression is detected (Ottolenghi et al., 2007; Chassot et al., 2008b).

In contrast to wild type ovaries, SOX9-expressing cells were detected at perinatal stages in *Wnt4* mutants (Fig. 5). SOX9-expressing cells were variable in number and individual SOX9-expressing cells were often identified in close proximity to FOXL2-expressing cells (Fig. 5B-C). While most ovaries had only a few SOX9-expressing cells, some exhibited larger areas of SOX9 expression. Occasional FOXL2/SOX9 double-positive cells were detected (Fig. 5C), likely representing a transitional state and suggesting that SOX9-positive cells were derived from the FOXL2-expressing pregranulosa cells, consistent with other reports of transdifferentiation between the granulosa and Sertoli fates (Uhlenhaut et al., 2009; Matson et al., 2011). Although SOX9-positive cells were present, AMH-expressing cells were always found in excess of cells expressing SOX9 (Fig. 5D-F). These results suggest that transdifferentiation of the pregranulosa cell lineage occurs only after the cells transition through a differentiated granulosa cell state;.

### Gonads lacking R-spondin1 resemble *Wnt4* mutants near birth

The phenotype of *Rspo1* mutants is similar to *Wnt4* mutants (Chassot et al., 2008b), therefore, we investigated whether the phenotype of *Rspo1* mutants follows a similar pattern of pregranulosa cell activation prior to transdifferentiation that is also dependent upon the presence of germ cells. Co-immunolabeling with AMH and SOX9 revealed some AMH/SOX9 double-positive somatic cells arranged into cord structures in *Rspo1*<sup>-/-</sup> ovaries, as previously described (Fig. 6A-B and (Chassot et al., 2008b). However, we observed a far greater number of AMH-expressing cells throughout the *Rspo1*<sup>-/-</sup> ovaries, similar to the *Wnt4* mutant. In postnatal *Rspo1* mutants (14 dpp), the phenotype progressed such that few primordial follicles or FOXL2-positive clusters were present and SOX9/AMH-expressing cells occupied the bulk of the ovary (Fig. S4).

Consistent with previous observations, 17.5 dpc *Rspo1*<sup>-/-</sup> ovaries had much higher numbers of germ cells compared to *Wnt4*<sup>-/-</sup> ovaries, and germ cells were distributed throughout the ovary (i.e., not posteriorly restricted as in *Wnt4*-null ovaries) (Fig. 6C-D). Nonetheless, AMH was expressed in the ovary with an anterior bias, similar to *Wnt4*<sup>-/-</sup> ovaries (Fig. 6B, D, F). The increased number of germ cells in *Rspo1*<sup>-/-</sup> ovaries mimics the situation created by *Bax*-mediated germ cell rescue of the *Wnt4* mutant (Fig. 4), further supporting our conclusion that the loss of germ cells is not a prerequisite for aberrant differentiation of pregranulosa cells in either the *Wnt4* or *Rspo1* mutants.

Depletion of germ cells from *Wnt4*-null ovaries blocked aberrant differentiation of pregranulosa cells (Fig. 4). To determine whether pregranulosa cells lacking *Rspo1* behaved



similarly, we treated *Rspo1* mice with busulfan and examined pregranulosa cells for AMH expression. Immunostaining for CDH1 revealed a clear reduction in germ cells between control and busulfan-treated samples (Fig. 6E-H). Following busulfan treatment, fewer AMH-positive cells were observed in *Rspo1*<sup>-/-</sup> ovaries compared to busulfan-treated controls (Fig. 6G, H), consistent with *Wnt4* mutants. These results suggest that, similar to the *Wnt4* mutant, *Rspo1* deficient pregranulosa cells resemble differentiating granulosa cells of the postnatal ovary prior to exhibiting signs of transdifferentiation and this transition is dependent on the presence of germ cells.

## Discussion

Beginning with the generation of the *Wnt4* mutation over 10 years ago (Vainio et al., 1999), canonical Wnt signaling has emerged as the predominant pathway controlling ovarian development (Chassot et al., 2008a; Tevosian et al., 2008; Liu et al., 2010a). While loss of several sex-determining genes in the XY gonad, including *Sry*, *Sox9*, or *Fgf9*, leads to male-to-female sex reversal, no single gene mutation leads to primary female-to-male sex reversal in the XX gonad (reviewed in Eggers et al., 2012). *Wnt4*-null embryos undergo partial sex reversal near the sex-determining stage (Vainio et al., 1999), but evidence of Sertoli differentiation (e.g., SOX9 expression) only occurs in sporadic cells near birth (Fig. 5). It is possible that expression of SOX9 requires an additional activating signal (such as SRY) not present in XX gonads at early stages. However, the fact that SOX9 is activated near birth in *Wnt4* and *Rspo1*-null ovaries argues against this explanation. Alternatively, additional mechanisms may function independently of Wnt signaling to repress expression of SOX9 in XX supporting cells. One possibility is that cells are only competent to activate SOX9 when proliferative; cell fate determination and cell cycle regulation may be interdependent in bipotential supporting cells.

