REPRODUCTION

REVIEW

R-spondin1, WNT4, and the CTNNB1 signaling pathway: strict control over ovarian differentiation

Anne-Amandine Chassot[1,2], Isabelle Gillot[1,2] and Marie-Christine Chaboissier[1,2]

[1]*University of Nice-Sophia Antipolis, Parc Valrose, F-06108 Nice, France and* [2]*UMR–INSERM1091, IBV, F-06108 Nice, France*

Correspondence should be addressed to A-A Chassot; Email: chassot@unice.fr or to M-C Chaboissier; Email: marie-christine.chaboissier@unice.fr

Abstract

Sex differentiation is a unique developmental process. Starting from a bipotential gonad, it gives rise to the ovary and the testis, two highly specialized organs that differ morphologically and physiologically despite sharing common reproductive and endocrine functions. This highlights the specific plasticity of the gonadal precursors and the existence of complex antagonistic genetic regulation. Mammalian sex determination is controlled by paternal transmission of the Y-linked gene, sex-determining region Y (*SRY*). Using mouse models, it has been shown that the main role of *Sry* is to activate the expression of the transcription factor *Sox9*; either one of these two genes is necessary and sufficient to allow testicular development through Sertoli cell differentiation. Thus, defects in *SRY/Sry* and/or *SOX9/Sox9* expression result in male-to-female sex reversal of XY individuals. Molecular mechanisms governing ovarian differentiation remained unknown for a long time, until the discovery of the roles of R-spondin1 (RSPO1) and WNT4. In XX individuals, activation of the β-catenin signaling pathway by the secreted proteins RSPO1 and WNT4 is required to allow granulosa cell differentiation and, in turn, ovarian differentiation. Thus, mutations in *RSPO1* result in female-to-male sex reversal of XX patients, and mouse models have allowed the identification of genetic cascades activated by RSPO1 and WNT4 to regulate ovarian development. In this review, we will discuss the respective roles of RSPO1, WNT4, and the β-catenin signaling pathway during ovarian differentiation in mice.

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WNT4 and R-spondin1: state of the art

WNT/β-catenin signaling and the ovary: a woman's story?

It has long been assumed that the female outcome was the default state during sexual development. This came from the work of the endocrinologist Alfred Jost, who carried out castration experiments of rabbit embryos and observed that the external genitalia of both male and female fetuses developed as female (Jost 1947). As a result, the majority of laboratories focused their research on finding the molecular clues triggering male development. In the 1990s, the male-determining factor was discovered and it has been shown that male fate of the gonad depends on expression of the sex-determining region Y (*SRY*) gene, localized to the Y chromosome transmitted by the father (Sinclair *et al*. 1990). While male differentiation began to be elucidated, the study of ovarian development received very little attention and remained quite mysterious. More recently, however, the view of a 'default' female pathway has been challenged by the description of genetic mutations leading to masculinization of XX gonads despite the absence of any 'male' genes in human XX patients (Parma *et al*. 2006, Mandel *et al*. 2008), in mice (Vainio *et al*. 1999), and in goats (Pailhoux *et al*. 2001). Most of these mutations affect the WNT/β-catenin signaling pathway that controls various steps in mammalian organogenesis and organ homeostasis during adulthood (Niehrs 2012, de Lau *et al*. 2014). In 2006, Prof. Giovanna Camerino *et al*. identified R-spondin1 (RSPO1), a WNT signaling pathway activator, as a novel key factor involved in sexual and ovarian differentiation (Parma *et al*. 2006). After 2 years, Prof. Blanche Capel *et al*. showed that genetic induction of WNT/β-catenin signaling can modify the fate of the gonad by promoting the ovarian development from a gonad programed to become a testis (Maatouk *et al*. 2008). This complemented their previous work on *Wnt4*, another player in the WNT signaling pathway, which showed that sex determination is governed by a balance between two antagonistic pathways (Kim *et al*. 2006) and firmly established WNT/β-catenin signaling as a key pathway involved in female differentiation. In this review, we will discuss the role of WNT4 and RSPO1, the best studied WNT signaling activators in sexual differentiation. We will

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also report the data obtained in other species and discuss some of the key questions that remain to be answered.

WNT4, a Wingless family gene involved in many biological processes

The first Wingless gene was discovered in the 1970s in genetic screens aiming to identify genes essential for segment patterning of the *Drosophila* embryo. Since then, numerous studies have shown the implication of Wingless in a wide spectrum of biological processes. WNT family members (for Wingless-type MMTV integration site family) are highly conserved secreted glycoproteins. Although it was assumed that Wingless was a long-range acting protein, recent data have indicated that, at least in *Drosophila*, it acts predominantly on neighboring cells (Alexandre *et al*. 2014). In mammals, 19 secreted WNT ligands have been identified whose function entails binding to Frizzled receptors and LRP co-receptors ((Niehrs 2012) and references herein). Depending on the WNT ligand and its association with Frizzled and LRP, different pathways may be activated.

WNT3A is known to activate the so-called canonical WNT/β-catenin signaling pathway that eventually leads to stabilization of CTNNB1 (β-catenin) and transcription of its targets. By contrast, other WNT proteins can induce CTNNB1-independent pathways including the planar cell polarity (PCP) and WNT/ROR pathways ((Niehrs 2012) and references herein). The cellular context is essential for the choice of the pathway to be activated as exemplified by WNT4. While WNT4 is able to activate canonical WNT signaling in gonadal development (Maatouk *et al*. 2008, Liu *et al*. 2010), it appears to induce CTNNB1-independent pathways in the kidney (Burn *et al*. 2011). In addition to its role in canonical WNT signaling, CTNNB1 is also an important component of adherent junctions, which regulates patterning and morphogenesis. This process is required for gonadogenesis (Fleming *et al*. 2012) and it has been suggested that WNT4 is involved in CTNNB1 relocalization to the cell membrane during ovarian development (Naillat *et al*. 2010).

In female patients, heterozygous missense mutations in the *WNT4* gene lead to a syndrome characterized by the absence of uterine and fallopian tubes and clinical signs of excess androgen, indicating that WNT4 is required for female reproductive tract development (Biason-Lauber *et al*. 2004). In mice, *Wnt4* has been shown to be important for multiple morphogenetic processes, including formation of the kidney, adrenal and mammary glands and the reproductive tract where it regulates endothelial and steroidogenic cell migration (Jeays-Ward *et al*. 2003), sex determination (Kim *et al*. 2006), and female development (Vainio *et al*. 1999).

RSPO family: four members with a similar structure, activating different receptors and regulating various signaling pathways

The activators of the WNT/β-catenin pathway include RSPO proteins. A few years ago, *Rspo1* (roof plate-specific spondin) was the first member of the RSPO family to be identified from screening of genes specifically expressed in the developing spinal cord. It was named for its expression in the roof plate of the neural tube of the mouse embryo and the presence of a protein domain that shares homology with thrombospondin (Kamata *et al*. 2004). That same year, *Xenopus* R-spondin2 was isolated in a functional screen for its property to activate WNT signaling (Kazanskaya *et al*. 2004). Since then, numerous studies have focused on the RSPO family. This family comprises four members (RSPO1, RSPO2, RSPO3, and RSPO4) with pleiotropic functions in embryogenesis, development, and tumorigenesis (Yoon & Lee 2012).

All four RSPOs are cysteine-rich (CR) secreted proteins containing a single thrombospondin type 1 repeat domain. The amino acid sequences of the RSPO proteins are highly conserved (40–60%), especially within vertebrate species, and the four members of the mammalian RSPO family have a similar domain organization (Kamata *et al*. 2004, Kazanskaya *et al*. 2004).

