R-spondin1 plays an essential role in ovarian development through positively regulating Wnt-4 signaling

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Received October 15, 2007; Revised and Accepted January 30, 2008

In mammals, female development has traditionally been considered a default process in the absence of the testis-determining gene, Sry. Recently, it has been documented that the gene for R-spondin1 (RSPO1), a novel class of soluble activator for Wnt/β-catenin signaling, is mutated in two Italian families with female-to-male (XX) sex reversal. To elucidate the role of Rspo1 as a candidate female-determining gene in a mouse model, we generated Rspo1-null (Rspo1<sup>−/−</sup>) mice and found that Rspo1<sup>−/−</sup> XX mice displayed masculinized features including pseudohermaphroditism in genital ducts, depletion of fetal oocytes, male-specific coelomic vessel formation and ectopic testosterone production in the ovaries. Thus, although Rspo1 is required to fully suppress the male differentiation program and to maintain germ cell survival during the development of XX gonads, the loss of its activity has proved to be insufficient to cause complete XX sex reversal in mice. Interestingly, these partial sex-reversed phenotypes of Rspo1<sup>−/−</sup> XX mice recapitulated those of previously described Wnt-4<sup>−/−</sup> XX mice. In accordance with this finding, the expression of Wnt-4 and its downstream genes was deregulated in early Rspo1<sup>−/−</sup> XX gonads, suggesting that Rspo1 may participate in suppressing the male pathway in the absence of Sry and maintaining oocyte survival through positively regulating Wnt-4 signaling.

INTRODUCTION

The R-spondin (Rspo) protein family is a recently described group of four distinct secreted proteins (1). Their ligand-type activities mimic those of the canonical Wnt ligands, resulting in transcriptional activation mediated by the β-catenin/T cell factor (TCF) signaling pathway (2,3). The spatiotemporal expression pattern of Rspo mRNAs in mouse embryos was shown to partially overlap with that of Wnt genes (4,5), and Rspo proteins synergize with Wnt ligands in the stabilization of cytoplasmic β-catenin and activation of downstream pathways (2,6), which suggest the close interplay between these two families of soluble ligands. Recent in vitro studies have shown that Rspo proteins can bind to the Wnt co-receptor LRP6 (6,7). In addition, it was reported by another group (8) that R-spondin1 (Rspo1) can regulate Wnt signaling by antagonizing dickkopf-1 (Dkk1)-dependent internalization of LRP6.

With regard to the physiological functions of Rspos, recent studies have reported that they play essential roles in various developmental processes, such as Xenopus myogenesis (2), mouse placental development (9), apical ectodermal ridge (AER) maintenance (10) and human nail morphogenesis (11). Human RSPO1 was first identified by its ability to stimulate the growth of intestinal epithelium in transgenic mice (3). The therapeutic potential of the RSPO1 protein was also demonstrated in mouse models of cancer-therapy-induced mucositis and experimental colitis (3,12). More recently, Parma et al. (13) showed that RSPO1 is the gene disrupted in a recessive syndrome characterized by XX sex reversal, palmo-plantar hyperkeratosis and predisposition to squamous cell carcinoma of the skin. They also reported that Rspo1 is

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expressed specifically in XX gonads of mice during the critical stage (12.5–14.5 days postcoitum) of gonad differentiation. This is the first report describing a single human gene mutation that causes complete female-to-male sex reversal and uncovered the role of Rsps1 as a candidate female-determining gene in mammals (14,15). However, limited clinical information is available for Rsps1-mutated XX men (16), and thus elucidation of the precise role of Rsps1 in the process of sex determination requires further investigation using knockout mice.

Here, we describe the results of initial phenotypic analyses in Rsps1-null (Rsps1−/−) female mice, thereby demonstrating the essential role of Rsps1 as a positive regulator of the Wnt-4 signaling pathway in suppressing male differentiation and maintaining germ cell survival during the development of XX gonads.

RESULTS

Rsps1-null XX mice do not show complete sex reversal but subfertility

To generate Rsps1-null mutant mice, we performed three step-wise genetic modifications of mouse embryonic stem (ES) cells to delete the 17.8 kb genomic region encompassing the entire protein coding sequence of the Rsps1 gene using the Cre-loxP recombination system (Fig. 1A). Intercrosses between mice heterozygous for the Rsps1-null allele (Rsps1+/−) yielded viable, outwardly normal homozygous (Rsps1−/−) offspring at the expected Mendelian frequency (+/+: 24.7%; +/−: 50.9%; −/−: 24.4%; n = 405) and normal male/female ratio (+/+: 52/48; +/−: 110/96; −/−: 53/46). The deletion of the entire coding sequence for the Rsps1 gene (Fig. 1B) and the absence of Rsps1 transcripts (Fig. 1C) were confirmed in the resultant Rsps1−/− mice. We then genotyped male and female Rsps1−/− mice by PCR with Sry-specific primers to test whether the deletion of the Rsps1 gene in mice leads to complete female-to-male sex reversal as seen in human patients (13). As a result, all phenotypic female Rsps1−/− mice, as determined by the morphology of external genitalia, were Sry-negative (n = 174), and all phenotypic males were Sry-positive (n = 115). Thus, the external sexual phenotype and Sry genotype are congruent, indicating that a complete XX sex reversal does not occur in Rsps1−/− mice. Mating studies (Table 1) showed normal fertility of Rsps1−/− males. On the other hand, female homozygotes exhibited severely reduced fertility. The appearance of copulatory plugs indicated that Rsps1−/− females successfully mated with fertility-proven C57BL/6 males; however, only one of six plugged females had litters after 1 month of continuous mating. The result led us to investigate further the female reproductive system in Rsps1−/− mice.