### Loss of *Wnt4* disrupts ovarian development asymmetrically along the anterior-posterior axis

The expression of *Wnt4*, by *in situ* hybridization or expression of a *Wnt4*-GFP reporter, shows no anterior-posterior bias (Vainio et al., 1999; Kim et al., 2006b; Maatouk et al., 2012). Therefore, the progressive disruption of pregranulosa cell quiescence and germ cell survival along the anterior-posterior axis in XX *Wnt4* mutants was a surprising finding.

It is well established that entry of germ cells into meiosis occurs in an anterior-posterior wave (Menke et al., 2003; Yao et al., 2003; Bullejos et al., 2004), possibly due to the delivery of the meiosis-inducing factor through the mesonephric tubules that attach to the anterior ovary (Bowles et al., 2006; Kumar et al., 2011). However, this factor is unaffected by loss of Wnt signaling, as evidenced by the widespread expression of SCP3 observed in *Wnt4* mutants (Fig. S1), *Rspo1* mutants (Fig. 6B) and in p-catenin conditional mutants (Liu et al., 2010b).

A few cases of somatic cell-specific genes expressed in a wave across the ovary also have been reported. *Sprr2d* is transiently expressed in both sexes and is downregulated from anterior-to-posterior in females (Hyunjoo et al., 2009). Additionally, *adamts19* expression is initiated in an anterior-to-posterior direction (Menke et al., 2002) and was found to be

downregulated in XX *Wnt4* mutants (Naillat et al., 2010). These observations suggest that regional differences exist in a gradient between the anterior and posterior ends of the ovary. Although *Wnt4* may disrupt pathways that promote ovarian differentiation specifically at the anterior end of the ovary, it is likely that over time the posterior end of the ovary would be progressively affected. Because of neonatal lethality, we could not analyze *Wnt4* mutants at later stages to make this determination; however, this appears to be the case in *Rspo1* mutants (Fig. S4).

### **Wnt4 is not required to initiate pregranulosa cell quiescence**

*Wnt4* transcripts are detected at 10.5 dpc (Nef et al., 2005), and are upregulated in pregranulosa cells by 12.5 dpc (Bouma et al., 2010; Jameson et al., 2012b). In the absence of *Wnt4*, the appearance of aberrantly differentiating pregranulosa cells is delayed with respect to the timing of *Wnt4* upregulation in XX gonads. If *Wnt4* was a direct regulator of pregranulosa cell mitotic arrest, an early disruption of mitotic arrest would be expected. The finding that pregranulosa cells prior to 15.5 dpc maintained expression of p27, as did a small population of cells at the posterior end of the ovary near birth, suggests that *Wnt4* is not directly responsible for initiating or maintaining pregranulosa cell quiescence. Failure to maintain mitotic arrest is likely a secondary consequence of other changes occurring in response to loss of *Wnt4* and, based on our data, may be related to disrupted interactions between germ cells and pregranulosa cells around the time of meiotic entry.

### **Meiotic germ cells may influence cell cycle regulation in pregranulosa cells**

The observation that germ cell loss in *Wnt4* mutants progresses in an anterior-posterior direction, in a manner resembling the progression of germ cell entry into meiosis, and the finding that mitotically-arrested pregranulosa cells were found in close proximity to surviving meiotic germ cells at the posterior end of the ovary, led us to question whether meiotic germ cells maintain mitotic arrest of pregranulosa cells.

In the XX *Wnt4* mutant, pockets of precociously differentiating pregranulosa cells were found in agametic regions at the anterior end of the ovary and near birth a subset of these cells began to transdifferentiate towards a Sertoli fate. Several previous studies have reported granulosa cell transdifferentiation associated with a prior loss of germ cells. Germ cell loss precedes transdifferentiation in the ER $\alpha$  KO and ArKO mouse models (Couse et al., 1999; Dupont et al., 2000; Britt et al., 2002), as well as in vivo and in vitro models of AMH overexpression (Vigier et al., 1987; Behringer et al., 1990), all of which undergo postnatal transdifferentiation. In  $\gamma$ -irradiated postnatal rat ovaries, primordial follicles lost germ cells and pregranulosa cells continued to differentiate; however, once granulosa cells began dividing and expressed AMH, transdifferentiation occurred and morphological characteristics of testis cords were observed (Guigon et al., 2005). The chronology of these transitions resembles the phenotype in the *Wnt4* mutant, but not all cases of transdifferentiation depend on a loss of germ cells. For example, in *Foxl2* mutants, granulosa cells begin expressing SOX9 one week after birth in the presence of germ cells (Ottolenghi et al., 2005; Uhlenhaut et al., 2009).