Although RSPOs contain an amino-terminal signal peptide, these secreted glycoproteins remain associated with the cell surface (Kazanskaya *et al*. 2004). This suggests that RSPOs act mainly as juxtacrine factors. Nevertheless, the missing link for clarifying how RSPOs activate the WNT signaling pathway remained yet to be found. In 2011, it has been demonstrated that RSPOs stimulate WNT signaling by binding to the leucine-rich repeat-containing G protein-coupled receptors LGR4, LGR5, and LGR6 (de Lau *et al*. 2011). RSPO can also bind the transmembrane RING-type E3 ubiquitin ligases ZNRF3 or its homolog RNF43, two negative-feedback regulators of WNT signaling leading to their clearance at the membrane level (Koo *et al*. 2012). Upon binding, degradation by ubiquitination of the WNT receptors can no longer occur and canonical WNT signaling becomes activated (de Lau *et al*. 2014). As RSPO1 can bind to ZNRF3 independent of LGR4, this suggests that RSPO1 could transduce WNT signaling without interacting with LGR receptors. However, RSPO1 binds ZNRF3 or RNF43 with a low affinity that is highly enhanced when RSPO1 is already bound to LGR. This implies that LGRs act as recruitment receptors for RSPOs at the membrane. The CR domain of RSPO is necessary and sufficient for this interaction ((de Lau *et al*. 2014) and references herein).

Moreover, RSPOs can also bind some cell membrane heparan sulfate proteoglycans such as syndecans. Thus, RSPO2 and RSPO3 bind syndecan 4 through their TSP domain (Glinka *et al*. 2011). In combination with

WNT5A, this leads to the activation of the MAP kinase JNK and WNT/PCP signaling pathways during *Xenopus* gastrulation.

LGRs can also activate non-canonical WNT signaling including the WNT/PCP, G-RhoGTPase pathways (Glinka *et al*. 2011) and can act independent of RSPO (Fafilek *et al*. 2013). Whether RSPOs have receptors other than LGR remains yet to be elucidated.

RSPO, a family of genes regulating morphogenesis and maintenance of numerous organs

RSPOs are involved in both embryonic development and homeostasis of adult tissue in many species. Disruption of the human *RSPO1* gene in a recessive syndrome is characterized by female-to-male sex reversal, palmo-plantar hyperkeratosis, a predisposition to squamous cell carcinoma, corneal opacity, onychodystrophy, and seminoma (Parma *et al*. 2006, Tomaselli *et al*. 2008). This suggests that RSPO1 functions as a tumor suppressor. However, RSPO1 can act as a potent specific mitogen by promoting the proliferation of intestinal cells (Kim *et al*. 2005). During development, *Rspo1* plays key roles in sex determination and ovarian differentiation (Chassot *et al*. 2008*a*, Tomizuka *et al*. 2008). *Rspo1* has also been shown to be involved in mammary gland formation in mice and angiogenesis in zebrafish. In addition to its different roles in embryogenesis, this gene can also promote organ homeostasis, as evidenced by its role in β-cell neogenesis in the pancreas (Gore *et al*. 2011, Yoon & Lee 2012).

The importance of *Rspo2* in development has also been revealed by loss-of-function experiments. Indeed, null mutations of *Rspo2* cause a variety of abnormalities in branchial arches, lungs, and limbs in mice (Yoon & Lee 2012). Abnormal expression of *RSPO2* or *RSPO3* has been identified in 10% of colorectal cancer cases (Wu *et al*. 2014). Moreover, *RSPO2* can also induce proliferation of another epithelium, the epidermis during keloid scarring (Chua *et al*. 2011). *Rspo3* ablation in mice is lethal at 10 days post coitum (dpc). Although this has led to the description of this gene's key roles in vasculogenesis, angiogenesis, and blood cell speciation (Kazanskaya *et al*. 2008), it has precluded, thus far, the analysis of its potential role in later development. The congenital mutations in *RSPO4*, the last member of the RSPO family, result in anonychia in humans (Blaydon *et al*. 2006).

In the future, it is likely that new physiological functions of RSPOs in organogenesis and maintenance of organs will be unraveled.

Sex determination: two sexes, common progenitors

Sex differentiation is a unique developmental process. Starting from an undifferentiated bipotential gonad, it gives rise to two very specialized organs, the ovary and the testis (Fig. 1). Initially, the morphogenesis of the gonadal primordium appears to be similar between male and female embryos, although the transcriptomic signature is different between both sexes in mice (Jameson *et al*. 2012*a*), in which the genital ridges are formed at ∼9.5 dpc (reviewed by Svingen & Koopman (2013)). Proliferation of coelomic epithelial cells, combined with the recruitment of precursor cells from the mesonephroi, leads to the outgrowth of future gonads. The early somatic precursors are specified and proliferate between 10.5 and 12.0 dpc, corresponding to

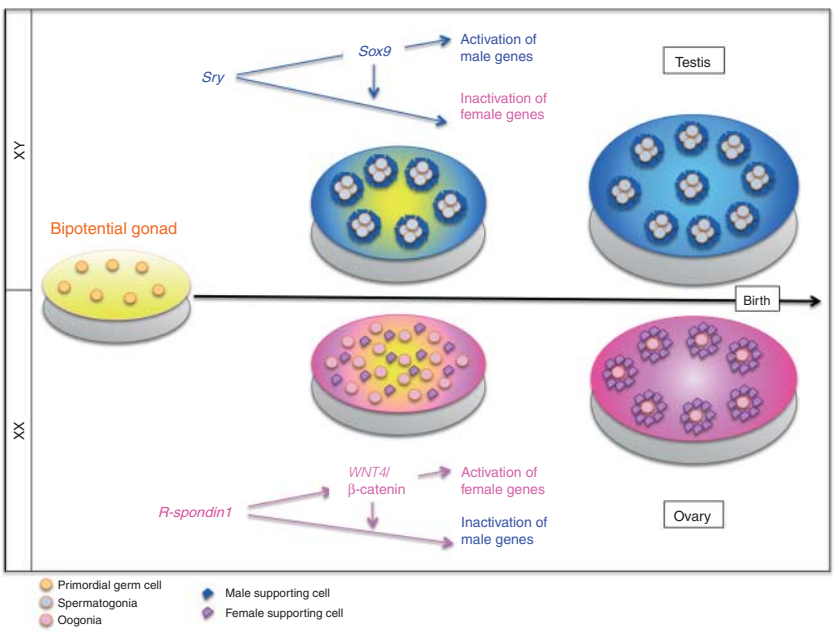

Figure 1 Current model of sex determination. In the embryonic testis (top), *Sry* promotes *Sox9* expression, which in turn induces Sertoli cell differentiation. In fetal ovaries (bottom), RSPO1, a direct WNT4/β-catenin pathway activator, regulates granulosa cell differentiation. Female genes are repressed while male genes are upregulated to trigger the development of a testis. By contrast, in the developing ovary, male genes need to be repressed, while female genes are upregulated (Jameson *et al*. 2012*b*).

~6th week of gestation in humans (Schmahl *et al*. 2004). At this stage, the gonadal primordium is morphologically similar in male and female embryos. The cells localized to the coelomic domain delaminate and enter the underlying mesenchyme to differentiate into Sertoli or pre-granulosa cells, and a few interstitial cells in XY and XX gonads respectively (Karl & Capel 1998). Thus, the supporting cell types, Sertoli cells in males and granulosa cells in females, stem from a common progenitor population present in the bipotential gonad before its commitment to a testicular or ovarian fate. In the same way, interstitial cell types, Leydig cells in males and theca cells in females, are thought to derive from a common progenitor (Albrecht & Eicher 2001). Supporting and interstitial cell types are responsible for germ cell support and hormone production in the gonad.

