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WNT4 and RSPO1 together are required for cell proliferation in the early mouse gonad

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SUMMARY

The gonad arises from the thickening of the coelomic epithelium and then commits into the sex determination process. Testis differentiation is activated by the expression of the Y-linked gene *Sry*, which promotes cell proliferation and differentiation of Sertoli cells, the supporting cells of the testis. In absence of *Sry* (XX individuals), activation of WNT/CTNNB1 signalling, via the upregulation of *Rspo1* and *Wnt4*, promotes ovarian differentiation. However, *Rspo1* and *Wnt4* are expressed in the early undifferentiated gonad of both sexes, and *Axin2-lacZ*, a reporter of canonical WNT/CTNNB1 signalling, is expressed in the coelomic region of the E11.5 gonadal primordium, suggesting a role of these factors in early gonadal development. Here, we show that simultaneous ablation of *Rspo1* and *Wnt4* impairs proliferation of the cells of the coelomic epithelium, reducing the number of progenitors of Sertoli cells in XY mutant gonads. As a consequence, in XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ foetuses, this leads to the differentiation of a reduced number of Sertoli cells and the formation of a hypoplastic testis exhibiting few seminiferous tubules. Hence, this study identifies *Rspo1* and *Wnt4* as two new regulators of cell proliferation in the early gonad regardless of its sex, in addition to the specific role of these genes in ovarian differentiation.

KEY WORDS: Gonad, Proliferation, Rspo1, Wnt4, Mouse

INTRODUCTION

In mammals, the gonadal primordium is a unique tissue able to undergo two divergent fates leading to the formation of either a testis or an ovary. In mice, the primordial gonads arise as linear ridges from the ventromedial surface of the mesonephroi between 8 and 16 tail somites (ts) [corresponding to 10.5 and 11.2 day post coitum (E10.5-E11.2) (Karl and Capel, 1998)]. It has been shown that the supporting and interstitial cells derive from the coelomic epithelium, a cell layer covering the coelomic cavity, including the gonadal primordium (DeFalco et al., 2011; Karl and Capel, 1998). The first coelomic cells entering the gonads are precursors of supporting cells and their fate depends on the expression of *Sry*, the sex-determining factor located on the Y chromosome (Karl and Capel, 1998; Koopman et al., 1991; Lovell-Badge and Robertson, 1990). *Sry* expression starts at E10.5 (11-12 ts), peaks at E11.5 (18 ts) and ceases at E12.5 (Bullejos and Koopman, 2001; Hacker et al., 1995; Jeske et al., 1995) in a center-to-poles wave (Hiramatsu et al., 2009). Moreover, *Sry* expression is restricted to the precursors of the supporting cells (Albrecht and Eicher, 2001; Sekido et al., 2004; Wilhelm et al., 2005). One of the earliest consequences of *Sry* expression is a dramatic increase in somatic cell proliferation, resulting in an increase in size of the embryonic testis relative to the embryonic ovary (Schmahl and Capel, 2003). Whereas somatic cell proliferation in XX gonads is constant between 8 and 29 ts, and localized to the coelomic region, in XY gonads, SF1-positive somatic cells of the coelomic region are highly proliferative between 16 and 18 ts (Schmahl et al., 2000).

The molecular signals regulating the early proliferation of the coelomic region are still unknown and are the main issue of the present study. Several genes regulate the expression of *Sry* to reach levels that are required for testis determination (for a review, see Kashimada and Koopman, 2010; Sekido and Lovell-Badge, 2009). So far, no direct effects of *Sry* on cell proliferation have been shown. Indeed, the main function of *Sry* is to activate directly the expression of *Sox9* (Sekido and Lovell-Badge, 2008), a transcription factor required for the differentiation of the supporting cells into Sertoli cells (Barrionuevo et al., 2006; Chaboissier et al., 2004). A crucial threshold number of Sertoli precursors is necessary to initiate testis differentiation (Palmer and Burgoyne, 1991) and mutations affecting the Sertoli cell threshold establishment can trigger sex reversal (Polanco and Koopman, 2007). Sertoli cells produce signalling molecules such as FGF9 (Colvin et al., 2001; Kim et al., 2006), which promotes further coelomic cell proliferation (Bagheri-Fam et al., 2008; Bradford et al., 2009; Kim et al., 2007; Schmahl et al., 2004). These cells will become additional Sertoli cells. Then, SF1-negative cells in this region proliferate between 19 and 25 ts (after 11.5 dpc) to give interstitial cells (Schmahl et al., 2000). Schmahl and Capel (Schmahl and Capel, 2003) have shown that inhibition of the early proliferation (between E10.8 and 11.2) results in smaller testes and reduced numbers of testis cords. From E12.0, the vascularisation of the XY gonads is involved in the formation of the testis cords (Combes et al., 2009; Coveney et al., 2008).

In XX gonads, there is no significant increase in proliferation during early embryogenesis (Schmahl et al., 2000). The simplest explanation is the absence of *Sry*. However, this might alternatively be associated with the female-specific expression of cell-cycle inhibitors (Nef et al., 2005). Ovarian differentiation is induced by activation of the WNT/CTNNB1 canonical signalling pathway (Maatouk et al., 2008) by *Rspo1* and *Wnt4* (Parma et al., 2006; Vainio et al., 1999). In mice, *Wnt4* is expressed in the undifferentiated gonad before becoming upregulated in the XX

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gonad after E11.5 (Jeays-Ward et al., 2004; Kim et al., 2006; Vainio et al., 1999). Similarly, *Rspo1* is specifically upregulated in XX foetal gonads at E11.5 (Nef et al., 2005; Parma et al., 2006). Loss of function of either *Rspo1* or *Wnt4* in XX gonads promotes: (1) ectopic steroidogenic precursors, endothelial cell migration and the formation of a coelomic vessel (Chassot et al., 2008b; Jeays-Ward et al., 2003); and (2) sex reversal of the supporting cell lineages with expression of the Sertoli cell markers, SOX9 or *Dhh*, and the development of ovotestes around birth (Chassot et al., 2008b; Tomizuka et al., 2008; Vainio et al., 1999). WNTs and R-spondins act synergistically by interaction of their respective receptors LRP5/6 and LGR4/5 (Carmon et al., 2011; de Lau et al., 2011; Glinka et al., 2011). R-spondin binding activates WNT/CTNNB1 signalling and can disrupt WNT/planar cell polarity signalling (Hao et al., 2012). How RSPO1 and WNT4 interact in the gonad remains to be elucidated; however, in absence of *Rspo1* and its effector CTNNB1, *Wnt4* upregulation is impaired, indicating that *Rspo1* is required for *Wnt4* upregulation in the ovary after 11.5 dpc (Chassot et al., 2008b; Liu et al., 2009; Manuylov et al., 2008; Tomizuka et al., 2008). WNT/CTNNB1 signalling is required for *Foxl2* upregulation (Manuylov et al., 2008), which in turn promotes follicular differentiation (Ottolenghi et al., 2005; Schmidt et al., 2004) and homeostasis in the ovaries (Uhlenhaut et al., 2009).

In addition to its role in ovarian development, *Wnt4* is also involved in testis differentiation. Indeed, in XY gonads, loss of function of *Wnt4* induces a delay in sex cord formation that is compensated at birth (Jeays-Ward et al., 2004). By contrast, *Rspo1*$^{-/-}$ males are normally fertile and do not exhibit gross gonadal abnormalities (Chassot et al., 2008a).

Here, we show that *Rspo1* is not only required for ovarian differentiation, but also for testis differentiation. The XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ foetuses exhibit hypoplastic testis, resulting from a dramatically reduced number of seminiferous tubules. Cell proliferation in the coelomic region is significantly impaired in XX and more severely in XY *Rspo1 Wnt4* knockout mutants, compared with control gonads. Hence, our findings support a synergic role for *Rspo1* and *Wnt4* in cell proliferation in the early gonad and, as a consequence, this pathway regulates the number of differentiating Sertoli cells and in turn testicular development.

MATERIALS AND METHODS

Mouse strains and genotyping

The experiments described here were carried out in compliance with the relevant institutional and French animal welfare laws; guidelines and policies and were approved by the French ethics committee Comité Institutionnel d'Ethique Pour l'Animal de Laboratoire (number: NCE/2011-12). All mouse lines were kept on a mixed 129/C57BL6/J background. *Rspo1*$^{-/-}$ and *Axin2*$^{+/LacZ}$ transgenic mice have been previously described (Chassot et al., 2008b; Lustig et al., 2002). *Wnt4*$^{+/-}$ mice (Vainio et al., 1999) were mated with *Rspo1*$^{+/-}$ mice and recombination events between both genes was selected by backcross. To obtain *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ embryos, we next mated *Wnt4*$^{+/-}$; *Rspo1*$^{+/-}$ mice together. Embryonic samples were collected from timed matings (day of vaginal plug = E0.5). Genotyping was performed as previously described (Chassot et al., 2008b; Hogan et al., 1994; Stark et al., 1994). Litter mates were used for all comparisons, except those between *Wnt4*$^{-/-}$ and *Wnt4*$^{-/-}$;*Rspo1*$^{-/-}$ embryos, as no litters were obtained containing both of these genotypes. This is because *Wnt4* and *Rspo1* are relatively close on the same chromosome making this recombination a rare event.

