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ORIGINAL ARTICLE

Amplification of R-spondin1 signaling induces granulosa cell fate defects and cancers in mouse adult ovary

M-C De Cian1,2,9, E Pauper1,9, R Bandiera3,9, VPI Vidal1, S Sacco1, EP Gregoire1, A-A Chassot1, C Panzolini1, D Wilhelm1, E Pailhoux5, SA Youssef6, A de Bruin6,7, K Teerds8, A Schedl1, I Gillot1 and M-C Chaboissier1

R-spondin1 is a secreted regulator of WNT signaling, involved in both embryonic development and homeostasis of adult organs. It can have a dual role, acting either as a mitogen or as a tumor suppressor. During ovarian development, Rspo1 is a key factor required for sex determination and differentiation of the follicular cell progenitors, but is downregulated after birth. In human, increased Rspo1 expression is associated with ovarian carcinomas, but it is not clear whether it is a cause or a consequence of the tumorigenic process. To address the role of Rspo1 expression in adult ovaries, we generated an Rspo1 gain-of-function mouse model. Females were hypofertile and exhibited various ovarian defects, ranging from cysts to ovarian tumors. Detailed phenotypical characterization showed anomalies in the ovulation process. Although follicles responded to initial follicle-stimulating hormone stimulation and developed normally until the pre-ovulatory stage, they did not progress any further. Although non-ovulated oocytes degenerated, the surrounding follicular cells did not begin atresia. Rspo1-induced expression not only promotes canonical WNT signaling but also alters granulosa cell fate decisions by maintaining epithelial-like traits in these cells. This prevents follicle cells from undergoing apoptosis, leading to the accumulation of granulosa cell tumors that reactivates the epithelial program from their progenitors. Taken together, our data demonstrate that activation of Rspo1 is sufficient in promoting ovarian tumors and thus supports a direct involvement of this gene in the commencement of ovarian cancers.

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INTRODUCTION

WNT/β-catenin (CTNNB1) signaling is involved in numerous biological processes from embryogenesis to stem cell activation. Deregulation of this pathway is responsible for many diseases, most notably colorectal cancer. Indeed, 90% of colorectal cancers result from APC mutations leading to the activation of this pathway.1 WNT signaling is tightly controlled by negative and positive modulators like APC and R-spondins (RSPO), a family of four members of secreted proteins. Fusions of RSPO2 and RSPO3 activate WNT signaling in colorectal cancers,2 and amplifications of RSPO1 have been associated with 8% of ovarian epithelial cancers.3,4 However, functional evidence for a direct involvement of Rspo1 in ovarian tumor development is still missing.

Depending on the cell type, Rspo1 may act either as a mitogen or a tumor suppressor.5,6 Variants of the Rspo1 locus have been reported in ovarian cancers highlighting its role as an oncogene.7,8 By contrast, Rspo1 loss-of-function mutations predispose to squamous cell carcinoma.9 On the molecular level, Rspo1 binds to the recruitment receptors LGR4, LGR5 or LGR6 leading to stabilization of CTNNB1, and in turn CTNNB1 interacts with transcription factors such as LEF/TCF in the nucleus and trans-activates target genes such as Axin2 (for a model, see ref. 7).

CTNNB1 is not only a key factor of the WNT signal-transduction pathway, but is also a component of adherens junctions linking cadherins via α-catenin to the actin cytoskeleton at the plasma membrane of epithelial cells.8 The membrane function of CTNNB1 can be partially substituted by the related protein JUP (Plakoglobin/γ-catenin), an important component of desmosomes that anchors epithelial cells to intermediate filaments and can also act as a signaling molecule at least in vitro.9,10 Several studies support a role for JUP in cancer.11 Although a direct link between Rspo1 and JUP has not yet been established, skin cells from patients affected by Rspo1 loss-of-function mutations exhibit defects reminiscent of desmosome abnormalities.6

