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Genetics of Ovarian Differentiation: *Rspo1*, a Major Player

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Abstract
In mammals, the sex of the embryo is determined during development by its commitment either to the male or female genetic program regulating testicular or ovarian organogenesis. Major steps towards unraveling sex determination in mammals are achieved by the identification of key genes involved in human pathologies and the application of mouse genetics to analyze their function. While the expression of *Sry* and *Sox9* is sufficient to induce the male developmental program, the molecular pathways that specify ovarian differentiation were unclear before the recent demonstration that mutations in the *RSPO1* gene induce female-to-male sex reversal in XX patients. By generating the corresponding mouse model, we have shown that *Rspo1* is so far the earliest known gene controlling the female genetic developmental program. Rspo1 activates the canonical β-catenin signaling pathway required for female somatic cell differentiation and germ cell commitment into meiosis. The aim of this review is to describe the roles of R-spondins (Rspo) in developmental processes and disorders and the current knowledge obtained from murine models. A particular focus will be on *Rspo1* and its crucial function in sex determination.

Overview of Testis Differentiation

The sex of an individual is determined by the fate of the gonad. This organ arises from two different structures: the coelomic epithelium and a mesenchymal part that forms from the mesonephros. The early embryonic gonad can differentiate into a testis or an ovary, thus suggesting that at an early stage the gonad is bipotential. Testis formation requires differentiation of Sertoli cells, which will form the supporting cell lineage of the seminiferous tubules. These cells synthesize Anti-Mullerian Hormone (AMH), which induces regression of the Mullerian duct, thus counteracting the development of female internal genitalia. Moreover, Sertoli cells favor recruitment of other somatic cell lineages migrating from the mesonephros that are also crucial for testis development to occur. The interstitial area of the testis contains the steroidogenic cells (Leydig cells), which have the function of producing androgens. These hormones stimulate the differentiation of internal and external genitalia of the male [for a review see Brennan and Capel, 2004].

Gonad differentiation depends on the paternal transmission of the sex chromosome. Thus, an XY embryo develops as a male, whereas an XX embryo becomes female. Most of the genes involved in this developmental pathway have been discovered from genetic studies of human XY sex-reversal. At a molecular level, the Y chromosome encodes a testis determining factor, SRY [Sinclair et al.,

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1990]. *SRY* (Sex determining Region of the Y chromosome) is so far the earliest known gene that is specifically expressed in the XY gonad. It encodes a protein that contains an HMG (High Mobility Group) DNA binding domain with high homology to the *SOX* gene family (*SRY*-related HMG-box gene). In mice, *Sry* becomes upregulated at E10.5 and its expression peaks at E11.5. *Sry* expression induces a variety of aspects of testis development including cell proliferation, migration, differentiation and glycogen accumulation. At a molecular level, Sry activates *Sox9,* a gene that also belongs to the *Sox* gene family. Previous genetic analysis shows that Sox9 is required for Sertoli cell differentiation [Chaboissier et al., 2004] and the expression of *Amh*, which represents a direct target of this transcription factor [De Santa Barbara et al., 1998].

Before E11.5, *Sox9* expression is weakly detected in the XY and XX bipotential gonad, but then becomes specifically up-regulated in the XY gonad. Up-regulation occurs both at the level of transcription and also by the Prostaglandin synthase (Pgds)/Prostaglandin D2 (PGD2) pathway [Malki et al., 2005]. After the transient expression of *Sry,* Fgf9 and Pgds signaling pathways are involved in the maintenance of *Sox9* expression in the Sertoli cells [Wilhelm et al., 2005; Kim et al., 2006].

In human, XY sex reversal is a genetically heterogeneous condition. Loss of function mutations of the *SRY* gene account for approximately 10 to 15% of the cases. In addition, mutations in *SOX9, SF1,* and at least twelve other loci have been implicated in XY sex reversal [Schafer et al., 1996; Achermann et al., 1999; Fleming and Vilain, 2005]. XX sex reversal is less common, and the majority of cases (80 to 85%) are caused by a translocation of *SRY* to another chromosomes. *SRY*-independent (*SRY* negative) XX males are extremely rare (approximately 1 in 200,000 newborn), with very few family cases and the majority arising in a sporadic manner. The low number of *SRY* negative XX sex reversals may explain why the identification of molecular pathways in ovarian differentiation has been lagging behind those of the male pathways.

Early Steps of Ovarian Development

In mice the first histologically detectable feature of ovarian differentiation is the commitment of germ cells to meiosis at E13.5. Meiosis progresses through leptotene and zygotene but then becomes blocked in pachytene I at E18.5. In sharp contrast, XY germ cells are quiescent G1 gonocytes at this stage. While histological features are only occurring at later stages, ovaries already show differences on a molecular level at a very early time point. For example *Wnt4* and *Foxl2,* being the most studied 'female genes' to date, are already up-regulated in the XX gonad between E11.5 and E13.5.

Molecular Basis of Ovarian Differentiation

Wnt4 is expressed in both sexes in the bipotential gonad, but becomes strongly up-regulated in the XX gonad at E11.5 [Kim et al., 2006]. Ablation of this gene triggers ectopic migration of endothelial cells from the mesonephros and steroidogenic cells from the adrenal anlage [Jeays-Ward et al., 2003]. Thus, XX *Wnt4*$^{-/-}$ embryos exhibit XY-like vascularization and produce androgens [Vainio et al., 1999; Heikkila et al., 2005]. At birth, Sertoli cell markers become expressed and rudimentary sex cords are formed. Further development is precluded by death of the embryos at birth due to kidney agenesis [Stark et al., 1994]. In human, a missense mutation in *WNT4* has been identified in an XX female patient with Mullerian-duct regression exhibiting virilization [Biason-Lauber et al., 2004].

