A novel function of R-spondin1 in regulating estrogen receptor expression independent of Wnt/β-catenin signaling

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Abstract R-spondin1 (Rspo1) has been featured as a Wnt agonist, serving as a potent niche factor for stem cells in many tissues. Here we unveil a novel role of Rspo1 in promoting estrogen receptor alpha (Esr1) expression, hence regulating the output of steroid hormone signaling in the mouse mammary gland. This action of Rspo1 relies on the receptor Lgr4 and intracellular cAMP-PKA signaling, yet is independent of Wnt/β-catenin signaling. These mechanisms were reinforced by genetic evidence. Luminal cells-specific knockout of Rspo1 results in decreased Esr1 expression and reduced mammary side branches. In contrast, luminal cells-specific knockout of Wnt4, while attenuating basal cell Wnt/β-catenin signaling activities, enhances Esr1 expression. Our data reveal a novel Wnt-independent role of Rspo1, in which Rspo1 acts as a bona fide GPCR activator eliciting intracellular cAMP signaling. The identification of Rspo1-ERα signaling axis may have a broad implication in estrogen-associated diseases.

Introduction

Estrogen and progesterone are the main players in mammary development and the progression of breast cancers (Hilton et al., 2018; Macias and Hinck, 2012). Both hormones act through their cognate receptors, estrogen receptor (ER) and progesterone receptor (PR) (Hilton et al., 2018). The mechanisms of ERα activity have been extensively studied (Carroll, 2016). However, the upstream regulation of ERα (Esr1) expression is much less understood.

The mammary gland is an epithelial organ profoundly influenced by estrogen and progesterone. The mammary gland is composed of basal and luminal cells, which can be separated by surface expression of CD24 and CD29/CD49f (Shackleton et al., 2006; Stingl et al., 2006). ER+ or PR+ cells, consisting 30 – 50% of luminal cells, can be enriched by surface expression of Sca1 (Regan et al., 2012; Shehata et al., 2012; Sleeman et al., 2007). Hormones exert their mitogenic effects primarily through induction of local growth factors (Asselin-Labat et al., 2010; Brisken et al., 2000; Cai et al., 2014; Joshi et al., 2010; Rajaram et al., 2015).

R-spondin1 (Rspo1) has been identified as a hormone-mediated local factor, whose expression is upregulated by estrogen and progesterone (Cai et al., 2020; Cai et al., 2014). R-spondin protein family (Rspo1-4) have been reported to function as niche factors for adult stem cells in multiple organs (Greicius et al., 2018; Han et al., 2014; Planas-Paz et al., 2016; Sigal et al., 2017), and Rspo1 has been implicated as critical growth factor in many in vitro stem cell expansion systems,
including intestine, stomach and liver (Barker et al., 2010; Huch et al., 2013; Kim et al., 2005; Sato et al., 2009). The role of Rspo1 in Wnt signaling has been extensively studied. Rspo1, through its interaction with its receptors Lgr4/5/6, enhances Wnt signaling by attenuating the turnover of Wnt receptors (Hao et al., 2012; Koo et al., 2012) and potentiating phosphorylation of the Wnt co-receptor Lrp (Carmon et al., 2011; de Lau et al., 2011; Glinka et al., 2011; Gong et al., 2012). In the mammary gland, Rspo1 synergizes with another niche factor, Wnt4, to promote mammary basal stem cell self-renewal (Cai et al., 2014). In line with the role of Rspo1 in MaSC regulation, Rspo1 expression is enhanced in the diestrus phase of the estrous cycle and during pregnancy (Cai et al., 2014), coinciding with the rise of progesterone level and the expansion of basal stem cells (Asselin-Labat et al., 2010; Joshi et al., 2010). Our recent study also reported the enhanced Rspo1 expression in estrus, a stage with high estrogen signaling activity (Cai et al., 2020). Another role of Rspo1 may exist besides maintaining basal stem cells.

In this study, we uncover a novel function of Rspo1 distinct from its previously reported role in stem cell regulation. We provide evidence that Rspo1 promotes ERα (Esr1) expression in luminal cells of the mammary gland. This action of Rspo1 is through activating G-protein coupled cAMP/ PKA pathway, while independent of Wnt/β-catenin signaling. Our data reveal a novel Wnt-independent role of Rspo1, and a new upstream regulatory axis for Esr1 expression.

Results

Rspo1 induces ERα expression and promotes ERα signaling

To investigate the potential role of Rspo1 in luminal cells, we isolated primary luminal cells (Lin+, CD24+, CD29lo) by FACS (fluorescence-activated cell sorting), and cultured them in 3D Matrigel in the presence of RSP01 (0.5 μg/ml) (Figure 1—figure supplement 1a). Transcriptome and Gene ontology (GO) analysis identified enrichment of various features, including estrogen receptor activity (Figure 1a and b). qPCR analysis verified that the expression of ERα signaling target genes, including Pgr (progesterone receptor, PR), Ctsd1 (Cathepsin D1) (Meneses-Morales et al., 2014), and Wisp2 (Zhang et al., 2012b) are enhanced in the presence of RSPO1 (Figure 1—figure supplement 1b).