In addition to the skin diseases, such as palmoplantar hyperkeratosis, patients carrying mutations in Rspo1 exhibit female-to-male sex reversal, and predisposition to squamous cell carcinoma and seminoma.6,12 Using functional analysis in mice, the role of Rspo1 and canonical WNT signaling has been unraveled in female ovarian development.13–15 Prior to sex determination, Rspo1 is involved in the proliferation of progenitor cells that are located within the surface epithelium of the undifferentiated gonads.16,17 Following sex determination, cells of the ovarian surface epithelium (OSE) continue to proliferate and enter the gonads to give rise to precursors of the granulosa cells.18,19 At this stage, Rspo1 is required to maintain granulosa precursors in the undifferentiated state.14,20 Accordingly, LGR4- and LGR5-expressing precursor cells contribute to the formation of the follicle pool from which follicles are recruited

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defects and eventually cancer development. Using a gain-of-function hypothesis that RSPO1 misexpression could be a cause of ovarian disorders, the juvenile and the adult form.29 Although less common than OSE-derived cancers in women, GCTs are categorized into two distinct subtypes, the juvenile and the adult form.28 Approximately 3% of ovarian cancers are derived from germ cells, 7% come from sex-chord stromal cells and 90% arise from OSE or fallopian tube epithelium.28 GCTs are categorized into two distinct subtypes, the juvenile and the adult form.29 Although less common than OSE-derived cancers in women, GCTs are categorized into two distinct subtypes, the juvenile and the adult form.28 Approximately 3% of ovarian cancers are derived from germ cells, 7% come from sex-chord stromal cells and 90% arise from OSE or fallopian tube epithelium.28 GCTs are categorized into two distinct subtypes, the juvenile and the adult form.28

RESULTS

RSPO1 expression is downregulated in adult follicles

RSPO1/Rspo1 is highly expressed in somatic cells of the ovary during embryogenesis.6,8 In the 2 weeks following birth, however, RSPO1 expression is downregulated as evidenced by qRT–PCR analysis.22 To obtain a more detailed expression profile and identify the cell types that express Rspo1 late in development and during the postnatal period, we performed in situ hybridization experiments. Strong expression of Rspo1 was found within the fetal ovary at 16.5 days post coitum (dpc), which significantly decreased after birth and remained at low levels in granulosa cells of cortical follicles in adult ovaries (Figures 1a–c). In contrast, Rspo1 was robustly expressed in the OSE at all stages (Figures 1a–c). To understand whether Rspo1 was able to activate canonical WNT signaling, we next analyzed whether the Axin2″LacZ reporter of this pathway31 was expressed in the adult ovary. β-Galactosidase staining was detected in the OSE and in the ovarian stroma as well as in oocytes (Figures 1e–g). By contrast, granulosa cells were weakly stained after birth, indicating that RSPO1/WNT signaling is downregulated in this compartment of the follicle.

Increased RSPO1 expression activates canonical WNT signaling in adult ovaries

To assess the importance of Rspo1 expression in the postnatal ovary, we next investigated the effect of induced Rspo1 expression on ovarian homeostasis. To this end, we generated Sf1-CreTg/+; R26Rspo1/− mice (Supplementary Figure S1). We first established the ability of female Sf1-CreTg/+; R26Rspo1/− mice to ectopically express Rspo1 throughout the ovary (Figures 1c and d). Next, we assessed that induced expression of Rspo1 led to a strong, although variable, follicular activation of canonical WNT signaling by detecting β-galactosidase in ovaries from Sf1-CreTg/+; R26Rspo1/−; Axin2″LacZ mice (Figures 1g and h). This confirmed that sustained expression of Rspo1 activates canonical WNT signaling in ovaries.

Figure 1. Pattern of Rspo1 expression and activation of WNT signaling in ovaries. Expression of Rspo1 revealed by in situ hybridization in R26Rspo1/− mouse ovaries at 16.5 dpc (a), 4 dpp (b) and 12 months (c). Rspo1 is expressed at low level in the R26Rspo1/− mouse adult ovary, mainly in the epithelial surface and in granulosa cells of some follicles in the ovarian cortex, whereas adult Sf1-CreTg/+; R26Rspo1/− ovaries display Rspo1 expression throughout the whole organ (d). Activation of the canonical WNT signaling by Rspo1 visualized by β-galactosidase staining of ovaries expressing the Axin2-LacZ reporter. Axin2-LacZ is expressed at 2.5 and 12 months in pre-antral follicles and the stroma (e and g) and strongly expressed in oocytes and the epithelial surface surrounding the ovary (f and arrow) in R26Rspo1/− mice. Induced Rspo1 expression leads to Axin2-LacZ expression in the whole Sf1-CreTg/+; R26Rspo1/− ovary (h) with strong staining in granulosa cells (arrow) and weaker staining in aberrant follicular structures lesions (*). Scale bars: 300 μm.
Rspo1 activation induces ovarian defects, subfertility and cancer