In the gonadal primordium, the insulin/insulin-like growth factor (IGF) pathway promotes the expression of key genes involved in gonad formation and sex determination, such as the orphan nuclear receptor SF1 (Pitetti *et al*. 2013), also known as nuclear receptor subfamily group A member 1 (NR5A1). In turn, *Sf1/Nr5a1* expression controls the differentiation into gonadal precursor cells. Moreover, MAP kinase signaling cascades, especially p38MAPK and MAP3K4, lead to the expression of *Sry* specifically in the XY gonad (Warr *et al*. 2012). SIX1 and SIX4 transcription factors are also involved both in gonadal precursor differentiation through *Sf1/Nr5a1* upregulation, and in *Sry* upregulation through direct regulation of *Zfpm2* (*Fog2*) expression, a zing finger protein interacting with GATA4 to stimulate *Sry* transcription (Fujimoto *et al*. 2013).

Following *Sry* expression, a genetic cascade is induced, triggering a testis-specific wave of proliferation of pre-Sertoli cells. Together with SF1, SRY promotes the expression of another Sry-related HMG box (*SOX*) gene, *Sox9*. SOX9 induces Sertoli cell differentiation and, in turn, testis differentiation (Chaboissier *et al*. 2004, Sekido & Lovell-Badge 2008). Their expression is then relayed by additional factors ensuring testis development ((Svingen & Koopman 2013) and references herein).

From the bipotential gonad to the ovary: roles of RSPO1 and WNT4

In the bipotential gonad, the insulin family of growth factors and its related receptors are required for gonadal differentiation in both sexes (Pitetti *et al*. 2013). XX or XY embryos, deficient for both insulin receptor (*Insr*) and IGF receptor 1 (*Igf1r*) genes, are characterized by smaller gonads that remain undifferentiated for several days before activation of the ovarian genetic program. In this delayed gonad, expression of *Sf1* is decreased and proliferation of the somatic cell progenitors severely affected, indicating that the insulin/IGF signaling is participating in early proliferation of the somatic precursor cells (Pitetti *et al*. 2013). Similarly, ablation of both *Six1* and *Six4* genes triggers the development of hypoplastic gonads, suggesting that they are also required for the proliferation of Sertoli or granulosa cell progenitors (Fujimoto *et al*. 2013).

Rspo1 and *Wnt4* also participate together in the early coelomic proliferation and mutant mice, deficient for both of these genes (*Rspo1*$^{-/-}$*; Wnt4*$^{-/-}$ embryos), display a significant decrease in coelomic cell proliferation between 10.5 and 11.5 dpc (Chassot *et al*. 2012). Nevertheless, *Sf1* expression is not significantly downregulated in the *Rspo1*$^{-/-}$*; Wnt4*$^{-/-}$ gonads, suggesting that RSPO1 and WNT4 act downstream of SIX1, SIX4, and SF1. Moreover, *Wnt4* expression is significantly reduced in *Insr; Igf1r* double mutant ovaries (Pitetti *et al*. 2013), implying either that *Wnt4/Rspo1* expression depends directly on the insulin signaling pathway, or that the low level of *Wnt4* expression indirectly results from decreased cell proliferation of *Insr; Igf1r* mutant gonads. However, *Igf1r* expression is strongly reduced in the *Rspo1*$^{-/-}$*; Wnt4*$^{-/-}$ gonads (Chassot *et al*. 2012), suggesting that potential feedback interactions may exist between these two signaling pathways.

The proliferation defect of the *Rspo1*$^{-/-}$*; Wnt4*$^{-/-}$ gonads is observed independent of the sex of the embryo. Nevertheless, this process is more severely impaired in mutant XY embryos than in mutant XX embryos. In normal XX embryos, the pre-granulosa cells, which are devoid of *Sry* expression, display ovarian-specific upregulation of cell cycle inhibitors such as p21^{kip1}, p57^{kip1}, and p27^{kip1} at 11.5 and 12.5 dpc, which may maintain their proliferation rates at a lower level than the proliferation of XY precursor cells (Cederroth *et al*. 2007). In addition, in XY embryos, proliferation of coelomic epithelial cells is activated following the expression of *Sry* and its downstream effectors, leading to an increase in testis size (Schmahl *et al*. 2004). Defects of proliferation in XY *Rspo1*$^{-/-}$*; Wnt4*$^{-/-}$ gonads lead to hypoplastic testis development.

As described above, RSPO1 is an activator of the β-catenin signaling pathway. Activation of the WNT/β-catenin signaling pathway is detected in the coelomic epithelium of the bipotential gonad in XY and XX embryos at 11.5 dpc, suggesting that WNT4/RSPO1 may activate proliferation of these cells through WNT signaling (Chassot *et al*. 2012). At this early stage, *Lgr5*, a recruitment receptor of RSPO1, and *Lrp4*, a WNT co-receptor involved in epithelial–mesenchymal cell communication during development, are specifically enriched in gonadal XY somatic precursor cells (Jameson *et al*. 2012*a*). However, one cannot exclude that RSPO1 and WNT4 act through different receptors. Nevertheless, no functional study of potential receptors has been carried out in the gonad and it remains to be shown that LGRs act as RSPO1 receptors in this organ. How RSPO1

and WNT4 signal synergistically in the bipotential gonad remains another open question.

Rspo1, *Wnt4*, and female sex determination

RSPO1, WNT4, and WNT/β-catenin signaling: an ovarian-specific activation

At 12.5 dpc, *Rspo1* and *Wnt4* expression becomes ovary specific (Vainio *et al*. 1999, Parma *et al*. 2006). RSPO1 and WNT4 are secreted by the somatic cells of the ovary. In addition, RSPO1 expression is detected at the membrane of both somatic and germ cells at 12.5 and 14.5 dpc (Kocer *et al*. 2008, Smith *et al*. 2008), suggesting that it may act on both cell types (see below).

At 12.5 dpc, *Wnt4* expression is abolished in the *Rspo1*$^{-/-}$ gonad, whereas *Rspo1* expression is maintained in the *Wnt4*$^{-/-}$ gonad, suggesting that RSPO1/WNT signaling promotes the maintenance of *Wnt4* expression (Chassot *et al*. 2008*a*, Tomizuka *et al*. 2008). This loss of *Wnt4* expression might highlight abnormal differentiation of pre-granulosa cells.

Following *Rspo1* and *Wnt4* expression, WNT/β-catenin signaling is activated in a sex-specific manner in somatic and germ cells of the ovary from 12.5 dpc onwards, as evidenced by *Axin2* expression, a universal activation marker for this signaling pathway (Chassot *et al*. 2011, Jameson *et al*. 2012*a*). In XY gonads, WNT/β-catenin signaling is downregulated following *Sry* expression. Consistent with this observation, *in vitro* studies have shown that SRY can antagonize CTNNB1 by physically interacting with it at the protein level, thus targeting CTNNB1 to nuclear bodies, to trigger its degradation and inhibit CTNNB1-mediated transcriptional activity (Bernard *et al*. 2008). Nevertheless, this mechanism has not yet been confirmed *in vivo* during sex determination. Conversely, ectopic activation of CTNNB1 in XY somatic cells results in male-to-female sex reversal by disrupting the testis fate and promoting ovarian development. This study established that β-catenin signaling is a female-determining pathway (Maatouk *et al*. 2008; Fig. 1). However, the conditional knockout of *Ctnnb1* in somatic tissue of the ovary does not impair ovarian differentiation (Manuylov *et al*. 2008, Liu *et al*. 2009), suggesting that either the ablation of *Ctnnb1* must occur within all ovarian cell types (somatic and germ cells) to promote sex reversal, or *Rspo1* and *Wnt4* activate pathways other than the WNT/CTNNB1 signaling pathway.