Internal pseudohermaphroditism in Rsps1-null XX mice

Consistent with the data showing normal fertility in Rsps1−/− males, the necropsy of newborn and adult (6- to 8-week-old) Rsps1−/− male mice indicated that the male internal reproductive organs were morphologically indistinguishable from wild-type and Rsps1+/− male littersmates (data not shown).
In contrast, marked alterations in the morphology of female reproductive organs were observed in both newborn and adult Rspo1−/− females (Fig. 2). The Rspo1−/− ovaries (Fig. 2E and K) were surrounded by highly coiled ductal structures resembling the male epididymis (Fig. 2F and N). They also had additional vas deferens-like ducts that extended from an epididymis-like structure and tracked parallel to the seminiferous tubules of the testis. A few sex-cord-like structures were detectable at birth (Fig. 3B), and tubular structures were more prominent at later stages (Fig. 3D and G). Within these tubes, cells resembling the Sertoli cells (Fig. 3H), characterized by an alignment with the basal lamina, tripartite nucleioloi and numerous veil-like cytoplasmic processes (19), were readily observed. Ectopic expression of a Sertoli cell differentiation marker, Dhh (desert hedgehog), in newborn Rspo1−/− ovaries was also demonstrated by RT-PCR (Fig. 3I) and in situ hybridization analyses (Fig. 3J). It has been suggested that continuous interaction between oocytes and supporting cells is required for the maintenance of ovarian phenotypes, and thus the loss of oocytes from the ovary frequently results in a secondary transdifferentiation of supporting cell lineages (20,21). Although we cannot now rule out the possibility that Sertoli cell differentiation in Rspo1−/− ovaries occurs because of cell-intrinsic problem within the supporting cells, it is presumed that this postnatal masculinization may be a secondary consequence of oocyte depletion in fetal Rspo1−/− XX gonads.

To investigate the fetal germ cell loss in Rspo1−/− ovaries, we examined the expression of a meiosis-specific, synaptonemal complex protein SCP3 in developing ovaries (Fig. 4). On day 14.5 dpc, almost all germ cells in the ovaries normally enter meiosis and most of the nuclei at the leptotene/zygotene stage are positive for SCP3 staining (22). Although we readily detected SCP3-positive oocytes throughout the wild-type ovaries at this stage (Fig. 4), their distribution was apparently restricted to a region other than the coelomic domain of 14.5 dpc Rspo1−/− ovaries. Furthermore, at both 14.5 and 16.5 dpc, the numbers of SCP3-positive cells were markedly reduced in Rspo1−/− ovaries as compared with wild-type ovaries. Staining for apoptosis by TUNEL assay in 16.5 dpc wild-type ovaries revealed that apoptosis is observed mainly within the medullary region; few germ cells undergo apoptosis in the coelomic (cortical) domain. In contrast, apoptotic cells appeared throughout the Rspo1−/− ovaries, and the distribution of apoptotic cells apparently overlaps with that of SCP-positive cells, implying that a substantial fraction of meiotic germ cells undergoes apoptosis in both the medulla and coelomic domains of Rspo1−/− ovaries.

### Table 1. Reproductive performance of Rspo1−/− mice

<table>
<thead>
<tr>
<th></th>
<th>Wild-type</th>
<th>Rspo1+/−</th>
<th>Rspo1−/−</th>
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<tbody>
<tr>
<td>Number of males tested</td>
<td>3</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Number of females mated</td>
<td>6</td>
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<td>12</td>
</tr>
<tr>
<td>Number of litters</td>
<td>5</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>Average litter size</td>
<td>8 ± 1.4</td>
<td>7.6 ± 2.3</td>
<td>8.5 ± 1.5</td>
</tr>
<tr>
<td>Number of females tested</td>
<td>8</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>Number of females plugged</td>
<td>8</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>Number of litters</td>
<td>8</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>Average litter size</td>
<td>6.1 ± 1.1</td>
<td>7.0 ± 2.4</td>
<td>7.0 ± 1.6</td>
</tr>
</tbody>
</table>

*aResults of average litter size are expressed as the mean ± SD.

*bThe time between the set-up of the mating and the detection of the first copulatory plugs was 2.9 ± 1.3 days (n = 8), 2.0 ± 1.3 days (n = 10) and 2.7 ± 1.4 days (n = 6) in wild-type, Rspo1+/− and Rspo1−/− females, respectively, indicating that the estrous cycle length in Rspo1−/− females is normal.

*cThese pups died shortly after birth, reasons unknown.

Oocyte depletion in masculinized Rspo1-null ovaries

We next examined the phenotypic changes of Rspo1−/− XX gonads at various time points (14.5 days postcoitum, postnatal day 1, 3- and 7-week-old). Histological studies showed that Rspo1−/− XX gonads from 14.5 dpc embryos have elongated shapes, typical of normal ovaries at this stage (Fig. 3A). Moreover, Rspo1−/− XX gonads showed no sign of testicular cord formation, an apparent morphological modification of an early stage of testis organogenesis. In normal XY gonads, the expression of Sry between 10.5 and 11.0 dpc initiates the differentiation and proliferation of Sertoli cells. Subsequently, the Sertoli cells participate in the formation of testicular cord architectures by 12.5 dpc and then produce anti-Müllerian hormone to inhibit the development of Müllerian duct-derived female reproductive tracts (17). Therefore, the current data showing the lack of sex-cord formation in early XX gonads and the persistence of fully developed female reproductive tracts suggest that the Rspo1−/− XX gonads have not undergone a primary sex reversal that depends on the Sertoli cell differentiation, an initial event crucial for male development. Although wild-type XX gonads have many detectable cortical oocytes at birth, Rspo1−/− XX gonads contained only a few oocytes (Fig. 3B), which was evidenced by reduced levels of transcripts for newborn ovary homeobox gene (Nobox, Fig. 3C), an oocyte-specific marker gene at this stage (18). Although some healthy maturing follicles were still present in prepubertal (3-week-old; Fig. 3D) and pubertal (7-week-old; Fig. 3F) Rspo1−/− ovaries, the number of follicles was also markedly reduced in both prepubertal and pubertal Rspo1−/− females. Thus, in Rspo1−/− ovaries, marked oocyte depletion was apparent by birth, but a few oocytes were able to survive to form healthy, mature follicles at later stages.