Premature ovarian failure associated with craniofacial and eyelid abnormalities are symptoms encountered in XX Blepharophimosis/Ptosis/Epicanthus inversus Syndrome (BPES) type I patients. BPES is caused by mutations in the *FOXL2* gene leading to the production of a non functional truncated protein [Crisponi et al., 2001]. *FOXL2* belongs to the Forkhead box transcription factor family and is predominantly expressed in fetal and adult ovaries. FOXL2 activates the *CYP19* (aromatase) gene, which is expressed in the fetal ovary of goat and human, but is absent before birth in the mouse [Pannetier et al., 2006]. Two animal models recapitulate human BPES: the Polled/Intersex Syndrome (PIS) in goat, in which a deletion close to the *FOXL2* locus affects *FOXL2* transcription [Pailhoux et al., 2001], and mice engineered for *Foxl2* ablation [Schmidt et al., 2004; Uda et al., 2004]. In goat, the *PIS*$^{-/-}$ mutation leads to XX female-to-male sex reversal. In XX *Foxl2*$^{-/-}$ mouse, although *Foxl2* expression usually occurs in the medulla part of the XX gonad from E12.5, the first abnormalities appear at birth with arrested ovarian somatic cell differentiation. Subsequently, mutant mice exhibit ovarian atresia and infertility.

During the last few years Giovanna Camerino and colleagues have studied XX (*SRY* negative) men from a consanguineous Italian family. This recessive trait segregated with palmoplantar hyperkeratosis and an associated

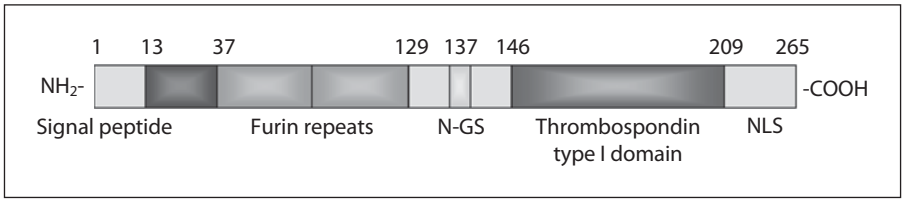

Fig. 1. Schematic representation of the Rspo1 structure. N-GS: putative N-glycosylation site; NLS: Nuclear Localization Signal.

Table 1. Disorders linked to *R-spondin* mutations in patients and their corresponding mouse models

Gene	Human diseases	Mouse phenotype	References
Rspo1	Sex reversal, palmoplantar hyperkeratosis, squamous cell carcinoma, upregulated in ovarian and stomach tumor samples	Sex reversal (*Rspo1* KO); intestinal crypt cell proliferation (*Rspo1* ectopic expression)	Parma et al., 2006 Kazanskaya et al., 2004 Chassot et al., 2008 Kim et al., 2005
Rspo2	Downregulated in colon, rectum, small intestine, lung and breast tumor samples	Malformations of laryngeal-tracheal cartilages, limbs, kidney and palate Lung hypoplasia (*Rspo2* KO)	Kazanskaya et al., 2004 Theodorou et al., 2007 Bell et al., 2008 Nam et al., 2007b Aoki et al., 2008
Rspo3	Downregulated in many tumor samples, upregulated in breast cancer	Placental defects (*Rspo3* KO)	Kazanskaya et al., 2004 Theodorou et al., 2007 Aoki et al., 2007
Rspo4	Anonychia		Blaydon et al., 2006

predisposition to squamous cell carcinoma. Genetic linkage analysis followed by sequencing led to the identification of a mutation within the *RSPO1* gene [Parma et al., 2006]. The proof that *RSPO1* mutations indeed underlie XX sex reversal came from the identification of a second independent mutation in a sporadic case with a similar case history. On the gonadal level, patients with *RSPO1* mutations have male external genitalia with hypospadias, severe hypogenitalism, or severe ambiguity of external genitalia. Gonadal biopsies showed the presence of testicular structures with Leydig cell nodular hyperplasia in the propositus and others of ovotestis with reminiscence of ovarian tissue [Micali et al., 2005; Radi et al., 2005; Tomaselli et al., 2008]. The variable phenotype and the presence of hypogenitalism, however, may point towards an insufficiency of male steroidogenic hormones.

The *R-Spondin* Family

Initial studies identified *Rspo1* as a gene expressed in the dorsal part of the neural tube, predominantly between the roof plate and the neuroepithelium in mice and it has consequently been named *Roof Plate-Specific Spondin* [Kamata et al., 2004]. *Rspo1* expression appears to be down-regulated in *Wnt1/3a* double knockout mice [Kamata et al., 2004]. Four members of the *R-spondin* family have been identified in the human and mouse genome [Chen et al., 2002; Kamata et al., 2004; Kazanskaya et al., 2004]. The structure of the 4 paralogs is very similar as all R-spondins contain an N-terminal signal peptide (SP), 2 furin-like domains (FU), one thrombospondin type 1 domain (TSP1), and a C-terminal low complexity region enriched with positively charged amino acids. This C-terminal region also contains a putative nuclear localization signal (NLS) (fig. 1).

TSP1 and FU proteins are involved in various molecular pathways. The relevant function of the TSP1 domain in *RSPO1* remains to be elucidated, but it is noteworthy that TSP1 has anti-angiogenic activity [Volpert et al., 2002]. The furin domains in R-spondins have been shown to be necessary for Wnt/β-catenin signaling [Kazanskaya et al., 2004]; both of these functions are impaired during gonadal development in XX *Rspo1*$^{-/-}$ mice (see further).

In situ hybridization analysis of these four genes demonstrated overlapping domains of expression in some places [Nam et al., 2007a]. Since all four members are able to activate the canonical signaling pathway at least in

vitro, it is likely that functional redundancy between individual R-spondins exists. However, certain expression domains are very specific, which is exemplified by *RSPO4* showing highly specific expression within the developing nails. In agreement, mutations in *RSPO4* lead to anonychia [Blaydon et al., 2006]. Moreover all *R-spondins* are crucial in developmental processes and their mutations result in dramatic phenotypes (table 1). *Rspo2* is required for the skeletal and respiratory system development, as well as limb and nail morphogenesis [Nam et al., 2007b; Aoki et al., 2008; Bell et al., 2008]. At a low penetrance, mutations in this gene are also associated with kidney agenesis and reduced fertility in aging heterozygous females. *Rspo2* mutant mice die at birth due to pulmonary defects.