It should also be noted that, in contrast to *Axin2*$^{+/LacZ}$ reporter, no positive staining was observed with the TOPGAL mouse model, another reporter line for β-catenin signaling (reporter lines are described by Barolo (2006)). TOPGAL mice carry three multimerized LEF/TCF consensus binding sites in front of the *LacZ* reporter, which may suggest that CTNNB1 in the developing gonad may act in a LEF/TCF-independent way (A A Chassot, unpublished data).

Rspo1 and *Wnt4* control WNT/β-catenin activation in the XX gonads as evidenced by rescue experiments (Chassot *et al*. 2008*a*, Maatouk *et al*. 2008, Liu *et al*. 2010). However, in the absence of *Wnt4* or *Rspo1*, *Ctnnb1* transcriptional activity is only partially decreased as *Axin2* remains expressed in the coelomic region of the XX gonads (Chassot *et al*. 2012), suggesting that there remain other positive regulators of WNT/β-catenin signaling to be identified.

Sex determination is governed by two antagonistic pathways, taken together, involving, on the one hand, WNT signaling, which promotes female differentiation, and, on the other hand, *Sry/Sox9/Fgf9*, which activate male development (Sekido & Lovell-Badge 2008, Jameson *et al*. 2012*b*, Lavery *et al*. 2012; Fig. 1).

Lack of Rspo1 *and* Wnt4 *both leading to partial sex reversal*

Human 46, XX *SRY*-negative patients carrying mutations in the *RSPO1* gene display male-to-female sex reversal with hypospadia and hypogenitalism (Parma *et al*. 2006). Histological analysis of one gonad from another XX patient carrying an *RSPO1* homozygous mutation revealed the presence of both testicular and ovarian tissues (Tomaselli *et al*. 2008), indicating that sex reversal was partial. Moreover, seminiferous tubules yield germ cell neoplasia and tumor cells were found in the stroma. The ovarian part contained corpora albicans, indicating the presence of a former follicle. Consistent with this finding, adult XX *Rspo1*$^{-/-}$ mice display sex reversal and are characterized by ovotestes in which the testicular part, evidenced by the presence of male-like seminiferous tubules with Sertoli cells and interstitial tissue, can form the main part of the mutant gonad, giving it the appearance of a hypoplastic testis (Chassot *et al*. 2008*b*). Within the ovarian part, the presence of large abnormal cysts was noticed in 11-week-old mice (Chassot *et al*. 2008*a*). No tumors have been described in these mutant mice; this could be due, however, to genetic background susceptibility as shown for other mutant models (Cook *et al*. 2011).

Homozygous mutations of *WNT4* in XX patients are associated with developmental defects such as renal and adrenal agenesis and female-to-male sex reversal (Mandel *et al*. 2008). Histological analysis of a 19-week-old 46, XX fetus revealed testicular development similar to a control testis, suggesting that complete sex reversal might have occurred. The gonad of another 24-week-old 46, XX affected fetus from the same family displayed partial sex reversal with both ovarian and testicular tissues. Both fetuses carried the same mutation affecting the stability of *WNT4* mRNA, a fact that could explain the phenotypic variability (Mandel *et al*. 2008). In the *Wnt4* mouse model, sex reversal with the appearance of

cord-like structures is observed around birth. These testis cords are similar to those observed in *Rspo1*-deficient females and express male markers such as DHH and SOX9 (Vainio *et al*. 1999, Maatouk *et al*. 2013). However, in mice, no adrenal agenesis has been described. The differences between humans and mice may be due to functional redundancy between *Wnt* genes in mice. *Wnt2b* and *Wnt9a* are expressed in early gonadal development (Cederroth *et al*. 2007, Jameson *et al*. 2012*a*) and could activate WNT signaling in the absence of WNT4. Moreover, *Rspo1* and *Wnt4* are also involved in maintaining the quiescent state in pre-granulosa cells after germ cells have entered meiosis ((Maatouk *et al*. 2013) and below). Their role at this later stage may account for the late appearance of sex reversal approximately at the time of birth, rather than at the time of sex determination.

Absence of Rspo1 *or* Wnt4 *stimulates a male-like vascularization of the gonad*

The first morphological abnormality observed in the XX *Rspo1*$^{-/-}$ embryo is the presence of a blood vessel at 12.5 dpc that is characteristic of male development. This coelomic vessel is usually formed at the surface of the embryonic testis and is required to pattern the gonad and guide sex cord formation (Cool *et al*. 2011). The male-like vasculature of mutant XX gonads suggests that *Rspo1* is required to inhibit the crosstalk between mesenchymal and endothelial cells. Interestingly, screening performed on isolated endothelial cells indicates that, at 12.5 and 13.5 dpc, *Axin2* and known RSPO1 receptors, such as *Lgr4*, *Lgr5*, or *Lgr6*, are not expressed in this cell population (Jameson *et al*. 2012*a*), suggesting that RSPO1 acts independent of canonical WNT/β-catenin signaling or indirectly in this context. Thus, the role of *Rspo1* in vasculature development remains to be analyzed.

Wnt4 deficiency also induces male-like vascularization of the embryonic XX gonad. Jeays-Ward *et al*. (2003) demonstrated that WNT4 is required to antagonize both endothelial and steroidogenic cell migration from the mesonephros within the XX gonad, thus preventing the formation of the testis-specific coelomic vessel and the production of steroids. WNT4 is required to stimulate follistatin (*Fst*) expression. FST is an activin-binding protein expressed in the XX gonad from 11.5 dpc onwards, whereas it is absent in XY gonad. FST is known to antagonize Activin B action to ensure that no testis-specific vasculature is formed (Yao *et al*. 2004). To date, it is not clear whether the effect of *Wnt4* on *Fst* and endothelial cells is direct or indirect. In *Rspo1*-deficient XX gonads, *Wnt4* and *Fst* expressions are abolished. The maintenance of *Rspo1* expression in *Wnt4*-deficient gonads indicates that the appearance of the coelomic vessel is not a direct effect of *Rspo1* (Chassot *et al*. 2008*a*). Therefore, it appears that *Rspo1*, *Wnt4*, and *Fst* have an epistatic relationship during ovarian development, with *Rspo1* acting at the top of this genetic cascade. In accordance with this model, XX *Rspo1*–*Wnt4*-deficient mice (double mutant *Rspo1*$^{-/-}$; *Wnt4*$^{-/-}$ mice) are not more severely affected in their phenotypes than the single XX *Rspo1*$^{-/-}$ or *Wnt4*$^{-/-}$ mutants (Chassot *et al*. 2012), indicating that *Rspo1* and *Wnt4* act along the same pathway, at least in somatic cells, at the time of sex determination.