Histological analysis

Urogenital organs were dissected, fixed in Bouin's solution overnight and processed to obtain 5 μm paraffin sections. For each genotype, five sections of three different embryos were processed for Haematoxylin and Eosin staining, and quiescent germ cells were analysed with a light microscope, using a 100× objective. The quiescent state was identified by a uniform size of the gonocytes and the absence of any heterochromatin in the nuclei, whereas the proliferative state was characterized by the variation in nuclear size, perinuclear heterochromatin (late S phase and G2 phase), and appearance of chromosome condensation and chromosomal threads (mitosis). Pictures were taken with an Axiocam Mrm camera (Zeiss) and processed with Adobe Photoshop.

X-gal staining and immunological analyses

Samples were fixed with 4% paraformaldehyde overnight or 2 hours and then processed for paraffin embedding or equilibrated in sucrose and embedded in Cryomount (Histolab) for cryosection. Samples for X-Gal staining were processed as described previously (Moore et al., 1998). For each genotype, five cryostat or microtome sections of 8 μm thickness of two different embryos were processed for X-Gal staining or/and immunostaining. The following dilutions of primary antibodies were used: DDX4/MVH (catalogue code 13840, Abcam), 1:200; SOX9 (kindly provided by Michael Wegner, Institute für Biochemie, Erlangen, Germany), 1:1500; AMH/MIS (C-20, catalogue code sc6886, Santa Cruz), 1:200; FOXL2 (kindly provided by Eric Pailhoux, INRA Jouy-en-Josas, France), 1:250; PECAM1 (H300, catalogue code sc8306, Santa Cruz), 1:200; Ki67 (clone SP6, catalogue code 9106, Thermo-Scientific), 1:200; laminin (catalogue code L9393, Sigma), 1:150; phospho-histone H3 (catalogue code ab14955, Abcam), 1:100; SF1 (kindly provided by Ken Morohashi, Kyushu University, Japan), 1:1500, FGFR2 (Bek C17 catalogue code sc122, Santa Cruz) 1:100, SRY [kindly provided by Dagmar Wilhelm (Bradford et al., 2007)], 1:50.

DAPI (blue) was used to detect nuclei. For histology, 5 μm sections of three embryos of each genotype were stained with Hematoxylin and Eosin. Fluorescent studies were performed with a motorized Axio ImagerZ1 microscope (Zeiss) or a Zeiss LSM-510 confocal microscope, and pictures were taken with an Axiocam Mrm camera (Zeiss) and processed with Axiovision LE or LSM Image Browser for confocal pictures.

After immunostaining, SRY-positive cells and total cells (DAPI-positive cells) of the gonad were manually quantified on eight sections from three embryos of each genotype (XY control and XY *Wnt4*$^{-/-}$ *Rspo1*$^{-/-}$), and the percentage of SRY-positive versus total cells was plotted on a graph.

In situ hybridization

In situ hybridization was carried out essentially as described previously (Wilkinson, 1992). *Sox9* riboprobes were synthesized according to Morais da Silva et al. (Morais da Silva et al., 1996), and *P450scc*, *Wnt4* and *Rspo1* riboprobe synthesis was carried out as described previously (Chassot et al., 2008b). *Bmp2* riboprobe was a gift from Richard Behringer (University of Texas M. D. Anderson Cancer Center, Houston, USA).

Quantitative PCR analysis

Individual gonads without mesonephros were dissected in PBS from E11.5 embryos (19 tail somites). RNA was extracted using the RNeasy Qiagen kit, and reverse transcribed using the RNA RT-PCR kit (Stratagene). Primers and probes were designed by the Roche Assay Design Center (https://www.rocheappliedscience.com/sis/rtpcr/upl/adc.jsp): *Hprt1*, 5′-tcctcctcagaccgctttt-3′ and 5′-cctggttcatcatcgctaatc-3′ (probe 95); *Sry*, 5′-agcctcatcggagggcta-3′ and 5′-aggcaactgcaggctgtaaa-3′ (probe 82); *Wt1*, 5′-caccaaaggagacacagaggt-3′ and 5′-ttcacttgtttttacctgtat-3′ (+*KTS* isoform) or 5′-gggcttttcacctgtatgag-3′ (-*KTS* isoform) (probe 47); *Cbx2/M33*, 5′-ggccgaggaaacacacag-3′ and 5′-atttggatggcgcatctg-3′ (probe 88); *Igf1r*, 5′-gagaatttccttcacaattccatc-3′ and 5′-cacttgcatgacgtctctcc-3′ (probe 104); *Sf1*, 5′-gcctgcatcaaggaaaagg-3′ and 5′-aagaggaccagagggaggag-3′ (probe 10); *Gata4*, 5′-actatgggcacagcagctc-3′ and 5′-gggacagcttcagagcagac-3′ (probe 58); *Fog2*, 5′-gcgaagacgtggagttcttt-3′ and 5′-ggctgtccccatctgattc-3′ (probe 92); *Cited2*, 5′-atcgcaaagacggaagga-3′ and 5′-tgctgctggtgatgatgc-3′ (probe 77); *Sox9*, 5′-cagcaagactctgggcaag-3′ and 5′-tccacgaagggtctcttctc-3′ (probe 66); *Fgf9*, 5′-tgcaggactggatttcatttag-3′ and 5′-ccaggcccactgctatactg-3′ (probe 60); *Axin2*, 5′-gcaggagcctcacccttc-3′ and 5′-tgccagtttctttggctctt-3′ (probe 50).

All real-time PCR assays were carried out using the LC-Faststart DNA Master kit Roche. QPCR was performed on cDNA from one gonad and

compared with a standard curve. QPCR were repeated in at least two independent runs. Relative expression levels of each sample were determined in the same run and normalized by measuring the amount of *Hprt1* (total gonadal cells) cDNA. For each genotype, n=12 gonads (six pairs of gonads).

Statistical analysis

QPCR experiments

For each sample, relative expression levels were quantified and normalized. For each genotype (n=12), the mean of these 12 absolute expression levels (i.e. normalized) was calculated and then divided by the mean of the 12 absolute expression levels of the XY samples considered as the reference (=1 when divided by itself), leading to the fold of change. Graphs of QPCR results show fold of change +1 s.e.m.

Proliferation experiments

For each of the four genotypes, four sagittal sections of three embryos were processed for confocal experiments. Immunostaining experiment followed by confocal analysis was performed four times (16 pictures per genotype). For each picture, the total number of cells in the coelomic epithelium (positive for SF1) and the proliferating cells (both SF1 and Ki67- or Phospho-Histone H3-positive cells) localized in the coelomic epithelium delimitated by the laminin staining were manually quantified on the entire section. Then the percentage of proliferating cells versus total cells in the coelomic epithelium was determined. For each genotype (16 pictures), the mean and mean +1 s.e.m. of these percentages were calculated and reported on a graph after statistical analysis.

Statistical analysis

The normalized expression of genes of interest for each pair of gonads for QPCR experiments, and the percentage of proliferating cells versus total cells in the coelomic epithelium in each embryo for the proliferation experiments, were analyzed using Graphpad for statistical relevance. Asterisks highlight the pertinent comparisons and indicate levels of significance: *P<0.05, **P<0.01 and ***P<0.001. Statistical significance was assessed using one-way ANOVA followed by Tukey-Kramer post test for selected pairs of genotypes.

RESULTS

WNT/CTNNB1 signalling is activated in the coelomic region of the gonad at E11.5

As expected for a gene involved in ovarian differentiation, *Rspo1* is robustly expressed in embryonic ovaries. However, in situ hybridization analysis has shown that *Rspo1* is also strongly expressed in the coelomic region of XY gonads at E12.5 (Parma et al., 2006). This prompted us to study the role of *Rspo1* during testis development in more detail. To characterize *Rspo1* and *Wnt4* expression during early gonad differentiation, we performed whole-mount in situ hybridization in embryos at 15 ts (E11.25). *Rspo1* and *Wnt4* were expressed in both XX and XY gonads at this stage (Fig. 1A). These results are consistent with earlier studies reporting that *Wnt4* is initially expressed in both sexes (Vainio et al., 1999).