To further investigate how Rspo1 regulates ERα signaling, we isolated ERα+ luminal cells (Lin+, CD24+, CD29lo, Sca1+) and ERα- luminal cells (Lin+, CD24+, CD29lo, Sca1-) based on Sca1 expression (Figure 1c), and cultured them in 3D. RSP01 treatment resulted in the upregulation of ERα targets, Pgr, Ctsd1 and Wisp2 in ERα+ luminal cells, indicating the further activation of ERα signaling (Figure 1d). Interestingly, the expression of ERα itself (Esr1) is also enhanced (Figure 1e). In contrast, ERα- luminal cells did not respond to RSP01 stimulation (Figure 1—figure supplement 1c). Estrogen (Estradiol-E2, E2) is one of the few known upstream regulator of Esr1 (Chu et al., 2007; Kanaya et al., 2019). Thus, E2 (1 μM) was used as control to show the extent of Esr1 activation. We found that in this ERα+ luminal cell culture system, RSP01 elevated the expression of Esr1 and its target Pgr to a level comparable with E2 treatment (compare Figure 1d–e with Figure 1f). The upregulation of ERα protein by RSP01 was confirmed by Western blot analysis (Figure 1g). This role of RSP01 was further validated in mouse mammary Eph4 cells. RSP01 upregulates the expression of Esr1 and ERα signaling targets Pgr and Greb1 (growth regulation by estrogen in breast cancer 1) in a dose-depending manner (Figure 1—figure supplement 2a–c).

To investigate whether Rspo1 regulates Esr1 transcription, we utilized a luciferase reporter driven by the proximal promoter (promoter A) of human ESR1 (Tanimoto et al., 1999). We found that RSP01 can induce luciferase expression in a dose-dependent manner, while the control reporter lacking ESR1 promoter was not activated in any conditions (Figure 1h). Together, these data suggest that Rspo1 enhances Esr1 transcription.

Rspo1-induced ERα expression is dependent on Lgr4

To investigate the mechanisms through which Rspo1 regulates Esr1, we first examined which receptor of Rspo1 is involved. qPCR analysis indicated that all three Lgr receptors, Lgr4/5/6 are expressed in basal cells, but only Lgr4 is expressed in luminal cells (Figure 2a), suggesting that Rspo1 may rely on Lgr4 to signal in luminal cells in the context of Esr1 induction. Within the luminal compartment, Lgr4 was evenly distributed in ERα+ (Sca1+) and ERα- (Sca1-) luminal cells (Figure 2a). In situ
Figure 1. Rspo1 enhances Esr1 transcription and ERα signaling activities. (a) RNA-seq of 3D cultured luminal cells in the presence of RSPO1 (0.5 µg/ml) or vehicle. Increased expression of ERα target genes (Pgr, Greb1) and Esr1 were enlisted in heatmap of differentially expressed genes (DEGs). (b) GO analysis was conducted on upregulated genes and estrogen receptor activity was enhanced in the presence of RSPO1. (c) Sca1+ luminal cells were FACS-isolated. (d, e) qPCR analysis of cultured cells in day two indicating increased expression of Esr1 (e) and its target genes (d) in the presence of RSPO1 (0.5 µg/ml). (f) E2 (1 µM) treatment was used as positive control indicating the upregulation of Esr1 and its target Pgr. (g) Western analysis of cultured cells in day 2 showing increased ERα protein levels after RSPO1 treatment. (h) A luciferase reporter driven by the ESR1 promoter was constructed and transfected into HEK293T cells. RSPO1 treatment activated the ESR1 promoter-luciferase reporter activities in a dose dependent manner. (d–h) Data are presented as mean ± s.e.m. of three independent experiments. Student’s t test: ***p<0.001, **p<0.01, *p<0.05.

The online version of this article includes the following figure supplement(s) for figure 1:

Figure supplement 1. Rspo1 promotes ERα signaling activities.

Figure supplement 2. RSPO1 induces Esr1 expression in a dose-dependent manner in Eph4 cell line.

hybridization validated the expression pattern of Lgr4 in both basal and luminal layers (Figure 2b). We next investigated whether Lgr4 mediates Rspo1’s action on Esr1 expression. We generated Lgr4 shRNA and validated its knockdown efficacy in primary luminal cells by qPCR analysis (Figure 2c). Lgr4 knockdown suppressed the upregulation of Esr1 induced by RSPO1 (Figure 2d). In an ESR1-luciferase reporter assay using T47D (a human breast cancer cell line), LGR4 Knockdown also inhibited the luciferase activities induced by RSPO1 (Figure 2e). The effect was validated using two different shRNAs (Figure 2e, Figure 2—figure supplement 1). Results suggest that Rspo1 relies on Lgr4 to activate Esr1 expression.
ERα induction by Rspo1 is independent of Wnt/β-catenin signaling