Impaired Rspo1 *or* Wnt4 *expression results in ectopic appearance of adrenal-like steroidogenic cells*

The concomitant feature of abnormal vascularization in the XX *Rspo1*$^{-/-}$ or *Wnt4*$^{-/-}$ gonad is the appearance of steroidogenic cells expressing markers such as *Cyp11a1* (*Cyp11α1*) or *P450scc*, and *Hsd17b3* (*Hsd17β3*) from 12.5 dpc onwards (Jeays-Ward *et al*. 2003, Chassot *et al*. 2008*a*). In addition, *Cyp21a1* (*Cyp21*), a marker of adrenal steroidogenic cells, is also expressed in the *Wnt4*$^{-/-}$ mutant ovaries, suggesting that these ectopic steroidogenic cells migrate from the neighboring adrenals into the gonad in the absence of *Wnt4* (Heikkila *et al*. 2002, Jeays-Ward *et al*. 2003). The origin of these cells is not as clear in the XX *Rspo1*$^{-/-}$ mutants, where *Cyp21a1* does not seem to be expressed (Lavery *et al*. 2012). In the XX *Wnt4*$^{-/-}$ mutant ovaries, testosterone is produced from 12.5 dpc onwards and several genes encoding enzymes regulating steroidogenesis (*Hsd17b1*, *Hsd17b3*, and *Cyp11a1*) are upregulated (Heikkila *et al*. 2002). Similarly, *Hsd17b3* and *Cyp11a1* are strongly upregulated in the *Rspo1*$^{-/-}$ mutant (Chassot *et al*. 2008*a*). It is likely that these steroidogenic cells produce enough hormones to stimulate the development of epididymis, vas deferens, and hypoplastic seminal vesicles in both mutant situations.

RSPO1, WNT4, and the sex determination of female germ cells

Although gonadal somatic cells are specified *in situ* within the developing gonad, the primordial germ cell precursors, future spermatocytes and oocytes, are committed to the germ cell lineage much earlier during embryonic development. Commitment occurs at ∼6.5 dpc in mice, in the proximal epiblast. Subsequently, germ cells migrate from the allantois through the primitive forming gut to reach and colonize the gonads between 10.5 and 11.0 dpc. Once they have reached their destination, they proliferate and initiate divergent sexual differentiation programs depending on their somatic environment, as well as on intrinsic signals (reviewed by Kocer *et al*. (2009)). Thus, at ∼13.5 dpc for female embryos, germ cells progressively enter meiosis, whereas they become quiescent in male embryos at ∼14.5 dpc. Reinitiation of proliferation, differentiation,

and meiosis occurs in males only after birth, at the onset of puberty.

Time- and sex-specific activation of several genes controls these different events (for a review, see Kocer *et al.* (2009)). Both *Rspo1* and *Wnt4* mutants display defects in germ cell development (see below).

RSPO1 regulates proliferation while WNT4 acts as a survival factor in female germ cells

The *Rspo1*-deficient XX gonad is characterized by a reduced number of germ cells already evidenced at 12.5 dpc and caused by a decrease in germ cell proliferation (Chassot *et al.* 2011). This is not surprising as *Rspo1* has been shown previously to stimulate the proliferation of crypt cells (intestinal progenitor cells) in gain-of-function experiments (Kim *et al.* 2005). However, proliferation affects only about half of the germ cells and remains partial in *Rspo1* mutant mice, indicating that RSPO1 is not the only factor controlling the cell cycle of female germ cells. Interestingly, at 16.5 dpc, germ cells in the *Rspo1*-mutant ovary resemble G0–G1-arrested gonocytes, and thus are similar to male germ cells at the same stage (Chassot *et al.* 2011). This suggests that *Rspo1* is also required for germ cell sex determination. Again, this is an incomplete phenotype, suggesting that *Rspo1* is not the only factor implicated in ovarian germ cell fate. This demonstrates that RSPO1 is likely to affect germ cells in two ways: i) it stimulates their proliferation and ii) it inhibits their entry into a quiescent phase.

In contrast to the *Rspo1*-deficient ovary, the number of germ cells in the $Wnt4^{-/-}$ ovary is very similar to that of WT XX gonads between 11.5 and 15.5 dpc (Yao *et al.* 2004), indicating that they proliferate normally.

Germ cell apoptosis is considered to be a normal process in ovarian development. It is restricted to the medullar region of the ovary and occurs at ∼16.5 dpc in the developing gonad in mice (De Felici *et al.* 2005). This apoptosis contributes to eliminating oocytes having undergone meiotic defects. Germ cell death also occurs in case of somatic defects, as these supporting cells are crucial for germ cell survival. At 16.5 dpc, in the XX *Wnt4*-deficient gonad, germ cells undergo massive apoptosis throughout the entire gonad, with ∼90% being lost at this stage, compared with 30% in a WT gonad (Yao *et al.* 2004). By contrast, no increase in apoptosis was detected in the germ cells of *Rspo1* mutant gonads before birth. Thus, 17.5 dpc $Rspo1^{-/-}$ ovaries display much higher germ cell numbers compared with $Wnt4^{-/-}$ ovaries, and these germ cells are distributed throughout the entire gonad (Chassot *et al.* 2011, Maatouk *et al.* 2013). This indicates that *Wnt4* is required for female germ cell survival, unlike *Rspo1*. In addition, this suggests that downregulation of *Wnt4* in *Rspo1* KO gonads is not complete, and that persistently low levels of *Wnt4* may be sufficient to support germ cell survival (Chassot *et al.* 2008*a*, Tomizuka *et al.* 2008).

RSPO1 and WNT4 regulate meiosis entry of female germ cells, potentially through distinct signaling pathways

Loss of *Rspo1* expression also impairs germ cell entry into meiosis (Chassot *et al.* 2011), and at 14.5 dpc, different germ cell populations can be observed in the mutant XX gonad: about half of the germ cells still express premeiotic and meiotic markers such as *Stra8* and *Ctdspl* (*Sycp3*). The other half expresses markers of pluripotency, such as *Pou5f1* (*Oct4*), or of quiescence in male germ cells, including *Nanos2* and *Dnmt3L*. In the *Wnt4* mutant gonads, the expression of *Stra8* is also weak while the expression of *Pou5f1* is maintained at 14.5 dpc, indicating a delay in meiosis entry (Naillat *et al.* 2010). However, the number of meiotic germ cells in the $Wnt4^{-/-}$ ovaries is normal when compared with a WT ovary at 15.5 dpc (Yao *et al.* 2004) and surviving germ cells usually express meiotic markers (Maatouk *et al.* 2013), before undergoing apoptosis through increasing Activin βb expression (Liu *et al.* 2010). The precise mechanisms of how *Rspo1* and *Wnt4* affect meiosis entry remain yet to be elucidated.

The retinoic acid (RA) pathway is a key regulator of meiosis entry and ectopic addition of exogenous RA on cultured testis stimulates premature meiosis entry and induces *Stra8* expression (Bowles *et al.* 2006, Koubova *et al.* 2006). Stimulated by retinoic acid 8 (STRA8) gene is necessary for DNA replication preceding meiosis. Despite these convincing *in vitro* data, a study aiming to monitor the endogenous role of RA in fetal mouse ovary *in vivo* reported that RA is required to induce neither *Stra8* nor meiosis (Kumar *et al.* 2011). Cyp26B1 is a RA-metabolizing cytochrome P450 enzyme expressed in the male embryonic gonad from 12.5 dpc onwards and absent from the ovary. In XY mice deficient for *Cyp26b1* ($Cyp26b1^{-/-}$ mice), germ cells prematurely enter into meiosis only to undergo massive apoptosis from 13.5 dpc onwards, despite generally normal Sertoli and Leydig cell specification (MacLean *et al.* 2007). Thus, CYP26B1 appears to maintain low RA levels in the male embryonic gonad, in order to inhibit entry of germ cells into meiosis and prevent their apoptosis.