Ablation of *Rspo3* is lethal at about E10 in mice, due to defective development of the labyrinthine layer of the placenta and placental vasculature [Aoki et al., 2007]. This early lethality precludes the analysis of *Rspo3* function at later stages of embryogenesis.

R-Spondins Are Activators of the Wnt/β-Catenin Signaling Pathway

Recent reports show that R-spondins are involved in the control of the Wnt/β-catenin signaling pathway. Binding of Wnt ligand to its receptors Frizzled and its transmembrane partners LRP triggers the formation of protein complexes containing Wnt/Frizzled/LRP/Dishevelled/Axin at the cell surface. This inhibits the phosphorylation function of a second complex containing Axin/GSK-3/APC proteins. β-catenin is no longer phosphorylated and subsequently is not degraded. After nuclear translocation, β-catenin can interact with TCF/LEF transcription factors to promote the expression of target genes.

Rspo2 has been identified in a screen for modulators of the Wnt/β-catenin pathway in *Xenopus* [Kazanskaya et al., 2004]. *Rspo2* acts by synergizing with the Wnt pathway to activate β-catenin. This study showed that *Rspo2* is associated with the cell surface. Moreover their data suggest that all the members of the R-spondin family are capable of regulating the Wnt/β-catenin pathway.

Kim et al. [2005] corroborate these findings by demonstrating that ectopic expression of *Rspo1* induces proliferation in the crypts of the intestine. Activation of canonical β-catenin signaling has been proposed to occur upon direct binding of Rspo1 to Lrp6 [Nam et al., 2006]. Further studies, however, indicate that Rspo1 is able to counteract Dkk1-mediated inhibition of Wnt/β-catenin signaling. Dkk1 inhibition is achieved by binding to Kremen, which results in downregulation of Lrp on the cell surface. R-spondins compete with Dkk1 for the binding to Kremen, which allows the Lrp6 receptor to become available to mediate Wnt signaling [Binnerts et al., 2007].

Induction of Male Development in the Absence of *Rspo1*

Analysis of *Rspo1* expression in the developing E11.5 gonads of mice showed that this gene is predominantly expressed in somatic cells of the XX gonads. In contrast, in XY gonads expression of *Rspo1* is mostly restricted to the coelomic epithelium, with only some interstitial cells showing weak staining from E12.5 onwards [Parma et al., 2006; Chassot et al., 2008] (fig. 2).

Loss of function studies showed that ablation of *Rspo1* triggers sex reversal of XX mice with the formation of ovotestis and hermaphroditism of male and female internal genitalia. Penetrance of the phenotype was slightly variable from one individual to another, and gonad morphology ranged from hypoplastic gonads to an ovotestis. Interestingly, the left gonad appears to be always more severely affected than the right in the same individual suggesting an effect of left/right asymmetry. To know whether reminiscent mechanisms of L/R asymmetry shown in birds are conserved in mice warrants further investigations [Guioli and Lovell-Badge, 2007]. At birth, histological analysis of XX *Rspo1*$^{-/-}$ gonads shows that they are smaller, contain clear seminiferous cords, but also arrangements of cells that only vaguely resemble rudimentary cords. In these cords, only few gonocytes could be detected. Interestingly, quiescent G1 gonocytes remained outside of cord structures. The gonadal phenotype was somewhat complex, with parts of the gonad showing cord structures, whereas others parts contained few nests of pachytene oocytes. This is also illustrated in Tomizuka et al. [2008] who used Scp3 as a marker to demonstrate that only a part of the germ cells initiated meiosis. In the adult 'male' part of the gonad, seminiferous tubules were devoid of germ cells (fig. 3). Indeed, dramatic death of these XX germ cells occurs shortly after birth, which is consistent with previous observations [Isotani et al., 2005].

Interestingly, somatic cells in XX *Rspo1*$^{-/-}$ gonads did not show male differentiation before E18.5. If activated at an early time of development, *Sox9* is able to induce male development, as shown in XX mice with ectopic expres-

sion of this gene [Bishop et al., 2000; Vidal et al., 2001]. Weak expression of *Sox9* was observed at earlier stages using qPCR, but the signal appeared to be insufficient to be visualized by in situ or immunostaining experiments [Chassot et al., 2008].

Strong *Sox9* activation and a concomitant differentiation of somatic cells into Sertoli cells only occurred around birth. Some questions however remain: why does *Sox9* become expressed at such a late time? Is there a signal that needs to build up to allow consistent expression of *Sox9?* To answer these questions it will be important to identify the activator of *Sox9* in a *Sry* negative environment. Furthermore, *Fgf9,* a gene required for testicular proliferation and cell migration, is not activated in the early stages of XX *Rspo1*$^{-/-}$ gonad development [Colvin et al., 2001], which may explain the abnormal growth and hypoplastic gonad (fig. 3).

Rspo1 Acts at the Beginning of the Female Determination Pathway

The phenotype of the XX *Rspo1*$^{-/-}$ mice shows similarities with the XX *Wnt4*$^{-/-}$ mutants suggesting that these genes act in the same molecular pathway. In order to test this hypothesis we studied the level of *Wnt4* expression in the XX *Rspo1*$^{-/-}$ gonads from E11.5 until 14.5. A dramatic down-regulation of *Wnt4* was observed compared to XX wild type gonads at E11.5 and after. The low level of *Wnt4* expression in XX *Rspo1*$^{-/-}$ gonads is actually due to the absence of up-regulation of *Wnt4* specifically in XX gonads, the basal non-specific level of *Wnt4* expression being unaffected. This indicates a requirement of Rspo1 for *Wnt4* up-regulation in XX gonads. Interestingly, *Wnt9a* expression also seems to be under the control of Rspo1. Redundant action of Wnt4 and Wnt9a in joint formation has been demonstrated [Später et al., 2006] and it is tempting to speculate that a similar overlapping role may also exist during ovarian development. It will therefore be interesting to analyze the gonadal phenotype in double mutant animals.