As Rspo1 is known for amplifying Wnt/β-catenin signaling, we investigated whether Wnt ligands have a synergistic influence on Erα expression. We first examined Wnt4, a major Wnt ligand in the mammary gland that can activate Wnt/β-catenin signaling (Cai et al., 2014; Rajaram et al., 2015). The activation of Axin2 expression indicated that Wnt/β-catenin signaling was activated in primary luminal cell culture in the presence of Wnt4 (Figure 3a). Wnt4+RSPO1 combination further stimulated Axin2 expression (Figure 3a). Intriguingly, addition of Wnt4 alone was ineffective in activating Erα expression in these cells (Figure 3b), and Wnt4+RSPO1 combination was unable to further increase Erα level compared to RSPO1 alone (Figure 3b). These results suggest that canonical Wnt signaling may not be involved in this regulatory axis. Furthermore, we used either Wnt3a or a GSK3 inhibitor CHIR99021 (CHIR) to stimulate Wnt/β-catenin signaling in primary luminal cell culture. Although Wnt-signaling activators markedly increased the expression levels of its target gene Axin2 (Figure 3c), they could not stimulate Erα expression (Figure 3d). It is noteworthy that the combination of RSPO1 with CHIR did not further induce Axin2 level (Figure 3c), probably due to the Wnt/β-catenin signaling activity induced by CHIR or Wnt3a had reached plateau. In contrast to their
Figure 3. Esr1 expression induced by Rspo1 is independent of Wnt/β-catenin signaling. (a–b) qPCR analysis of cultured luminal cells indicating that Wnt4 alone or in combination with Rspo1 can activate Wnt target Axin2 expression (a). While Rspo1 alone promoted Esr1 expression, Wnt4 was ineffective for Esr1. Combination of Rspo1 and Wnt4 displayed no difference compared with Rspo1 alone (b). (c–d) qPCR analysis of cultured luminal cells indicating that Wnt signaling activators (Wnt3a and GSK3β inhibitor CHIR) cannot activate Esr1 expression, and that Esr1 expression induced by Rspo1 cannot be suppressed by addition of Wnt signaling inhibitor (FzCRD or β-catenin inhibitor XAV939) (d). In contrast, Wnt-target gene Axin2 expression was activated in the presence of Wnt signaling activators, and was suppressed by adding the signaling inhibitors (c). (e–f) HEK293T cells with transiently expressing ERα-luciferase reporter were cultured in the presence of Rspo1, or in combination with Wnt inhibitors (XAV939 and IWP2). Wnt inhibitors cannot suppress ERα-luciferase activities induced by Rspo1. Data in (a–f) are pooled from more than three independent experiments and presented as mean ± s.e.m. Student’s t test. ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05; ns, not significant.

stimulating effect to Axin2, Wnt3a and CHIR treatment suppressed Esr1 expression (Figure 3d), an observation in line with a previous report, in which Wnt/β-catenin signaling represses the expression of luminal differentiation genes, mainly Esr1 (Lindley et al., 2015). Inhibition of the Frizzled receptor using its soluble CRD domain (FzCRD) (Hsieh et al., 1999) or stimulating β-catenin degradation using XAV939 (Huang et al., 2009) effectively suppressed Axin2 expression induced by Wnt3a (Figure 3c), still, they could not suppress Esr1 upregulation by Rspo1 (Figure 3d). To further verify, we used HEK293T cells transiently expressing ERα-luciferase reporter and cultured them in the presence of Rspo1 or Rspo1 in combination with XAV939 or IWP2. Consistently, inhibition of WNT signaling did not affect ERα promoter activities induced by Rspo1 (Figure 3e and f). Together, these data suggest that Rspo1 induces ERα expression independent of Wnt/β-catenin signaling.

Loss of luminal Rspo1 results in decreased ERα expression in vivo

To investigate the role of Rspo1 in vivo, we generated a conditional Rspo1 knockout allele in which the second Rspo1 exon is subjected to removal upon Cre recombination, resulting in frame-shift of the remaining exons (Figure 4a, also see Figure 4—figure supplement 1a–b). Of note, Rspo1 is predominantly expressed in ER- luminal cells as described previously (Cai et al., 2014), while Esr1 is expressed in ER+ luminal cells. Thus, this Rspo1-Esr1 regulation is likely achieved through a paracrine
manner in vivo. A luminal cells-specific BAC transgenic CreER line, Keratin8-CreER (Krt8-CreER) (Zhang et al., 2012a), was used to generate luminal cells-specific Rspo1 knock-out mice (Krt8-CreER;Rspo1fl/fl) (Rspo1-cKO) (Figure 4b). Tamoxifen was administered into 8-week-old nulliparous female mice, and mammary glands were examined 4 weeks later. Whole-mount carmine staining showed significantly reduced side branches in Rspo1-cKO mice when compared with the control (Rspo1fl/fl) (Figure 4c and d). These results are consistent with previous observation in a Rspo1-knockout mammary transplantation model (Chadi et al., 2009). The knockout efficacy of Rspo1-cKO was validated. ERα luminal cells (Lin−, CD24+, CD29lo, Sca1−), where Rspo1 is expressed, were isolated.

Figure 4. Loss of Rspo1 in mammary luminal cells results in reduced side branching and decreased ERα expression. (a) Schematic illustration of Rspo1flox knock-in allele generation (see also Figure 4—figure supplement 1). (b) Krt8-CreER;Rspo1fl/fl inducible model specifically knocked down Rspo1 in luminal cells. (c–d) 8-week-old adult virgin mice were Tamoxifen injected twice, 1 day apart (2 mg/25 g body weight per injection). Mammary glands were obtained 4 weeks later. Whole-mount imaging (d) showing decreased side branches in Rspo1-cKO mice. n = 3. Scale bar, 2 mm. More than six views were used for quantification. (e–f) FACS gating strategy for mammary basal and luminal cell isolation. Luminal ERα+ and ERα− subpopulations were separated based on Sca1 (e). qPCR analyses of luminal cells showing efficient Rspo1 knockdown in Krt8-CreER;Rspo1fl/fl (f). (g–h) Immunostaining indicated decreased ERα+ cell number after Rspo1 knockdown (g). Scale bar, 40 μm. Quantification of ERα+ cells were performed in (h). (i) qPCR analyses of ERα+ luminal cells indicated downregulation of ESR1 transcription after Rspo1 knockdown in ERα+ cells. (j) Sca1− (ERα+) luminal cells were FACS isolated and Western blot was performed to indicate decreased ERα expression after Rspo1 knockdown. (k) qPCR analyses of ERα+ luminal cells indicated downregulation of ERα target genes after Rspo1 knockdown in ERα+ cells. Data are presented as mean ± s.e.m. of more than three independent experiments. Student’s t test: ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05. L.N. Lymph node.