Another morphological alteration in postnatal Rspo1−/− ovaries was the development of structures resembling the seminiferous tubules of the testis. A few sex-cord-like structures were detectable at birth (Fig. 3B), and tubular structures were more prominent at later stages (Fig. 3D and G). Within these tubules, cells resembling the Sertoli cells (Fig. 3H), characterized by an alignment with the basal lamina, tripartite nucleioloi and numerous veil-like cytoplasmic processes (19), were readily observed. Ectopic expression of a Sertoli cell differentiation marker, Dhh (desert hedgehog), in newborn Rspo1−/− ovaries was also demonstrated by RT-PCR (Fig. 3I) and in situ hybridization analyses (Fig. 3J). It has been suggested that continuous interaction between oocytes and supporting cells is required for the maintenance of ovarian phenotypes, and thus the loss of oocytes from the ovary frequently results in a secondary transdifferentiation of supporting cell lineages (20,21). Although we cannot now rule out the possibility that Sertoli cell differentiation in Rspo1−/− ovaries occurs because of cell-intrinsic problem within the supporting cells, it is presumed that this postnatal masculinization may be a secondary consequence of oocyte depletion in fetal Rspo1−/− XX gonads.
Taken together, these morphological studies demonstrate that the absence of Rspo1 activities in female mice results in the masculinization of both genital ducts and ovaries. The subfertility observed in \( \text{Rspo1}^{+/ -} \) females may be because of these abnormalities in the reproductive organs. It is also important to note that these phenotypic features resemble female-specific, partial sex reversal in \( \text{Wnt-4}^{+/ -} \) mice (23). Besides the phenotypes described above, ectopic formation of the coelomic blood vessel, which is normally specific to testis development, is among the earliest phenotypic changes in \( \text{Wnt-4}^{-/-} \) XX gonads (24). Our examination of the 13.5 dpc gonads revealed that this vessel arises ectopically in \( \text{Rspo1}^{-/-} \) XX gonads (Fig. 5). On the other hand, other urogenital defects (kidney and Müllerian duct deficiencies) common to both sexes of \( \text{Wnt-4}^{+/ -} \) mice (23) were not observed in \( \text{Rspo1}^{-/-} \) mice, indicating that Rspo1 is not involved in the developmental process of these organs.
Persistence of Wolffian ducts is because of ectopic testosterone production in Rspo1-null XX mice

Previous analyses of Wnt-4−/− XX gonads revealed the expression of several genes involved in steroidogenesis and production of testosterone (23,25). These enzymes are normally expressed in the testis, but suppressed in the ovary at birth. This implies that ectopic production of testosterone in the fetal ovary allows for differentiation of the Wolffian duct in Wnt-4−/− females (25). To determine whether this is also the case in Rspo1−/− mice, we examined the expression of transcripts for Hsd3b1 (3β-hydroxysteroid dehydrogenase), Hsd17b1 (type-1 isoform of 17β-hydroxysteroid dehydrogenase) and Cyp17 (17α-hydroxylase/C17-20 lyase: P450c17) in...
newborn Rspo1−/− XX ovaries by semi-quantitative RT-PCR (Fig. 6A) and in situ hybridization (Fig. 6B–D) analyses. The expression of these three mRNAs was elevated in the Rspo1−/− ovaries compared with the levels seen in wild-type ovaries. We also detected the presence of testosterone in wild-type and Rspo1−/− male testes and in the Rspo1−/− ovaries of newborn mice, but not in wild-type ovaries (Fig. 6E). Furthermore, the treatment of pregnant females with the anti-androgen flutamide (25) frequently resulted in Rspo1−/− female pups without extra, male sex ducts (data not shown). Thus, these results indicate that the impaired regression of the Wolffian duct in Rspo1−/− females is because of the ectopic production of testosterone in their ovaries, which is in accord with the effects seen in Wnt-4−/− females.

**Rspo1 is required to establish the female-specific expression pattern of Wnt-4 and its downstream genes in early XX gonads**

As described, our data show a striking similarity between the phenotypic features of Rspo1−/− and Wnt-4−/− female mice, implying a possible physiological interplay between these two soluble factors in suppressing the male differentiation program and maintaining the oocyte development during the organogenesis of XX gonads. Both Wnt4 and Rspo1 are specifically expressed in XX-gonads (13,23) between 12.5 and 13.5 dpc, and the loss of Wnt4 resulted in an altered XX-specific expression pattern of its downstream genes including follistatin (Fst), bone morphogenic protein 2 (Bmp2) (26) and inhibin beta B (Inhbb) (27). We therefore performed quantitative real-time PCR analyses of these genes in addition to genes important for male development (Sox9, Dhh) in 13.5 dpc gonads to determine the epistatic relationship between the Rspo1 and the Wnt-4 signaling pathways. As expected from the results showing the lack of a sex-cord structure at this stage (see Fig. 3A), pre-Sertoli cell markers Sox9 and Dhh were not detected in Rspo1−/− XX gonads (Fig. 7A). This result corroborates that primary sex reversal does not occur in Rspo1−/− XX gonads as seen in Wnt-4−/− XX gonads (23).