At 13.5 dpc, germ cells initiate meiosis and progress through prophase I before arresting at diplotene around birth (McLaren, 1984). Shortly after meiotic entry, germ cell numbers are reduced in *Wnt4*-null ovaries and, to a lesser extent, *Rspo1*-null ovaries. Using the *Bax* mutation to block germ cell death in *Wnt4* mutants, we found that pregranulosa cells became activated even in the presence of near-normal numbers of germ cells, suggesting that the absence of meiotic germ cells was not the cause of the phenotype. However, when pre-meiotic germ cells were depleted by busulfan, aberrant differentiation was blocked. While we cannot exclude the possibility that busulfan treatment disrupted pregranulosa cells in a manner that blocked their ability to resume proliferation, we favor the interpretation that the differentiation of XX germ cells around the stage of meiotic entry triggers a maturation step in pregranulosa cells, rendering them competent for activation and transdifferentiation. This concept has been proposed previously based on the effect of germ cell loss on ovarian development in various models that exhibit germ cell depletion (Guigon et al., 2006). It is also possible that progression through meiosis is disrupted in *Wnt4* mutants even though SCP3 expression and localization appear unaffected (Fig. S1). Other components of the meiotic machinery may be affected, or the timing of meiotic progression may be altered. Alternatively, it is possible that prenatal mutant germ cells begin to express markers of genes normally restricted to postnatal oogonia, such as *Gdf9* or *Bmp15* (Knight et al., 2006) that could trigger pregranulosa cells to undergo activation. After birth, communication between germ cells and granulosa cells is critical for folliculogenesis (reviewed in Matzuk et al., 2002). Such an interaction has not been defined during fetal stages, but disrupted adhesion between germ cells and somatic cells in *Wnt4* mutants has been observed (Naillat et al., 2010). Our results suggest that germ cell-somatic cell interactions at prenatal meiotic stages are critical to maintain both pregranulosa cell quiescence and germ cell survival.

### **Precociously differentiating pregranulosa cells are found specifically in *Wnt4*- and *Rspo1*-null mutants and not in conditional $\beta$ -catenin mutants**

While *Wnt4*, *Rspo1* and  $\beta$ -catenin conditional mutants all undergo a similar partial sex reversal near the sex-determining stage and a similar loss of germ cells, discrepancies exist among these mutants near birth. Contrary to *Wnt4*-null and *Rspo1*-null ovaries, loss of  $\beta$ -catenin in gonadal somatic cells causes a loss of FOXL2 expression by 13.5 dpc (Manuylov et al., 2008). Yet, even in the absence of FOXL2, pregranulosa cells maintain expression of p27 (Fig. S5). Furthermore, conditional loss of  $\beta$ -catenin in somatic cells did not lead to precocious differentiation of pregranulosa cells or perinatal sex reversal;  $\beta$ -catenin mutants lack both SOX9 and AMH expression near birth (Fig. S5 and Manuylov et al., 2008).

The lack of a similar perinatal phenotype in  $\beta$ -catenin conditional mutants is curious. In addition to somatic cells,  $\beta$ -catenin signaling is active in germ cells of wild type ovaries as evidenced by the expression of its downstream target, *Axin2*, by localization of an *Axin2-LacZ* reporter (Chassot et al., 2011) and by microarray (Jameson et al., 2012b). While the *Wnt4*- and *Rspo1*-null alleles disrupt canonical Wnt signaling in all cells of the gonad, the *Sfl1-Cre-driven* conditional  $\beta$ -catenin mutation is limited to somatic cells and does not disrupt canonical Wnt signaling in germ cells. It is possible that active  $\beta$ -catenin signaling in germ cells of the  $\beta$ -catenin conditional mutant may maintain normal germ cell-somatic cell interactions, preventing precocious activation of pregranulosa cells.

## Transdifferentiation of *Wnt4*-null pregranulosa cells towards a Sertoli fate occurs after disruption of mitotic arrest

In the bipotential gonad, components of the male and female pathways antagonize one another; *Sox9* and *Fgf9* in males versus *Wnt4*, *Rspo1* and  $\beta$ -catenin in females (reviewed in Kim et al., 2006a). In the testis, it is clear that Wnt-signaling antagonizes SOX9 expression. Stabilization of  $\beta$ -catenin in XY gonadal somatic cells leads to downregulation of SOX9 (Maatouk et al., 2008). Additionally, while XY *Fgf9* mutants are completely sex reversed (Colvin et al., 2001; Kim et al., 2006b), XY gonads that are null for both *Fgf9* and *Wnt4* develop as males (Jameson et al., 2012a). This complete rescue of sex reversal suggests that the primary function of *Fgf9* is to repress *Wnt4* in XY gonads, and in the absence of *Fgf9*, derepression of *Wnt4* leads to the downregulation of SOX9. However, loss of canonical Wnt signaling is not sufficient for SOX9 upregulation in early XX gonads.

