Testicular Differentiation Occurs in Absence of R-spondin1 and Sox9 in Mouse Sex Reversals

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Abstract

In mammals, male sex determination is governed by SRY-dependent activation of Sox9, whereas female development involves R-spondin1 (RSPO1), an activator of the WNT/beta-catenin signaling pathway. Genetic analyses in mice have demonstrated Sry and Sox9 to be both required and sufficient to induce testicular development. These genes are therefore considered as master regulators of the male pathway. Indeed, female-to-male sex reversal in XX Rspo1−/− mice correlates with Sox9 expression, suggesting that this transcription factor induces testicular differentiation in pathological conditions. Unexpectedly, here we show that testicular differentiation can occur in XX mutants lacking both Rspo1 and Sox9 (referred to as XX Rspo1−/− Sox9−/−), indicating that Sry and Sox9 are dispensable to induce female-to-male sex reversal. Molecular analyses show expression of both Sox8 and Sox10, suggesting that activation of Sox genes other than Sox9 can induce male differentiation in Rspo1−/− Sox9−/− mice. Moreover, since testis development occurs in XY Rspo1−/− Sox9−/− mice, our data show that Rspo1 is the main effector for male-to-female sex reversal in XY Sox9−/− mice. Thus, Rspo1 is an essential activator of ovarian development not only in normal situations, but also in sex reversal situations. Taken together these data demonstrate that both male and female sex differentiation is induced by distinct, active, genetic pathways. The dogma that considers female differentiation as a default pathway therefore needs to be definitively revised.

Introduction

Mammalian sex determination depends on the primary developmental decision of the gonad to differentiate as testis or ovary. The gonad develops as a bipotential organ with the capacity to respond to two different genetic stimuli: the activation of the SRY/Sox9 pathway that induces testicular development, or the expression of the R-spondin1 (RSPO1)/beta-catenin pathway that regulates ovarian differentiation [1]. Indeed in humans and mice, male sex determination is initiated by the expression of the Y-linked gene Sry [2,3,4]. Sry expression in turn activates the transcriptional regulator Sox9 [5]. Subsequently, Sox9 initiates Sertoli cell differentiation, the supporting cell of the testicular sex cords [6,7]. Signaling pathways initiated in these cells contribute to the organization of the XY gonads [8], as well as to the differentiation of other testicular cell lineages such as the Leydig steroidogenic cells [9,10] and the pro-spermatagonia [11,12], ultimately leading to tests formation and, in turn, male development. In 46,XY patients, loss-of-function mutations in Sry and Sox9 promote male-to-female sex reversal [13,14], whereas translocations of the Sry locus to another chromosome can yield 46,XX patients with female-to-male sex reversal [3]. Loss-of-function mutations [6,7,15,16] and gain-of-function mutations [4,17,18] of Sry and Sox9 have been generated in mouse models, showing that Sry and Sox9 are necessary and sufficient to induce testis differentiation and the associated male development. As a consequence, these genes have been considered as the master inducers of testis differentiation and male development.

In the absence of Sry [XX individuals], up-regulation of Rspo1, an activator of the WNT/beta-catenin signaling pathway, promotes ovarian differentiation. Mutations in Rspo1 are responsible for skin disorders and female-to-male sex reversal in 46,XX patients [19]. Similarly, ablation of Rspo1 in mice yields female-to-male sex reversal and promotes Sox9 up-regulation correlated with differentiation of Sertoli cells and formation of testis cords at birth [20]. This gonadal dysgenesis yields development of an ovotestis, a gonad displaying both testicular and ovarian regions [20,21]. Rspo1 expression in turn activates expression of Wnt4 [21], another activator of the WNT/beta-catenin signaling pathway involved in ovarian differentiation [22,23]. When the canonical beta-catenin signaling pathway is activated in XY gonads, this induces male-to-female sex reversal.
Author Summary

Mammalian sex determination is controlled by the paternal transmission of the Y-linked gene, SRY. Using mouse models, it has been shown that the main, if not the only, role of Sry is to activate the transcription factor Sox9, and these two genes are necessary and sufficient to allow male development. Indeed, defects in Sry and/or Sox9 expression result in male-to-female sex reversal of XY individuals. In XX individuals, Rspo1 is important for ovarian development as evidenced by female-to-male sex reversal of XX Rspo1 mutants. Since testicular differentiation appears concomitantly with Sox9 expression, it was assumed that Sox9 is the inducer of testicular differentiation in XX Rspo1 mutants. Our genetic study shows that i) neither Sry nor Sox9 are required for female-to-male sex reversals; ii) other masculinizing factors like Sox8 and Sox10 are activated in sex reversal conditions; iii) Rspo1 is the main effector of male-to-female sex reversal in the XY Sox9 mutants. Together these data suggest that male and female genetic pathways are both main effectors involved in sex determination and that the long-standing dogma of a default female pathway should definitively be revised.

indicating that this pathway acts on top of ovarian differentiation [23]. Indeed, activation of WNT/beta-catenin is required for expression of Foxl2 [24], a transcription factor involved in folliculogenesis [25,26] and homeostasis of the ovary [27]. Thus Rspo1 appears to be the gene instructing the molecular network leading to ovarian development.