It is interesting that real-time PCR and in situ hybridization analyses showed that Wnt-4 expression was clearly diminished in Rspo1−/− XX gonads as compared with wild-type XX gonads (Fig. 7A and B). Furthermore, transcripts for Fst and Bmp2, both of which have been previously shown to be activated downstream of Wnt4 (26), were also diminished in Rspo1−/− XX gonads (Fig. 7C), suggesting that Rspo1 is required for sustaining Wnt4 expression to activate its downstream signal in early XX gonads. It has been shown that Wnt4 reduces Inhbb expression at the transcriptional level in XX gonads (27). In Rspo1−/− XX gonads, Inhbb expression was elevated as seen in Wnt4−/− XX gonads (Fig. 7C). Taken together, these results indicate that Rspo1 is required to establish and maintain the XX-specific expression pattern of genes downstream of Wnt-4 through positively regulating Wnt-4 expression in early XX gonads.
DISCUSSION

Sex determination is an unusual developmental process that directs differentiation of the embryonic bipotential gonads to either testes or ovaries (17). In mammals, it has been well established that the differentiation of XY gonads depends on the signaling cascade triggered by the expression of Sry, the testis-determining gene on the Y chromosome. The mouse XY gonad undergoes dramatic morphological changes including Sertoli cell differentiation, testis cord formation and male-specific vascularization by 12.5 dpc. In contrast, the XX gonad normally appears dormant until germ cells enter into first meiosis between 13.5 and 14.5 dpc, and thus ovarian development had long been considered a default process in the absence of Sry. Despite an apparently quiescent feature of early XX gonads, the XX-specific gonadal gene expression pattern is normally established between 11.5 and 12.5 dpc, and it has been postulated that these XX-specific genes play important roles in differentiation of bipotential gonads into functional ovaries (28). The two female overexpressed genes, Wnt-4 and Fst, are of particular interest because genetic experiments using knockout mice revealed that...
Wnt-4 acts through Fst to inhibit male-specific vascularization and to maintain germ cell survival in early ovarian development (26). The expression of Rspo1, a gene for another female factor implicated in Wnt signaling, can also be detected in the somatic cells of bipotential gonads of both sexes and then becomes female-specific by 12.5 dpc as a result of down-regulation in males (13,29). Furthermore, a recent study by Parma et al. (13) showed the female-to-male sex reversal in human patients carrying Rspo1 mutations, suggesting that Rspo1 may also be involved in regulating the ovarian pathway. Here, our current studies of Rspo1-null mice, in conjunction with the work on Rspo1-mutated human patients, have demonstrated the essential requirement for Rspo1 in mammalian ovary organogenesis. Importantly, the data presented here also suggest that Rspo1 actively suppresses some aspects of male differentiation in the absence of Sry and maintains oocyte survival through positively regulating the Wnt-4 signaling pathway.

Figure 7. Deregulation of Wnt-4 and its downstream genes in 13.5 dpc Rspo1−/− XX gonads. (A) TaqMan real-time PCR analyses of Sox9, Dhh, and Wnt-4 mRNA levels in 13.5 dpc wild-type/Rspo1+/− (n = 3/2), Rspo1−/− XX (n = 6) and wild-type XY (n = 3) gonads. The expression level of the tested genes was normalized to GAPDH. In each analysis, the averaged expression level in wild-type XY (for Sox9 and Dhh) or wild-type/Rspo1−/+ XX (for Wnt-4) gonads was arbitrarily defined as unit 100. The mean value for five real-time PCR experiments was calculated for each sample, and the data are presented as mean ± SD. (B) RNA in situ hybridization to 13.5 dpc gonads show that an ovary marker, Wnt-4 is expressed in the wild-type XX gonad, but undetectable in the wild-type XY gonad. The Rspo1−/− XX gonad shows only a weak Wnt-4 signal compared with the wild-type XX gonad. It should be noted that the developing kidney (23) and adrenal grand (31) normally express Wnt-4, and the Rspo1−/− kidney (k) and adrenal grand (ad) still express Wnt-4, suggesting that Rspo1 may not be involved in the regulation of Wnt-4 expression in these tissues. (C) TaqMan real-time PCR analyses of Fst, Bmp2 and Inhbb mRNA levels in 13.5 dpc wild-type/Rspo1+/− (n = 3/2), Rspo1−/− XX (n = 6) and wild-type XY (n = 3) gonads. The expression level of the tested genes was normalized to GAPDH. In each analysis, the averaged expression level in wild-type/Rspo1−/+ gonads was arbitrarily defined as unit 100. The mean value for five real-time PCR experiments was calculated for each sample and the data are presented as mean ± SD. P < 0.0001 (double asterisk); P < 0.001 (single asterisk).
critical timing of gonadal development. It is worth noting that the Rspo1 expression was diminished in dorsal spinal cords of Wnt-1/−/−, Wnt-3a/−/− and Wnt-1/−α double knockout embryos (4) and therefore the analyses of Rspo1 expression in Wnt4/−/− XX gonads in future experiments should be informative to determine the precise epistatic relationship between Rspo1 and Wnt-4. With regard to this point, Ottolenghi et al. (30) recently noted (the actual data was not shown) that Rspo1 expression was not affected in Wnt4/−/− XX gonads during fetal life, which is consistent with the former possibility that Rspo1 acts upstream of Wnt-4. Because it has been reported that the ligand-type activity of Rspo proteins shows synergy with canonical Wnt ligands in positive modulation of the β-catenin/TCF signaling (6, 7), it is also tempting to speculate that the cooperative action between Rspo1 and Wnt-4 might contribute to suppressing male differentiation and maintaining ovarian function during female development through β-catenin-mediated transcriptional activation. Nevertheless, the involvement of β-catenin/TCF signaling in gonadal development has yet to be defined, and further studies will therefore be required to elucidate the molecular mechanism underlying the actions of Rspo1 and Wnt-4.

The formation of a testis-specific artery, the coelomic vessel, is one of the earliest morphological changes during development of XY gonads. Immediately after the expression of Sry (11.5 dpc), the coelomic vessel forms through the mechanism of migration of endothelial cells from the mesonephros into the XY gonad (24). The Wnt-4/Fst signaling normally inhibits the formation of the coelomic vessel in the absence of Sry (26). Yao et al. (26) reported that Fst expression is absent in Wnt4/−/− XX gonads and that Fst/−/− XX mice share two unique gonadal defects (ectopic formation of the testis-specific coelomic vessel, fetal loss of meiotic germ cells) with Wnt4/−/− XX mice, indicating that Wnt-4 acts through Fst to control these cellular events in developing ovaries. Another report by Yao et al. (27) described that Inhbb contributes to forming coelomic vessels in XY gonads, and the expression of Inhbb is normally suppressed by Wnt-4 in XX gonads. Here, we show that the coelomic vessel appears ectopically on the surface of Rspo1/−/− XX gonads, as seen in Wnt4/−/− or Fst/−/− XX gonads. Consistent with this observation, we found that Wnt-4 and Fst were down-regulated, and that Inhbb was up-regulated in the Rspo1/−/− XX gonads, thereby indicating that, in normal ovaries, Rspo1 contributes to antagonizing the formation of testis-specific vasculature as a positive regulator of Wnt-4 expression.