The online version of this article includes the following figure supplement(s) for figure 4:

Figure supplement 1. Generation of Rspo1flox mouse model.

Figure supplement 2. Loss of Lgr4 reduces ESR1 expression and ERα signaling activities.
(Figure 4e). Rspo1 level in cKO group was significantly reduced shown by qPCR analysis (Figure 4f).
By whole-mount immunofluorescence staining, we observed the decreased ERα expression in Rspo1-cKO mammary gland (Figure 4g). Quantification indicated decreased percentage of ERα+ cells (Figure 4h), likely reflecting the overall reduction of ERα level in luminal compartment. Although we could not exclude the possible switching of ERα to ERβ cell fate due to other indirectly reasons, we tested a more direct possibility—whether it is the reduction of ERα expression in ER+ compartment that results in loss of ER+ cells. To this end, we isolated ER+ luminal cells (Lin−, CD24−, CD29lo, Sca1+), and analyzed ERα levels as well as ERα signaling activities. We found that ERα levels were reduced in this compartment as shown by qPCR (Figure 4i) and Western analysis (Figure 4j). Consistently, ERα signaling target genes, including Pgr, Wisp2 and Ctsd1 were declined in Rspo1-cKO group (Figure 4k). Therefore, together these results suggest that loss of Rspo1 results in reduced ERα expression and its signaling activities in luminal cells.

The Esr1 expression was also examined in Lgr4ac2 lacZ mouse model, a hypomorphic allele of Lgr4, (Mazerbourg et al., 2004). Mammary glands of Lgr4 homozygous mutant (Lgr4ac2 lacZ) were isolated for whole mount imaging. At 9 weeks, Lgr4ac2 lacZ mammary glands displayed significantly less side branches (Figure 4—figure supplement 2a–b). Immunostaining revealed decreased ERα expression in Lgr4ac2 lacZ mammary gland (Figure 4—figure supplement 2c–d). When ERα luminal cells (Lin−, CD24+, CD29lo, Sca1+) were isolated, we found that Esr1 was significantly reduced in Lgr4 mutant, so were the ERα downstream targets Pgr, Ctsd1 and Wisp2 (Figure 4—figure supplement 2e). Lgr4 expression was markedly decreased in Lgr4ac2 lacZ mammary gland as a validation of the hypomorphic nature of the allele (Figure 4—figure supplement 2e). These results support that Lgr4 plays a role in mediating Rspo1-induced ERα expression.

Genetic evidence supports that Esr1 regulation is independent of luminal Wnt4
To investigate whether Wnt/β-catenin signaling affects Esr1 in vivo, we also generated a Wnt4 conditional knockout mouse. In this model, the second Wnt4 exon is flanked by flox, and is removed upon Cre recombination, which leads to frame shift of the remaining exons (Figure 5a, Figure 5—figure supplement 1a–b). We generated inducible, luminal cells-specific Wnt4 knock-out mice (Krt8-CreER; Wnt4fl/fl) (Figure 5b). Tamoxifen was administered into 8-week-old nulliparous female mice, and mammary glands were examined 4 weeks later. Loss of Wnt4 resulted in reduced side branching (Figure 5c and d), consistent with a previous report using MMTV-Cre;Wnt4fl/fl model (Rajaram et al., 2015).
To address whether loss of Wnt4 affects Esr1, we isolated ER+ luminal populations from both Wnt4-cKO (Krt8-CreER;Wnt4fl/fl) and control (Krt8-CreER;Wnt4fl/fl) mammary gland. qPCR and Western analyses both indicated that loss of Wnt4 increases ERα level in ER+ luminal cells (Figure 5f–g), as well as ERα signaling activities showed by increased target gene expression (Figure 5h). These were in contrast to the reduced Esr1 level and ERα signaling activity observed in Rspo1-cKO mouse (Figure 4i–k). These were consistent with the in vitro results that Wnt3a and CHIR treatment suppressed Esr1 expression (Figure 3d), and consistent with the previous report, in which Wnt-controlled transcripational regulator LBH repress luminal genes, mainly Esr1 (Lindley et al., 2015). The successful deletion of Wnt4 in cKO group was validated by significantly reduced Wnt4 level in ER+ luminal cells (Figure 5e), as well as reduced expression of Wnt/β-catenin signaling targets Axin2 and Lgr5 in basal cells (Figure 5i). Together, in vivo genetic evidence supports that Esr1 regulation is independent of luminal Wnt4.