Since ablation of Rspo1 promotes SOX9 expression concomitantly with Sertoli cell differentiation [20], it was assumed that Sox9 is the sex reversal inducer in XX Rspo1 KO mutants. We now show that i) testicular differentiation occurs in XX Rspo1 KO; Sox9 KO mutants indicating that neither Sry nor Sox9 are required for female-to-male sex reversals; ii) testicular differentiation also occurs in XY Rspo1 KO; Sox9 KO mutants indicating that Rspo1 is required for male-to-female sex reversal in XY Sox9 KO mutants.

Results/Discussion

Rspo1 is required for ovarian development in XY Sox9 KO mice

Sox9 is required for Sertoli cell differentiation, testis formation and male development. Indeed, deletion of Sox9 in XY Sox9 KO; Sf1creTg/+, referred to as XY Sox9 KO, triggers male-to-female sex reversal [16]. However the factor(s) inducing sex reversal in XY Sox9 KO remained to be identified. Given i) the prominent role of Rspo1, an activator of beta-catenin signaling, in female sex determination [19], and ii) the fact that ectopic activation of beta-catenin in XY gonads can induce male-to-female sex reversal [23], we hypothesized that Rspo1 expression induced male-to-female sex-reversal in XY Sox9 KO gonads. According to this scenario, neither testicular (which is Sox9-dependent) nor ovarian (which is Rspo1/beta-catenin-dependent) differentiation should occur in XY Sox9 KO gonads additionally lacking Rspo1. To test this hypothesis, we have generated and analyzed double loss-of-function mice (i.e. XY Rspo1 KO; Sox9 KO; Sf1creTg/+, referred to as XY Rspo1 KO; Sox9 KO, Sox10 KO mice was equivalent to that of XX controls but the internal genitalia contained both male and female organs including oviducts, uterine horns and vaginal tissues, as well as epididymides, vasa deferens, seminal vesicles and prostate (Figure S1). The XY Sox9 KO developed as ovaries (Figure 1b, 1g, 1l and Figure S1), as expected from a previous report [16]. Interestingly, XY Rspo1 KO; Sox9 KO gonads developed as hypoplastic testes containing well-defined seminiferous tubules as evidenced by histological analysis (Figure 1c, 1h and Figure S1). We next examined whether the supporting cells forming the seminiferous tubules differentiated as granulosa cells, the ovarian supporting cells expressing FOXL2 [25,26] or as Sertoli cells expressing DMRT1 [27]. In P21 gonads, immunostaining experiments showed that the supporting cells forming the seminiferous tubules in XY Rspo1 KO; Sox9 KO gonads were DMRT1-expressing Sertoli cells (Figure 1f), even though SOX9 was clearly missing (Figure 1m). However, a few FOXL2-positive granulosa cells were found within the alignment of the Sertoli cells forming the seminiferous tubules (Figure 1m, r) and in a few XY Rspo1 KO; Sox9 KO mice (3 out of 18), rare and abnormal follicles were observed (Figure S2A). The mixed genetic background of Rspo1 KO; Sox9 KO mice is a likely factor causing the variation of this phenotype.

Altogether this shows that a genetic pathway activated by Rspo1 is required for the female-to-male sex-reversal of XY Sox9 KO and indicates that Sertoli cell differentiation and seminiferous tubules formation can occur in the absence of SOX9.

Sertoli cell differentiation occurs without Sry and Sox9 in XX Rspo1 KO gonads

Our study also allowed us to evaluate the effect of Sox9 removal in a female-to-male sex reversal context (i.e. in XX Rspo1 KO; Sox9 KO). Given that homozygous mutations of Rspo1 promote Sertoli cell differentiation around birth, a process that is associated with Sox9 up-regulation in these cells [20], we hypothesized that Sox9 is the inducing factor of testicular differentiation in XX Rspo1 KO mice. If Sox9 is indeed the main switch for female-to-male sex reversal in XX individuals, one expects an impaired differentiation of Sertoli cell and seminiferous tubules in the absence of both Rspo1 and Sox9 in XX Rspo1 KO; Sox9 KO gonads. Unexpectedly, at P60, these XX double mutants displayed hermaphroditism of the reproductive tracts (Figure S1). Histological analysis revealed that XX Rspo1 KO; Sox9 KO mice exhibited oovestes with an extensive presence of sex cords (Figure 1d, 1i and in Figure S2B, i) as do XX Rspo1 KO gonads (shown in Figure S2B, S2e and in previous analyses [20,21]). Thus, the development of XX Rspo1 KO; Sox9 KO mouse genitalia is indistinguishable from that of XX Rspo1 KO mice indicating that the additional deletion of Sox9 in XX Rspo1 KO; Sox9 KO gonads does not change the fate of XX Rspo1 KO gonads. We next examined whether the supporting cells forming the sex cords differentiated as granulosa cells, the ovarian supporting cells expressing FOXL2 [25,26] or as Sertoli cells expressing DMRT1 [29]. In three weeks old mice (P21), Sox9-depleted cells forming the seminiferous tubules generally lacked the follicular cell marker FOXL2 and instead expressed DMRT1 (Figure 1n, 1s). These data clearly indicate that Sertoli cell, seminiferous tubule and testis differentiation can occur in the absence of Sry and Sox9 in XX Rspo1 KO gonads.