As RSPO1 and WNT4 are WNT/CTNNB1 activators during gonadal development (Chassot et al., 2008b; Maatouk et al., 2008), we next tested whether CTNNB1 signalling is activated at an early stage of development. As a readout, we have made use of the *Axin2:lacZ* line that is considered to be a universal reporter of nuclear CTNNB1 activity (Lustig et al., 2002). Whole-mount X-Gal blue staining (Fig. 1B) did not show a strong expression of the *Axin2:lacZ* reporter at E10.5, suggesting that either the *Axin2:lacZ* reporter is not yet upregulated or the canonical β-catenin signalling pathway is not activated at this stage. At E11.5, X-Gal staining revealed blue cells in the coelomic region of XX and XY gonads (Fig. 1B), and staining was confirmed in cells of the coelomic region on gonadal sections. Thus, CTNNB1 activation occurs in the coelomic region of the early gonad in both XX and XY gonads. At this stage, XY gonads exhibited high levels of cell proliferation within the coelomic epithelium, which appeared as multiple cell layers. By contrast, the coelomic epithelium of XX gonads was markedly thinner (Schmahl et al., 2000). The stronger blue staining (*Axin2:LacZ*) in XY gonads when compared with XX gonads may reflect the difference in thickness of the coelomic epithelium at this stage of development. These results show that *Rspo1* is expressed in the XY gonad and that the WNT/CTNNB1 signalling pathway is activated in both sexes during early gonadogenesis.

XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ mice exhibit impaired testis development

It has been shown that XY *Wnt4*$^{-/-}$ gonads exhibit a delay in Sertoli cell differentiation and sex cord formation that is compensated at birth (Jeays-Ward et al., 2004). As RSPO1 is an activator of the WNT/CTNNB1 signalling pathway (Binnerts et al., 2007; Carmon et al., 2011; de Lau et al., 2011; Glinka et al., 2011), and *Rspo1* and *Wnt4* are expressed in the early developing gonad, we asked whether RSPO1 activates WNT4 signalling to promote testicular development. At E18.5, the XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ mice exhibited descended hypoplastic testes in comparison with XY controls (Fig.2A). Macroscopic analysis of XY control, *Wnt4*$^{-/-}$, *Rspo1*$^{-/-}$ and *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads at birth showed that XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ foetuses exhibit smaller gonads than the single mutants or controls (Fig. 2B), suggesting that *Rspo1* and *Wnt4* act synergically during testicular development. The size reduction of XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads was variable (see Fig. 5), probably owing to the mixed genetic background.

To address whether ablation of both *Wnt4* and *Rspo1* leads to a general developmental defect, we compared the body size of littermates at birth. *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ double mutants and control littermates were similar in size (Fig. 2C), indicating that the gonadal hypoplasia observed in XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ foetuses is organ specific.

Given that in ovarian differentiation, upregulation of *Wnt4* is initially promoted by *Rspo1* expression (Tomizuka et al., 2008), XX *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads may not exhibit more dramatic phenotypes than the single mutant gonads. As expected, XX *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads were grossly similar to the XX *Rspo1*$^{-/-}$ gonads (data not shown), confirming that *Rspo1* and *Wnt4* act along the same pathway.

Proliferation of the coelomic epithelium is impaired in *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads

Given that RSPO1 can stimulate proliferation (Chassot et al., 2011; Kim et al., 2005) and that proliferation of the coelomic epithelium is crucial for gonadal development (Schmahl and Capel, 2003; Schmahl et al., 2000), we hypothesised that *Rspo1* and *Wnt4* are involved in gonadal cell proliferation. To address whether cell proliferation in the coelomic epithelium is affected by *Rspo1* and *Wnt4* ablation in the gonads, we have performed immunostaining experiments followed by statistical analyses at 12-14 ts and 16-17 ts in three different embryos of the different genotypes: XY and XX *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$, and controls. At this stage, the SF1-positive cells of the coelomic epithelium are actively proliferating and will become Sertoli cells (Schmahl et al., 2000). To quantify proliferation within the coelomic epithelium, we have used antibodies against either KI67 (Fig. 3A,B) or phosphorylated histone H3 (data not shown), as a marker of proliferation, and laminin to delimit the basal membrane of the coelomic epithelium (Fig. 3B). At 12-14 ts (E10.5), the coelomic epithelium is made of

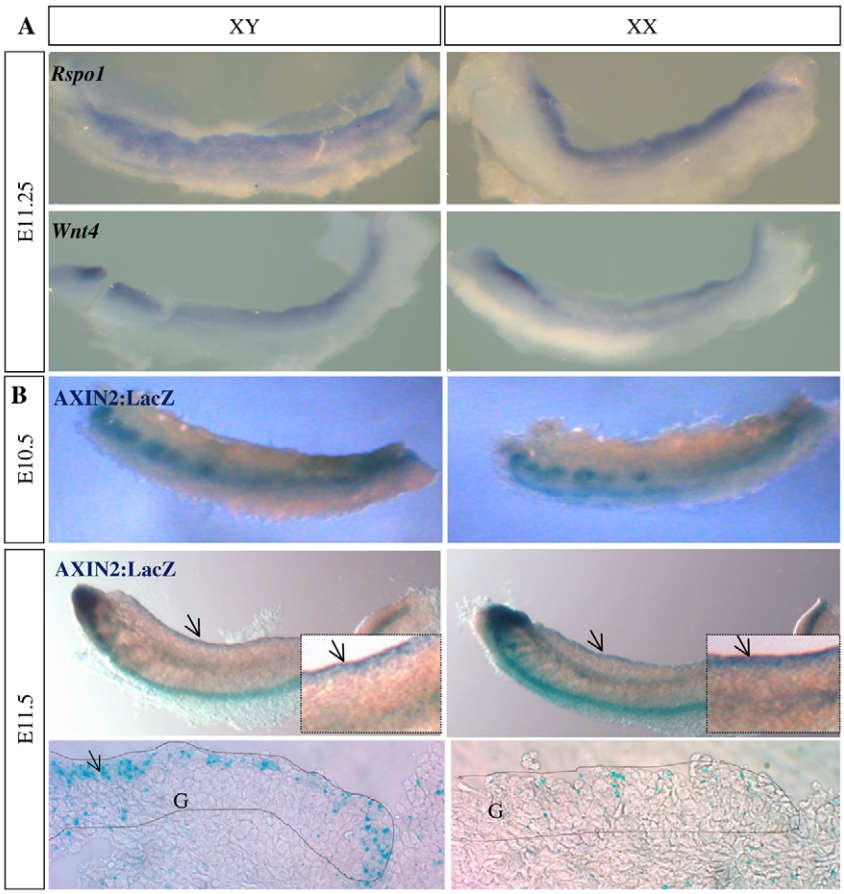

Fig. 1. Canonical CTNNB1 signalling is activated in the coelomic epithelium of the male gonad. (**A**) Upper panels: Rspo1 and Wnt4 whole-mount in situ hybridization E11.25 (16-17 ts) in XX and XY wild-type gonads. (**B**) X-Gal staining (AXIN2) (blue) in XY *Axin2*$^{+/LacZ}$ and XX *Axin2*$^{+/LacZ}$ gonads at E10.5 and E11.5. Arrows indicate coelomic epithelium positive for X-Gal staining. G, gonad.

a single cell layer. Proliferation in this cell layer was reduced in both XX and XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads (15 and 11% of proliferating cells, respectively) when compared with XY and XX control gonads (Fig. 3A) (29 and 26%, respectively). At 16-17 ts (E11.25), proliferation in the coelomic epithelium was dramatically and highly significantly reduced in XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads, and only 42% of cells were positive for proliferation markers compared with 79% in XY controls. This percentage of proliferating cells was even lower than in XX control gonads (62%) (Fig. 3B). Proliferation in the coelomic epithelium of XX *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads was also significantly decreased, i.e. 53% of proliferative cells compared with 62% in XX controls. However, this reduction was less severe than in XY gonads. Although the proliferative activity stays constant in XX gonads (Schmahl et al., 2000), it is amplified as early as 14-15 ts in XY gonads by the action of FGF9, a growth factor secreted by the first differentiated Sertoli cells (Schmahl et al., 2004). This suggests that the greater disparity in proliferation between XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ and control gonads compared with that of the XX gonads is due to an impairment of the male-specific FGF9 effect. Indeed, the level of expression of *Fgf9* was significantly reduced in XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads (Fig. 4C), indicating that the decrease in *Fgf9* expression amplified the defects in proliferation in XY mutant gonads relative to XY controls. Note that we could not analyse the proliferation levels in the *Wnt4*$^{-/-}$ single mutant embryos from the same litter owing to the fact that *Rspo1* and *Wnt4* are located on the same chromosome and recombination was a rare event. Taken together, these results show that RSPO1 and WNT4 synergistically regulate early cell proliferation in the coelomic epithelium in both sexes.

Number of SRY-positive cells number is reduced in XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads

Sry is expressed within pre-Sertoli cells that are derived from the coelomic epithelium (Sekido et al., 2004; Wilhelm et al., 2005). Consequently, the proliferation of the coelomic epithelium is crucial for the production of sufficient pre-Sertoli (SRY-positive cells) and Sertoli cells (SOX9-positive cells). To investigate whether SRY expression was modified in the XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads, we performed immunostaining followed by quantification of the SRY-expressing cells in XY double mutant and control gonads at E11.25 (16±1 ts). Statistical analysis revealed that the number of SRY-expressing cells was significantly decreased to half of the number of SRY-positive cells in the control gonad (Fig. 4A) by quantitative PCR was significantly reduced to around half of the level measured in the XY control gonads (Fig. 4C). To verify whether SRY expression is delayed in the mutant gonad, we next analysed SRY expression at E12.5 (Fig. 4A). Whereas only a few cells still expressed SRY in the control gonad, SRY expression was maintained in the mutant gonad, but the number of SRY-expressing cells never reached the number observed earlier in the control gonads. This suggests that, in addition to the proliferation defects, the gonadal development is partly delayed in XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ embryos.