The first striking phenotype in XX *Rspo1*$^{-/-}$ mice is the formation of a coelomic vessel, as it was previously described for XX *Wnt4*$^{-/-}$ gonads. This raises the question whether *Rspo1* may have a similar anti-angiogenic activity as TSP1. Jeays-Ward et al. [2003] noticed the formation of an ectopic vessel in adrenal glands of *Wnt4*$^{-/-}$ embryos [Jeays-Ward et al., 2003]. *Rspo1* is not expressed in the adrenals and *Rspo1*$^{-/-}$ animals did not exhibit obvious abnormalities of the adrenals suggesting that the coelomic vessel formation results from lack of up-regulation of *Wnt4* in the *Rspo1*$^{-/-}$ gonads rather than a direct role of Rspo1.

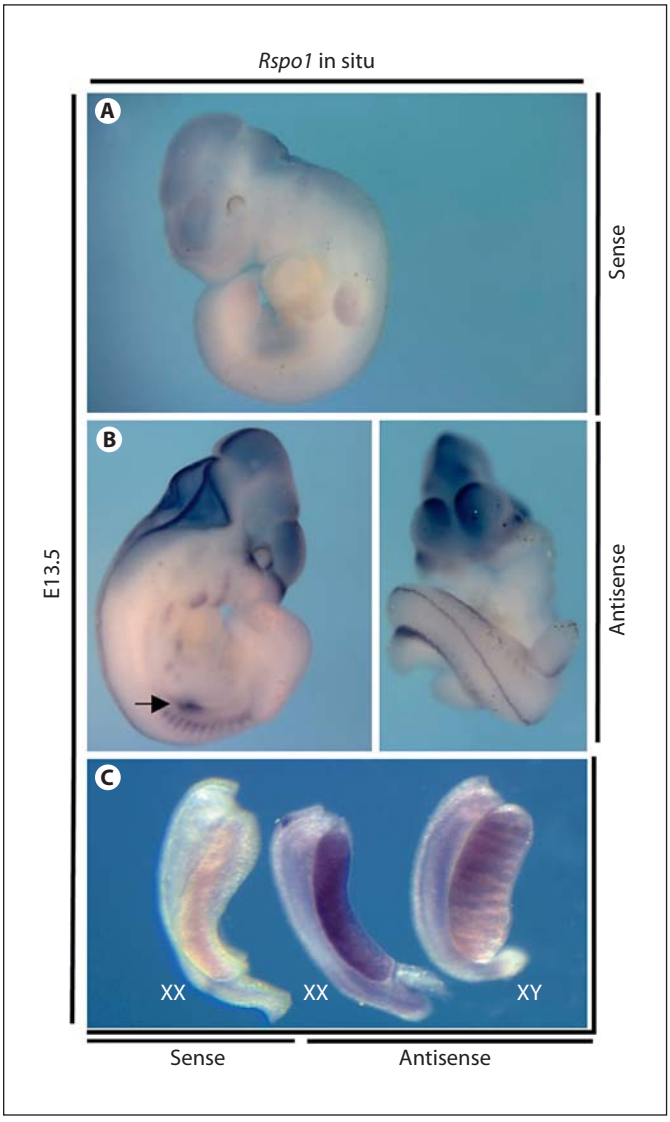

Fig. 2. *Rspo1* is specifically expressed in the XX gonad. Whole-mount in situ hybridizations of *Rspo1* with **A** sense (negative control) and **B** antisense *Rspo1* probes on whole XX embryo at E11.5 and **C** on dissected gonads and mesonephros from XX and XY embryos at E13.5. *Rspo1* was expressed throughout the XX gonads, whereas it was restricted to the coelomic epithelium and some interstitial cells in XY gonads. Arrow indicates the XX gonad in the whole embryo (**B**).

Another striking phenotype is the early presence of functional steroidogenic cells within the XX *Rspo1*$^{-/-}$ gonads. It is likely that these steroidogenic cells result from the lack of up-regulation of *Wnt4* and this phenotype re-