The disparate effects of *Wnt4* on *Sox9* expression in the testis versus the ovary may relate to different cofactors or downstream transcriptional regulators in XX versus XY gonads. Another possibility is that supporting cells are not competent to activate SOX9 unless they are actively dividing. In support of this hypothesis, transdifferentiation near birth occurred in regions of the *Wnt4*- and *Rspo1*-null ovaries where pregranulosa cells exited mitotic arrest and resumed cycling. Additionally, most other cases of granulosa cell transdifferentiation occur postnatally when granulosa cells normally resume proliferation (Vigier et al., 1987; Behringer et al., 1990; Couse et al., 1999; Dupont et al., 2000; Britt et al., 2002; Ottolenghi et al., 2005; Uhlenhaut et al., 2009). Indeed, a detailed analysis of transdifferentiation in  $\gamma$ -irradiated rat ovaries showed that transdifferentiation was restricted to granulosa cells in growing follicles, and did not occur in mitotically arrested primordial follicles (Guigon et al., 2005).

We suggest that XX supporting cells may be incapable of upregulating SOX9 while mitotically arrested, even in the absence of its repressor, *Wnt4*. Mitotic arrest may contribute to the specification and maintenance of female supporting cell fate during primary sex determination. Although disruption of p27 expression leads to premature activation of pregranulosa cells in primordial follicles and increased granulosa cell proliferation after birth (Rajareddy et al., 2007), there is no evidence of sex reversal at prenatal or postnatal stages. Therefore, disruption of mitotic arrest alone is not likely to cause sex reversal. However, we predict that disruption of the mitotic arrest program in conjunction with mutations that disrupt *Wnt4*, or other genes responsible for maintenance of the pregranulosa cell fate, may result in a primary female-to-male sex reversal at the sex-determining stage.

Our results suggest that female sex determination is not solely dependent on Wnt signaling. Instead, multiple independent pathways function in the early XX gonad to maintain pregranulosa cell fate. Preservation of mitotic arrest and later signals from meiotic germ cells may cooperate to protect pregranulosa cells from precocious differentiation and sex reversal. This may explain why cases of female-to-male sex reversal have not been observed at the primary sex-determining stage.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

We thank members of the Capel lab for their helpful comments and suggestions and David Zarkower and Dr. Shoji Tajima for kindly sharing the DMRT1 antibody and DNMT3L antibodies, respectively. Funding was provided by the National Institutes of Health (5R01-HD039963-13 to B.C. and F32-HD055791 to D.M.M.) and by Agence Nationale pour la Recherche (ANR-09-GENM-009-03 GENIDOV to M.-C.C.).

