Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Original research article

Amphiregulin mediates the hormonal regulation on Rspondin-1 expression in the mammary gland



DEVELOPMENTA BIOLOG

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ARTICLE INFO	A B S T R A C T
Keywords: Hormone Estrogen Rspol Areg Egfr	The steroid hormones are instrumental for the growth of mammary epithelial cells. Our previous study indicates that hormones regulate the expression of Rspondin-1 (Rspo1). Yet, the regulatory mechanism remains unknown. In the current study, we identify Amphiregulin (Areg) as a novel upstream regulator of Rspo1 expression mediating the hormonal influence. In response to hormonal signaling, Areg emanating from estrogen receptor (ER)-positive luminal cells, induce the expression of <i>Rspo1</i> in ER-negative luminal cells. The paracrine action of Areg on <i>Rspo1</i> expression is dependent on Egfr. Our data reveal a novel Estrogen-Areg-Rspo1 regulatory axis in the mammary gland, providing new evidence for the orchestrated action of systemic hormones and local growth factors

1. Introduction

The Rspondins (Rspo) belong to a superfamily of thrombospondin type 1 repeat (TSR-1)-containing proteins that are secreted agonist of the Wnt/ β -catenin signaling pathway. There are 4 members within the Rspo family (Rspo1-4), all of which share similar functional structure composed of two furin-like cysteine-rich domain at the N-terminus followed by a thrombospondin domain and a basic charged C-terminal tail (Kim et al., 2006). Although Rspondins are unable to initiated Wnt signaling, they can uniquely synergize with Wnt proteins and potently enhance receptor response to low-dose Wnt protein, as reviewed (de Lau et al., 2014). Rspo1 enhances Wnt signaling through interaction with their receptors Lgr4/5/6 to slow down their turnover (Hao et al., 2012; Koo et al., 2012) therefore potentiate Lrp phosphorylation (Carmon et al., 2011; de Lau et al., 2011; Glinka et al., 2011; Gong et al., 2012).

Rspos have been identified as niche factors for adult tissue stem cells (Greicius et al., 2018; Han et al., 2014; Planas-Paz et al., 2016; Sigal et al., 2017), and have been implicated as a critical growth factor in many *in vitro* stem cell culture systems, such as intestine, stomach and liver (Sato et al., 2009; Huch et al., 2013; Barker et al., 2010; Kim et al., 2005).

Rspo1 is the major form of Rspo family protein identified in mammary epithelium. Our previous study has demonstrated an important role of Rspo1 in mammary stem cell (MaSC) self-renewal (Cai et al., 2014). However, little is known about the upstream regulation of Rspo1.

The mammary gland is an epithelial organ, consisting of a basal layer of myoepithelial cells and an inner layer of luminal cells. The ovarian steroid hormones are instrumental for mammary epithelial cells growth throughout development. Starting at puberty, through oscillated estrogen and progesterone in recurrent estrous cycles, the mouse ductal epithelial tree undergoes elongation and increases in complexity with the addition of side branches. Estrogen is the primary ovarian steroid that triggers allometric growth of the gland (Hovey et al., 2002), while Progesterone is specifically required for tertiary side branching (Brisken et al., 1998; Atwood et al., 2000; Humphreys et al., 1997). Hormone receptor-positive cells are predominantly in luminal layer and can be enriched and isolated by their surface Sca1 expression (Shehata et al., 2012). In addition to the cell autonomous influence, hormones exert their mitogenic effects primarily through induction of local growth factors (Sternlicht et al., 2006).

Our previous study indicates that hormones positively regulate Rspo1

https://doi.org/10.1016/j.ydbio.2019.10.006

Received 12 April 2019; Received in revised form 23 September 2019; Accepted 8 October 2019 Available online 11 October 2019 0012-1606/© 2019 Elsevier Inc. All rights reserved.



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expression (Cai et al., 2014). Since Rspo1 is expressed in luminal cells that are negative for hormone receptors, the regulation is predicted to be indirect. It remains unknown which paracrine factor can mediate the hormonal influence on Rspo1 expression. In this study, we uncover a novel upstream regulatory mechanism of Rspo1 in response to estrogen. Through a transcriptome-based screen, we identify Amphiregulin (Areg) as the mediating factor, and unveil an Areg/Egfr dependent paracrine regulation of Rspo1 in the mammary gland.