Steroidogenic cells are present in Rspo1 KO; Sox9 KO embryonic gonads

Previous studies clearly show that the development of male genitalia depends on androgens secreted by the embryonic testis
In XX Rspo1KO gonads, steroidogenic cells appear before Sertoli cell differentiation [20,31] and this was also observed in Wnt4KO gonads [22,32], Wnt4 being up-regulated upon Rspo1 expression in XX gonads [20,21]. In addition, lack of Wnt4 expression was shown to allow ectopic migration of steroidogenic cells from the neighboring adrenals into gonads [32,33] and subsequent androgen synthesis [34], which explains the development of male genitalia in these mutants. When investigating whether steroidogenic cells were present in XX and XY Rspo1KOSox9cKO gonads, we found that P450Scc, a gene encoding for a precursor involved in androgen synthesis was expressed at 14.5 dpc in XY controls, XY and XX Rspo1KOSox9cKO gonads and XX Rspo1KO gonads, but not in XX controls (Figure S3A). However, Sox9 deletion no longer allows ovarian cells differentiation when Rspo1 is deleted in the XY (m, n, p, r, s) XY (a, f, k, q) and XX (e, j, o, t) Rspo1KO; Sox9cKO controls, XY Sox9KO gonads (b, g, l, q) and XX (d, i, n, s) Sox9KO gonads (Figure 2j, 2k), a factor whose

Figure 1. Testicular differentiation in XY and XX Rspo1KO; Sox9cKO mice. Macroscopic views of gonads of 2 month-old mice show hypoplastic tests and ovotestis development in XY (c) and XX (d) Rspo1KO; Sox9cKO mice, respectively. Seminiferous tubules are revealed by PAS histological analysis of XY (h) and XX (i) Rspo1KO; Sox9cKO gonadal sections. They are less abundant than in XY controls (f). XY Sox9KO gonads (g) develop as ovaries (j). (T: testicular region, O: ovarian region, scale bar: 200 μm). Immunofluorescence of SOX9 (k–o) or DMRT1 (p–t) (a Sertoli cell marker, in red), FOXL2 (k–t) (a follicular cell marker, in green) and DAPI (a nuclear marker in blue) (scale bar, 50 μm). Deletion of Sox9 with Sft1:cre (Sox9cKO) eliminates SOX9 expression in Sertoli cells (l, m, n) and promotes male-to-female sex reversal in XY Sox9KO gonads as highlighted by robust FOXL2 expression (l, q). However, Sox9 deletion no longer allows ovarian cells differentiation when Rspo1 is deleted in the XY (m, n, p, r, s) XY (a, f, k, q) and XX (e, j, o, t) Rspo1KO; Sox9cKO controls, XY Sox9KO gonads (b, g, l, q) and XX (d, i, n, s) Sox9KO gonads (Figure S2B).
expression is normally down-regulated between 13.5 and 16.5 dpc in the ovary [41] (Figure 2l), also suggests that the XX \textit{Rspo1KOSox9cKO} gonads are still undifferentiated or have differentiated as testis. However, with respect to the latter, the absence of AMH expression shows that no Sertoli cell differentiation has occurred (Figure 2c, 2g). Altogether these data indicate that the \textit{Rspo1KOSox9cKO} gonads are still undifferentiated at 13.5 dpc.

The first signs of Sertoli cell differentiation appeared at 16.5 dpc in \textit{Rspo1KOSox9cKO} gonads, with some rare DMRT1-positive cells in comparison to XY controls (Figure S3B). Then, few rudimentary testis cords were observed around 17.5 dpc (Figure S3B). At P0, Sertoli cells aligned to form sex cords as evidenced by the localization of DMRT1-positive cells (Figure S4A c, d). Quantitative PCR experiments further confirmed that \textit{Dmrt1} expression was strongly expressed in XY \textit{Rspo1KOSox9cKO} gonads and weakly in XX \textit{Rspo1KOSox9cKO} gonads at P0, highlighting that more Sertoli cells were present in XY \textit{Rspo1KOSox9cKO} gonads (Figure S4C). In addition, some FOXL2-positive cells were also detected in \textit{Rspo1KOSox9cKO} gonads (Figure S4A c, d). However, quantitative PCR experiments showed that \textit{Foxl2} expression was significantly reduced in comparison to XX control or XY \textit{Sox9cKO} gonads (Figure S4B) as expected for a gonad developing as ovotestis or testis.