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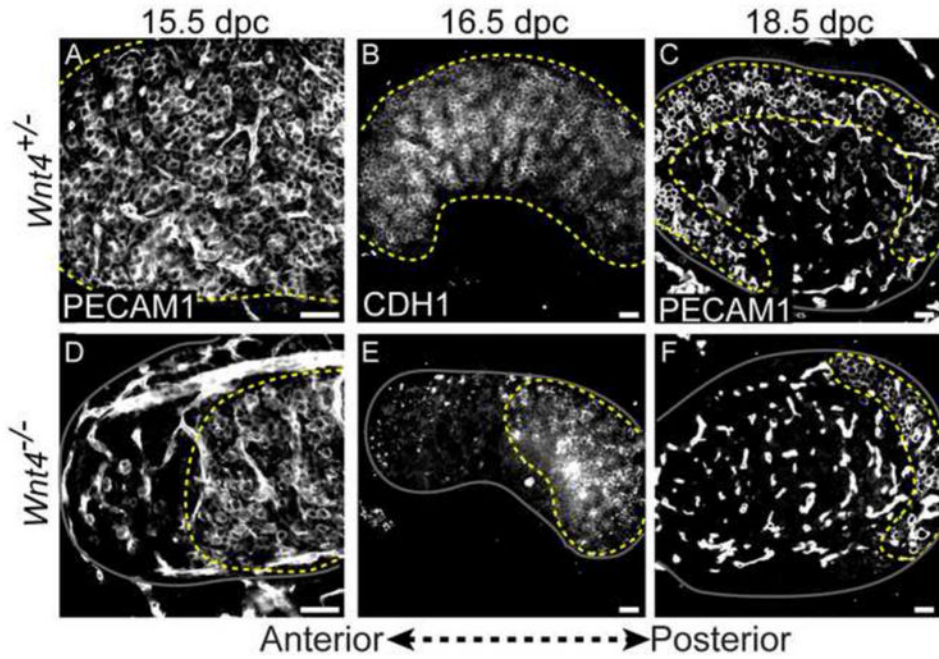
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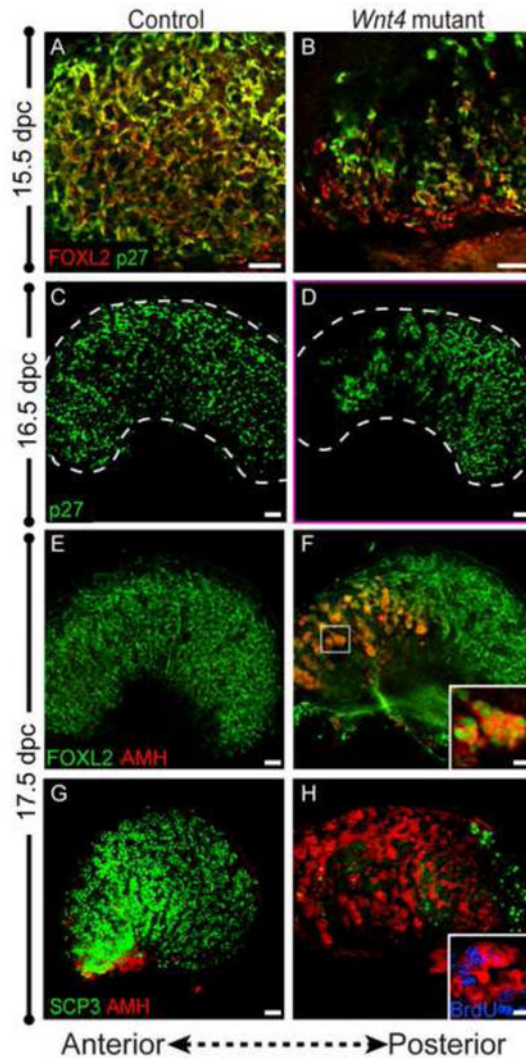
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### Highlights

- Ovary development is progressively disrupted across the A-P axis in *Wnt4* mutants.
- Pregranulosa cells transdifferentiate to Sertoli cells in perinatal *Wnt4* mutants.
- Pregranulosa cells become proliferative prior to transdifferentiating.
- Interactions with meiotic germ cells may trigger pregranulosa cell differentiation.
- Mitotic arrest may prevent transdifferentiation at earlier developmental stages.