Moreover, the level of *Axin2* expression in XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads collected at E11.5 (18±1 ts) measured by quantitative PCR was significantly reduced (Fig. 4B), compared with the XY gonad, suggesting that *Wnt4* and *Rspo1* act through the canonical β-catenin signalling pathway during early gonadogenesis. These results imply that *Rspo1* and *Wnt4* together, possibly through canonical β-catenin signalling, stimulate proliferation in the bi-

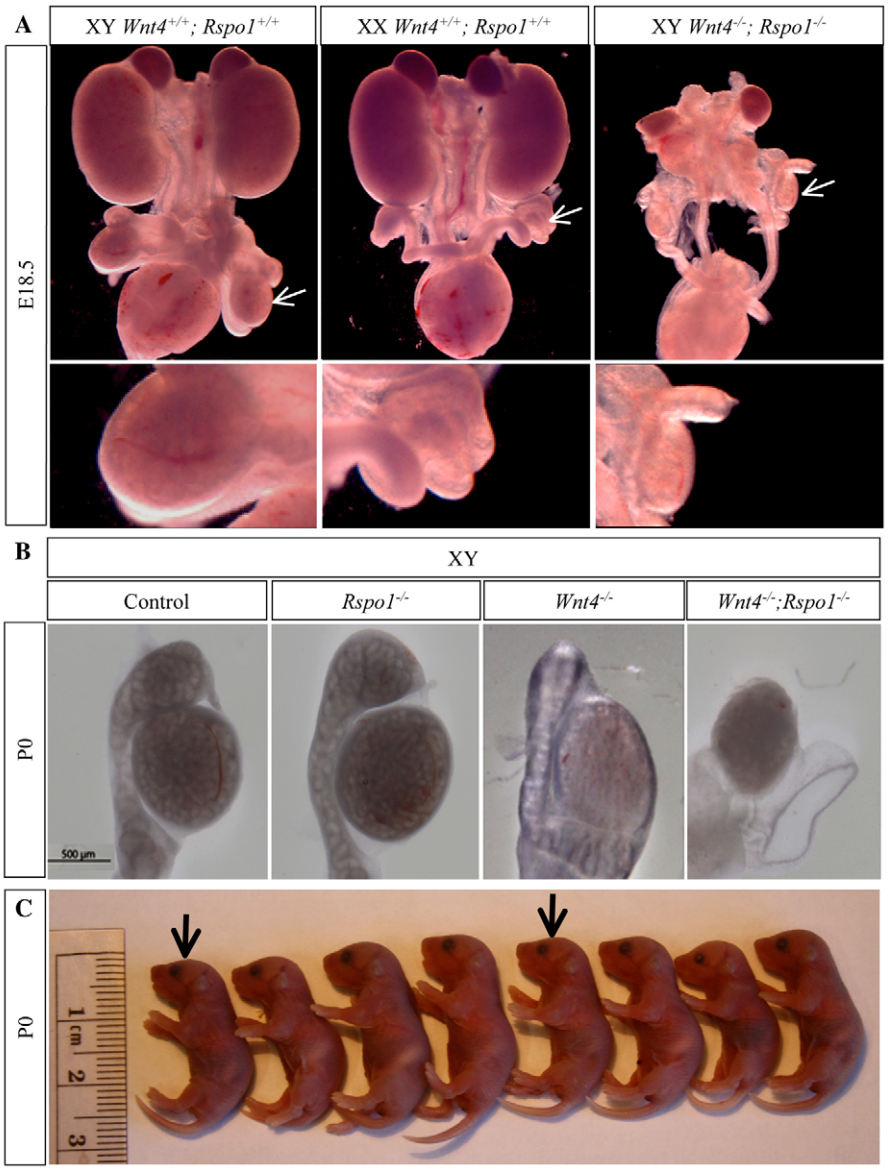

Fig. 2. XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ foetuses exhibit testicular hypoplasia. (**A**) Upper panels: macroscopic views of the reproductive tract of XY, XX control and XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ foetuses at E18.5. Arrows indicate the gonad. Lower panels: macroscopic views of XY and XX control, and XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads at E18.5. (**B**) Macroscopic view of XY, XY *Rspo1*$^{-/-}$, XY *Wnt4*$^{-/-}$ and XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads at birth. Scale bar: 500 µm. (**C**) Macroscopic view of a litter at birth (P0). Arrows indicate XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ foetuses.

potential gonad and are directly or indirectly involved in *Sry* expression. This shows that these two genes – previously described as 'female genes' – are unexpectedly participating in regulating the development of the male gonad.

We next asked whether RSPO1, together with WNT4, can regulate the expression of genes involved in *Sry* expression. Several genetic interactions implicated in *Sry* transcriptional or post-transcriptional regulation have been identified, including *Wt1* (*–KTS* and *+KTS* isoforms), *CBX/M33* (*Cbx1* and *Cbx2* – Mouse Genome Informatics), *Igf1r*, *Gata4*, *Fog2* (*Zfpm* – Mouse Genome Informatics), *Sf1* and *Cited2* (Barbaux et al., 1997; Buaas et al., 2009; Hammes et al., 2001; Katoh-Fukui et al., 1998; Nef et al., 2003; Tevosian et al., 2002). In XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads collected at E11.5 (18±1 ts), *Wt1+KTS*, *Wt1-KTS*, *CBX/M33* and *Igf1r* exhibited significantly reduced levels of expression when measured by quantitative PCR, whereas *Gata4*, *Fog2* and *Sf1* showed a tendency to reduced levels of expression in the XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads relative to XY controls (Fig. 4C). This general reduction of gene expression suggests that the number of pre-Sertoli cells expressing *Sry* is reduced in the XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads, which is consistent with a defect in proliferation in the coelomic epithelium.

SOX9 expression is downregulated in the XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads

In XY gonads, SRY upregulates *Sox9* that in turn induces Sertoli cell differentiation and male development (Sekido and Lovell-Badge, 2008; Vidal et al., 2001). In mice, *Sox9* is upregulated in XY gonads at E11.5 (Kent et al., 1996; Morais da Silva et al., 1996) and initiates the expression of a genetic network, including the genes *Amh*, *Fgf9* and *L-Pdgs* that are required for sex cord formation (Arango et al., 1999; De Santa Barbara et al., 1998; Kim et al., 2006; Malki et al., 2005; Moniot et al., 2009). *Sox9* expression is therefore an important feature of testis differentiation, prompting us to analyze *Sox9* expression in XY *Wnt4*$^{-/-}$, *Rspo1*$^{-/-}$ and *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads at different developmental stages.

No gonadal phenotype has been observed in XY *Rspo1*$^{-/-}$ adult mice and these mice are normally fertile (Chassot et al., 2008b). However, we cannot exclude that a delayed testicular development