We then studied SDMG1 expression, a cytoplasmic marker of Sertoli cells and of granulosa cells when follicles form [Best et al. 2008]. Using this marker, sex cords were evident at P0 (Figure 3c, 3d and Figure S5c, S5d) and, at puberty (P12), development of the seminiferous tubules appeared complete in \textit{Rspo1KOSox9cKO} gonads (Figure 3h, 3i and Figure S5h, S5i). At puberty, androgen receptor (AR) immunostaining indicated that, in addition to Sertoli cells, peritubular myoid and Leydig cells were also present both in XY \textit{Rspo1KOSox9cKO} testes (Figure 4i) and in testicular parts of the XX \textit{Rspo1KOSox9cKO} ovotestes (data not shown). In addition, follicle development appeared at P12 in XX \textit{Rspo1KOSox9cKO} ovotestes and XX control ovaries (Figure S2B d, f). Together our results indicate that seminiferous tubule development is delayed in the absence of Sox9 and \textit{Rspo1}, thereby explaining the small size of the XY \textit{Rspo1KOSox9cKO} testes (Figure 1c).

**Figure 2. Non-differentiated XY and XX \textit{Rspo1KOSox9cKO} gonads at 13.5 dpc.** Immunofluorescence of SOX9 (Sertoli cell marker, in red) and AMH (Sertoli cell marker green) (a–d), AMH (Sertoli cell marker, in green) and SRY (pre-Sertoli and Sertoli cell marker in red) (e–h) and SF1 (undifferentiated supporting cell, Sertoli and Leydig cell marker) (i–l). Counterstain is DAPI (in blue). Lack of SOX9 and AMH expression in XY (b) and XX (c) \textit{Rspo1KOSox9cKO} gonads shows that Sertoli cell differentiation did not occur at 13.5 dpc. Note that the kidneys (K) are positive for SOX9. This is accompanied with the maintenance of SRY expression in the XY \textit{Rspo1KOSox9cKO} gonads (f) whereas SRY expression has ceased in XY controls (e). SF1 expression is maintained in absence of Sertoli cells differentiation in XY and XX \textit{Rspo1KOSox9cKO} gonads (j and k respectively) (scale bar: 100 \textmu m). Note that SF1 is also expressed in steroidogenic cells of the adrenals (A). XY (a, e, i) and XX (d, h, l) \textit{Rspo1KOSox9cKO} controls. XY (b, f, j) and XX (c, g, k) \textit{Sox9cKO Rspo1KO} respectively. doi:10.1371/journal.pgen.1003170.g002

**SOX9-negative Sertoli cells can support germ cell differentiation until initiation of meiosis**

We next investigated whether the Sertoli cells that differentiate in the \textit{Rspo1KOSox9cKO} gonads can support germ cell differentiation. Since XX germ cells cannot survive in a testicular environment [42,43], the analysis was only carried out in XY \textit{Rspo1KOSox9cKO} gonads (Figure 3h, 3i and Figure S5h, S5i). At puberty, androgen receptor (AR) immunostaining indicated that, in addition to Sertoli cells, peritubular myoid and Leydig cells were also present both in XY \textit{Rspo1KOSox9cKO} testes (Figure 4i) and in testicular parts of the XX \textit{Rspo1KOSox9cKO} ovotestes (data not shown). In addition, follicle development appeared at P12 in XX \textit{Rspo1KOSox9cKO} ovotestes and XX control ovaries (Figure S2B d, f). Together our results indicate that seminiferous tubule development is delayed in the absence of Sox9 and \textit{Rspo1}, thereby explaining the small size of the XY \textit{Rspo1KOSox9cKO} testes (Figure 1c).
gonads expressed Oct4 at 14.5 dpc (Figure S6o). Nonetheless, some cells had already committed to meiosis (Figure S6k) and expressed the meiotic marker Stra8 [47], possibly because of the low level of Cyp26b1 expression in XY Rspo1KOSox9cKO gonads (Figure S6g). The reduced level of Cyp26b1 expression is however not sufficient to allow all germ cells to enter meiosis in XY Rspo1KOSox9cKO gonads.

At P10, GATA1, Androgen Receptors (AR) and Clusterin (Clu) were normally expressed in Sertoli cells of XY Rspo1KOSox9cKO gonads (Figure 4c, 4i, 4l), suggesting that these cells have acquired their identity and may be capable to support spermatogenesis. Accordingly, XY germ cells had committed to meiosis at P10, as assessed by immunodetection of the pre-meiotic and meiotic markers STRA8 and cH2AX, respectively (Figure 4c, 4f, 4i). However, later stages of spermatogenesis cannot however be analyzed, as hypoplasia of germ cells occurred within the seminiferous tubules of adult XY Rspo1KOSox9cKO gonads (Figure 1h and Figure S1m), most likely because of cryptorchidism.