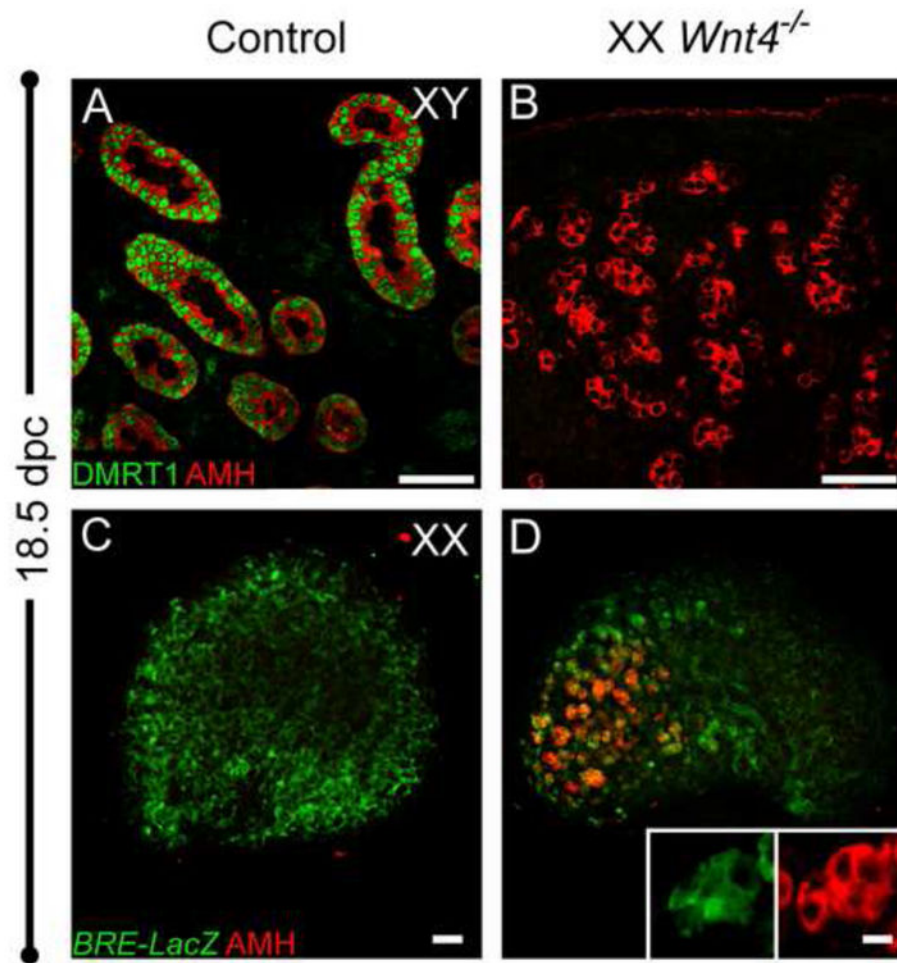
**Figure 1. Germ cell loss proceeds in an anterior-posterior wave in XX *Wnt4*-null ovaries** (A-C) Germ cells in *Wnt4*<sup>+/+</sup> ovaries were distributed throughout the ovary at 15.5-16.5 (A,B) and restricted to the ovarian cortex at 18.5 dpc (C). (D-F) In *Wnt4*<sup>-/-</sup> ovaries, germ cell loss was apparent at the anterior end of gonad at 15.5 dpc (D) and progressed posteriorly across the gonad at 16.5 dpc (E). Only a small population remained clustered at the posterior pole of the ovary at 18.5 dpc (F). Germ cells were labeled with two different markers: PECAM1 (A,C,D,F) or CDH1 (B,E) (PECAM1 labels both germ cells and endothelial cells). The boundary of the ovary is denoted by a solid gray line, and a dotted yellow line marks the region occupied by germ cells. Panels (A) and (D) show the anterior end of the gonad, and panels (E) and (F) are oriented with the anterior end on the left. Whole mount immunostaining was performed on samples in A, B, D and E. Cryosectioning followed by immunostaining was performed for samples in C and F. Scale bars represent 50  $\mu$ m in all panels.



**Figure 2. Pregranulosa cells lose p27 expression and precociously upregulate AMH in *Wnt4*-null ovaries near birth**

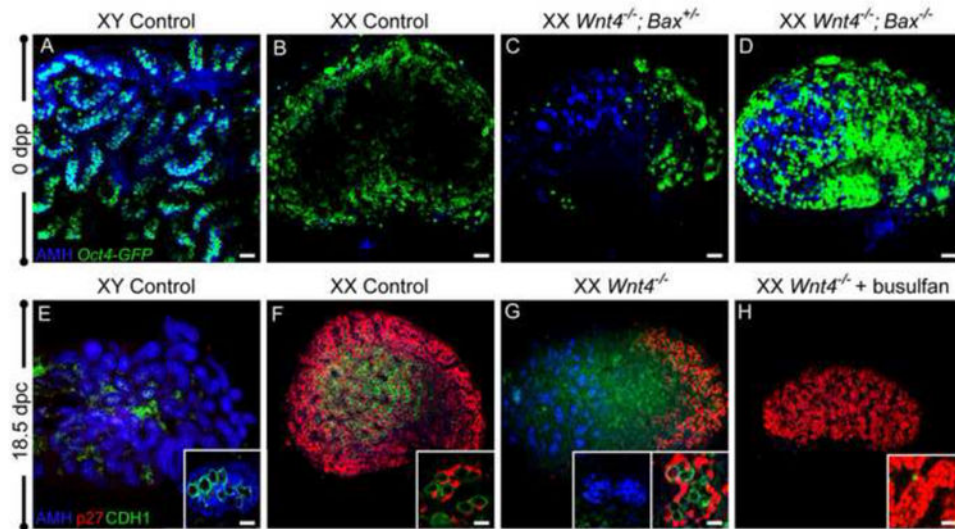
(A,B) At 15.5 dpc, FOXL2 (red) and p27 (green) are co-expressed in pregranulosa cells of control ovaries (A). In *Wnt4* mutants, a small group of pregranulosa cells at the anterior of the ovary downregulate p27 (B) (Panels A and B show the anterior end of the ovary). (C,D) By 16.5, the population of pregranulosa cells that downregulated p27 (green) has expanded in an anterior-posterior direction, and continues to do so until birth (data not shown). The border of the ovary is marked by a white dotted line. (E,F) By 17.5 dpc, FOXL2-expressing pregranulosa cells (green) ectopically express AMH (red) in the anterior end of the ovary (see high magnification of boxed region, F, inset). (G,H) AMH-expressing cells (red) are found throughout the ovary near birth, but are excluded from the posterior end of the ovary occupied by meiotic germ cells (SCP3, green). These AMH-positive cells are actively proliferating and are labeled by BrdU (inset in H; blue). Whole mount immunostaining was performed for all samples. Scale bars represent 50  $\mu\text{m}$  in main panels, and 5.5  $\mu\text{m}$  and 2.75  $\mu\text{m}$  in insets in F and H, respectively.