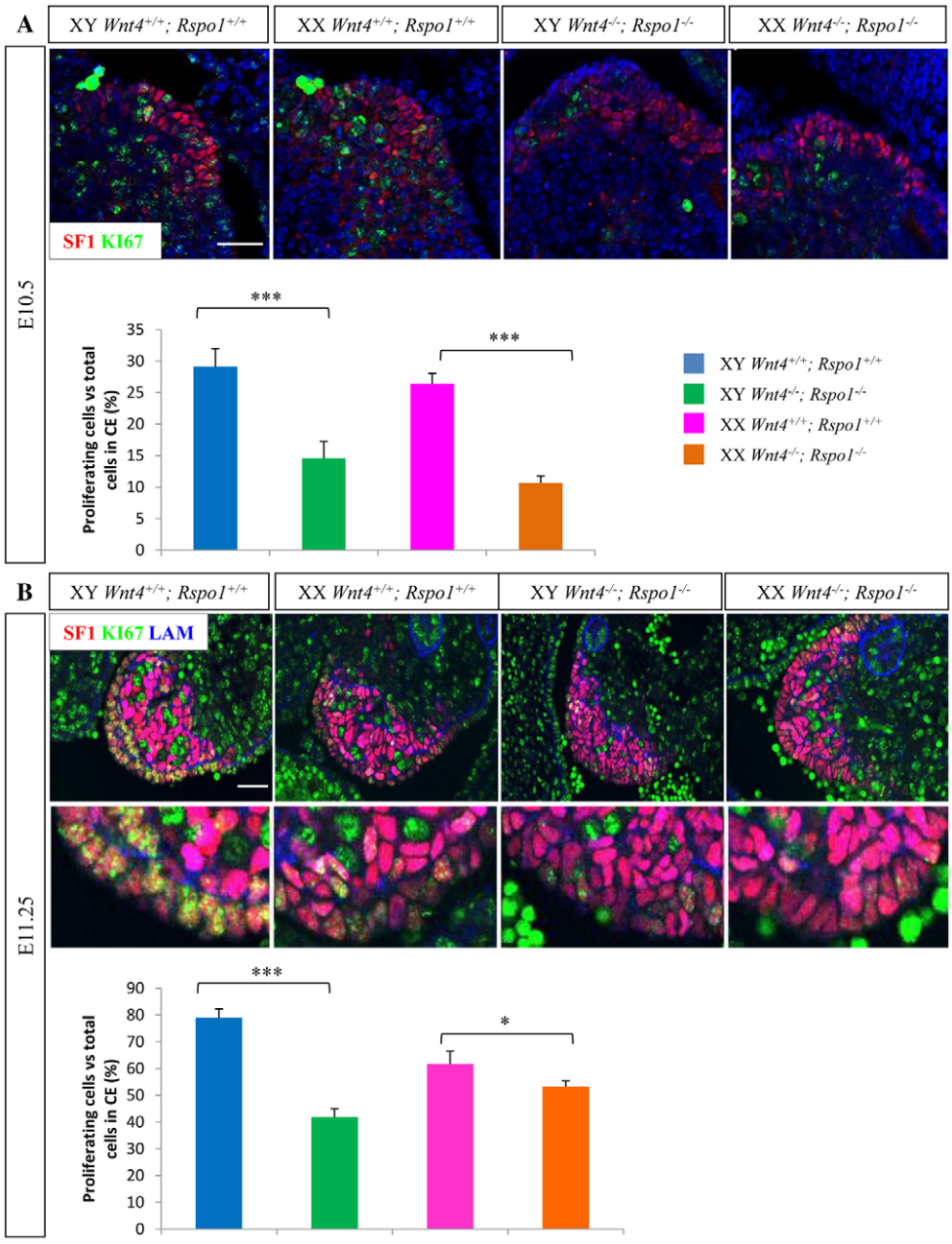

Fig. 3. The proliferation in the coelomic epithelium is reduced in the *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads. (**A**) KI67 (green) and SF1 (red) immunostaining in XY, XX control, XY and XX *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads at E10.5 (12-14 tail somites). Scale bar: 50 μm. Graph shows quantification of the proliferating cells versus the total cells in the coelomic epithelium. ***P<0.001. Data are mean±s.e.m. (**B**) KI67 (green), laminin (blue) and SF1 (red) immunostaining in XY, XX control, XY and XX *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads at E11.25 (16-17 tail somites). Scale bar: 50 μm. Graph shows quantification of the proliferating cells versus the total cells in the coelomic epithelium. *P<0.1; ***P<0.001. Data are mean±s.e.m.

is compensated at birth, as described in the XY *Wnt4*$^{-/-}$ embryos (Jeays-Ward et al., 2004). *Sox9* expression was downregulated in the XY *Wnt4*$^{-/-}$ gonad at E11.5 (Jeays-Ward et al., 2004) (Fig.5A), but returned to normal levels between E14.5 and E18.5. To determine whether a similar delay occurs in the XY *Rspo1*$^{-/-}$ embryos, we analysed *Sox9* expression at E11.5, E12.5 and E14.5 (Fig. 5), these stages corresponded to the most obvious defects in the *Wnt4* mutant (Jeays-Ward et al., 2004). However, analysis of *Sox9* expression in XY *Rspo1*$^{-/-}$ gonads did not reveal any differences compared with XY controls at E11.5, nor any defect in gonadogenesis and sex cord formation at E12.5 and E14.5. This suggests that ablation of *Rspo1* alone does not trigger a developmental delay in sex cord formation.

In XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads collected at E11.5 (18±1 ts), *Sox9* expression level was reduced to around one third of the level found in normal XY gonads when measured by quantitative PCR (Fig. 4C). Moreover, in situ hybridization experiments revealed that at E11.5 (19-21 ts) in XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads, *Sox9* expression was dramatically decreased in comparison with XY controls (Fig. 5A). At E12.5, when sex cords are forming, XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads were thinner than XY controls and fewer SOX9-expressing cells could be detected in XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads relative to XY controls (Fig. 5B). In the XY mutant gonads at E14.5, sex cords were less abundant relative to XY gonads, as evidenced by whole-mount in situ hybridization for *Sox9* (Fig. 5C). Although there was some variability between individuals, fewer sex cords were always observed in XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads compared with XY controls, leading in some cases to the development of very small testes (Fig. 5C). In addition, histology and immunochemistry using the Sertoli cell markers FGFR2, SDMG1 (TMEM184A – Mouse Genome Informatics) and AMH (supplementary material Fig. S1; Fig. 7B) showed that fewer sex cords are formed in XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads, when compared with XY gonads, at E14.5 and E18.5. This indicates that the

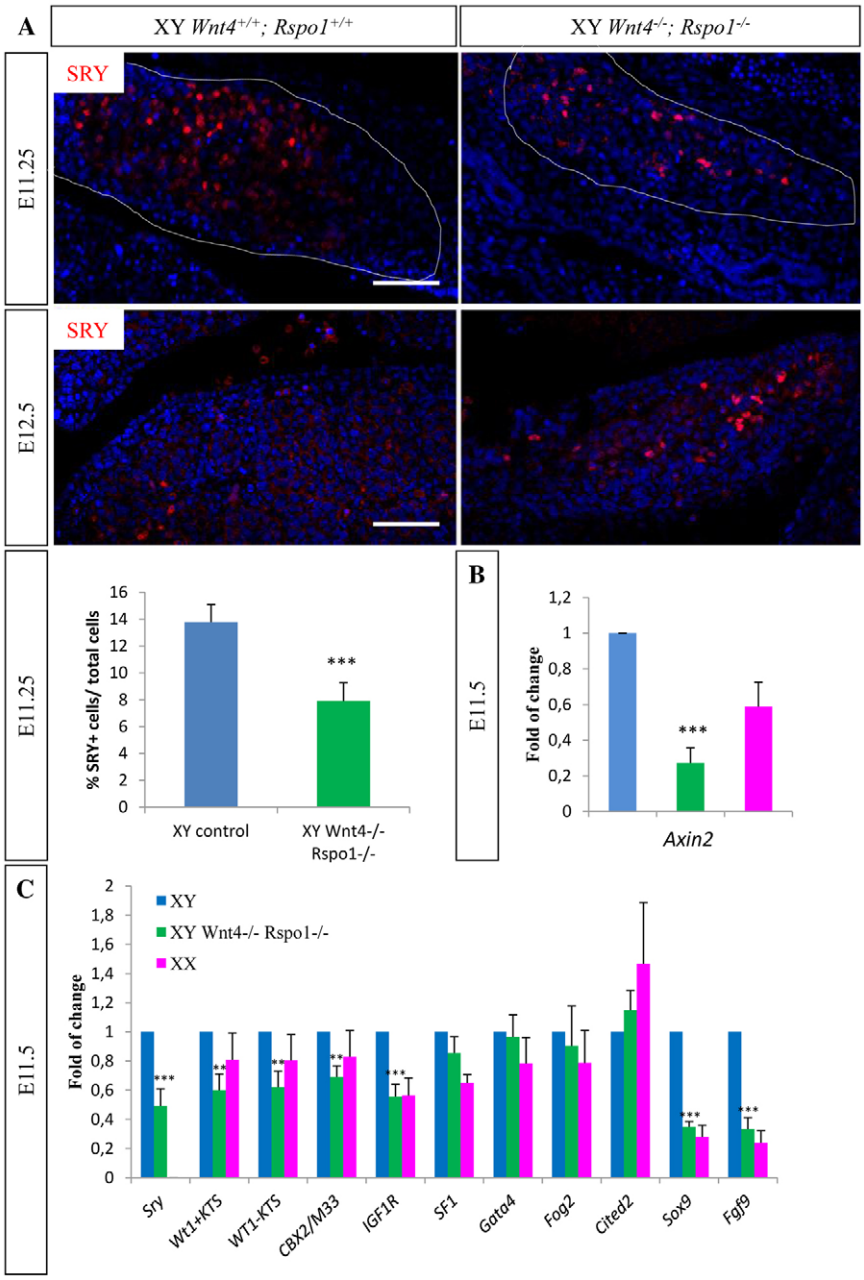

Fig. 4. SRY expression is downregulated in the XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads. (**A**) SRY (red) immunostaining in XY, XX control and XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads at E11.25 (16-18 tail somites) and E12.5. Scale bars: 200 µm. Graph shows quantification of the percentage of SRY-positive cells versus the total cells in the gonad at E11.25 (16-18 tail somites). ***P<0.001. Data are mean±s.e.m. (**B**) Quantitative PCR for Axin2 at E11.5. The y-axis represents the fold-change between the levels of expression quantified in XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonad (green) or in the XX control gonad (pink) versus the levels in the XY control gonad (blue). For each genotype, n=6. ***P<0.001, relative to XY controls. Data are mean±s.e.m. (**C**) Quantitative PCR for genes involved in pre-Sertoli and Sertoli cell differentiation at E11.5. The y-axis represents the fold-change between the levels of expression quantified in XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonad (green) or in the XX control gonad (pink) versus the levels in the XY control gonad (blue). For each genotype, n=12. **P<0.01; ***P<0.001, relative to XY controls. Data are mean±s.e.m.

developmental defects observed in XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads are not compensated for at birth.