Sox8 and Sox10 are expressed in the absence of Sox9

Interestingly, we found that AMH was expressed in Sertoli cells of both XX and XY Rspo1KOSox9cKO gonads at P12 (Figure 5A). Given that [i] Anh is a target-gene of SOX9 [48,49], [ii] Anh expression can be induced by SOX8 [50] and SOX10 [51], and [iii] Sox10 ectopic up-regulation in XX gonads can induce testis differentiation [51], we hypothesized that a Sox factor distinct from Sox9 could have induced late AMH expression in Rspo1KOSox9cKO gonads and delayed testicular differentiation. In agreement with this possibility, expression of both Sox8 and Sox10 was activated in Rspo1KOSox9cKO mutants at P12 and P0 respectively (Figure 5B, 5C). Previous data have shown that Sox8 becomes crucial from 14.5 dpc onwards, for the maintenance of testis development [52], suggesting that Sertoli cell differentiation can be induced by Sox genes other than Sox9 during late embryogenesis. However, the function of these Sox genes during late development is likely not sufficient to replace the role of Sox9 in early Sertoli cells development, thus leading to the formation of an hypoplastic testis, as is the case in the XY Rspo1KOSox9cKO mice. To date, the only factors that have been shown to be able to induce Sertoli cell differentiation are Sox genes [51,53], while other factors such as Dmrt1 are required after birth (P7) for the maintenance of Sertoli cell identity [54]. Further studies are required to address whether DMRT1 is able to allow Sertoli cell differentiation from undifferentiated supporting cells. Given that Sox9 expression is controlled by Wil when Sry expression has ceased [55], we can speculate that Wil might also be involved in Sox8 and Sox10 expression in these mutants. Furthermore, FGF9 or PGD2, two secreted factors synthesized in the undifferentiated gonads, [56,57] can also contribute to Sertoli cell differentiation [58,59,60]. Whether Wil, PGD2 or FGF9 signaling also regulate Sox8 and Sox10 remains to be investigated.

Figure 3. Post-natal development of sex cords in XY and XX Rspo1KOSox9cKO mice. Immunofluorescence of SDMG1 (in red). Counterstain is DAPI (in blue). SDMG1 is expressed in Sertoli cells (XY controls a, f, k, p) and in follicular cells of growing ovaries as evidenced at P12 onwards (j, o, t). Sertoli cells are present and formed sex cords in both XY and XX Rspo1KOSox9cKO gonads, with more developing sex cords in XY Rspo1KOSox9cKO testis (c, h, m, r) in comparison to XX Rspo1KOSox9cKO ovotestis (d, i, n, s). At P12, the sex cords are fully developed in both XY (h) and XX (i) Rspo1KOSox9cKO mice. In XY Sox9cKO (b, g, l, q) and XX control (e, j, o, t) gonads, ovarian follicles express SDMG1 at P12, P21 and P60. At these stages, SDMG1 is also expressed in the follicles of the XX double mutant ovotestes (see n) and in XY double mutant follicles when they develop (scale bars: 100 μm). XY (a, f, k, p) and XX (e, j, o, t) Rspo1KOSox9cKO controls, XY Sox9cKO gonads (b, g, l, q), XY (c, h, m, r) and XX (d, i, n, s) Rspo1KO Sox9cKO gonads respectively.

doi:10.1371/journal.pgen.1003170.g003
In addition, when XX and XY Rspo1^KOSox9cKO gonads are compared at the same stage, XY gonads always appear more masculinized than XX gonads (Figure 1, Figure 3, Figure S1, Figure S3, Figure S5), because they contain more sex cords/seminiferous tubules. At a molecular level, the main difference between XX and XY Rspo1^KOSox9cKO gonads is the expression of SRY in XY gonads (Figure 2). Indeed, SRY expression is

|     | XY Control | Sox9cKO | Rspo1^KOSox9cKO |
|-----|------------|---------|-----------------|
| STRA8 | GATA1 | ![a](image) | ![b](image) | ![c](image) |
| STRA8 | γH2AX | ![d](image) | ![e](image) | ![f](image) |
| AR | γH2AX | ![g](image) | ![h](image) | ![i](image) |
| Clu | DAPI | ![j](image) | ![k](image) | ![l](image) |

Figure 4. Sertoli cells support germ cell differentiation in XY Rspo1^KOSox9cKO gonads. Immunofluorescence (a–i) of GATA1 (Sertoli cell marker, in green), AR (Androgen Receptor) (Sertoli, peritubular myoid and Leydig cell marker, in red), STRA8 (a premeiotic marker, in red), and γH2AX (a meiotic marker, in green) at P10. Counterstain is DAPI (in blue). In situ hybridization (j–l) using a probe for Clu transcripts, another marker for mature Sertoli cells, illustrated as computer-generated bright field superimpositions of the blue counterstain (DAPI) with the hybridization signal (red false color). GATA1, AR and Clu expression show that the Sertoli cells mature in XY controls (a, g, j) and XY Rspo1^KOSox9cKO testes (c, i, l), and are able to support germ cell differentiation until meiosis initiation as revealed by STRA8 (a, c, d, f) and γH2AX (d, f, g, i) expression. Note that both Sertoli, peritubular myoid and Leydig cells of XY Sox9^KOSox9cKO mutant gonads normally expressed AR (h). (scale bars: 50 μm). XY (a, d, g, j) Rspo1^+/+; Sox9^flox/flox controls, XY Sox9^KOSox9cKO gonads (b, e, h, k) and XY Rspo1^KOSox9cKO (c, f, i, l) gonads.