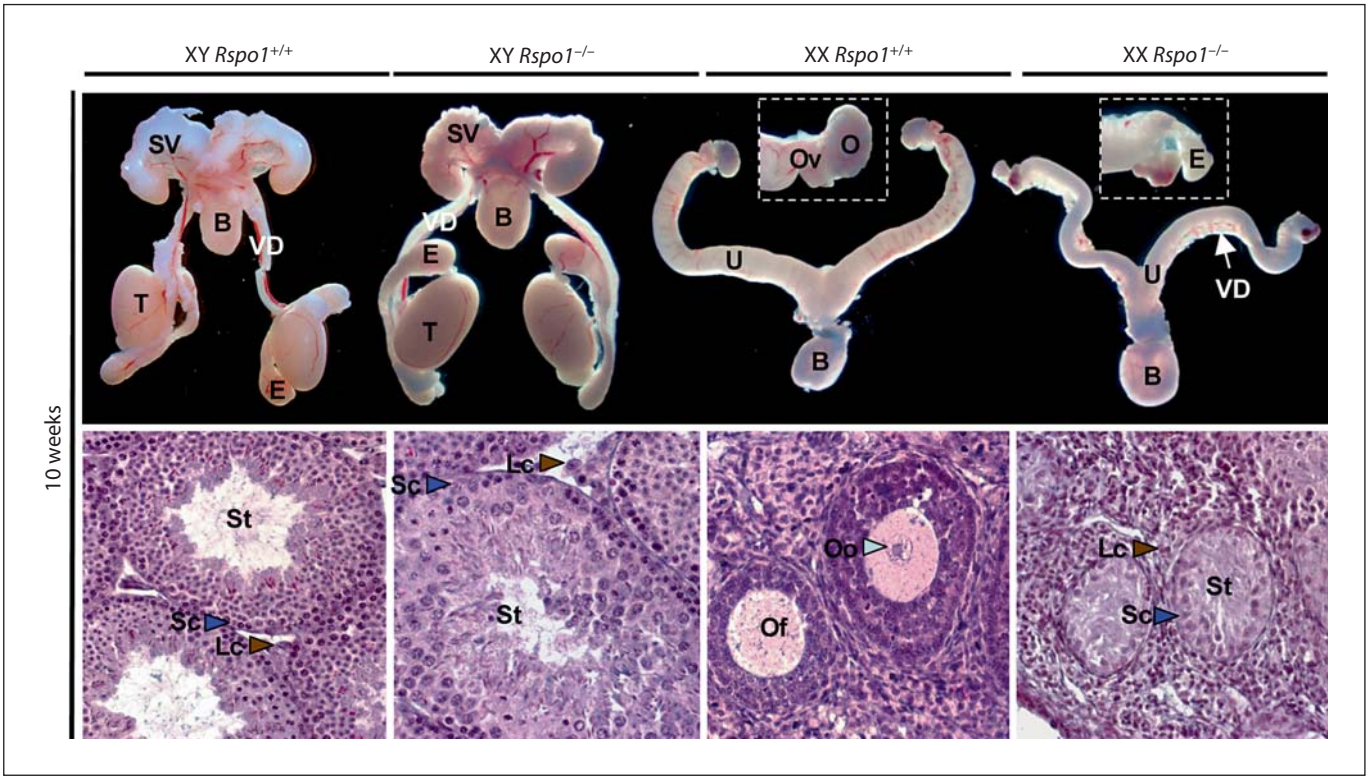

Fig. 3. Induction of a male development in the absence of *Rspo1*. Upper panels: Macroscopic view of XX and XY urogenital systems of 10 weeks old wild-type and *Rspo1*$^{-/-}$ mice from pure 129SV genetic background (magnification ×5; inset ×20). Note the persistence of Wolffian and Mullerian ducts and the presence of an ovotestis surrounded by an epididymis in XX *Rspo1*$^{-/-}$ animals (insets). B: bladder; E: epididymis; SV: seminal vesicles; O: ovary; Ov: oviduct; T: testis; U: uterus; VD: vas deferens. Lower panels: Haematoxylin and eosin staining of the gonads from the same animals (magnification ×40). Lc: Leydig cells; Of: ovarian follicle; Oo: oocyte; Sc: Sertoli cells; St: seminiferous tubules.

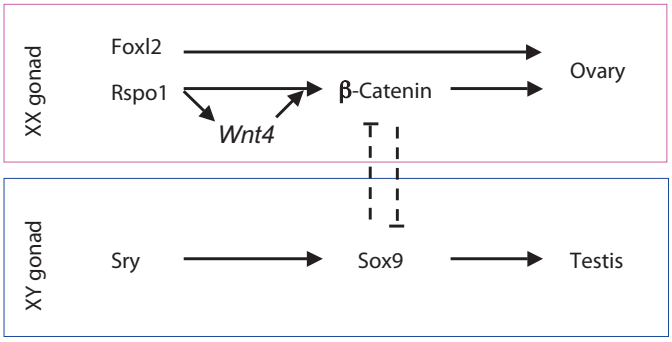

Fig. 4. A genetic model for sex determination. Sex determination is controlled by a balance between two antagonist pathways: while *Sry* stimulates *Sox9* expression leading to male differentiation, *Rspo1* induces *Wnt4* expression and β-catenin signaling to tip the balance to the female side. *Foxl2* acts through an independent pathway. Solid lines denote stimulations, dashed lines denote inhibitions.

capitulates that described in XX *Wnt4*$^{-/-}$ mice where adrenal precursors migrate into the gonads. In contrast to the complete absence of female genitalia in *Wnt4*$^{-/-}$ embryos, *Rspo1* knockout mice show hermaphrodism of the genitalia. This is in agreement with the fact that *Rspo1* does not seem to be expressed in the mesonephros, indicating that activation of *Wnt4* in this organ is independent of Rspo1.

In rare occasions (2 in our hands out of 10 tested), XX knockout animals are fertile and can have litters of a normal size. Analysis of the ovaries of these females shows the presence of ovotestis and hermaphrodism of the ducts. Despite normal litter sizes these females cannot nurture their pups due to defects in mammary gland development. Whether this is due to a direct effect of Rspo1 on Wnt/β-catenin signaling or as a result of perturbed hormonal signaling due to the abnormal gonads remains to be investigated.

Rspo1 Activates the Canonical β-Catenin Signaling Pathway

Given the involvement of *RSPO1* in sex reversal and its role as a modulator of the Wnt/β-catenin pathway, the question of β-catenin activation in XX differentiation became predominant. Expression analysis of various known targets of β-catenin demonstrated that this signaling pathway is mostly activated in somatic cells of XX gonads. In agreement with in vitro data, Rspo1 therefore acts as an activator of the canonical signaling pathway in ovarian development. Moreover, ectopic activation of β-catenin in gonads rescued the XX *Rspo1*$^{-/-}$ gonadal phenotype and resulted in fertile females with functional ovaries [Chassot et al., 2008]. These data clearly demonstrated that β-catenin signaling plays a key role in female sex determination.