Several lines of evidence suggest that female germ cells appear to be a critical organizer of normal ovary organogenesis (21). Progressive loss of meiotic germ cells during fetal life is another common feature between Wnt-4/−/− (23,26), Fst/−/− (26) and Rspo1/−/− XX gonads, indicating that these somatic cell-derived factors contribute to providing the environment for supporting germ cell survival in the ovaries. It has been proposed that the Wnt-4/Fst signaling may be involved in the formation of a protective niche in the ovarian cortex where meiotic germ cells escape from the extensive apoptosis that occur in the medulla of the ovaries (26). In 16.5 dpc Rspo1/−/− ovaries, the number of SCP3-positive meiotic cells was markedly diminished, and the apoptotic cells were observed in both the medulla and the coelomic domains as seen in Wnt-4/−/− and Fst/−/− ovaries (26), which suggests that the Rspo1 may also play an essential role in establishing this protected domain to support meiotic germ cell survival, possibly through Wnt-4/Fst signaling. It has been reported that the numbers of germ cells in Wnt4/−/− and Fst/−/− XX gonads are similar to those in wild-type XX gonads from 11.5 to 15.5 dpc (26). On the other hand, our data from the SCP3-staining at 14.5 dpc showed abnormal distribution and reduced numbers of meiotic germ cells in Rspo1/−/− ovaries (Fig. 4), implying that the onset of germ cell loss is slightly earlier in Rspo1/−/− XX gonads than in Wnt4/−/− and Fst/−/− XX gonads. Given that no obvious increase in apoptosis was observed in Rspo1/−/− XX gonads at both 13.5 and 14.5 dpc (data not shown), it is possible that, at earlier stages, Rspo1 plays unique roles in the maintenance of primordial germ cells in XX gonads independent of Wnt-4 and Fst. Nevertheless, it is still an open question if Rspo1 is involved in the maintenance of primordial germ cells in XX gonads, and to clarify these issues, more detailed comparative analyses between early Wnt4/−/− and Rspo1/−/− XX gonads in the same genetic background may be required.

In addition to the phenotypes mentioned above, abnormal steroid synthesis and Wolffian duct formation in Rspo1/−/− females are also reminiscent of those in Wnt4/−/− females. Although the initial analysis of Wnt4/−/− mice suggested that Wnt-4 normally suppresses Leydig cell differentiation in XX gonads, further studies revealed that steroidogenic cells in Wnt4/−/− XX gonads are likely to be adrenal-derived because they express the adrenal marker, Cyp21, and are located in the anterior region of the Wnt-4 ovary (31). Steroid-producing cells are absent in Fst/−/− XX gonads, and thus Wnt-4 signaling may control pathways that affect mesonephric steroidogenic cell migration into XX gonads independent of Fst. At present, the origin of steroidogenic cells detected in Rspo1/−/− XX gonads still remains to be elucidated. Nevertheless, on the basis of the attenuated expression of Wnt-4 in Rspo1/−/− XX gonads, it is possible that they are also adrenal-derived. In Rspo1/−/− ovaries, we also found elevated expression of some other genes encoding steroidogenic enzymes, which may contribute to testosterone synthesis. The development of Wolffian duct-derived, male sex ducts in Rspo1/−/− female mice may be because of the ectopic production of testosterone because the inhibition of testosterone action with the anti-androgen flutamide during gestation frequently leads to regression of extra sex ducts in Rspo1/−/− females. It should be noted that, although the loss of Wnt-4 causes a defect in the formation of Müllerian ducts, Rspo1/−/− females have apparently normal female sex ducts. Thus, these results indicate that Rspo1 is not directly involved in the development of both male and female sex ducts. It is also interesting to note that the newborn Rspo1/−/− ovary was surrounded by a thick membrane (data not shown) that resembles the tunicia albuginea seen in the testis and in Wnt4/−/− ovaries (25).

Micali et al. reported that 46 XX men carrying the RSP01 mutation exhibited severe hypogonadism with hypospadias and gynecomastia, and ultrasound evaluation did not reveal any abnormalities indicating the persistence of Müllerian
duct derivatives. In addition, testicular biopsy specimens showed hyperplasia of Leydig cells. Based on this limited information available, it is difficult to conclude whether primary sex reversal associated with Sertoli cell differentiation in the early gonads is induced in Rspo1-mutated patients. Nevertheless, anomalies observed in the reproductive organs of Rspo1−/− female mice (partial female-to-male sex reversal) do not completely phenocopy those of the Rspo1−/− patients. This may not be surprising because discrepant phenotypes between human and mouse mutants have often been observed in genes involved in sexual development (32,33).

The reason for this discrepancy is unclear at present; however, truncated Rspo1 polypeptides probably produced in the Rspo1−/− patients (13) could alter the human phenotypes and be a possible cause. Alternatively, the requirement of an appropriate dosage balance of these extracellular, soluble signaling molecules for the proper control of sex determination may be different between humans and mice. That the loss of function would lead to complete female-to-male sex reversal in the absence of Sry has been one of the criteria for the long-sought ‘female-determining gene’ (15). Given that the sex-reversed features in Rspo1−/− XX mice are only partial as seen in Wnt-4−/− XX mice, at least in mice, Rspo1 does not satisfy this criterion.