doi:10.1371/journal.pgen.1003170.g004
Figure 5. AMH and SOX genes are expressed in XY and XX Rspo1KO Sox9cKO gonads. A- AMH expression in absence of SOX9. Immunofluorescence of SOX9 (in red) and AMH (in green). Counterstain is DAPI (in blue). SOX9 and AMH are synthesised in Sertoli cells of the testis (a, f). SOX9 is expressed in theca cells (white star in e) and AMH in follicular cells of the ovary at P12 (e, j). Deletion of Sox9 with Sf1:cre eliminates SOX9 expression in Sf1:cre positive cells of the gonads, which are Sertoli cells in XY (c) and XX (d) Rspo1KO Sox9cKO gonads and theca cells of the ovarian region of XX Rspo1KO Sox9cKO gonads (d) and XX Sox9cKO gonads (b). AMH expression is observed in Sertoli cells of the XY (c, h) and XX (d, i) Rspo1KO Sox9cKO gonads even the absence of SOX9. (scale bar: 50 μm). Immunofluorescence of FOXL2 (in red) and AMH (in green). Counterstain is DAPI (in blue). Most of the AMH positive cells in the testicular cords of Rspo1KO Sox9cKO gonads (h, i) are negative for FOXL2 indicating that they are not granulosa cells, some AMH/FOXL2 positive cells were observed outside of these cords indicating that they are granulosa cells (h, i). (scale bar: 100 μm). XY (a, f) and XX (e, j) Rspo1KO Sox9cKO control, XY Sox9cKO gonads (b, g), XY (c, h) and XX (d, i) Rspo1KO Sox9cKO gonads respectively. B- Sox8 is expressed in XY and XX Rspo1KO Sox9cKO gonads. In situ hybridization of Sox8 transcripts. Sox8 is expressed in Sertoli cells at P5 in XY control.
maintained in XY Rspo1KO Sox9KO gonads at 13.5 dpc, while at this time point its expression has ceased in XY control gonads. This suggests that SRY participates in the masculinization of the XY Rspo1KO Sox9KO gonads by inducing the expression of genes other than Sox9 to promote sex cord formation.

In summary, here we have shown that (i) both SRY and SOX9 are dispensable for female-to-male sex reversal in Rspo1KO, (ii) Rspo1 signaling is required for male-to-female sex reversal in Sox9KO, (iii) Sertoli cell differentiation and seminiferous tubule formation can occur in the absence of Sox9, possibly because of a functional redundancy with other SOX proteins such as SOX8 and SOX10. Indeed, ectopic absence of SOX9, possibly because of a functional redundancy with cell differentiation and seminiferous tubule formation can occur in the female-to-male sex reversal in pathophysiological conditions (Figure 6). Not the only masculinizing factors, since other SOX proteins can induce development in XY gonads by inducing the expression of genes other than Sox9, which are the SOX genes on the one hand and the RSPO1/WNT/FOXL2 paracrine signaling is required for male-to-female sex reversal in gonads by inducing the expression of genes other than Sox9.

**Materials and Methods**

Mouse strains and genotyping

The experiments here described were carried out in accordance with the relevant institutional and French animal welfare laws, guidelines and policies. They have been approved by the French ethics committee (Comité Institutionnel d’Éthique pour l’Animaux de Laboratoire; number NCE/2011-12). All mouse lines were kept on a mixed 129SV/C57BL6/J background. Gonad samples were collected from timed pups (day of birth = P0).

Gonads were dissected in PBS from P0 animals (day of birth = P0). Individual gonads were dissected in PBS from P0 animals (day of birth = P0). Individual gonads were dissected in PBS from P0 animals (day of birth = P0). Individual gonads were dissected in PBS from P0 animals (day of birth = P0). Individual gonads were dissected in PBS from P0 animals (day of birth = P0). Individual gonads were dissected in PBS from P0 animals (day of birth = P0).

Histological analysis

Urogenital organs were dissected, fixed in Bouin’s solution overnight, and then embedded in paraffin. Micron sections of 5 μm thickness were stained with periodic acid Schiff (PAS) or hematoxylin and eosin (H&E) according to standard procedures. Pictures were taken with an AxioCam mrm camera (Zeiss) and processed with Adobe Photoshop.