Given these findings, it is important to further test whether β-catenin activation is sufficient to counteract male development. We have shown that ectopic expression of *Sox9* in XX transgenic gonads (XX *Wt1:Sox9*$^{Tr/+}$) is able to block β-catenin signaling [Chassot et al., 2008]. Moreover, a recent study demonstrates that stabilization of β-catenin in Sertoli cells from E14.5 onwards totally compromised expression of *Sox9* and its downstream target *Amh*. Subsequently, these embryos exhibit a progressive disruption of well-formed seminiferous tubules [Chang et al., 2008]. Interestingly, expression of the ovarian somatic marker *Wnt4* became up-regulated in these mice. These findings support a model of direct antagonism between the female and male pathways and is thus in line with an earlier study [Kim et al., 2006] and may suggest a direct competition between Sox9 and β-catenin as previously described for chondrogenesis [Akiyama et al., 2004] (fig. 4). Whether β-catenin activation is also sufficient to antagonize the early male sex determination process remains to be tested.

Germ Cells Fail to Enter Meiosis in XX *Rspo1*$^{-/-}$ Gonads

While the somatic phenotype in XX *Rspo1*$^{-/-}$ and *Wnt4*$^{-/-}$ gonads is comparable, there exist obvious differences in the fate of germ cells between these two mouse models. In XX *Rspo1*$^{-/-}$ gonads the majority of germ cells survives until birth, whereas ablation of *Wnt4* results in survival of only 10% of these cells. At first glance this may seem surprising since Rspo1 is required for female specific activation of *Wnt4*. However, detailed analysis showed that *Wnt4* expression is retained in both XX and XY gonads at a basal level in *Rspo1* mutants. This low level of expression might be sufficient to allow germ cells to survive in the XX *Rspo1*$^{-/-}$ gonads and thus explain the different phenotype. These data also indicate that *Wnt4* and possibly *Wnt9a* have a second, *Rspo1*-independent function.

While germ cells survive in early XX *Rspo1*$^{-/-}$ embryos, they are clearly affected, as they do not enter meiosis. Whether Rspo1 is directly or indirectly required to induce commitment to meiosis remains to be tested, but these findings emphasize the importance of germ cell environment in determining their fate. Previous studies suggested that a widespread factor favors meiosis commitment in XX gonads [McLaren and Southee, 1997]. Knockout experiments in mice demonstrated that *Stra8*, a gene activated by retinoic acid, is also required to induce meiosis [Baltus et al., 2006]. qPCR experiments did not show significant differences in *Stra8* expression between XX *Rspo1*$^{-/-}$ and control gonads. However, the variability of the phenotype prevents us to exclude the possibility that the observed meiotic block in *Rspo1* mutant mice is due to a direct perturbation of retinoid acid signaling.

Germ cells in XX *Rspo1*$^{-/-}$ gonads at E12.5–14.5 exhibit cell-cell adhesion complexes reminiscent of those found in prospermatogonia [Di Carlo and De Felici, 2000; Kimura et al., 2006; Chassot et al., 2008] and appear to be blocked in G1 phase at birth. Various experiments have shown the importance of the male environment in prospermatogonia differentiation. Thus, defects within Sertoli cell differentiation can allow XY germ cells to enter meiosis [Chaboissier et al., 2004; Best et al., 2008]. There is no doubt that ablation of *Rspo1* prevents correct differentiation of the somatic lineages in XX gonads. Thus, these cells could secrete a factor involved in meiosis blockage. However, XY germ cells that are lost in the mesonephros do not enter meiosis [McLaren et al., 1984] suggesting that the Sertoli environment is not the only environment that can prevent meiosis.

Moreover, the XX *Rspo1*$^{-/-}$ somatic lineage at E12.5–14.5 does not show robust expression of Sertoli markers *(Sox9, Amh)*. Since G1 gonocytes are visible before formation of obvious sex cords, the signal suppressing meiosis may be Sertoli-cell independent. Further studies will unravel the crosstalk that exists between somatic and germ cells in an XX *Rspo1*$^{-/-}$ gonad.

Rspo1 and *Foxl2*: Two Independent Pathways?

In mice, *Rspo1, Wnt4,* and *Foxl2* have a common role in antagonizing testis differentiation. *RSPO1* expression is not affected in *PIS*$^{-/-}$ goat and Kocer et al. [2008] suggested that *RSPO1* and *FOXL2* could act in distinct cell types in the early differentiating goat ovary. Interestingly, XX *Wnt4*$^{-/-}$; *Foxl2*$^{-/-}$ gonads also show a sex reversal phenotype, which appears to occur at an earlier time point than that of the simple mutant [Ottolenghi et al., 2007]. While *Wnt4* is required for early somatic cell differentiation, *Foxl2* is necessary for follicle formation. Thus, while male sex determination results from a single genetic pathway in which *Sry* activates *Sox9,* ovarian development appears to be a more complex process controlled by at least 2 parallel genetic programs: the Wnt4 and the Foxl2 pathway.

The identification of *RSPO1* by Giovanna Camerino and colleagues [Parma et al., 2006] has been incredibly important in advancing our understanding of sex determination. Not only has it become clear that sex differentiation is based on the antagonistic relationship [Kim et al., 2006; Chassot et al., 2008] between two very different molecular pathways (Sry/Sox9 and Rspo1/β-catenin), but it has also shed new light on the understanding of the molecular basis of what makes us a woman or a man. Mouse genetics have allowed us to place *Rspo1* as the most upstream gene required for ovarian differentiation, but the following years should unravel what controls the expression of *Rspo1* and how the Wnt/β-catenin pathway is influencing sex determination.

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References

Achermann JC, Ito M, Ito M, Hindmarsh PC, Jameson JL: A mutation in the gene encoding steroidogenic factor-1 causes XY sex reversal and adrenal failure in humans. Nat Genet 22: 125–126 (1999).

Akiyama H, Lyons JP, Mori-Akiyama Y, Yang X, Zhang R, et al: Interactions between Sox9 and beta-catenin control chondrocyte differentiation. Genes Dev 18:1072–1087 (2004).