Although the interplay between male and female signaling pathways appears to be central to the sexually dimorphic differentiation of indifferent gonads, the molecular mechanisms involved in this process still remain elusive. Recently, Kim et al. (34) proposed that the fate of the gonad is directed by mutual antagonism between two soluble signaling molecules, Fgf9 and Wnt-4. Experiments using in vitro cultured XX gonads showed that the addition of exogenous Fgf9 induced Sox9 expression, but blocked Wnt-4 expression. They also found that the loss of Wnt-4 caused transient up-regulation of both Sox9 and Fgf9 in XX gonads between 11.5 and 12.0 dpc, thus indicating that Wnt-4 may normally act as a repressor of the male pathway by interfering with the up-regulation of Sox9. However, this de-repression of Sox9 was not maintained at later stages, which was insufficient to give rise to the differentiation of Wnt-4−/− XX gonads into the testis. In this context, it is now of interest how Rspo1 participates in this cross-talk between the signaling for sex determination. Although we have not examined Sox9 expression in Rspo1−/− XX gonads between 11.5 and 12.0 dpc, the induction of Sox9 expression was not detected at 13.5 dpc and there was no sign of Sertoli cell differentiation or sex cord formation in the early Rspo1−/− XX gonads, which are consistent with those seen in Wnt-4−/− XX gonads. Given that Rspo1 appears to act as a positive regulator of Wnt4 expression and the balance between Fgf9 and Wnt4 should be important for sex determination, it is also possible that Rspo1 contributes to maintain the appropriate balance between these male and female factors during normal gonadal development. The essential requirement for Rspo1 in Wnt-4 expression in early XX gonads supports a simple model that the phenotypes observed in Rspo1−/− XX mice represent the consequence of the loss of both Rspo1 and Wnt-4 activities during the development of XX gonads. However, at present, it is also possible that the phenotypic features in Rspo1−/− XX mice are influenced by residual activities of Wnt-4 in the absence of Rspo1. Thus, future studies could possibly focus on the elimination of both activities in Rspo1/Wnt-4 double knockout mice, and thus the Rspo1-null mice and such combined mutants should be valuable models for providing insight into the role of balanced interaction between extracellular signaling molecules during sexually dimorphic differentiation of mammalian reproductive organs.

MATERIALS AND METHODS

Generation of Rspo1-null mutant mice

To replace the 50 bp sequence of exon 2 containing the initiator ATG of Rspo1 gene with a loxp-flanked neomycin-resistant (neo', STneo) cassette (35), we engineered the replacement-type, first targeting (1st-KO) vector. To create the 5′ homologous arm, a BAC clone (RP23-325M4)-derived, 4.8 kb, Apal DNA fragment upstream of the ATG codon was subcloned into the pBluescript (pBS, Clonetech). The subcloning of 2.3 kb of a 3′ arm was carried out using the DNA fragment generated by PCR with the same BAC clone as the template. The A subunit of diptheria toxin (DT) was used as a counter-selection marker. We then constructed the second targeting (2nd-KO) vector to replace the 2.0 kb sequence encompassing exons 4–6 with a loxp-flanked puromycin-resistant (puro', PGKpuro) cassette (35). The 5.0 kb 5′ arm and 2.0 kb 3′ arm were generated by PCR with the BAC clone as the template and subcloned into the pBluescript (pBS) vector. The A subunit of the DT fragment was used as a counter-selection marker. In both the 1st-KO and 2nd-KO vectors, the loxp sequence was inserted in the same orientation, allowing for the deletion of the 17.8 kb genomic sequence encompassing the entire protein-coding region of the Rspo1 gene using Cre recombinase.

As the first step of the genetic modification, TT2 (C57BL/6 x CBA-F1) ES cells (36) were electroporated with the 1st-KO vector, and correct recombination events were verified in G418-resistant ES clones by Southern blot analysis of genomic DNA digested with EcoRI using a 5′ external probe (5′-1). The resultant targeted clones were further analyzed by Southern blot analysis of Apal-digested genomic DNA using a 3′ external probe (3′-1). The forward (f) and reverse (r) primers for amplifying 5′-1 and 3′-1 probes from the BAC clone were as follows: 5′-1f-CTAGACACCTTGCCTGCCTCCT, 5′-1r-CTGTGTGGCCAGGAGTAAGTT, 3′-1f-CC ATAGGAGCAGAGCAGGAT, 3′-1r-GAGCATCGGAAAGATCCTGA. The first targeted ES clones were then electroporated with the 2nd-KO vector, and correct recombination events were verified in G418- and puromycin-resistant ES clones by Southern blots of XbaI-digested genomic DNA with a 5′ external probe (5′-2). The resultant double-KO clones were further analyzed by Southern blots of NcoI-digested genomic DNA, using a 3′ external probe (3′-2). The primer pairs for amplifying 5′-2 and 3′-2 probes from the BAC clone were as follows: 5′-2f-GGAGAGAAGAGCTGGGAGTTCAAGAC, 5′-2r-GATGCGCACATCACCATTGTTACAC, 3′-2f-CAGCCTCCAGGGTACTCGTCTT and 3′-2r-AAGTATGGTGGCTAGGCTTTT. In the final step of the genetic modification of ES cells, the...
double-KO clones were electroporated with a Cre expression vector constructed by inserting a Cre recombinase cDNA into the site between the cytomegalovirus immediate-early enhancer-chicken β-actin hybrid promoter and a 3′-flanking sequence of the rabbit β-globin gene of the pCAGGS vector. The correct recombination events with Cre recombinase were verified in G418- and puromycin-sensitive ES clones by Southern blots of Xbal-digested genomic DNA using the 5′ probe (5′-2) for second targeting as described above. The resultant recombinated clones were further analyzed by Southern blots of Apal-digested genomic DNA using the 3′ probe (3′-2) for second targeting. Deletion of the 17.8 kb sequence from the Rspo1 gene was also confirmed by PCR analysis using the following five primer pairs: a (forward: 5′-AAGACTTCTCATGTGACCCCTCTGAG; reverse: 5′-ACCTCCTGAAACGAATGGA; 96°C for 20 s, 55°C for 30 s, 72°C for 30 s, 35 cycles) to produce an 18.0 kb band from the wild-type allele and a 377 bp band from the deleted allele; b (forward: 5′-AATCTCATCCATCATCCATCCA TGCA; reverse: 5′-ACACCTGTAACGAAATGGA; 96°C for 20 s, 55°C for 30 s, 72°C for 30 s, 35 cycles) to produce a 475 bp band from the wild-type allele and no band from the deleted allele; c (forward: 5′-CTAACAGCACCAT GGAGTG; reverse: 5′-GCATGGAGCACTCTGCGTGTC; 94°C for 30 s, 59°C for 30 s, 72°C for 40 s, 40 cycles) to produce a 561 bp band spanning exons 4–5 from the wild-type allele and no band from the deleted allele; d (forward: 5′-ACAGGCGCCTACATCATAGTG; reverse: 5′-GCAG TTTCAGTCAAGTGCTAC; 94°C for 30 s, 59°C for 30 s, 72°C for 40 s, 40 cycles) to produce a 561 bp band from the wild-type allele and no band from the deleted allele; e (forward: 5′-CTACCCCTGCAGTCAAGTGCTAG; reverse: 5′-GGAGCCTAGCTGTGACAATGG; 94°C for 30 s, 59°C for 30 s, 72°C for 40 s, 40 cycles) to produce a 561 bp band from the wild-type allele and no band from the deleted allele.