Immunological analyses

Gonad samples were fixed with 4% (w/v) paraformaldehyde overnight and then processed for paraffin embedding. Gonad samples for cryosectioning were successively fixed for 2 hours in 4% (w/v) paraformaldehyde, washed in cold phosphate-buffered saline (PBS), equilibrated in 10% (w/v) sucrose solution during 3 hours, then in 30% (w/v) sucrose solution overnight at 4°C, embedded in Cryomount (Histolab) and stored at −80°C. For paraffin-embedded and Cryomount-embedded samples, sections of 5 and 8 μm thickness were processed, respectively. The following dilutions of primary antibodies were used: AMH/MIS (C-20, sc-6886, Santa Cruz), 1:200; AR (sc-816, SantaCruz), 1:100; DMRT1 (kindly provided by David Zarkower), 1:500; FOXL2 (ab5096, Abcam), 1:250; γH2AX (US-636, Upstate), 1:500; GATA1 (sc-265, SantaCruz), 1:50; SDF1 (a kind gift from Ian Adams) 1:2000; SF1 (kindly provided by Ken Morohashi) 1:1500; SOX9 (HPA-001758, Sigma) 1:250 and SRY [59,66] 1:100; STRA8 (ab49602, Abcam), 1:100. Counterstain with 4′,6-diamidino-2-phenylindole (DAPI) was used to detect nuclei (in blue). Fluorescent studies were performed with a motorized Axio ImagerZ1 microscope (Zeiss) and pictures were taken with an AxioCam mrm camera (Zeiss) and processed with AxiosVision LE.

In situ hybridization

Embryos were fixed with 4% paraformaldehyde (PFA) in 1×PBS at 4°C overnight. Further processing of embryos and in situ hybridization were carried out essentially as described [67]. Sox9 riboprobe was synthesized according to [68] and Sox8 to [69], P450sc, Stra8 and Oct4 riboprobe synthesis were carried out as described previously [20]. In situ hybridisation (ISH) with digoxigenin-labelled probes was performed as described [70], using 10 μm-thick cryosections. Each experiment was repeated on at least two gonads. Post-hybridization washes were done in 100 mM maleic acid pH 7.5, 150 mM NaCl, 0.1% (v/v) tween–20 (MBT). To increase the sensitivity, 5% (v/v) polyvinyl alcohol (Sigma) was added to the staining solution [71]. Nuclei were counterstained with DAPI diluted in the mounting medium at 10 μg/ml (Vectorshied, Vector laboratories). ISH signals corresponding to Csta-positive cells were converted into a red false color on the merged pictures. The plasmids containing Lgals1 (366 bp-long; exons 2–4; MGI:96777) or Cua (942 bp-long; exons 5–9; MGI:88423) cDNA fragments were linearized and used as templates for the synthesis of the sense or antisense riboprobes.

Quantitative PCR analysis

Individual gonads were dissected in PBS from P0 animals (day of birth) and immediately frozen at −80°C. RNA was extracted using the RNeasy Qiagen kit, and reverse transcribed using the RNA RT–PCR kit (Stratagene). Primers and probes were designed at Roche Assay design center (https://www.rocheappliedscience.com/sis/rtcprt/upl/adc.jsp). Primers are 5′-TCCTCTCAGACCGGCTTTTC-3′ and 5′-CCTGGTTCATCATCGGTAATGTC-3′ (probe 95) for Hprt1, and 5′-ATGTCAGATGGAAACCCAGA-3′ and 5′-GCTTGGGGGGGGTGGTTGGAG-3′ (probe 21) for Sox10, 5′-aagaagatgctcattggtc-3′ and 5′-gggtgctgatcactctg-3′ (probe 40) for Dmnt1, and 5′-
ggctgctgtaactcctaca-3'

and 5′-gcagatgatgtgcgtgag-3′ (probe 51) for Foxl2. All real-time, quantitative, PCR assays (QPCR) were carried out using the LC-Faststart DNA Master kit Roche, according to the manufacturer’s instructions. QPCR was performed on cDNA from one gonad and compared to a standard curve. QPCR were repeated at least twice. Relative expression levels of each sample were quantified in the same run, and normalized by measuring the amount of Hprt1 cDNA (which represents the total amount of gonadal cells).

Statistical analysis

For each genotype (n = 6 individuals), the fold-change was the mean normalized expression levels divided by the mean normalized expression levels of the XY samples considered as the reference. Graphs illustrate fold-changes +1 s.e.m. The results were analyzed using Graphpad for statistical significance that was assessed using one-way ANOVA followed by Tukey-Kramer post-test for selected pairs of genotypes. Asterisks indicate: * p<0.05, ** p<0.01 and *** p<0.001.

Supporting Information

Figure S1 Testicular differentiation in XY and XX Rspo1KO-Sox9KO mice. External genitalia of XX control mice (e) is similar to XY Sox9KO mice (b), XY and XX Rspo1KO-Sox9KO mice (c and d respectively) at 2 months of age. The internal genitalia of XY and XX Rspo1KO-Sox9KO mice (h, i) show epididymides (E), vasa deferentia (VD) and seminal vesicles (SV), as in XY males (f) but also uterine horns (U) and oviducts (Ovi) as in XX Sox9KO mice (g). PAS stained histological sections of XY and XX Rspo1KO-Sox9KO gonads (m, n) show seminiferous tubules lacking germ cells because hypoplasia of germ cells occurred in these tubules. XY Sox9KO gonads (l) are similar to ovaries (o) (scale bar, 50 μm). XY (a, f, k) and XX (e, j, o) Rspo1KO−/−;Sox9flox/flox controls, XY Sox9KO gonads (b, g, l), XY (c, h, m) and XX (d, i, n) Rspo1KO-Sox9KO gonads respectively.