To evaluate the effect of Rspo1 expression on ovarian function we first assessed the fertility of Sf1-Cre\(^{G^{p}}\); R26\(^{R^{p}}\)/+ female mice. Litter sizes were significantly reduced with only 1.25 pups/female (±1.58) compared with 7.25 pups/female (±1.16) for R26\(^{R^{p}}\)/+ littersmates (n=8 plugged female per genotype). By the age of 6 months double-transgenic mice were all sterile (n=8). Macroscopic observation of dissected ovaries revealed a marked increase in the size of mutant ovaries when compared with those of the control littersmates (Figures 2a–c). The size of the transgenic ovaries increased over time (data not shown).

To gain insights into the ovarian phenotype, we examined 170 haematoxylin and eosin-stained slides containing serial sections from 14 murine ovaries from Sf1-Cre\(^{G^{p}}\); R26\(^{R^{p}}\)/+ mice. Histological analysis revealed a variety of abnormalities, ranging from 100% penetrate of dense follicle-like lesions to tumors in about 10% of ovaries (Figure 2). The ovarian tissue presented multifocal aggregates of neoplastic cells that arrange either in discrete solid aggregates, solid sheets or in microfollicular pattern (Figures 2f–h) reminiscent to granulosa cells. They are bland, ovoid, have distinct borders, scant amount of cytoplasm and devoid nuclei. On the basis of these observations, we conclude that GCTs accumulate in Sf1-Cre\(^{G^{p}}\); R26\(^{R^{p}}\)/+ mouse ovaries. None of these abnormalities were detected in control ovaries (Figure 2d). A proportion of these follicle-like structures developed into blood-filled cysts (Figures 2i and j) surrounded by cuboidal cells (Figure 2j) that were never observed in ovaries from control animals. In addition, fewer corpora lutea (CLs) were observed which is in agreement with the reduced fertility of the Sf1-Cre\(^{G^{p}}\); R26\(^{R^{p}}\)/+ females (Figure 2e). A proportion of regressing CLs contained erythrocytes infiltration (Figures 2i and m). One-year-old animals carried bigger cysts (Figures 2k and n), which, in some cases, contained layered keratinocytes in the cavity (Figure 2n). In addition to the accumulation of GCT, solid tumors were observed in 12–18-month-old females with 1 tumor/13 Sf1-Cre\(^{G^{p}}\); R26\(^{R^{p}}\)/+ and 3/19 Sf1-Cre\(^{G^{p}}\); R26\(^{R^{p}}\)/+R26\(^{R^{p}}\)/+ females, whereas 0/22 control littersmates were scored (Figure 2c). The severity of the different defects varied between animals and was not linked to whether they were heterozygous or homozygous for the Rosa26-Rspo1 transgene.

We next analyzed whether GCTs were proliferative or undergoing apoptosis in 40 days post partum (dpp) Sf1-Cre\(^{G^{p}}\); R26\(^{R^{p}}\)/+ ovaries using immunostaining for MKI67 and active CASP3, respectively, two markers of these processes. Only occasionally, we observed a cell expressing one of these markers in the GCT, implying that cell proliferation and apoptosis were negligible in ovaries using immunostaining for MKI67 and active CASP3, respectively, two markers of these processes. Only occasionally, we observed a cell expressing one of these markers in the GCT, implying that cell proliferation and apoptosis were negligible in ovaries (Figures 5c and d), whereas they were absent in the untreated females (Figures 5a and b). This indicates that Rspo1 does not impair follicular maturation by increasing proliferation of PMG5 to pre-pubertal females (21–24 dpp). Forty-eight hours after PMG administration, numerous large antral follicles in both Sf1-Cre\(^{G^{p}}\); R26\(^{R^{p}}\)/+ and R26\(^{R^{p}}\)/+ ovaries were observed (Figures 5c and d), whereas they were absent in the untreated females (Figures 5a and b). In laboratory mouse strains, puberty occurs between 30–40 dpp. Although GCT accumulation arises at the onset of puberty, we hypothesized that gonadotropins are involved in GCT formation in Sf1-Cre\(^{G^{p}}\); R26\(^{R^{p}}\)/+ females. Thus, we stimulated follicular maturation by injecting hCG to pre-pubertal females (21–24 dpp). Forty-eight hours after hCG injection, numerous large antral follicles in both Sf1-Cre\(^{G^{p}}\); R26\(^{R^{p}}\)/+ and R26\(^{R^{p}}\)/+ ovaries were observed (Figures 6a and b). This indicates that Rspo1 does not impair follicular maturation by hCG injection. However, most of these large antral follicles in Sf1-Cre\(^{G^{p}}\); R26\(^{R^{p}}\)/+ mice exhibited structural abnormalities ranging from asymmetric repartition of granulosa cells to naked or abnormal oocytes (Figures 6c–g) as described in ref. 34.