The establishment of an XY specific vasculature with the formation of the coelomic vessel is one of the first cellular events observed during testis differentiation (Brennan et al., 2002; Coveney et al., 2008). This process seems to be disturbed in XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads, as highlighted by PECAM1 immunostaining at E12.5 (Fig. 5B). Such alteration was not observed in the single *Wnt4*$^{-/-}$ and *Rspo1*$^{-/-}$ XY gonads. It has been shown that endothelial cells migration is required in male-specific proliferation (Cool et al., 2011). However, the defects/delays in coelomic vessel formation in XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads at E12.5 are therefore likely to result from the early proliferation defects. This suggests that the male-specific proliferation and vascularisation of the gonad are two interdependent processes.

Taken together, these data indicate that the proliferation defect in the coelomic epithelium in absence of RSPO1 and WNT4 leads to a reduction in Sertoli cell numbers, evidenced by reduced expression of a range of Sertoli cell-specific genes, as well as a defect/delay in vascularisation of the gonad and a deficit in testis cord formation.

No sex reversal in the XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads

Subsequently, we investigated the identity of the different cell lineages forming the XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads. To verify whether or not the somatic cells of the XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads have adopted a female cellular fate, we analyzed the expression of the ovarian markers FOXL2 at E12.5 and E14.5, and *Bmp2* at E14.5. Neither *Bmp2* nor FOXL2 expression was detected in the XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads (Fig. 6A), indicating that no sex reversal occurred. Moreover, expression of female markers was not detected at the poles of the mutant gonad, as illustrated by the FOXL2-SOX9 co-immunostaining (Fig. 6A). The histological analysis did not reveal any follicular structures that could be

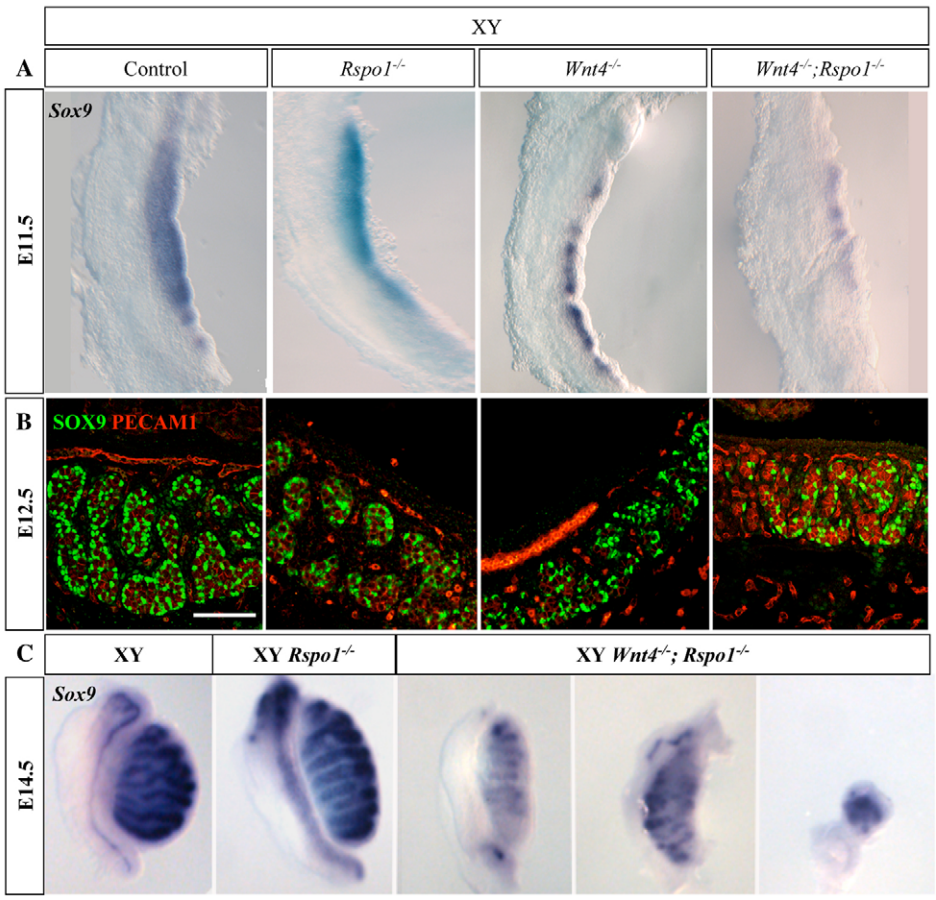

Fig. 5. *Sox9* expression is downregulated in the XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads. (**A**) *Sox9* whole-mount in situ hybridization in XY control, XY *Rspo1*$^{-/-}$, XY *Wnt4*$^{-/-}$ and XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads at E11.5 (18-21 tail somites). (**B**) *Sox9* (Sertoli cells in green) and PECAM (endothelial cells and germ cells in red) immunostaining in XY control, XY *Rspo1*$^{-/-}$, XY *Wnt4*$^{-/-}$ and XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads at E12.5. Scale bar: 200 μm. (**C**) *Sox9* whole-mount in situ hybridization in XY, XY *Rspo1*$^{-/-}$ and XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads at E14.5.

reminiscent of an ovotestis (Fig. 7A). Given that testis development requires not only the expression of male-specific genes such as *Sry*/*Sox9,* but also the repression of female-specific genes, such as *Wnt4* (Jameson et al., 2012a), ablation of *Wnt4* prevents both *Sox9* expression and male-to-female sex reversal in XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads.

We next investigated whether Leydig cells, the steroidogenic cells of the testis (Yao et al., 2002), differentiate normally within the XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads. Indeed, *P450scc* (a steroidogenic marker) was expressed in XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads at E14.5 (Fig. 6B). Moreover, Leydig cells were visible in histological sections (Fig. 7A), suggesting that differentiation of the Leydig cells occurs. In the foetal gonad, the Leydig cell population is required to produce androgens that promote the development of the Wolffian duct into the epididymis, vas deferens and seminal vesicles. In *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ foetuses, the reproductive tracts were similar to those of XY controls and testes were descended (data not shown), indicating the presence of hormones synthesised by the Leydig cells during development. These results demonstrate that in the male gonad a *Wnt4* and *Rspo1* deficiency does not affect Leydig cell differentiation. Altogether, these results show that in XY gonads, ablation of *Rspo1* and *Wnt4* promotes hypoplastic testis formation and prevents male-to-female sex reversal from occurring.

Germ cells are committed to male differentiation as soon as they become enclosed by Sertoli cells in developing sex cords, suggesting that there sexual fate is determined by factors provided by their somatic environment (for a review, see Kocer et al., 2009). However, when sex cord development is prevented in vitro (Yao and Capel, 2002), XY germ cells can develop into male gonocytes outside of the sex cords. Histological analysis and immunostaining for MVH (germ cells) and AMH (Sertoli cells) showed that XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads contain germ cells both within and outside of the sex cords (Fig. 7A,B). Similar to XY controls, the majority of germ cells were quiescent male gonocytes. FGF9 has been identified as a differentiation promoting factor for gonocytes (Bowles et al., 2010). *Fgf9* is expressed in the XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads (Fig. 4C) and thus may participate in the differentiation into gonocyte/pro-spermatogonia. Surprisingly, of those germ cells outside the seminiferous cords only very few exhibited some pachytene-like structures of meiotic germ cells (data not shown). This shows that the close contact with testis cords is not absolutely required for male germ cell differentiation and germ cells can adopt a male fate and survive when localized outside of the sex cords [as shown in vivo here and elsewhere (Tanaka et al., 2000)].

DISCUSSION

One of the earliest morphological changes to occur during sex determination is a dramatic increase in size of the XY gonad coinciding with a peak of *Sry* expression at E11.5 (Brennan et al., 1998; Schmahl et al., 2000). This increase is due to male-specific cell proliferation beginning within the coelomic epithelial cell layer, and the subsequent entry of precursors of the supporting cells from this layer into the gonad (Karl and Capel, 1998). This rapid male proliferation is essential to allow the production of a crucial number of Sertoli cells.