2. Experimental procedures

2.1. Experimental animals and estrous staging

Nude, CD1 and BALB/c strains were purchased from B&K universal (Shanghai). Animals were housed under conditions of 12-h dav/night cycle. Vaginal smear of nulliparous female BALB/c mice aged 12-week or otherwise specified age was collected as previously described (Fata et al., 2001; Butts et al., 2010). Estrous cycle stage was determined by cytology of the smear based on proportion among three cell types: round, nucleated epithelial cells; irregular-shaped, anucleated cornified cells; and small, round leukocytes. Proestrus is characterized by predominance of round, nucleated vaginal epithelium (ovoid cells); estrus primarily consists of large irregular-shaped, anucleated cornified epithelial cells; metoestrus is identified by the presence of cornified vaginal epithelial cells and polymorphonuclear cells (leukocytes); and diestrus primarily consists of leukocytes. After assigning stages, animals were anaesthetized at mid-day, blood samples and mammary glands were removed. Since estrus occurs at night, animals for this stage were examined in late afternoon and the mammary glands were removed in early evening.

2.2. Antibodies

Mouse anti β -Actin (1:3000; Sigma), Rabbit anti R-spondin1 (1:500; Abcam), and Rabbit anti Amphiregulin (1:500; Abcam) were used in Western blot analyses.

2.3. Administration of Erlotinib

10 mg/ml stock solution was prepared by dissolving Erlotinib hydrochloride (Selleck, s1023) in DMSO. For *in vivo* study, 2 mg/ml working solution was prepared by diluting stock into diluent buffer (30% PEG+15% propylene glycol+5% Tween-80). For animal experiments, mice were randomly divided into two groups of eight and administered Erlotinib at a dosage of 25 mg/kg/day for 6 days. Mice administered with 20% DMSO in diluent buffer were used as control. On day 7, mice were sacrificed and mammary glands were excised for consequent analysis.

2.4. Primary cell preparation

Mammary glands from 8- to 12-wk-old virgin or pregnant female mice were isolated. The minced tissue was placed in culture medium (RPMI 1640 with 25 mM HEPES, 5% FBS, 1% PSQ, 300U mL⁻¹ Collagenase III [Worthington]) and digested for 2 h at 37 °C. After lysis of the red blood cells in NH4Cl, a single cell suspension was obtained by sequential incubation with 0.05% Trypsin-EDTA for 5 min at 37 °C and 0.1 mg/mL DNase I (Sigma) for 5 min with gentle pipetting followed by filtration through 70 μ m cell strainers.

2.5. Cell labeling and flow cytometry

The following antibodies in 1:200 dilutions were used: FITC conjugated CD31, CD45, and TER119 (BD PharMingen); and Sca1-PE, CD24-PE/cy7, and CD29-APC (Biolegend). Antibody incubation was performed on ice for 30 min in HBSS with 10% FBS. All sortings were performed using a FACS Aria or FCAS Jazz (Becton Dickinson). The purity of sorted population was routinely checked and ensured to be >95%.

2.6. In vitro culture assay

FACS-sorted cells were resuspended in chilled 100% growth factor-reduced Matrigel (BD Bioscience), and the mixture was allowed to polymerize before covering with culture medium (DMEM/F12; ITS [1:100; Sigma]; 50 ng mL⁻1 EGF; plus either vehicle [1% CHAPS in PBS], 200 ng Wnt3A [Willert et al., 2003], 1 μ M E2, 2.5 μ M Pg, 10 μ M XAV-939 or amphiregulin (R&D) 20 ng mL⁻1. Culture medium was changed every 24 h. Cell samples were collected after 6–7 days in culture for RT-qPCR and western blot.

2.7. Lentiviral vector and infection

Areg-shRNAs were synthesized and subcloned into plko backbone with EGFP. Lentivirus was produced by transient transfection in 293T cells. Mammary cells were isolated from 8- to 12-wk-old virgin female glands as described above, followed by sorting into luminal cells. The infection protocol was modified from methods described in Welm et al. (2008). Sorted cells were collected and cultured in a low adherent plate in 10% FBS, EGF and ITS-supplemented DMEM-F12 with virus. At 12 h after infection, cells were collected and resuspend in Matrigel for consequent *in vitro* culturing. Sequence of Areg-shRNA is GAAACGATACTTCAGGGAATA.

2.8. In situ hybridization

In situ hybridization was performed using the RNAscope kit (Advanced Cell Diagnostics) following the manufacturer's instructions. Rspo1, ER, and Areg 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.