In order to test whether the antral follicles present in PMG-treated pre-pubertal Sf1-Cre\(^{G^{p}}\); R26\(^{R^{p}}\)/+ females are competent for ovulation, superovulations were induced by injecting human chorionic gonadotropin (hCG) 48 h after PMG administration. Ovulation takes place about 12 h post-hCG injection. We first examined the expression of Fshr and Foxo1, two factors involved in follicular development, 4 h after hCG injection and found no significant differences between the two genotypes (Figure 6a). Twenty hours after hCG injection, control ovaries were mainly constituted by post-ovulatory CLs, but none or only a few were identified in Sf1-Cre\(^{G^{p}}\); R26\(^{R^{p}}\)/+ females (Figures 6b and c). Strikingly, in Sf1-Cre\(^{G^{p}}\); R26\(^{R^{p}}\)/+ ovaries, follicular lesions began to emerge and many large antral follicles were still apparent, some of which still contained oocytes (Figure 6c), suggesting that ovulation did not process efficiently. To confirm this observation, we quantified ovulated oocytes in superovulated females. Indeed, the number of ovulated oocytes was significantly reduced in Sf1-Cre\(^{G^{p}}\); R26\(^{R^{p}}\)/+ females (Figure 6d) and when ovulated, the oocytes were devoid of a cumulus oophorus (data not shown).