Rspo1 relies on cAMP-PKA pathway to induce Esr1 expression
To further investigate the downstream mechanisms through which RsPo1/Lgr4 regulate Esr1, we conducted an inhibitor-based screen. HEK293T cells with transiently expressing ESR1-luciferase reporter were cultured in the presence of RSPO1, and screened for molecules that could suppress luciferase activity using a GPCR inhibitor library (Figure 6a, Figure 6—figure supplement 1, Figure 6—source data 1). Amongst over 250 inhibitors, the cAMP inhibitor Bupivacaine HCl (Bup), effectively suppressed ESR1-luciferase activities induced by RSPO1 (Figure 6a). Considering that the major downstream effector of cAMP in mammalian cells is Protein Kinase A (PKA), we examined the effect of inhibition of PKA. Consistently, H89, an inhibitor of PKA effectively repressed ESR1-
luciferase activities induced by RSPO1 (Figure 6a). The inhibitory effects of Bup and H89 were further examined in primary luminal cell culture. Both inhibitors suppressed Esr1 expression stimulated by Rspo1 as shown by qPCR (Figure 6b), but were ineffective on Axin2 expression (Figure 6c). Considering the cAMP-PKA pathway can also be activated by estrogen and ERα (Castoria et al., 2008), we further examined whether Esr1 induction by Rspo1 involves ERα. We found that the ERα inhibitor ICI (ICI182, 780) does not affect ESR1 promoter activities that are induced by RSPO1 (Figure 6d), suggesting that Esr1 induction by Rspo1 does not involve ERα.

The transcription factor CREB (cAMP response element binding protein) is the best-characterized nuclear protein that mediates stimulation of transcription by cAMP. CREB binds to the conserved consensus cAMP response element (CRE, sequence TGACATCA) (Rosenberg et al., 2002). A CRE was found at the proximal promoter of ESR1 (−991 to −984 bp). Therefore, we examined whether this CRE is responsible for induction of ESR1 by RSPO1. While RSPO1 induced the wild type promoter-luciferase in a dose-dependent manner, it could not activate the reporter with CRE mutations.

**Figure 5.** Loss of Wnt4 increases Esr1 expression in luminal cells. (a) Schematic illustration of Wnt4flox knock-in allele generation (see also Figure 5—figure supplement 1). (b) Krt8-CreER;Wnt4flox inducible model specifically knockdown Wnt4 in luminal cells. (c–d) 8-week-old adult virgin mice were Tamoxifen administered for 2 courses at 1 day apart, 2 mg/25 g body weight per injection and harvested 4 weeks later. Whole-mount imaging of the mammary epithelium showing decreased side branches in Wnt4-cKO mice (c). Scale bar, 1 mm. More than six views were used for quantification. (e) qPCR of isolated ER+ luminal cells validated efficient Wnt4 knockdown in cKO mice. (f) qPCR analysis of ER+ luminal cells indicated Wnt4 loss increased Esr1 expression levels. (g) Western blot analysis indicated increased ERα protein level in Wnt4-cKO. (h) qPCR analysis of ER+ luminal cells indicated increased ERα signaling pathway activity after Wnt4 knockdown. (i) qPCR analysis of basal cells showed Wnt signaling pathway was decreased after Wnt4 knockdown. Data are presented as mean ± s.e.m. Student’s t test: ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05; ns, not significant.

The online version of this article includes the following figure supplement(s) for figure 5:

**Figure supplement 1.** Generation of Wnt4flox mouse model.

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Figure 6. Rspo1 inducing Esr1 expression relies on cAMP-PKA pathway. (a) HEK293T cells transfected with ESR1-luciferase reporter were cultured in the presence of Rspo1, and in combination with pharmaceutical compounds from a GPCR inhibitor library (Selleck). Bupivacaine HCl (Bup), a cAMP inhibitor, and H89, a PKA inhibitor, suppressed Esr1-luciferase activities induced by Rspo1 (see Figure 6—figure supplement 1a–b). (b–c) qPCR analysis of cultured luminal cells indicating both Bup and H89 counteracted the upregulation of Esr1 expression induced by Rspo1 (b), while Axin2 expression is not affected (c). (d) HEK293T cells with transiently expressing ESR1-luciferase reporter were cultured in the presence of Rspo1 alone or with ERα inhibitor ICI182, 780. Luciferase activities were measured. ICI did not affect the ESR1 upregulation induced by Rspo1. (e) CRE site on ESR1 promoter-luciferase reporter was mutated, and Rspo1 could not activate the reporter with CRE mutation. (f) Rspo1-FL, Rspo1-R66A/Q71A mutant could, but Rspo1-N137Q and Rspo1-F110A/F106A mutants could not induce Esr1 promoter luciferase activities. (g) Dluminescence was read out after Eph4 cells were treated with forskolin (FSR) or Rspo1 for 30 min, Dluminescence was calculated as Luminescence_{treated}—Luminescence_{untreated}. Rspo1 treatment induced cAMP production in Eph4 cells in a dose dependent manner. (h) Illustration of Rspo1 regulated Esr1 expression mediated by cAMP-PKA pathway. Data in (a–d) are pooled from three independent experiments and presented as mean ± s.e.m. Student’s t test: ***p<0.001, **p<0.01, *p<0.05; ns, not significant.

The online version of this article includes the following source data and figure supplement(s) for figure 6:

Source data 1. ESR1-luciferase activities induced by Rspo1 in combination with a GPCR inhibitor library.