The resultant engineered ES clones were used to produce chimeras (35), and the mating between male chimeras and the C57BL/6J females resulted in a germline transmission of the deleted allele of the Rspo1 gene. Male and female mice heterozygous for Rspo1 gene deletion (Rspo1+/−) were subjected to further breeding, and the resultant pups were genotyped and used in this study. Genotyping of mice carrying the deleted allele of Rspo1 was performed by PCR reactions on genomic tail DNA using the a, b, and e primer pairs as described in Figure 1A. We produced and maintained two independent Rspo1-null mutant lines, each of which was derived from independent ES clones. No phenotypic variation was evident between these two lines, so that both lines were used in this study. The SRY genotyping was performed according to a previous report (37). All animal experiments were performed using protocols approved by the Institutional Animal Care and Use Committee of the Discovery Research Laboratories, Research Division, Kirin Pharma Co., Ltd.

Fertility assessment
The reproductive capacities of 7- and 8-week-old male wild-type, Rspo1+/− and Rspo1−/− mice were investigated by mating one male with one 8-week-old C57BL/6J female for 1 week. Two successive sets of 1-week pairing, with two different female mice, were performed for each male. The females were examined for copulatory plugs each morning and litter sizes were recorded. To examine the reproductive performance of female Rspo1−/− mice 6- to 8-week-old virgin female wild-type, Rspo1+/− and Rspo1−/− mice were mated with fertility-proven C57BL/6J males for 1 month. The females were examined for copulatory plugs each morning to determine if and when copulation occurred, and the size of the firstborn litter was recorded.

RT-PCR analysis
Ovaries and testes were prepared from Rspo1−/− and wild-type newborn mice. The separated ovaries and testes were frozen immediately in liquid nitrogen and stored at −80°C until used for the extraction of RNA. Total RNA was purified using the RNeasy Kit (Qiagen, Chatsworth, CA) according to the manufacturer’s instructions. First-strand cDNA was synthesized with Superscript III (Invitrogen, Carlsbad, CA) using random hexamers or oligo dT primer (only for Rspo1) and 250 ng of total RNA. Semi-quantitative RT-PCR analysis was carried out using the cDNA at specific annealing temperatures for each primer pair. PCR products were resolved on 2% agarose gels and stained with ethidium bromide. Murine gene for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a control to verify the integrity of RNA and to ensure that equal amounts of templates were used for RT-PCR. The forward (f) and reverse (r) primers and conditions for RT-PCR analyses are as follows: Rspo1(Ex2-3), f-TCTATCTTGGGGGTGTCTCTGC, r-TCCGGGTTTGCGG CATCAAA, 94°C for 30 s, 60°C for 30 s, 72°C for 30 s, 40 cycles; Rspo1 (Ex2-6), f-CTGAGGTGAGCAACATCGCAGGGCCT, r-CTGTCAGATGCTGCTCCAGA, 94°C for 30 s, 62°C for 30 s, 72°C for 30 s, 2 min, 40 cycles; Rspo1 (Ex4-6), f-CTAACAGCACCATGAGTGTC, r-CCGACGT CCCAGTGCCATCC, 94°C for 30 s, 58°C for 30 s, 72°C for 30 s, 3 cycles; GAPDH, f-CACCATGGAGAAGGCCGGGGGCAGGCA, r-ACCCATCTTGGGAGTTTTCAGAG, 94°C for 30 s, 65°C for 30 s, 72°C for 30 s, 30 cycles; Hsd17b1, f-CAGATCTCTCTAGCTGTCCTT, r-GTCCTGAC CCAGTTATCCCG, 94°C for 30 s, 60°C for 30 s, 72°C for 30 s, 32 cycles; Rspo1 (Ex2-3), f-CTCTCTCCAGCCTGACAGAC, r-CTGTCAGATGCTGCTCCAGA, 94°C for 30 s, 62°C for 30 s, 72°C for 30 s, 2 min, 40 cycles; Rspo1(Ex6-6), f-CTAACAGCACCATGAGTGTC, r-CCGACGT

Taqman quantitative RT-PCR
Gonads (free of mesonephros) were carefully dissected from five Rspo1−/− XX, three wild-type XX, two Rspo1+/− XX
and three wild-type XY embryos at 13.5 dpc. Total RNA was isolated using the RNeasy Mini Kit (Qiagen). For each sample, first-strand cDNA was prepared using 150 ng of total RNA using PrimeScript first-strand cDNA Synthesis Kit (TaKaRa). Real-time PCR reactions and analyses were performed with ABI Prism 7900HT Sequence Detection System (Applied Biosystems) using TaqMan Universal PCR master mix (Applied Biosystems) and commercially available primers and labeled TaqMan probes (TaqMan Gene Expression Assays, product number: 4331182, Applied Biosystems). PCR amplification was performed using a 1:20 dilution of each first-strand cDNA plus primer and probe master mix containing 900 nM of each primer and 250 nM TaqMan probe in TaqMan Universal PCR Master Mix. Amplifications were performed using 2 min at 50°C, 10 min at 95°C, and then 50 cycles of denaturation at 95°C for 15 s, annealing and extension at 60°C for 1 min. TaqMan Rodent GAPDH Control Reagent (Applied Biosystems) was used according to the manufacturer’s instructions. The relative amount of each mRNA was determined using the standard curve method and was normalized to the level of GAPDH in each sample.