Moreover, we observed a dramatic reduction of the level of expression of Amphiregulin (Areg) and Epiregulin (Ereg), two EGF-like growth factors (Figures 3a and b). In addition, in Sf1-Cre\(^{G^{p}}\); R26\(^{R^{p}}\)/+ mice, we observed a cell expressing one of these markers in the GCT, implying that cell proliferation and apoptosis were negligible in ovaries stained for MKI67 and active CASP3, respectively, two markers of these processes. Only occasionally, we observed a cell expressing one of these markers in the GCT, implying that cell proliferation and apoptosis were negligible in ovaries stained for MKI67 and active CASP3, respectively, two markers of these processes. Only occasionally, we observed a cell expressing one of these markers in the GCT, implying that cell proliferation and apoptosis were negligible in ovaries stained for MKI67 and active CASP3, respectively, two markers of these processes. Only occasionally, we observed a cell expressing one of these markers in the GCT, implying that cell proliferation and apoptosis were negligible in ovaries stained for MKI67 and active CASP3, respectively, two markers of these processes. Only occasionally, we observed a cell expressing one of these markers in the GCT, implying that cell proliferation and apoptosis were negligible in ovaries stained for MKI67 and active CASP3, respectively, two markers of these processes. Only occasionally, we observed a cell expressing one of these markers in the GCT, implying that cell proliferation and apoptosis were negligible in ovaries stained for MKI67 and active CASP3, respectively, two markers of these processes. Only occasionally, we observed a cell expressing one of these markers in the GCT, implying that cell proliferation and apoptosis were negligible in ovaries stained for MKI67 and active CASP3, respectively, two markers of these processes. Only occasionally, we observed a cell expressing one of these markers in the GCT, implying that cell proliferation and apoptosis were negligible in ovaries stained for MKI67 and active CASP3, respectively, two markers of these processes. Only occasionally, we observed a cell expressing one of these markers in the GCT, implying that cell proliferation and apoptosis were negligible in ovaries stained for MKI67 and active CASP3, respectively, two markers of these processes.
Figure 2. Ovarian defects and tumors in Sf1-CreTg/+; R26Rspo1/+ ovaries. Macroscopic view of 18-month-old R26Rspo1/+ (a) and Sf1-CreTg/+; R26Rspo1/+ ovaries (b and c). The size of the Sf1-CreTg/+; R26Rspo1/+ ovaries is increased in comparison with the control ovary owing to the presence of prominent tumor tissue (c) and/or blood-filled cysts (b). Magnification 10×. Histological sections (haematoxylin and eosin staining) analysis of 12-month-old ovaries. Sf1-CreTg/+; R26Rspo1/+ ovaries (e) contain numerous multifocal aggregates of neoplastic cells that arrange either in discrete solid aggregates (*) or in microfollicular pattern (arrows) in comparison with R26Rspo1/+ ovaries (d). Scale bars, 500 μm. Magnifications of structural abnormalities in Sf1-CreTg/+; R26Rspo1/+ ovaries (f–n); neoplastic cells arranged in microfollicular or rosette-like pattern (f; *) with central pale eosinophilic globule (Call-Exner body; f and g arrow); neoplastic cells arranged in solid sheets composed of bland, ovoid, scant amount of cytoplasm and ovoid nuclei granulosa cells. (h, arrow); hematocysts (i and j), with columnar cells (j, arrow), CL or regressing CL with erythrocytes infiltration (I and m, arrow), Ovarian epithelial cyst composed of cystic and papillary structures lined with tall columnar ciliated epithelium. (k, arrow) and ovarian epithelial inclusion cyst composed of keratinized squamous epithelium (n, arrow). Scale bars: 100 μm. a, antral follicle; cl, corpus luteum.
RSPO1 and ovarian disorders
M-C De Cian et al

Figure 3. RSPO1 promotes GCT. Histological analysis (haematoxylin and eosin staining) of R26Rspo1/+ and Sf1-CreTg/+; R26Rspo1/+ ovaries at 28 dpp (a and b) and 40 dpp (c and d). Follicle-like structures (*) consisting of nests of disorganized, pleiomorphic granulosa cells become evident at 28 dpp (b) in Sf1-CreTg/+; R26Rspo1/+ ovaries, whereas they are not observed in R26Rspo1/+ ovaries (a). They enclose oocytes at 28 dpp (b), whereas they lack an antrum and contain no visible or a degenerating oocyte at 40 dpp (d). Scale bar: 300 μm. Immunolabelling of FOXL2 (e and f), AMH (g and h) and Vimentin (VIM) (i and j) in R26Rspo1/+ (left panel) and Sf1-CreTg/+; R26Rspo1/+ ovaries (right panel) at 40 dpp. FOXL2 staining in the GCT (f, *) confirms the presence of granulosa cells in these follicle-like structures in R26Rspo1/+ ovaries (e). The absence of AMH staining (h) and the presence of VIM staining (j) in GCT (*) suggest that follicular maturation can occur up to the antral stages. Nuclei are labeled with DAPI (blue). Scale bars: 100 μm. a, antral follicle; pa, pre-antral follicle, cl, corpus luteum.
factors involved in cumulus cell-oocyte complex expansion and thus required for ovulation (Figure 6a), which could at least partially explain the improper ovulation phenotype in Sf1-CreTg/+; R26Rspo1/+ females. The lack of CLs in Sf1-CreTg/+; R26Rspo1/+ ovaries (Figure 6c) suggests that RSPO1 expression impairs the terminal differentiation of antral follicles. Indeed, the expression of Lhr, Cyp11a1 and Star, key genes of luteinization process of the granulosa cells which occurs upon LH/hCG surge, was significantly downregulated in Sf1-CreTg/+; R26Rspo1/+ ovaries (Figure 6a). As a result, although R26Rspo1/+ ovaries were filled with CLs expressing CYP11A1, only few CYP11A1-positive CL were observed in Sf1-CreTg/+; R26Rspo1/+ ovaries (Figures 6f and g). Interestingly, the newly formed follicular lesions were devoid of CYP11A1 (Figure 6g). In addition, 21–24 dpp superovulated Sf1-CreTg/+; R26Rspo1/+ ovaries contained aberrant follicles including degenerating oocytes as observed in 40 dpp non-superovulated Sf1-CreTg/+; R26Rspo1/+ ovaries (Figures 3d and 6e), suggesting that follicular lesions become apparent in the later stages of follicular development.