Figure supplement 1. ESR1-luciferase activities induced by Rspo1 in combination with a GPCR inhibitor library HEK293 cells with transiently expressing ESR1-luciferase reporter were cultured in the presence of Rspo1, and screened for the molecules that could suppress the luciferase activities using a GPCR inhibitor library (Selleck, L2200).
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Rspo1 activates G-protein coupled cAMP signaling in regulating Esr1

CAMP is a well-known intracellular mediator of protein hormones including FSH (follicle-stimulating hormone), LH (luteinizing hormone), and TSH (thyroid stimulating hormone), which bind to LGR1, LGR2 and LGR3 respectively (de Lau et al., 2014). These known hormone receptors belong to the class-A LGRs. Class-B LGRs, including LRG4-6, are reported to promote phosphorylation of Lrp5/6 and stabilization of β-catenin without the G-protein-coupled cAMP production (Carmon et al., 2011; de Lau et al., 2011). There have been a few reports that suggest differently, in that Lgr4 activates CAMP/PKA signaling in bone (Luo et al., 2009), and in the male reproductive system (Li et al., 2010). Independently, our data demonstrate that Rspo1/Lgr4 relies on the cAMP/PKA axis to maintain proper Esr1 expression during mammary development. This action is highly likely cell type specific. In vivo, either conditional KO of Rspo1 or Lgr4 hypomorphic mutant leads to reduced Esr1 expression. The latter is in line with previous reports in the male reproductive system, in which deficiency of Lgr4 results in reduced Esr1 in the efferent ducts and epididymis (Hoshii et al., 2007; Li et al., 2010). The current study, for the first time, demonstrates that Rspo1 can activate CAMP/PKA signaling.

A new hormonal regulation feed forward mechanism

Our previous studies find that hormones indirectly activate Rspo1 expression in ER+ luminal cells (Cai et al., 2014), and identify Areg (in ER+ cells) as the intermediate paracrine factor for the hormonal regulation of Rspo1 expression (in ER+ cells) (Cai et al., 2020). Moreover, the elevated levels of Areg and Rspo1 are also detected in estrus, a stage with high estrogen signaling activity.
In this study, we found that Rspo1 in turn enhances ERα expression in ER⁺ cells. This may represent a feed forward mechanism engaging estrogen-ERα-Rspo1-ERα, highlighting the impact of local growth factors for the amplification of hormonal signaling output. This additional layer of ERα regulation by Rspo1 could be hijacked during tumor initiation or progression. Elucidating the molecular mechanisms on how estrogen engages with ERα in the mammary gland is the key for advancing current knowledge over breast cancer progression and resistance to hormone therapy.

In conclusion, our study demonstrated a novel Wnt-independent role of Rspo1, revealed a novel Rspo1-Lgr4-cAMP-ERα regulatory axis. As ERα is crucial for the development and diseases of various tissues, this new Rspo1 signaling axis may have broader implication in estrogen-associated diseases.

### Materials and methods

#### Key resources table

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**Experimental animals**

`Rspo1^floxed/+` and `Wnt4^floxed/+` mice were constructed as illustrated in the text. In all conditional knockout experiments, mice were maintained on a C57BL/6 genetic background and at least three animals were analyzed for each genotype. `Lgr4^lacZ/+` ([Mazerbourg et al., 2004](https://doi.org/10.7554/eLife.56434)) and `Krt8-CreERT2` ([Zhang et al., 2012a](https://doi.org/10.7554/eLife.56434)) strains were used in this study. Nude, CD1 and BALB/c strains were purchased from B and K universal (Shanghai). Animals were housed under conditions of 12 h day/night cycle.

For Cre recombination induction experiments induced in adult mice, animals received intraperitoneal injection of 2 mg tamoxifen (TAM; Sigma-Aldrich; T5648) diluted in sunflower oil. The Animal Care and Use Committee of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences approved experimental procedures.

**Antibodies**

Rabbit anti Gapdh (1:3000; Proteintech; 10494–1-AP), Mouse anti β-Actin (1:2000; Sigma; A2228) and Rabbit anti ERα (1:1000; Millipore; 06–935) were used in Western blot analyses.

**Primary cell preparation**

Mammary glands from 8- to 12-wk-old virgin female mice were isolated. Minced tissues were placed in digestion buffer (RPMI 1640 [Gibco; C11875500BT] with 25 mM HEPES [Sigma; H4034-500G], 5% FBS [HyClone], 1% PSQ [Gibco; 15140122], 300 U mL^−1 Collagenase III [Worthington; LS004183]) and digested for 2 hr at 37°C. After lysis of the red blood cells in red blood cell lysing buffer (Sigma;
R7757), a single cell suspension was obtained by sequential incubation with 0.05% Trypsin-EDTA (Gibco; 25300–062) for 5 min at 37˚C and 0.1 mg/mL DNase I (Sigma; D4263) for 5 min with gentle pipetting followed by filtration through 70 μm cell strainers (Falcon; 352350).

RNA extraction and RNA sequencing
Total RNA from day two cultured luminal cells (Lin, CD24+, CD29lo) were extracted with RNAiso Plus (Takara) following manufacturer’s protocol. Total mRNA concentration was determined with NanoDrop ND-1000 and RNA-seq libraries were prepared according to manufacturer’s instruction (Illumina) followed by applying to sequencing on Illumina nova-seq, which was performed by ANOR-OAD (http://en.annoroad.com, Beijing). Differential gene expression analysis was carried out and genes with significant alteration were extracted and further analysed using DAVID Bioinformatics Resources. RNA-seq data can be viewed online at http://www.biosino.org/node/index, under accession number OEP000754.