Stra8 is downregulated in the $Rspo1^{-/-}$ XX gonad, while *Cyp26b1* is not upregulated (Chassot *et al.* 2011). The absence of *Cyp26b1* expression suggests that RA is usually present in mutant $Rspo1^{-/-}$ gonad and that the reduced expression of *Stra8* is independent of RA. Nevertheless, further experiments using RA reporter assays are required to confirm definitively RA presence in mutant gonads. It has been recently shown that germ cell differentiation and entry into meiosis are two dissociable events that contribute to the development of a functional oocyte (Dokshin *et al.* 2013). We currently hypothesize that *Rspo1* participates in establishing female germ cell identity, allowing germ cells to become competent to respond to meiotic signals.

Another hypothesis would be that RA regulates RSPO1 signaling in the developing ovary to promote meiotic gene expression. Further experiments will be required to analyze the relative importance of the two pathways and determine their relationship.

RSPO1, WNT4, and β-catenin signaling in the female germ cell: transcriptional activity and cell adhesion

Canonical β-catenin signaling is activated in XX germ cells, as evidenced by *Axin2* expression (Chassot *et al.* 2011, Jameson *et al.* 2012*a*). The active form of CTNNB1 and its transcriptional activity detected in the nuclei of control XX germ cells are lost in germ cells of the *Rspo1*$^{-/-}$ mutant ovary. Thus, RSPO1 appears to activate canonical β-catenin signaling in both somatic cells and germ cells of the ovary (Chassot *et al.* 2011). Consistent with this model, RSPO1 protein is detected on the membrane of both cell types (Kocer *et al.* 2008, Smith *et al.* 2008), suggesting that CTNNB1 is directly activated in female germ cells to regulate their fate. Consistent with this hypothesis, germ cells of the XX embryos normally enter meiosis when *Ctnnb1* is specifically ablated in somatic cells of the developing ovary (*Sf1:cre; Ctnnb1*$^{flox/flox}$ mice; Manuylov *et al.* 2008, Liu *et al.* 2009). This suggests that WNT/β-catenin signaling in somatic ovarian cells does not affect germ cell fate. Instead, the intrinsic activation of CTNNB1 within the germ cells contributes to female germ cell fate.

Early gonadal patterning is strictly correlated with the cell-specific membrane expression of CTNNB1 and CDH (cadherins) in adherent junctions during sex determination (Fleming *et al.* 2012). In the absence of *Rspo1*, β-catenin signaling is downregulated in the nucleus and CTNNB1 seems to be relocalized to the cell membrane, where it may function as an adhesion molecule to organize germ cells in clusters and establish cell–cell contacts, a feature characteristic of testes at the same stage (Chassot *et al.* 2008*a*). RSPO1 could also directly act on cell surface adhesion molecules through its thrombospondin 1 domain to regulate germ cell adhesion differentially between XX and XY gonads. Interestingly, *Wnt4*-deficient gonads also display enhanced cell-to-cell contacts (Naillat *et al.* 2010), suggesting that *Wnt4* is also involved in cell adhesion. Whether RSPO1 and WNT4 directly regulate the expression and/or localization of cell adhesion molecules or whether this increased adhesion is a consequence of an abnormal germ cell identity remains yet to be investigated.

Rspo1/Wnt4 pathways prevent pro-spermatogonia differentiation and sex reversal, in turn

It has been demonstrated that the decision of germ cells to enter meiosis or to become quiescent depends on their somatic environment (McLaren 1991). Conversely, the involvement of germ cells in the differentiation of supporting cells is less documented.

In *Rspo1*$^{-/-}$ and *Wnt4*$^{-/-}$ mutant ovaries, *Pou5f1* expression is maintained as it is in quiescent male germ cells, and the expression of *Nanos2*, a marker of male germ cells, is upregulated (Chassot *et al.* 2011). This indicates that in both mutants, germ cells have undergone abnormal differentiation. In addition, germ cells of the *Rspo1*$^{-/-}$ and *Wnt4*$^{-/-}$ ovary have the morphology of G0–G1 arrested gonocytes at 16.5 and 18.5 dpc respectively ((Chassot *et al.* 2011) and unpublished data). This suggests that, while proliferation is not affected by the absence of *Wnt4* expression, a proportion of germ cells does not usually progress in meiosis and become quiescent. It is noteworthy that these masculinized germ cells are always found within or in close vicinity of developing testicular cords. However, these masculinized germ cells differentiate before becoming mature Sertoli cells. This is in contrast to normal gonadal development during which somatic cells determine the sexual fate of germ cells (Adams & McLaren 2002).

Meiotic germ cells antagonize male development in embryonic mouse ovaries (Yao *et al.* 2003) and female germ cells exert strict control on their surrounding environment under the influence of RSPO1 and WNT4 (Maatouk *et al.* 2013). Thus, *Wnt4*-deficient as well as *Rspo1*-deficient ovaries fail to undergo sex reversal, when germ cells are depleted from the gonad at mid-gestation by bisulfan treatment (Maatouk *et al.* 2013). This implies that abnormally differentiated germ cells of these mutants participate in Sertoli cell differentiation and sex reversal. Moreover, the improvement of cell–cell contacts through sex-specific localization of adhesion molecules such as CTNNB1 and CDH1 (E-cadherin) may play a role in this process. Thus far, the signals generated by germ cells upon RSPO1 and WNT4 action and the pathways induced by these signals are unknown.

Lack of Rspo1 *or* Wnt4 *triggers Sertoli cell differentiation around birth*

In the developing ovary, pre-granulosa cells are usually in a quiescent state: they are mitotically arrested and they express cell cycle inhibitors such as p27^{kip1}. In pubescent females and throughout reproductive life, quiescent pre-granulosa cells are organized in ovarian follicles that resume proliferation when the follicle is recruited and matures (Edson *et al.* 2009). In *Rspo1* and *Wnt4* mutant gonads, the pre-granulosa cells are not quiescent and differentiate precociously and transiently into proliferative granulosa cells in an anterior-to-posterior wave across the ovary. Then, approximately at the time of birth, germ cells promote these cells to transdifferentiate into Sertoli cells (Maatouk *et al.* 2013) that, with the onset of *Sox9* expression, form tubular structures reminiscent of seminiferous tubules (Vainio

et al. 1999, Chassot *et al*. 2008*a*; Fig. 2). Surprisingly, this transdifferentiation can still occur in the absence of *Sry* or *Sox9*, indicating that other genes can induce female-to-male sex reversal (Lavery *et al*. 2012). Together, these data indicate that *Rspo1* and *Wnt4* are involved in maintaining the immature progenitor state of the ovarian supporting cells.

Rspo1, Wnt4, *and* Foxl2 *relationship in ovarian development*

Mutations in *FOXL2* in humans lead to blepharophimosis/ptosis/epicanthus inversus syndrome (BPES), an autosomal dominant genetic disorder characterized by drooping eyelids and/or premature ovarian failure in women (Crisponi *et al*. 2001). FOXL2 is required for the transition from primordial to primary follicle approximately at the time of birth and *Foxl2*$^{-/-}$ mice show progressive apoptosis of functional granulosa cells, leading to follicular depletion and ovary atresia (Schmidt *et al*. 2004). Interestingly, conditional ablation of *Foxl2* in adult mice induces reprograming of granulosa cells into Sertoli cells, demonstrating that maintenance of the sexual identity of supporting cells is determined by the antagonistic action of SOX9 and FOXL2 (Uhlenhaut *et al*. 2009).