Altogether our results demonstrate that follicular abnormalities observed in Sf1-CreTg/+; R26Rspo1/+ ovaries are driven by puberty, that Sf1-CreTg/+; R26Rspo1/+ ovaries are unable to properly respond to LH and that GCT formation is linked to FSH stimulation. These results show that superovulation experiments induce and accelerate the formation of GCT and that Rspo1 downregulation within adult ovary is required for the proper occurrence of the ovulation process and the subsequent formation of CLs.

Induced expression of Rspo1 promotes cell junction adherence and epithelial traits in GCT

Next, we searched for an explanation for the persistence of these abnormal follicular structures. RSPO1 is typically associated with canonical WNT signaling as corroborated by the expression of the Axin2+/LacZ reporter (Figures 1g and h). However, CTNNB1 is also a component of adherens junctions, where it associates with cadherins (CDH). Maintaining adherence junctions has been shown previously to inhibit apoptosis of granulosa cells thus preventing follicular degeneration. Therefore, the presence of adherens junctions was investigated in ovaries of 6-month-old Sf1-CreTg/+; R26Rspo1/+ and R26Rspo1/+ mice by immunostainings using the markers CTNNB1 and cell-adhesion molecule N-cadherin (CDH2). CTNNB1 was mostly detected at the membrane of granulosa cells of pre-antral follicles and in granulosa cells surrounding the antrum in larger antral follicles in control ovaries (Figures 7a and c). CTNNB1 was enriched at the membrane of the cells in the GCT in Sf1-CreTg/+; R26Rspo1/+ ovaries.
The presence of CDH2 was evident at all stages during maturation in both genotypes (Supplementary Figures S3A, B). GCT also strongly expressed membrane-bound plakoglobin (JUP), a component of desmosomes and intermediate junctions (Figures 7e and f), and the epithelial markers KRT18 and pan-cytokeratin (Figures 7g and h and Supplementary Figures S3C–F) that are absent or weakly expressed in follicles in control ovaries. These data reveal the presence of strong intercellular junctions in GCT in Sf1-CreTg/+;R26Rspo1/+ ovaries, indicating that induced expression of Rspo1 increases cellular adhesion, which in turn may promote epithelialisation of granulosa cells and the persistence of the follicular remnants.

**DISCUSSION**

Although RSP01 is crucial for ovarian development, here we show that its postnatal downregulation is critical for the maintenance of a healthy adult ovary. When RSPO1 expression is maintained, GCT appear at the onset of puberty in 100% of the cases. Prolonged RSPO1 expression allows follicular maturation until the late antral stage. Most follicles are capable of responding to FSH stimulation as previously reported for induced CTNNB1 expression, and fail to respond to LH. It has been shown that FSH increases the occurrence and onset of GCT formation. These GCT are classified as adult GCT. Accordingly, we observed high FSH levels that could contribute to the adult GCT formation in Sf1-CreTg/+;R26Rspo1/+ mice.