Mammary gland whole mount carmine staining
The 4th pair of mammary glands were dissected and fixed for 2 hr in 4% paraformaldehyde, and then washed the tissue three times in PBS for 15 min each time. Finally, the tissues were stained in carmine alum solution (2 mg/ml carmine [Sigma; C1022], 5 mg/ml KAl(SO4)2 in H2O) overnight at room temperature. After the staining, the tissues were washed in de-staining solution (50% ethanol, 2% HCl) for 2 hr, and then serial dehydrated in 75%, 85%, 95%, 100%, 100% ethanol and finally stored in Histoclear (National Diagnostics; HS-200). Whole mount analyses were performed under a dissection microscope (Leica).

Mammary gland whole mount immunostaining
Whole-mount staining was performed as previously described (Rios et al., 2014), with minor modification. In brief, mammary glands were dissected into small pieces, then processed in digestion buffer (RPMI 1640 with 25 mM HEPES, 5% fetal bovine serum, 1% penicillin–streptomycin–glutamine (PSQ), 300 U/ml collagenase III (Worthington)) for 30 min at 37˚C, then fixed in 4% paraformaldehyde for 30 min at 4˚C. Tissues were incubated with primary antibodies (Krt8; 1:500; DSHB, ER; 1:200; Millipore) at 4˚C overnight, followed by washes, incubated with secondary antibodies and DAPI (Life Technologies) at 4˚C overnight. Then the tissues were incubated in 80% glycerol overnight, before dissection for 3D imaging. Confocal images were captured using Leica SP8 laser confocal scanning microscope. Representative images were shown in the figures.

Cell labeling and flow cytometry
The following antibodies in 1:200 dilutions were used: biotinylated and FITC conjugated CD31, CD45, and TER119 (BD PharMingen; 553371; 553078; 553372; 553080; 557915); CD24-PE/cy7, CD29-APC (Biolegend; 101–822; 102216) Sca1-PE and Streptavidin-V450 (eBioscience; 12-5981-82; 48-4317-82). Antibody incubation was performed on ice for 25 min in PBS with 5% FBS. All sorting experiments were performed using a FCAS Jazz (Becton Dickinson). The purity of sorted population was routinely checked and ensured to be >95%.

In vitro culture assay
FACS-sorted cells were resuspended in chilled 100% growth factor-reduced Matrigel (BD Bioscience; 354230), and the mixture was allowed to polymerize before covering with culture medium (DMEM/F12 [Gibco; 11039–021]; ITS [1:100; Gibco; 41400045]; 50 ng ml1 EGF [Corning; 354001]), plus either 1 μM E2 (Sigma; E8875), 200 ng Wnt3A, 1:100 FzCRD, 2.5 μM IWP2 (Selleck; s7085), 3 μM CHIR (Selleck; S1263), 10 μM XAV-939 (Selleck; S1180), Rspo1 purified protein or Wnt4 conditioned media. Culture medium was changed every 24 hr. Cell samples were collected after 2–4 days in culture for RT-qPCR and western blot.

Maintenance of cell lines
293T and Eph4 cell lines were cultured in DMEM high glucose (4.5 g/L) (Gibco, C11995500BT) with 1% Penicillin/Streptomycin (Gibco, 15140) and 10% Fetal bovine serum (FBS) (Hyclone). Both cell lines were cultured in tissue culture dish, kept at 37˚C with 5% CO2, trypsinized, and split three times.
a week 1:4. T47D cell line was kindly provided by Dr. Gaoxiang Ge, Institute of Biochemistry and Cell Biology and was cultured in 1640 Medium (Gibco, C11875500BT) +10 mg/ml Insulin with 1% Penicillin/Streptomycin and 10% FBS. All cell lines were routinely negatively tested for mycoplasma.

**Conditioned media preparation**

Wnt4 conditional medium was prepared by culturing Wnt4-expressing Eph4 cells for 48 hr, followed by supernatant collect. Wnt4 conditional medium was stored at 4°C for short-term storage (up to 1 week). For long-term usage, conditional medium was aliquoted after collection and stored at −80°C.

**RSPO1 protein purification**

RSPO1-FC construct was cloned into expression vector with a C-terminal Fc tag. RSPO1-FC was transiently expressed in HEK293T cells and medium changed into CD293 medium (Gibco, 11913-019). One day after transfection, medium was collected by centrifugation and incubated with Protein A Agarose Beads (Santa Cruz, sc-2003). The bound recombinant protein was eluted using 500 μl 0.1M Glycine (pH = 3.0) and was collected in 1.5 ml tubes containing 30 ul 1 M Tris-HCl (pH = 9.5) buffer for neutralization. In total 5 tubes of elution were collected. The RSPO1 protein was subsequently purified and concentrated by Centrifugal Filter Volumes (Millipore, UFC803096).