Aoki M, Mieda M, Ikeda T, Hamada Y, Nakamura H, Okamoto H: R-spondin3 is required for mouse placental development. Dev Biol 301:218–226 (2007).

Aoki M, Kiyonari H, Nakamura H, Okamoto H: R-spondin2 expression in the apical ectodermal ridge is essential for outgrowth and patterning in mouse limb development. Dev Growth Differ 50:85–95 (2008).

Baltus AE, Menke DB, Hu YC, Goodheart ML, Carpenter AE, et al: In germ cells of mouse embryonic ovaries, the decision to enter meiosis precedes premeiotic DNA replication. Nat Genet 38:1430–1434 (2006).

Bell SM, Schreiner CM, Wert SE, Mucenski ML, Scott WJ, Whitsett JA: R-spondin 2 is required for normal laryngeal-tracheal, lung and limb morphogenesis. Development 135: 1049–1058 (2008).

Best D, Sahlender DA, Walther N, Peden AA, Adams IR: Sdmg1 is a conserved transmembrane protein associated with germ cell sex determination and germline-soma interactions in mice. Development 135:1415–1425 (2008).

Biason-Lauber A, Konrad D, Navratil F, Schoenle EJ: A *WNT4* mutation associated with Mullerian-duct regression and virilization in a 46,XX woman. N Engl J Med 351:792–798 (2004).

Binnerts ME, Kim KA, Bright JM, Patel SM, Tran K, et al: R-Spondin1 regulates Wnt signaling by inhibiting internalization of LRP6. Proc Natl Acad Sci USA 104:14700–14705 (2007).

Bishop CE, Whitworth DJ, Qin Y, Agoulnik AI, Agoulnik IU, et al: A transgenic insertion upstream of *Sox9* is associated with dominant XX sex reversal in the mouse. Nat Genet 26:490–494 (2000).

Blaydon DC, Ishii Y, O'Toole EA, Unsworth HC, Teh MT, et al: The gene encoding R-spondin 4 *(RSPO4)*, a secreted protein implicated in Wnt signaling, is mutated in inherited anonychia. Nat Genet 38:1245–1247 (2006).

Brennan J, Capel B: One tissue, two fates: molecular genetic events that underlie testis versus ovary development. Nat Rev Genet 5: 509–521 (2004).

Chaboissier MC, Kobayashi A, Vidal VI, Lutzkendorf S, van de Kant HJ, et al: Functional analysis of Sox8 and Sox9 during sex determination in the mouse. Development 131: 1891–1901 (2004).

Chang H, Gao F, Guillou F, Taketo MM, Huff V, Behringer RR: Wt1 negatively regulates β-catenin signaling during testis development. Development 135:1875–1885 (2008).

Chassot AA, Ranc F, Gregoire EP, Roepers-Gajadien HL, Taketo MM, et al: Activation of beta-catenin signaling by Rspo1 controls differentiation of the mammalian ovary. Hum Mol Genet 17:1264–1277 (2008).

Chen JZ, Wang S, Tang R, Yang QS, Zhao E, et al: Cloning and identification of a cDNA that encodes a novel human protein with thrombospondin type I repeat domain, hPWTSR. Mol Biol Rep 29:287–292 (2002).

Colvin JS, Green RP, Schmahl J, Capel B, Ornitz DM: Male-to-female sex reversal in mice lacking fibroblast growth factor 9. Cell 104: 875–889 (2001).

Crisponi L, Deiana M, Loi A, Chiappe F, Uda M, et al: The putative forkhead transcription factor *FOXL2* is mutated in blepharophimosis/ptosis/epicanthus inversus syndrome. Nat Genet 27:159–166 (2001).

De Santa Barbara P, Bonneaud N, Boizet B, Desclozeaux M, Moniot B, et al: Direct interaction of SRY-related protein SOX9 and steroidogenic factor 1 regulates transcription of the human anti-Mullerian hormone gene. Mol Cell Biol 18:6653–6665 (1998).

Di Carlo A, De Felici M: A role for E-cadherin in mouse primordial germ cell development. Dev Biol 226:209–219 (2000).

Fleming A, Vilain E: The endless quest for sex determination genes. Clin Genet 67:15–25 (2005).

Guioli S, Lovell-Badge R: PITX2 controls asymmetric gonadal development in both sexes of the chick and can rescue the degeneration of the right ovary. Development 134:4199–4208 (2007).

Heikkila M, Prunskaite R, Naillat F, Itaranta P, Vuoristo J, et al: The partial female to male sex reversal in Wnt-4-deficient females involves induced expression of testosterone biosynthetic genes and testosterone production, and depends on androgen action. Endocrinology 146:4016–4023 (2005).

Isotani A, Nakanishi T, Kobayashi S, Lee J, Chuma S, et al: Genomic imprinting of XX spermatogonia and XX oocytes recovered from XX<–>XY chimeric testes. Proc Natl Acad Sci USA 102:4039–4044 (2005).

Jeays-Ward K, Hoyle C, Brennan J, Dandonneau M, Alldus G, et al: Endothelial and steroidogenic cell migration are regulated by WNT4 in the developing mammalian gonad. Development 130:3663–3670 (2003).

Kamata T, Katsube K, Michikawa M, Yamada M, Takada S, Mizusawa H: R-spondin, a novel gene with thrombospondin type 1 domain, was expressed in the dorsal neural tube and affected in Wnts mutants. Biochim Biophys Acta 1676:51–62 (2004).

Kazanskaya O, Glinka A, del Barco Barrantes I, Stannek P, Niehrs C, Wu W: R-spondin2 is a secreted activator of Wnt/beta-catenin signaling and is required for *Xenopus* myogenesis. Dev Cell 7:525–534 (2004).

Kim KA, Kakitani M, Zhao J, Oshima T, Tang T, et al: Mitogenic influence of human R-spondin1 on the intestinal epithelium. Science 309:1256–1259 (2005).