In the embryonic mouse ovary, *Foxl2* expression is partly dependent on RSPO1 and CTNNB1 (Manuylov *et al*. 2008, Auguste *et al*. 2011), whereas it is normally expressed in the *Wnt4*$^{-/-}$ ovary (Manuylov *et al*. 2008), suggesting that *Foxl2* is expressed in two different somatic cell populations. Moreover, simultaneous deletion of *Foxl2*$^{-/-}$ and *Wnt4*$^{-/-}$, as well as *Foxl2*$^{-/-}$ and *Rspo1*$^{-/-}$, promotes early female-to-male sex reversal (Ottolenghi *et al*. 2007, Auguste *et al*. 2011), indicating that *Foxl2* and *Rspo1/Wnt4* have

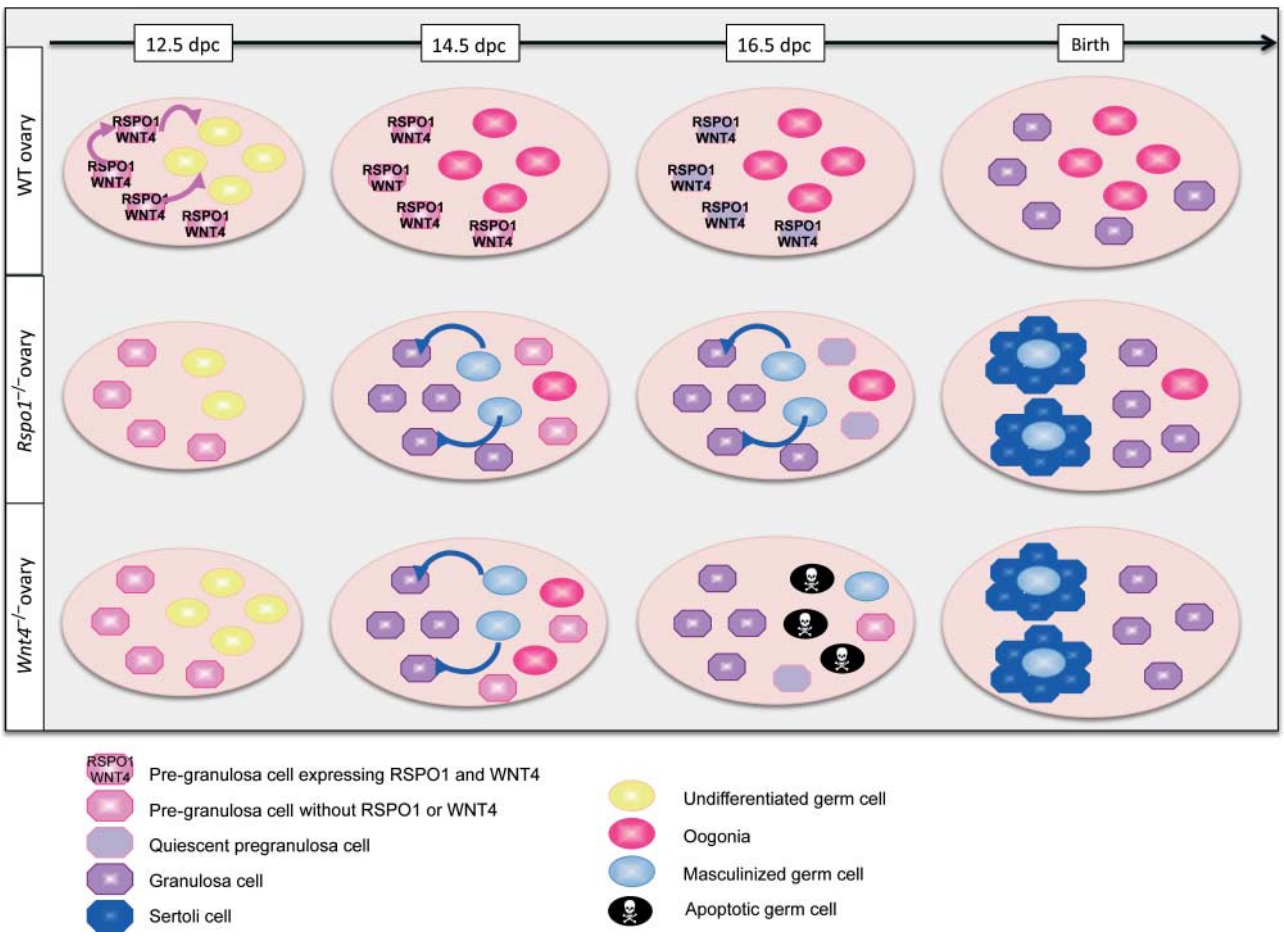

Figure 2 Role of *Rspo1* and *Wnt4* during sexual differentiation. During embryonic development of WT ovaries (top), *Rspo1* expression by somatic cells at 12.5 dpc first induces the proliferation, then differentiation of female germ cells into oogonia at 12.5 and 14.5 dpc respectively (pink arrows). Moreover, *Rspo1/Wnt4*-expressing somatic cells (pre-granulosa cells) become quiescent granulosa cells and resume folliculogenesis approximately at the time of birth. By contrast, in *Rspo1*$^{-/-}$ embryonic ovaries (middle), ~50% of the germ cell population do not proliferate at 12.5 dpc and become masculinized at 14.5 dpc. In *Wnt4*$^{-/-}$ embryonic ovaries (bottom), the germ cell population proliferates normally, then becomes masculinized at 14.5 dpc and undergoes apoptosis at 16.5 dpc. In both mutants, these masculinized germ cells inhibit the entry into quiescence of the pre-granulosa cells (blue arrows), which differentiate early into granulosa cells (Maatouk *et al*. 2013). These cells then transdifferentiate into Sertoli cells approximately at the time of birth. Some normal granulosa cells and oogonia are still present in the mutant gonads at birth.

complementary roles during ovarian development. *FOXL2* mutations are also sufficient to induce XX sex reversal in the goat (Pailhoux *et al*. 2001). The different phenotypes observed in *Foxl2* mutant mice and goats may indicate a difference in the identity/maturation time of pre-granulosa cells between these species.

R-spondin and *Wnt4* genes in ovarian differentiation throughout evolution

Despite the diversity of 'master sex-determining genes' among species, the key role of WNT signaling in sexual differentiation in humans and mice raises questions about the conservation of this pathway during evolution. Expression and potential roles of *WNT* genes in other species are largely unclear. WNT4 has been found in various metazoan phyla and the embryonic expression pattern is well conserved throughout vertebrate evolution ((Nicol *et al*. 2012) for references). RSPOs are expressed in vertebrates and invertebrates, such as hemichordates, chordates, and echinoderms, but screening of *Drosophila melanogaster* and *Caenorhabditis elegans* failed to identify any RSPO homolog (Yoon & Lee 2012). However, the WNT/β-catenin pathway is required for male cell fate commitment in the gonad of *C. elegans* (Kalis *et al*. 2010).

Mammals

RSPO1 is upregulated in humans and goats during critical stages of ovarian development. In goats, RSPO1 is expressed in both male and female mesonephroi and the level of expression peaks just before germ cell entry into meiosis (55 dpc; Kocer *et al*. 2008). In addition to RSPO1, RSPO2 is expressed at the time of folliculogenesis (from 70 dpc until before birth) in goat gonads, while RSPO4 begins to be very faintly expressed in 50 dpc ovaries (Kocer *et al*. 2008).