Endocrine functionality of CL involves LGR receptors. However, neither Rspo1 nor the CTNNB1 target Axin2 is expressed in these structures, which suggests that LGRs may act through an RSPO1/CTNNB1 independent pathway in this cell type. In contrast, RSPO1, stabilized CTNNB1 and LGR5 are expressed in the OSE stem cells that contribute to post-ovulatory repair, putting forward that these factors act together in the ovulation process. However, the ovulatory defects observed in our model prevented us from analyzing whether induced Rspo1 affects CL physiology and post-ovulatory OSE repair.
Figure 6. Induced Rspo1 expression prevents ovulation. Quantitative RT–PCR analysis of Fshr, Foxo1, Areg, Ereg, Lhr, Cyp11a1 and Star expression in R26Rspo1/+(black bars) and Sf1-CreTg/+;R26Rspo1/+(white bars) ovaries from 21–24 dpp superovulated mice, using Sdha1 as the normalization control (a). Bars represent mean ± s.e.m. (n=6 for each genotype) with significance set at P<0.05 (*), P<0.01 (**) and P<0.001 (**). Histological analysis (haematoxylin and eosin staining) of R26Rspo1/+ (b) and Sf1-CreTg/+;R26Rspo1/+ ovaries at 21–24 dpp stimulated by hCG (b, c and e). R26Rspo1/+ ovaries (b) contain many corpora lutea (cl) which are rarely observed in Sf1-CreTg/+;R26Rspo1/+ ovaries (c). Instead, these ovaries display several GCT (*) and naked oocytes (arrowhead) in antral (indicated as a) follicles (c) and degenerating follicles (arrowhead) (e). Superovulation experiments show a reduced number of ovulated oocytes (d) with an average of 8 and 0.6 oocyte/ovary (n=8–10) in R26Rspo1/+ and Sf1-CreTg/+;R26Rspo1/+ females, respectively. CYP11A1 revealed by immunostaining is highly expressed in CL of R26Rspo1/+ ovaries (f) but absent in the abnormal follicles (*) of Sf1-CreTg/+;R26Rspo1/+ ovaries (g). Nuclei are labeled with DAPI (blue). Scale bars: 100 μm.
In addition to the granulosa cell phenotype, we observed an enriched population of SF1-positive theca cells suggesting a role of CTNNB1 signaling in differentiation of steroidogenic lineages. The steroidogenic precursors of adrenal and gonadal cells have a common origin and both arise from a pool of WT1-positive cells. In adrenals, CTNNB1 is required for the formation and maintenance of the steroidogenic cells of the adrenal cortex, but the role of this signaling pathway in the fate of the theca cells has not been investigated to date.

Ablation of Rspo1 induces a precocious differentiation of granulosa cells during fetal development. This demonstrates that Rspo1 is required to maintain an undifferentiated state of the granulosa cell progenitors during early development, before Rspo1 expression decreases within the ovary around birth. Here, we show that when Rspo1 expression is maintained, it alters the differentiation of granulosa cells via their epithelialization. First, CTNNB1 and JUP are maintained at the plasma membrane of aberrant follicular structures and contribute to cell junction formation. Although follicular atresia is promoted by oocytes disruption, the maintenance of intercellular junctions can prevent degeneration of the granulosa cells. This suggests that stabilization of cell–cell contacts promote survival of the GCT in Scf1-CreTg/+; R26Rspo1/ mice. Interestingly, in a granulosa cell dominant-stable CTNNB1 mutant mouse model, GCTs develop via a mechanism that mainly implies canonical WNT signaling. Here, we show that RSPO1 activation in adult ovaries induces formation of GCTs by regulating not only canonical WNT signaling activation but also intercellular junction homeostasis in granulosa cells.

Figure 7. RSPO1 expression maintains intercellular junctions and epithelial identity in GCT. Immunostaining for CTNNB1 (a–d), JUP (e and f), and KRT18 (g and h) in 6-month-old R26Rspo1/+ (left panel) and Scf1-CreTg/+; R26Rspo1/ (right panel) ovaries. Membrane staining for CTNNB1 and JUP underline cellular junctions in these follicle-like structures (h). Nuclei are labeled with DAPI (blue). Scale bars: 100 μm.
Ovaries, including fat pad, oviduct and partial uterine tube, were fixed in 4% paraformaldehyde or Bouin’s solution overnight. For qPCR analyses, ovaries were carefully isolated from the genital tract and quickly frozen in liquid nitrogen. For oocyte counting, immature females (*n* = 8 (*R26Rtam/+) and 10 (*Sfi-CreF95; R26Rtam/+) ovaries) were similarly superovulated and killed 20 h after the hCG injection. Oviducts were isolated from ovary and uterus and opened to collect the oocytes.