Figure S2 A- XY Rspo1KO Sox9KO gonad containing a single follicle. XY Rspo1KO Sox9KO gonad with a single grossly large follicle was located near the entrance of the oviduct (a). This follicle contained three oocytes and was observed in XY Rspo1KO Sox9KO gonads on rare occasions (b). B- Comparison of XX Rspo1KO and XX Rspo1KO Sox9KO gonads. Immunofluorescence detection of SDMG1 in Sertoli cells (cytoplasmic) of XX Rspo1KO (b) and XX Rspo1KO Sox9KO (c) gonads at P0. Some sex cords are clearly visible in XX Rspo1KO and XX Rspo1KO Sox9KO in contrast to XX control gonads (a). Histological analysis of XX Rspo1KO and XX Rspo1KO Sox9KO gonads at P12 and P21 (c, h and f, i respectively) show the presence of seminiferous tubules and follicles in comparison to XX controls containing only follicles.
(d, g). Empty and filled arrowheads indicate testis cords and follicles, respectively. (TIF)

**Figure S3** A-Expression of the steroidogenic marker P450sc and Cyp21 in XY Rspo1KO Sox9KO gonads. Whole-mount in situ hybridization of gonads at 14.5 dpc and 13.5 dpc. P450sc is expressed in XY Rspo1KO Sox9KO gonads (b), and in XY controls (a) but not in XX controls (c). P450sc, was expressed in cells at the anterior part of the XX Rspo1KO Sox9KO and XX Rspo1KO gonads (c and d respectively) at 14.5 dpc. Cyp21 was strongly expressed in the adrenals (f, g, h, i, j), whereas no signal was detected in the gonads at 13.5 dpc (f, g, h, i, j). Ad: adrenal. G: gonad. K: kidney. B: Delayed testicular cords formation in XY and XX controls (e).

**Figure S4** Detection of DMRT1 and FOXL2 in XY and XX Rspo1KO Sox9KO gonads at P0. A- Immunofluorescence analysis of DMRT1 and FOXL2 in XY and XX Rspo1KO Sox9KO gonads at P0. DMRT1 (red), a marker of postnatal Sertoli cells, was detected in the XY control (a), XY Rspo1KO Sox9KO (b) and XY Rspo1KO gonads (c and d respectively) whereas no signal was detected in the XX Rspo1KO Sox9KO (g) gonads. Haematoxylin and eosin stained histological sections of XY and XX Rspo1KO Sox9KO gonads at 17.5 dpc show that some sex cords are forming in the XY Rspo1KO Sox9KO (b) gonads in contrast to the XY controls (a) containing already formed sex cords. In the littermates XX Rspo1KO Sox9KO and Rspo1KO gonads (c and d respectively), no sex cords were observed at this stage (scale bar, 10 μm). Insets in a and b show AMH (Red) and DMRT1 (green), two markers of Sertoli cells highlighting sex cords at 16.5 dpc. Sex cords were rare in XY Rspo1KO Sox9KO gonads and absent from XX Rspo1KO Sox9KO gonads at this stage. (TIF)

**Figure S5** Histological analysis of XY and XX Rspo1KO Sox9KO gonads at P0, P12 and some until P21 in the XY Rspo1KO Sox9KO seminiferous tubules (h and m respectively) (scale bar: 50 μm). Controls XY (a, f, k) and XX (c, j, o) XY Sox9KO (b, g, l) and XY Rspo1KO Sox9KO (c, h, m) XX Sox9KO (d, i, n) and XX Rspo1KO Sox9KO (e, l, o).

**Figure S6** Mixed germ cell differentiation in XY Rspo1KO Sox9KO gonads at 14.5 dpc. In situ hybridization using a riboprobe for Sox9 (a-d), Cyp26b1 (e-h), Stmnt (i-l) and Oct4 transcripts (m-p) shows lack of Sox9 expression in the XY Sox9KO (b) XY Rspo1KO Sox9KO (c) and the XX control (d) gonads. Germ cells in XY Sox9KO (j) and XX (i) gonads have entered meiosis as evidenced by expression of primordial germ cell marker Oct4 (n and o respectively). XY Rspo1KO Sox9KO mutants (k) showed Stmnt expression at the periphery of the E14.5 gonad indicating these few cells have undergone meiosis (k) while the remaining germ cells were quiescent, thus, express Oct4 (o).

**Acknowledgments** We would like to thank Richard Behringer and Robin Lovell-Badge for helpful discussions; Ian Adams, Ken Morohashi, Dagmar Wilhelm, and David Zarkower for the SDMG1, SF1, FOXL2, and DMRT1 antibodies, respectively; and Stephen Bradford, Dagmar Wilhelm, and Peter Koopman for the SRY antibody.

**Author Contributions** Conceived and designed the experiments: RL A-AC NBG M-CC. Performed the experiments: RL A-AC EP EPG MK MM M-CC. Analyzed the data: RL A-AC EP EPG DGI R MM NBG M-CC. Wrote the paper: AS NBG M-CC.

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