In humans, RSPO1 is expressed between 6 and 9 weeks after conception in the fetal ovary (Tomaselli *et al*. 2011), whereas *WNT4* is detected in both the fetal and adult ovary at different follicular stages (Jaaskelainen *et al*. 2010) as in mice. In marsupials, the *WNT4* mRNA level increases after birth, reaching a peak of expression by days 9–13 *post partum* when the ovarian cortex and medulla become distinguishable (Yu *et al*. 2006).

Birds

The amniotes split into the ancestral lineages of mammals and reptiles ∼320 million years ago. In birds, some reptiles, and insects, sex determination depends on the ZZ/ZW chromosomes with ZZ homogametic males and ZW heterogametic females. In ZZ embryos, *Rspo1* and *Wnt4* expression levels remain low while *Rspo1* and *Wnt4* are specifically expressed in chicken ovaries (Smith *et al*. 2008). *Wnt4* mRNA is detected in oocytes of postnatal chick ovaries. Moreover, *Rspo1* expression is downregulated when sex reversal is induced by hormone manipulation of ZW embryos (Lambeth *et al*. 2013). Thus, *Rspo1* and *Wnt4* also seem to be key determinants of the female differentiation pathway in the chicken.

Reptiles

Besides XY or ZW genetic sex determination, temperature has a dominant role in the establishment and maintenance of sexual fate in some reptiles. The thermosensitive period of the red-eared slider turtle (*Trachemys scripta*) spans from embryonic stages 14–19/20, during which a 31 °C temperature of incubation triggers *Rspo1* upregulation and the production of female hatchlings (Smith *et al*. 2008). Moreover, in this species, *Wnt4* transcription appears to be upregulated following estrogen signaling (Mork & Capel 2013).

Amphibians

In *Rana rugosa*, *Wnt4* is not expressed in a sexually dimorphic fashion in the early stages of gonadal development. *Wnt4* is transcribed in the embryos at the late gastrula stage, and its expression is maintained until the undifferentiated gonad differentiates into a testis or an ovary (Oshima *et al*. 2005).

Teleost fishes

In mammals, testes and ovaries retain the ability to transdifferentiate in adult life, as documented using mouse mutants (Uhlenhaut *et al*. 2009, Matson *et al*. 2011). The plasticity of the adult gonad is most evident in fish species that spontaneously change sex in adult life, shifting from male to female (protandrous) or female to male (protogynous). In the protandrous Black Porgy (*Acanthopagrus schlegeli*), *wnt4* expression is increased during the late ovarian growth and during the transition from male to female (Wu & Chang 2009), suggesting a conserved role of *wnt4* in ovarian development. While in mammals, the transdifferentiation of adult gonads not only affects the somatic cells, but also is associated with sterility; sex-changing fishes retain their fertility. Zebrafish, salmonids, and medakas are teleost fishes, but they have been separated for 115–200 million years. In zebrafish, Rspo1 expression starts to be detected at 30 day post fertilization (dpf) in the somatic cells of the undifferentiated gonads, until 150 dpf in the ovary (granulosa cells and theca cells) and in the testis (Leydig cells), while its expression is present only from 30 to 60 dpf in female germ cells and until 150 dpf in male germ cells (Zhang *et al*. 2011). Unlike other teleost fishes, the salmonid rainbow trout (*Oncorhynchus mykiss*) has a

third *wnt4* gene (Nicol *et al*. 2012). Protein comparisons with WNT4 from other vertebrates show a high level of amino acid identity (>80%). However, *wnt4* is not strongly expressed in the ovary during early gonadal differentiation in the rainbow trout. Thus, gonadal differentiation may involve other members of the *Wnt* gene family. Indeed, other *wnt* genes have been shown to display a sex-specific expression at different stages of development in the rainbow trout (Nicol & Guiguen 2011). Moreover, transgenic inhibition of *wnt* signaling results in male-biased sex ratios, suggesting that Wnt signaling is a conserved key pathway during gonadal differentiation in zebrafish (Sreenivasan *et al*. 2014). In medaka (*Oryzias latipes*), Rspo1 and Rspo2 are prominently expressed in both germ cells and somatic cells (Zhou *et al*. 2012). Their functions in this species remain yet to be clarified. Recently, three genes belonging to the WNT pathways, *wnt4*, *rspo1*, and *ctnnb1*, have been identified in *Latimeria menadoensis* transcripts and in the *Latimeria chalumnae* genome, two coelacanths considered to be true 'living fossils' (Forconi *et al*. 2013).

Despite the diversity of sex determinants in these different phyla, WNT/RSPO1/β-catenin signaling appears as an ancient, conserved pathway of ovarian determination.

Remaining questions and prospects

It is clear today that the RSPO1/WNT/β-catenin pathway acts at the top of the ovarian differentiation cascade (Fig. 1). The development of male or female traits appears to be antagonistic, and two key events orient the developing gonad toward a male or a female fate: female (or male) genes need to be expressed and male (or female) genes actively repressed (Jameson *et al*. 2012*b*; Fig. 1). The molecular mechanisms regulating the expression of *Rspo1* and *Wnt4* in the bipotential gonad, as well as those involved in their ovary-specific upregulation remain yet to be discovered.

Thus far, it is speculated that *Rspo1* and *Wnt4* act through the WNT signaling pathway during gonadal development. However, when *Ctnnb1* is deleted in somatic cells (*Sf1cre; Ctnnb1*$^{flox/flox}$ mice), the gonads do not undergo sex reversal at 18.5 dpc while sex reversal of somatic cells highlighted by *Sox9* expression is already noticeable in the *Rspo1*$^{-/-}$ and *Wnt4*$^{-/-}$ XX gonads at this stage (Chassot *et al*. 2011, Maatouk *et al*. 2013). It is likely that sex reversal is promoted by a signal derived from abnormally differentiated germ cells in *Rspo1* and *Wnt4* mutants, whereas this signal is not produced in *Sf1cre; Ctnnb1*$^{flox/flox}$ embryos. Thus, the differentiation of germ cells is unaffected before they undergo apoptosis in *Sf1cre; Ctnnb1*$^{flox/flox}$ gonads unlike in *Rspo1*$^{-/-}$ and *Wnt4*$^{-/-}$ gonads (Manuylov *et al*. 2008, Liu *et al*. 2009, Naillat *et al*. 2010, Chassot *et al*. 2011). However, it is not clear as to how germ cells promote sex reversal to occur in *Rspo1* and *Wnt4* knockout XX gonads (Maatouk *et al*. 2013). Germ cells might be involved in the early differentiation of granulosa cells via a paracrine signal after which these granulosa cells could transdifferentiate into Sertoli cells. Whether pre-granulosa cells differentiate early in *Sf1cre; Ctnnb1*$^{flox/flox}$ XX gonads has not been investigated thus far.

The most obvious difference between *Rspo1*$^{-/-}$ and *Wnt4*$^{-/-}$ XX mutant embryos is the differentiation of germ cells. Thus, RSPO1 appears to contribute to female germ cell proliferation, while WNT4 rather acts as a survival factor, protecting them from apoptosis, either directly or indirectly. Although both secreted factors are able to activate WNT/β-catenin signaling, it becomes obvious that they may act through different receptor complexes (LGR/RNF43 for RSPO1 and LRP/Fzl for WNT4) that may be differentially expressed depending on the cell type. Nevertheless, we cannot exclude that these molecules act through distinct signaling pathways in the gonad, which remains yet to be identified. Moreover, their specific receptors and the genetic network they are regulating during sex determination still need to be clarified.

Declaration of interest

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

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