**X-gal staining and immunological analyses**

Tissue samples were fixed in 4% paraformaldehyde overnight and then processed for paraffin embedding or equilibrated in sucrose and embedded in Cryomount (Histolab, Göteborg, Sweden) for cryosectioning. Cryostat or microtome sections of 10 μm and 5 μm thickness respectively were processed for X-Gal and immunostaining. Samples for X-Gal staining were processed as described previously.14 Immunohistochemical experiments were performed as described in. The following dilutions of primary antibodies were used: AMH (C-20, cat sc6886, Santa Cruz Biotechnology, Dallas, TX, USA) 1:200, CTHB1 (cat 610153, BD Biosciences, San Jose, CA, USA) 1:250, active CASP3 (cat AF835, R&D Systems) 1:200, CDH2 (cat 33-3900, Invitrogen) 1:200, CYP11A1 48 1:300, FOXL2 21 1:400, JUP (cat 610254, BD Biosciences) 1:250, KRT18 (cat ab52948, Abcam, Cambridge, UK) 1:1000, LHR (LHR-29, ATCC-CRL-2685, ATCC) 1:150, MKI67 (clone SP6, cat 9106, Thermo Fisher Scientific, Waltham, MA, USA) 1:200, SF1 (kindly provided by Professor Morohashi) 1:1000 and Vimentin (cat ab92547, Abcam) 1:200. Slides were counterstained with DAPI diluted in the mounting medium at 10 μg/ml (Vectorshield, Vector laboratories, Burlingame, CA, USA) to detect nuclei. Imaging was performed with a motorized Axioskop ImagerZ1 microscope (Zeiss) coupled with an Axioham Mrm camera (Zeiss) and processed with Axiovision LE (Zeiss) and Adobe Photoshop.

**Materials and methods**

Mouse strains and genotyping

The experiments were carried out in compliance with the relevant institutional and French animal welfare laws, guidelines and policies and have been approved by the French ethics committee (CIEPAL: NCE/2011-12).

*Rspo1* gain-of-function model (Supplementary Figure S1) carries an inducible *Rspo1* gene knockled into the Rosa26 locus. The construct is similar to the one reported in ref. 46 with the exception of the promoter. Here, the Rosa26 promoter controls *Rspo1* expression. R26Rtam/; mice were mated with the Sfi-CreF95 (*Sfi-CreF95*) strain, which drives expression of the Cre recombining enzyme in the gonads from 11.5 d.p.c toward 4.47 Axin2-LacZ mice were described previously,33 and crossed with *Sfi-CreF95/; R26Rtam/+ males to generate *Sfi-CreF95/; R26Rtam/+; Axin2LacZ animals. Fertility tests were performed using 3–4-month-old *Sfi-CreF95/; R26Rtam/+ females mated with R26Rtam/+ males (Supplementary Figure S1). Genotyping was performed using DNA extracted from tail tips or ear biopsies of mice. The presence of the Y chromosome and the Axin2-LacZ transgene was determined as described previously.14

**Histological analysis**

Ovaries were dissected, fixed in Bouin’s solution overnight, and embedded in paraffin. Five-micrometer-thick sections were stained with hematoxylin and eosin. To determine the ovarian phenotype in *Rspo1* expressing ovaries, consecutive sections were analyzed. Pictures were taken with an Axiostar 2 (Zeiss, Oberkochen, Germany) or MZ9.5 (Leica, Mannheim, Germany) microscope coupled with an Axiocam MRCS (Zeiss) or DHC490 (Leica) camera and Axiosvision 4.8 (Zeiss) or application suite V3.3.0 (Leica) software, and processed with Adobe Photoshop (San Jose, CA, USA).

**Serum collection and hormone assays**

Blood was collected from adult mice in the same stage of estrous cycle and allowed to clot for 90 min at room temperature. Samples were then centrifuged and the serum was collected and stored at −20 °C. Hormone analyses were performed by the Ligand Assay and Analysis Core Laboratory at The Center for Research and Reproduction, University of Virginia (Charlottesville, VA, USA). LH, FSH, estradiol and progesterone measurements were run in duplicates using specific assays (*n* = 5 for each genotype).

**Superovulations**

Immature 3–4-week-old mice were injected intraperitoneally (i.p.) with 5 IU/mouse pregnant mare serum gonadotropin (PMSG) (Sigma-Aldrich, St Louis, MO, USA) to promote follicle maturation, followed by 5 IU/mouse hCG (Sigma-Aldrich) 48 h later to induce ovulation. Mice were humanely killed 48 h after the first hormone injection for follicle maturation studies or 4 h and 20 h after the second injection for ovulation and luteinization studies.
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