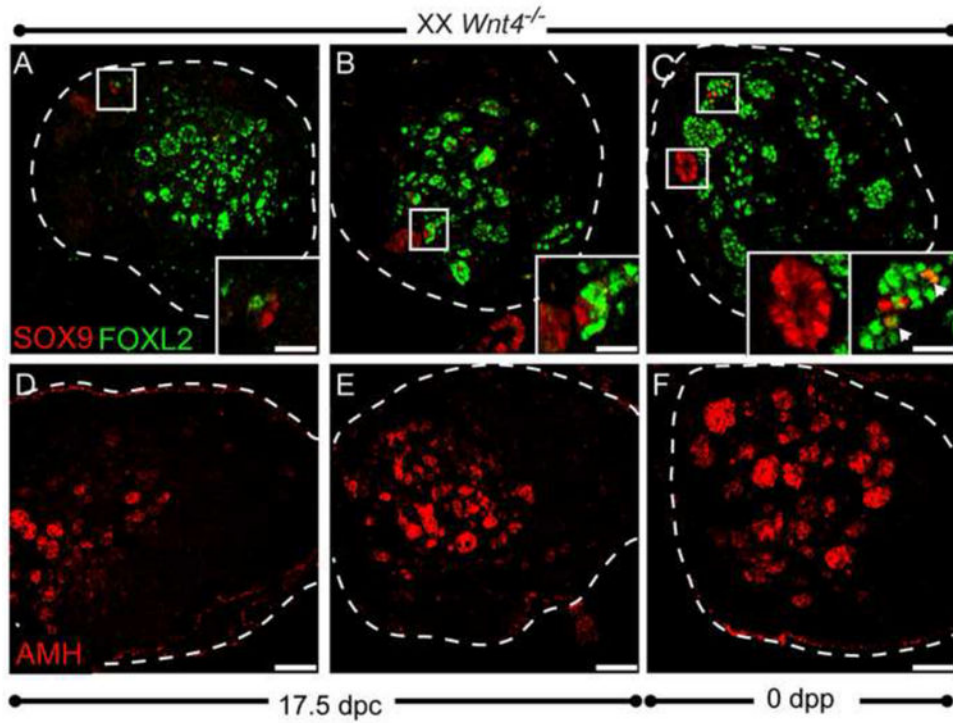


**Figure 3. Cells co-expressing FOXL2 and AMH in XX *Wnt4* mutants resemble activated granulosa cells, not Sertoli cells**

(A) At 18.5 dpc, Sertoli cells express DMRT1 (green) and AMH (red). DMRT1 expression was not detected in the AMH-expressing cells of *Wnt4*<sup>-/-</sup> ovaries (B). (C-D) cytoplasmic *BRE-LacZ* expression was detected by immunostaining for β-galactosidase (green). At 18.5 dpc, *BRE-LacZ* localized to somatic cells, including pregranulosa cells, of control ovaries (C) and is expressed in AMH-positive cells in *Wnt4* mutants (D and Fig. S3). Whole mount immunostaining was performed for all samples. Scale bars represent 50 μm in main panels and 14 μm in insets.