**Lentiviral vector and infection**

Lgr4-shRNA was synthesized and subcloned into plko backbone with EGFP. Lentivirus was produced by transient transfection in 293 T cells. Mammary cells were isolated from 8- to 12-wk-old virgin female glands as described above, followed by sorting into luminal cells. The sorted cells were collected and cultured in a low adherent plate in EGF, ITS-supplemented DMEM/F12 with virus. At 12 hr after infection, cells were collected and resuspended in Matrigel for consequent in vitro culturing. Sequences of Lgr4-shRNA are CGTAATCAAATCTCCCTGATA and CCTCCAGAACAATCAGTTGAA.

**Luciferase assay**

Oligonucleotide primers (nucleotides –1133 to –1107 and –1 to –24) based on previously published sequence information for the upstream region of the ESR1 were used to generate ESR1 promoter fragments from normal placental DNA by polymerase chain reaction (PCR) (Castles et al., 1997). A 1133 bp (promoter A) of ESR1 promoter expression vector (ERP) was created by cloning this PCR-generated product into the Xhol-HindIII sites of the promoterless luciferase reporter plasmid pGL4.17 basic respectively (Promega, Madison, WI, U.S.A.). Transfections of individual wells were performed using luciferase reporter plasmid (ERP or pGL4.17 basic vector alone), and pRL-TK Renilla luciferase control constructs as a correction for transfection efficiency, and also transfected with pcDNA3.1-RSPO1 overexpression (RSPO1-OE) plasmid from 0.5 μg/ml to 4 μg/ml) Cells were then harvested, the dual luciferase assays were performed using a commercial kit (Promega; E1910), Results are shown as fold activity over control activity of the promoterless pGL4.17 basic vector in each set of experiments. All transfections and assays were performed in duplicate with n ≥ 3 individual experiments. GPCR compound library (Selleckchem L2200) was used to for screening of inhibitors that suppress ESR1 upregulation by RSPO1. In each experiment, ESR1-luciferase reporter cells were treated with RSPO1 for 36–48 hr.

**In situ hybridization**

In situ hybridization was performed using the RNAscope kit (Advanced Cell Diagnostics) following the manufacturer’s instructions. Lgr4 probes were ordered from Advanced Cell Diagnostics. For in situ staining, at least three independent experiments were conducted. Representative images are shown in the figures.

**AMP-Glo assay to detect intracellular cAMP levels**

The intracellular cAMP concentration was measured using the cAMP-Glo assay kit (Promega, V1501) according to the manufacturer’s instruction. The cAMP standard curve was generated using purified cAMP, from which the relative intracellular level of cAMP was inferred. For each drug treatment, three biological repeats were used, and each experiment was repeated 2–3 times.
RT-qPCR
RNA was isolated with Trizol (Invitrogen; 9109). The cDNA library was prepared with the SuperScript III kit (Invitrogen; RR036A). RT–PCR was performed on a StepOne Plus (Applied Biosystems). RNA level was normalized to GAPDH. The primers used were as following:

Axin2-F, AGCCTAAAGGTCTTATGTGGCTA;
Axin2-R, ACCTACGTGATAAGGATTGACT;
Wnt4-F, GCAATTTGCTGTACCTGG;
Wnt4-R, GCACTGAGTCCATACCT;
Rspo1-F, GCAACCGACATGAAACAAAT;
Rspo1-R, GGTGCTGTATCGGCGCTGTAG;
Esr1-F, TCCAGCAATAGAGAAGAAAGGA;
Esr1-R, AGCCAGAGGCATAGTCTTG;
Pgr-F, GGCGGTGAGGTCGTACAAAG;
Pgr-R, GCGAGTGAATGACAGCTCCTT;
Lgr4-F, AGAACTCAAAAGCTCCTAACCCTCT;
Lgr4-R, ATGCGCAACTGACACGAG;
Lgr5-F, CCTACTCAAGACTTACCAAGT;
Lgr5-R, GCATTGCGGTGAATGATAGCA;
Lgr6-F, CTGTAGCCCTGCTGATGA;
Lgr6-R, GGTGAAGGACAGAGGTAG;
Ctsd1-F, GCTTCCGGTCTTTGACCAACT;
Ctsd1-R, CACCAAGGATTATTTCTGCCTCC;
Wisp2-F, TGTTGACCAGCGTCTGATG;
Wisp2-R, GTGCTCCAGTTTGACGAGG.

Statistical analysis
One-way ANOVA or Student’s t-test was performed, and the P-value was calculated in Prism on data represented by bar charts, which consisted of results from three independent experiments unless otherwise specified. For all experiments with error bars, the standard deviation (SD) was calculated to indicate the variation within each experiment. No statistical method was used to pre-determine sample size. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment.

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The funders conceived the study, wrote the manuscript and made the decision to submit the work for publication.

Author contributions
Ajun Geng, Data curation, Investigation, Visualization; Ting Wu, Validation, Investigation, Visualization; Cheguo Cai, Investigation; Wenqian Song, Validation, Methodology; Jiqiu Wang, Resources; Qing Cissy Yu, Supervision, Project administration, Writing - review and editing; Yi Arial Zeng, Conceptualization, Writing - original draft, Writing - review and editing

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Ethics
Animal experimentation: The Animal Care and Use Committee of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences approved experimental procedures (SIBCB-S335-1601-002-c4).

Decision letter and Author response
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Additional files
Supplementary files
• Transparent reporting form

Data availability
RNA-seq data can be viewed online at http://www.biosino.org/node/index, under accession number OEP000754.

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