Kim Y, Kobayashi A, Sekido R, Dinapoli L, Brennan J, et al: *Fgf9* and *Wnt4* act as antagonistic signals to regulate mammalian sex determination. PLoS Biol 4:e187 (2006).

Kimura T, Nakamura T, Murayama K, Umehara H, Yamano N, et al: The stabilization of beta-catenin leads to impaired primordial germ cell development via aberrant cell cycle progression. Dev Biol 300:545–553 (2006).

Kocer A, Pinheiro I, Pannetier M, Renault L, Parma P, et al: R-spondin1 and FOXL2 act into two distinct cellular types during goat ovarian differentiation. BMC Dev Biol 8:36 (2008).

Malki S, Nef S, Notarnicola C, Thevenet L, Gasca S, et al: Prostaglandin D2 induces nuclear import of the sex-determining factor SOX9 via its cAMP-PKA phosphorylation. EMBO J 24:1798–1809 (2005).

McLaren A, Southee D: Entry of mouse embryonic germ cells into meiosis. Dev Biol 187: 107–113 (1997).

McLaren A, Simpson E, Tomonari K, Chandler P, Hogg H: Male sexual differentiation in mice lacking H-Y antigen. Nature 312:552–555 (1984).

Micali G, Nasca MR, Innocenzi D, Frasin LA, Radi O, et al: Association of palmoplantar keratoderma, cutaneous squamous cell carcinoma, dental anomalies, and hypogenitalism in four siblings with 46,XX karyotype: a new syndrome. J Am Acad Dermatol 53: S234–239 (2005).

Nam JS, Turcotte TJ, Smith PF, Choi S, Yoon JK: Mouse cristin/Rspondin family proteins are novel ligands for the Frizzled 8 and LRP6 receptors and activate beta-catenin-dependent gene expression. J Biol Chem 281:13247–13257 (2006).

Nam JS, Turcotte TJ, Yoon JK: Dynamic expression of R-spondin family genes in mouse development. Gene Expr Patterns 7:306–312 (2007a).

Nam JS, Park E, Turcotte TJ, Palencia S, Zhan X, et al: Mouse R-spondin2 is required for apical ectodermal ridge maintenance in the hindlimb. Dev Biol 311:124–135 (2007b).

Ottolenghi C, Pelosi E, Tran J, Colombino M, Douglass E, et al: Loss of Wnt4 and Foxl2 leads to female-to-male sex reversal extending to germ cells. Hum Mol Genet 16:2795–2804 (2007).

Pailhoux E, Vigier B, Chaffaux S, Servel N, Taourit S, et al: A 11.7-kb deletion triggers intersexuality and polledness in goats. Nat Genet 29:453–458 (2001).

Pannetier M, Fabre S, Batista F, Kocer A, Renault L, et al: *FOXL2* activates P450 aromatase gene transcription: towards a better characterization of the early steps of mammalian ovarian development. J Mol Endocrinol 36: 399–413 (2006).

Parma P, Radi O, Vidal V, Chaboissier MC, Dellambra E, et al: R-spondin1 is essential in sex determination, skin differentiation and malignancy. Nat Genet 38:1304–1309 (2006).

Radi O, Parma P, Imbeaud S, Nasca MR, Uccellatore F, et al: XX sex reversal, palmoplantar keratoderma, and predisposition to squamous cell carcinoma: genetic analysis in one family. Am J Med Genet A 138:241–246 (2005).

Schafer AJ, Foster JW, Kwok C, Weller PA, Guioli S, Goodfellow PN: Campomelic dysplasia with XY sex reversal: diverse phenotypes resulting from mutations in a single gene. Ann NY Acad Sci 785:137–149 (1996).

Schmidt D, Ovitt CE, Anlag K, Fehsenfeld S, Gredsted L, et al: The murine winged-helix transcription factor Foxl2 is required for granulosa cell differentiation and ovary maintenance. Development 131:933–942 (2004).

Sinclair AH, Berta P, Palmer MS, Hawkins JR, Griffiths BL, et al: A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. Nature 346:240–244 (1990).

Später D, Hill TP, Gruber M, Hartmann C: Role of canonical Wnt-signalling in joint formation. Eur Cell Mater 12:71–80 (2006).

Stark K, Vainio S, Vassileva G, McMahon AP: Epithelial transformation of metanephric mesenchyme in the developing kidney regulated by Wnt-4. Nature 372:679–683 (1994).

Tomaselli S, Megiorni F, De Bernardo C, Felici A, Marrocco G, et al: Syndromic true hermaphroditism due to an Rspondin1 *(RSPO1)* homozygous mutation. Hum Mutat 29:220–226 (2008).

Tomizuka K, Horikoshi K, Kitada R, Sugawara Y, Iba Y, et al: Rspondin1 plays an essential role in ovarian development through positively regulating Wnt-4 signaling. Hum Mol Genet 17:1278–1291 (2008).

Uda M, Ottolenghi C, Crisponi L, Garcia JE, Deiana M, et al: *Foxl2* disruption causes mouse ovarian failure by pervasive blockage of follicle development. Hum Mol Genet 13:1171–1181 (2004).

Vainio S, Heikkila M, Kispert A, Chin N, McMahon AP: Female development in mammals is regulated by Wnt-4 signalling. Nature 397: 405–409 (1999).

Vidal VP, Chaboissier MC, de Rooij DG, Schedl A: *Sox9* induces testis development in XX transgenic mice. Nat Genet 28:216–217 (2001).

Volpert OV, Zaichuk T, Zhou W, Reiher F, Ferguson TA, et al: Inducer-stimulated Fas targets activated endothelium for destruction by anti-angiogenic thrombospondin-1 and pigment epithelium-derived factor. Nat Med 8:349–357 (2002).

Wilhelm D, Martinson F, Bradford S, Wilson MJ, Combes AN, et al: Sertoli cell differentiation is induced both cell-autonomously and through prostaglandin signaling during mammalian sex determination. Dev Biol 287:111–124 (2005).