In the undifferentiated gonads, there is a basal level of proliferation that stays constant in XX gonads. When entering the

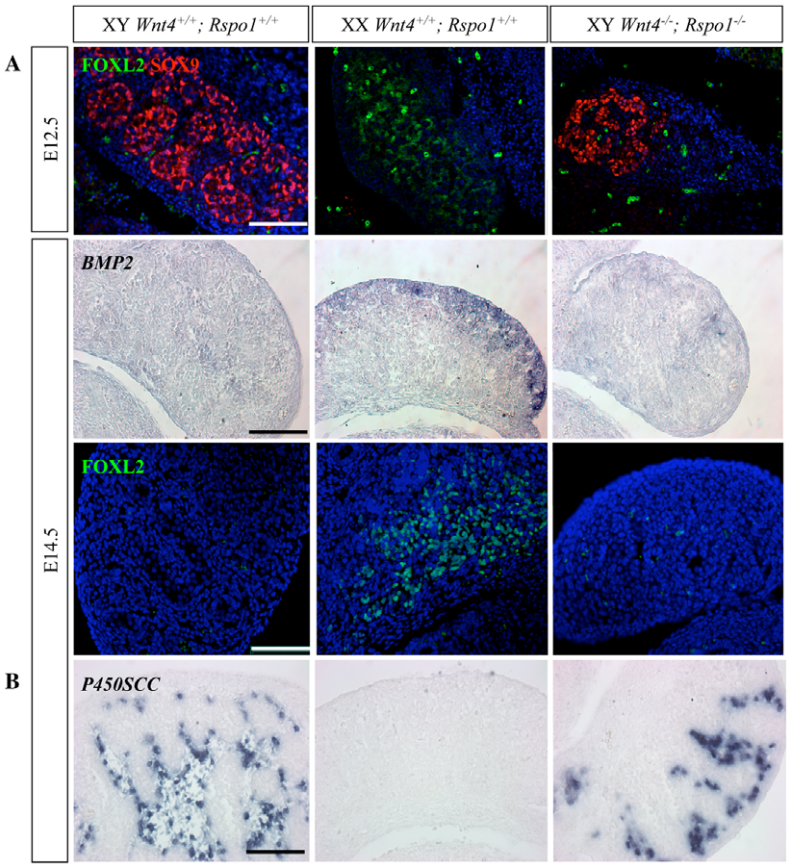

Fig. 6. No sex reversal in the XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads. (**A**) FOXL2 (green) and SOX9 (red) immunostaining at 12.5 in the XY, XX control and XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads. Scale bar: 200 μm. *Bmp2* in situ hybridization at E14.5 (upper panel) and FOXL2 immunostaining (green, lower panel) at 14.5 in the XY, XX control and XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads. BMP2 and FOXL2 are two ovarian markers. DAPI (blue) indicates nuclei. Scale bars: 200 μm. (**B**) P450SCC in situ hybridization at E14.5 in the XY, XX control and XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads. P450SCC is a marker of the steroidogenic Leydig cells at E14.5. Scale bar: 200 μm.

XY gonads, some of the cells will express *Sry*/*Sox9* and differentiate into Sertoli cells, synthesizing FGF9 that further stimulates proliferation and Sertoli cell differentiation (Fig. 8). In XY gonads, the coelomic epithelial cells next participate in the establishment of interstitial cell lineages before ceasing proliferation. Finally, proliferation is no longer located in the coelomic region, but occurs within the somatic cells (Karl and Capel, 1998; Schmahl and Capel, 2003; Schmahl et al., 2000).

XY *Wnt4*$^{-/-}$ gonads show reduced *Sox9* levels at E11.5 or E12.5 (Fig. 5) (Jeays-Ward et al., 2004), but the expression levels of *Sry*

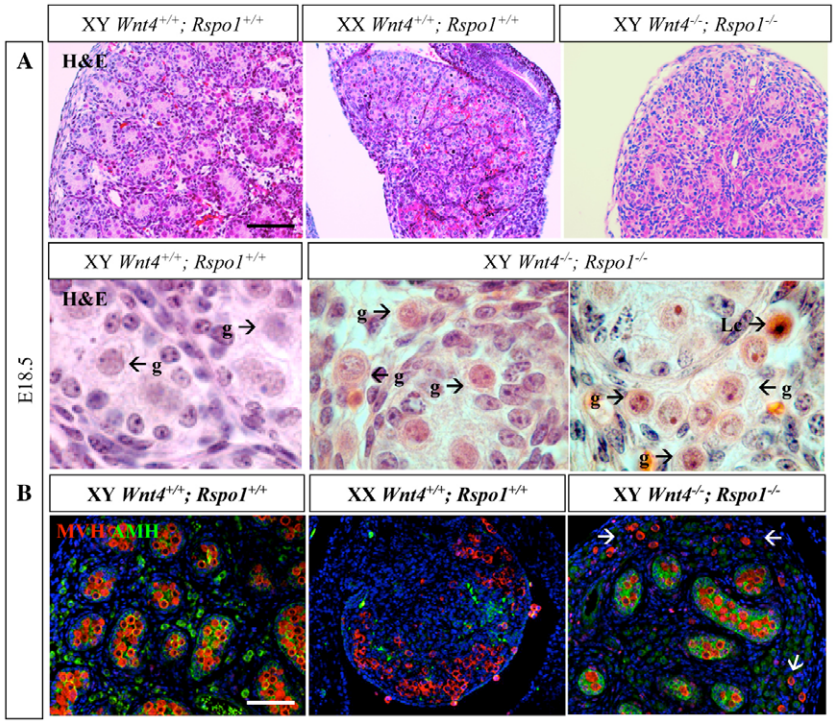

Fig. 7. XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ foetuses exhibit impaired testis development. (**A**) Haematoxylin and Eosin histological analysis of the XY, XX control and XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads at E18.5. g, gonocytes; Lc, Leydig cells. Scale bar: 100 μm. (**B**) Immunostaining for MVH (germ cells, red) and AMH (Sertoli cells, green) in the XY, XX controls and XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads at E18.5. DAPI (blue) indicates nuclei. Scale bar: 100 μm. White arrows indicate germ cells localized outside the sex cords in the mutant gonad.

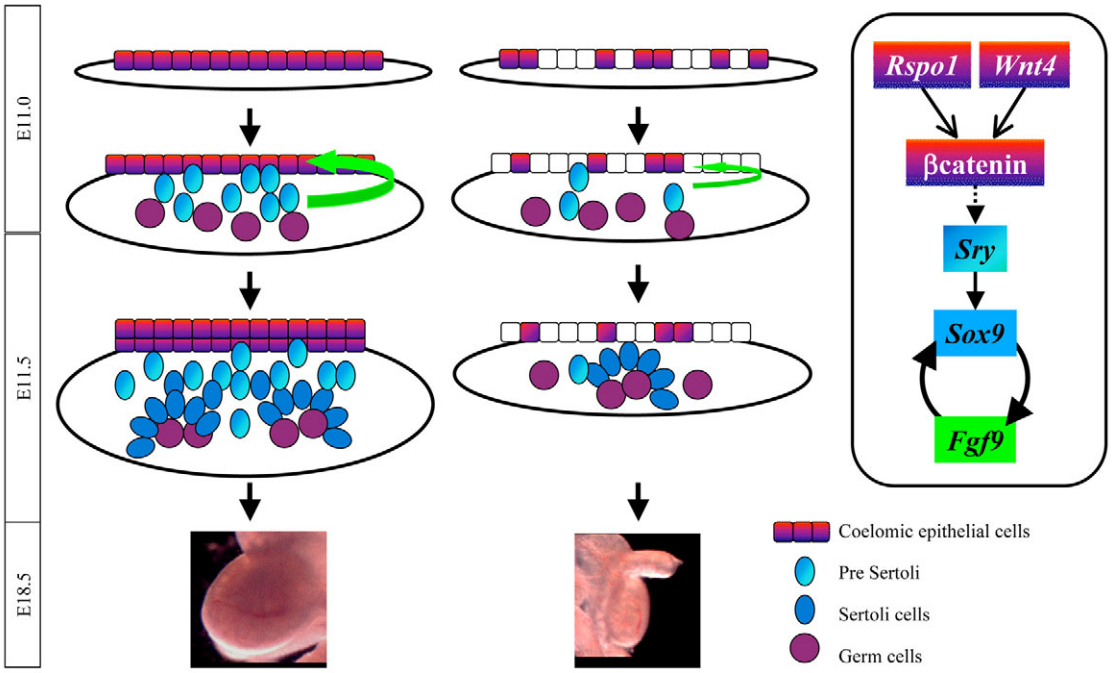

Fig. 8. Model recapitulating the role of Rspo1 and Wnt4 in the development of the early testis. At E11.0 in wild type (left), Rspo1 and Wnt4 contribute to the regulation of the proliferation of the coelomic epithelium. These cells enter the gonad and become pre-Sertoli cells expressing SRY (pale blue) that will then differentiate into Sertoli cells expressing SOX9 (blue) at E11.5. The Sertoli cells synthesise FGF9 (green arrow), which will amplify the proliferation of the coelomic epithelium and allow further differentiation of Sertoli cells. When they reach a critical number, the Sertoli cells form sex cords surrounding the germ cells (violet), which leads to the development of a normal testis. In the XY *Wnt4 Rspo1* mutants (middle), the proliferation of the coelomic epithelial cells is severely reduced. Fewer pre-Sertoli cells enter the gonad and express SRY, SOX9 and FGF9, leading to a deficit in the number of Sertoli cells. Thus, fewer sex cords can form and all germ cells cannot be encompassed within the sex cords. This triggers the formation of a hypoplastic testis that exhibits a reduced number of seminiferous tubules at E18.5.

at E11.5 appears to be unchanged in these mice (Jeays-Ward et al., 2004), indicating that the number of Sertoli cell precursors are not severely affected when deleting *Wnt4* alone. Similarly, loss of *Rspo1* alone does not significantly affect male development. However, ablation of both genes induces testicular hypoplasia characterized by a significant reduction of *Sry* levels and a dramatic drop in coelomic epithelium proliferation. We therefore conclude that *Wnt4* and *Rspo1* work in synergy to promote proliferation in the undifferentiated (E10.5-E11.25) gonad. In XY gonads, RSPO1/WNT4 signalling becomes downregulated when SRY/SOX9 expression occurs and Sertoli cells differentiate. Although the precise molecular mechanisms underlying this antagonism in the gonads remain to be elucidated, in vitro experiments suggest that SRY can repress CTNNB1 signalling (Bernard et al., 2008) and that FGF signalling represses, in vivo, the female-promoting gene *Wnt4*, thus allowing *Sox9* expression to occur (Jameson et al., 2012a).