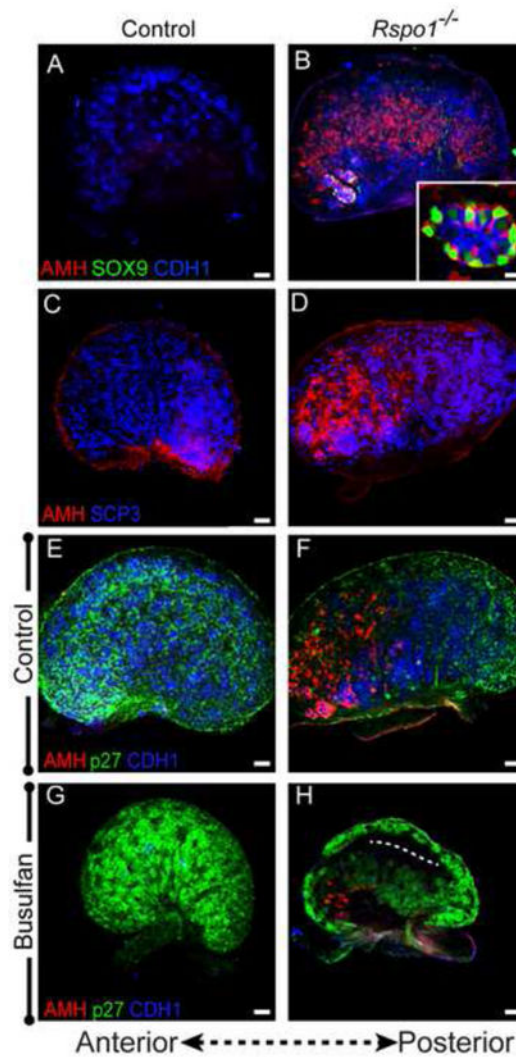


**Figure 4. Meiotic germ cells are required for precocious pregranulosa cell differentiation** (A-D) At birth (0 days post-partum, dpp), gonads were immunostained for AMH (blue), and germ cells were visualized with *Oct4-Gfp* (green) to determine whether rescue of germ cells in agametic ovarian regions blocked AMH activation. AMH is expressed in Sertoli cells (A) and not in control ovaries (B). *XX Wnt4<sup>-/-</sup>; Bax<sup>+/-</sup>* mutants phenocopied *XX Wnt4<sup>-/-</sup>* mutants, with ectopic activation of AMH in agametic regions (C). In double mutant *Wnt4<sup>-/-</sup>; Bax<sup>-/-</sup>* ovaries, rescued germ cells were present throughout the ovary, but did not prevent ectopic activation of AMH at the anterior end of the ovary (D). (E-H) To determine whether the absence of germ cells blocked or exacerbated ectopic AMH expression, germ cells were depleted by injection of busulfan at 11.5 dpc. As expected, male and female control gonads expressed AMH (blue) and p27 (red), respectively (E,F). *Wnt4<sup>-/-</sup>* ovaries had ectopic AMH at the anterior end and p27 expression at the posterior end, surrounding CDH1-positive germ cells (green) (G). Following busulfan treatment, *XX Wnt4<sup>-/-</sup>* ovaries did not ectopically activate AMH, and all pregranulosa cells, at both the anterior and posterior ends, maintained expression of p27 (H). Whole mount immunostaining was performed for all samples. For the samples in (A) and (E), a small piece of testis was used as a control for whole mount antibody immunostaining. Scale bars represent 50  $\mu\text{m}$  in main panels and 10  $\mu\text{m}$  in all insets.



**Figure 5. Transdifferentiation from a pregranulosa to Sertoli cell fate occurs sporadically in *Wnt4*-null ovaries**

(A-C) Representative perinatal ovaries (17.5 dpc or 0 dpp) were co-immunolabeled for SOX9 (red) and FOXL2 (green). SOX9 was detected in a variable number of cells near birth in *Wnt4* mutants (A-C). Insets in (A) and (B) show individual SOX9-expressing cells adjacent to FOXL2-expressing cells. The left inset in (C) shows a cluster of SOX-expressing cells that no longer express FOXL2, while the right inset shows a cluster of FOXL2-expressing cells with a few cells co-expressing SOX9 and FOXL2 (arrowheads). (D-E) Immunolabeling of representative samples with AMH shows that *Wnt4* mutants have more AMH-expressing cells than SOX9. Samples in B and C carried the *Kit*<sup>Wv/+</sup> mutation, which when heterozygous, reduces germ cell numbers, but does not alter the phenotype of *Wnt4* mutant ovaries (compare A to B). All samples were cryosectioned (18 μm), followed by immunostaining. The edge of the ovary is marked by a white dotted line. Scale bars represent 50 μm in main panels and 16.5 μm in all insets



**Figure 6. Pregranulosa cell activation precedes transdifferentiation in 17.5 dpc *Rspo1*-null ovaries near birth**

(A,B) AMH-expressing cells (red) are found in excess of SOX9-expressing cells (green) in 17.5 dpc *Rspo1*<sup>-/-</sup> mutant ovaries. (C,D) Immunolabeling of germ cells with SCP3 (blue) shows that XX *Rspo1*<sup>-/-</sup> mutants have many meiotic germ cells at 17.5 dpc. Contrary to wild type ovaries, *Rspo1*<sup>-/-</sup> ovaries show anteriorly restricted AMH expression (red). (E,F) p27 expression (green) is downregulated in the anterior end of *Rspo1*<sup>-/-</sup> ovaries, coinciding with regions of AMH upregulation (red). (G,H) Following busulfan treatment at 11.5 dpc, 17.5 dpc *Rspo1*<sup>-/-</sup> ovaries have fewer AMH-expressing cells. The dotted line in (H) marks the location of the ectopic vasculature. Samples were whole mount immunostained and oriented with the anterior end to the left. Scale bars represent 50  $\mu$ m in main panels and 11.5  $\mu$ m in the inset (D).