Immunohistochemistry and TUNEL staining

The mouse embryos were fixed with Tissue Fixative (GenoStaff Co., Ltd.), and then embedded in paraffin, and sectioned at 6 μm. Tissue sections were de-paraffinized with xylene and rehydrated through a ethanol series and Tris-buffered saline (TBS). Antigen retrieval was performed by microwave treatment for 10 min at 500 W in 1 mM ethylenediaminetetra-acetic acid (EDTA) buffer, pH 9.0. Endogenous peroxidase was blocked with 3% H2O2 in methanol for 15 min, followed by incubation with Protein Block (Dako). The sections were incubated with rabbit polyclonal SCP3 (abcam, ab15092) at 4°C overnight. After washing with TBS, the sections were treated with Biotin Blocking System (Dako) and biotin-conjugated goat anti-rabbit Ig (Dako) diluted 1:600, for 30 min at room temperature, followed by addition of peroxidase-conjugated streptavidin (Nichirei) for 5 min. Peroxidase activity was visualized by diaminobenzidine. The sections were counterstained with Kernechtrot stain solution (Muto), dehydrated and then mounted with Malinol (Muto). Whole-mount immunohistochemistry usingPECAM antibody (BD Pharmingen, 550274) was performed as described previously (24).

TUNEL staining was performed using an ApoTag Peroxidase in situ Apoptosis Detection Kit (Chemicon, S7100) according to the manufacturer’s instructions. Peroxidase activity was visualized by diaminobenzidine. The sections were counterstained with Mayer’s Hematoxylin (Muto), dehydrated and then mounted with Malinol (Muto). The information on DNA fragments used for probes is as follows: Dhh, 436 bp DNA fragment corresponding to the nucleotide positions 49–484 of the mouse gene for Desert hedgehog (GenBank accession number NM_007857); Hsd3b1, 305 bp DNA fragment corresponding to nucleotide positions 34–338 of the mouse gene for Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1 (GenBank accession number NM_008293); Hsd17b1, 391 bp DNA fragment corresponding to nucleotide positions 90–480 of the mouse gene for Hydroxysteroid (17-beta) dehydrogenase 1 (GenBank accession number NM_010475); Cyp17a, 540 bp DNA fragment corresponding to nucleotide positions 1169–1708 of the mouse gene for cytochrome P450, family 17, subfamily a, polypeptide 1 (GenBank accession number NM_007809); Wnt-4, 270 bp DNA fragment corresponding to nucleotide positions 1–270 of the mouse gene for Wingless-related MMTV integration site 4 (GenBank accession number NM_009523).

Testosterone measurement

Gonadal tissues were collected, frozen quickly and stored at −20°C. They were homogenized for 1 min in 1 ml of distilled water, and protein concentrations were measured using a BCA Protein Assay Kit (Pierce). Testosterone concentrations were measured using a Liquid Chromatography/Mass Spectrometry/Mass Spectrometry System (API5000, Applied Bio-systems) by Teikoku Zoki Pharmaceutical Medical Co., Ltd. (Kanagawa, Japan).

In situ hybridization

The DNA fragment for each gene was subcloned into pGEMT-Easy Vector (Promega) and was used for generation of sense or anti-sense RNA probe. Tissues were fixed with 4% paraformaldehyde, then embedded in paraffin and sectioned at 6 μm. For in situ hybridization, tissue sections were de-waxed with xylene and rehydrated through an ethanol series and PBS. The sections were fixed with 4% paraformaldehyde in PBS for 15 min and then washed with PBS. The sections were treated with 15 μg/ml ProteinaseK in PBS for 30 min at 37°C, washed with PBS, refixed with 4% paraformaldehyde in PBS, washed again with PBS and placed in 0.2 M HCl for 10 min. After washing with PBS, the sections were acetylated by incubation in 0.1 M triethanolamine–HCl, pH 8.0, 0.25% acetic anhydride for 10 min. After washing with PBS, the sections were dehydrated through a series of ethanol. Hybridization was performed with digoxigenin-labeled RNA probes at a concentration of 100 ng/ml in Probe Diluent (GenoStaff Co., Ltd.) at 60°C for 16 h. After hybridization, the sections were washed in 5× HybriWash (GenoStaff Co., Ltd.), equal to 5× SSC, at 60°C for 20 min and then in 50% formamide, 2× HybriWash at 60°C for 20 min, followed by RNase treatment in 50 μg/ml RNaseA in 10 mM Tris–HCl, pH 8.0, 1 M NaCl and 1 mM EDTA. Then the sections were washed twice with 2× HybriWash at 60°C for 20 min, twice with 0.2× HybriWash at 60°C for 20 min and once with TBS containing 0.1% Tween20 (TBST). After treatment with 0.5% blocking reagent (Roche) in TBST for 30 min, the sections were incubated with anti-DIG AP conjugate (Roche) diluted to 1:1000 with TBST for 2 h. The sections were washed twice with TBST and then incubated in 100 mM NaCl, 50 mM MgCl2, 0.1% Tween20, 100 mM Tris–HCl, pH 9.5. Coloring reactions were performed with BM purple AP substrate (Roche) overnight and then washed with PBS. The sections were counterstained with Kernechtrot stain solution (Muto), dehydrated and then mounted with Malinol (Muto).
SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

ACKNOWLEDGEMENTS


Conflict of Interest statement: None declared.

FUNDING

The work presented here was supported by Nuvelo, Inc., San Carlos, CA, USA.

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