Whereas the XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads exhibit a reduced number of Sertoli cells, the differentiation of the steroidogenic lineage is not affected. In addition, absence of gross abnormalities of the reproductive tract in these mutants suggests that the embryonic testes produced a sufficient level of androgens to allow male genitalia development. This is surprising, given that Leydig cells differentiation is dependent on Sertoli cell differentiation. However, Jeays-Ward et al. (Jeays-Ward et al., 2004) have shown that *Wnt4* ablation promotes an increase of steroidogenic cells (*Cyp11a1* positive) in the early developing gonads. This suggests that a potential deficit of Leydig cell differentiation in the XY double mutant gonads might be compensated for by an increase of steroidogenic cells that would normally be repressed by *Wnt4* expression in the early gonad.

In the female, the early proliferation is followed by a proliferation block occurring from E12.5 (Bouma et al., 2010; Mork et al., 2012; Nef et al., 2005). Transcriptional studies reveal that cell cycle inhibitors such as *p21kip1*, *p57kip1* and *p27kip1* are specifically expressed in XX gonads at E11.5 and E12.5 (Cederroth et al., 2007), suggesting that female-specific genetic networks also regulate proliferation in developing ovaries. Thus, *Rspo1* and *Wnt4* stimulate cell proliferation during early ovarian differentiation, which may then be regulated by the expression of the cell cycle inhibitors. At this stage, *Rspo1* and *Wnt4* become involved in ovarian differentiation (Chassot et al., 2008b; Vainio et al., 1999).

R-spondins are potent stem cell growth factors in the crypt of the intestine (Sato et al., 2011) but their mechanism of action appears to be complex. Indeed, R-spondins are activators of both WNT/CTNNB1 (Kazanskaya et al., 2004) and WNT/planar cell polarity (PCP) (Glinka et al., 2011; Ohkawara et al., 2011) signalling pathways. The expression of the R-spondins receptors/co-receptors complexes at the membrane of the cell at a given time may determine the signalling pathway they activate. Leucine-rich repeat-containing G protein-coupled receptor (LGR) 5 and LGR4 are essential for both RSPO1-induced β-catenin and PCP signalling (Carmon et al., 2011; de Lau et al., 2011; Glinka et al., 2011).

In the gonad, *Lgr4* and *Lgr5* are expressed during sex determination in a dynamic manner (Jameson et al., 2012b). Indeed, *Lgr5* is expressed in male somatic cells at E11.5, and *Lgr4* is expressed in female somatic cells at E12.5. At E13.5, no Lgr genes are detected in male somatic cells whereas both *Lgr4* and

Lgr5 are expressed in female somatic cells. It is presently unclear whether LGR5 is the receptor transmitting the RSPO1 signal in the coelomic region of the XX and XY early gonads, and whether LGR4 is the RSPO1 receptor in the XX gonads after sex determination, when *Rspo1* and *Wnt4* become upregulated.

One of the remaining questions is whether CTNNB1 ablation would completely prevent early cell proliferation in the gonad. To answer this question, conditional ablation of CTNNB1 is required, as CTNNB1 knockout embryos die at E7.5 (Haegel et al., 1995). So far, however, no transgenic line with restricted expression of the CRE recombinase in the coelomic domain has been described.

In conclusion, our study demonstrates that the RSPO1/WNT4 genetic pathway involved in ovarian differentiation is indeed also required for testicular development by stimulating proliferation of the coelomic epithelium of the undifferentiated gonad.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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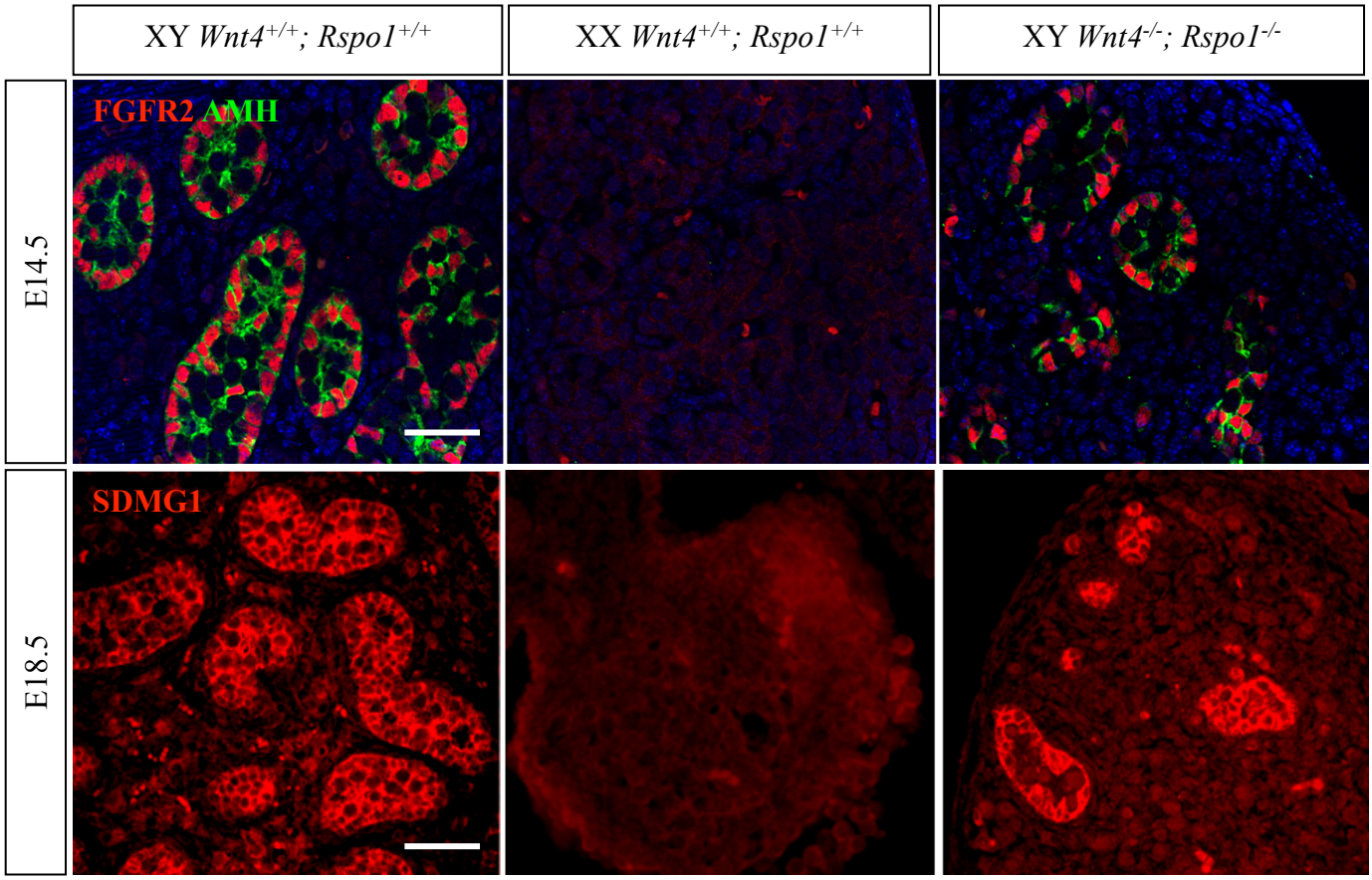

Fig. S1. FGFR2, AMH and SDMG1 immunostaining. Upper panels: immunostaining for FGFR2 (Sertoli cells in red) and AMH (Sertoli cells in green) at E14.5 in XY, XX and XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads. DAPI (blue) indicates nuclei. Scale bar: 50 µm. Lower panel: immunostaining for SDMG1 (Sertoli cells in red) at E18.5 in XY, XX and XY *Wnt4*$^{-/-}$; *Rspo1*$^{-/-}$ gonads. DAPI (blue) indicates nuclei. Scale bar: 50 µm.