2.9. Quantitative real-time PCR

RNA was isolated with Trizol (Invitrogen). The cDNA library was prepared.with the PrimeScript[™] RT Master Mix (TaKaRa). RT–PCR was performed on a StepOne Plus (Applied Biosystems). RNA level was normalized to GAPDH. The primers used were:

Axin2-F, AGCCTAAAGGTCTTATGTGGCTA; Axin2-R, ACCTACGTGATAAGGATTGACT; Wnt4-F, GCAATTGGCTGTACCTGG; Wnt4-R, GCACTGAGTCCATCACCT; Wnt5a-F, CGCTAGAGAAAGGGAACGAATC; Wnt5a-R, TTACAGGCTACATCTGCCAGGTT; Wnt7b-F, CTTCACCTATGCCATCACGG; Wnt7b-R, TGGTTGTAGTAGCCTTGCTTCT; Rspo1-F, GCAACCCCGACATGAACAAAT; Rspo1-R, GGTGCTGTTAGCGGCTGTAG; Esr-F, TCCAGCAGTAACGAGAAAGGA. Esr-R, AGCCAGAGGCATAGTCATTGC. Pgr-F, GGGGTGGAGGTCGTACAAG. Pgr-R, GCGAGTAGAATGACAGCTCCTT. Areg-F, CTGTTGCTGCTGGTCTTA. Areg-R, AGTAGTCGTAGTCCCCTGT. Sca1-F, GAGGCAGCAGTTATTGTGGAT. Sca1-R, CGTTGACCTTAGTACCCAGGA. Rankl-F, TGTACTTTCGAGCGCAGATG. Rankl-R, CCACAATGTGTTGCAGTTCC.

2.10. RNA-seq transcriptome analysis

Total RNA from freshly isolated sca1⁺ luminal cells (Lin⁻, CD24⁺, CD29^{lo}, Sca1⁺), sca1⁻ luminal cells (Lin⁻, CD24⁺, CD29^{lo}, Sca1⁺) and cultured luminal cells (Lin⁻, CD24⁺, CD29^{lo}) were extracted with Trizol. RNAseq libraries were prepared according to the manufacturer's instructions (Illumina) and then applied to sequencing on Illumina HiSeq

2000 in the CAS-MPG Partner Institute for Computational Biology Omics Core, Shanghai. In total, around 20 million 1×100 bp reads for each sample were obtained. RNA-seq reads were mapped to GRCm38/mm10 reference genome using HISAT2 (v 2.1.0) (Kim et al., 2015). Expression for each known gene from RefGene (downloaded from http://genome .ucsc.edu) (Pruitt et al., 2007) was determined by uniquely mapped reads and normalized with RPKM (reads per kilo base per million mapped reads) using Cuffdiff (v 2.2.1) (Trapnell et al., 2012). DEG (differential expressed gene) was defined with fold change ≥ 1.5 or ≤ 0.67 , and RPKM ≥ 1 in at least one sample. DEGs were identified between sca1⁺ luminal cells and sca1⁻ luminal cells, as well as between cultured luminal cells + E2+Pg and cultured luminal cells + vehicle for further analysis. RNA-seq data can be accessed under GEO accession number GSE100664.

2.11. 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 predetermine sample size. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment.

2.12. Key resources table

Reagent or resource	Source	Identifier
Antibodies		
Mouse monoclonal Anti-β-Actin antibody	Sigma-Aldrich	Cat#A2228;
Rabbit polyclonal anti-Rspo1	abcam	Cat#ab106556;
antibody		RRID:
		AB_10891945
Rabbit polyclonal anti-	abcam	Cat#ab33558;
Amphiregulin antibody		RRID: AB_722761
FITC 45	BD	Cat#553080
FITC 31	BD	Cat#553372
FITC TER119	BD	Cat#557915
PE Cy7-CD24	Biolegend	Cat#101-822
APC anti-mouse/rat CD29	Biolegend	Cat#102216
Bacterial and Virus Strains		
Plko.1-Areg-shRNA-EGFP	addgene	N/A
Biological Samples		
Chemicals, Peptides, and Recombinant	Proteins	
Recombinant mouse amphiregulin	R&D systems	Cat#989-AR-100
Erlotinib HCl inhibitor	Selleck Chemicals	Cat#S1023; CAS:
		183319-69-9
Canertinib inhibitor	Selleck Chemicals	Cat#S1019; CAS:
		267243-28-7
β-Estradiol	Sigma	Cat# E8875 ;CAS:
Progesterone	Sigma	Cat#P0130;
		CAS:57-83-0
Insulin, Teansderrin, Selenium	ThermoFisher	Cat#41400-045;
Solution (ITS-G) 100X		
Egf Mouse Natural Cult	BD	Cat#354001
Critical Commercial Assays		
FS Universal SYBR Green MasterRox	Roche	Cat# 04913914001
Deposited Data		
Raw and analyzed data	This paper	GEO: GSE100664
Experimental Models: Cell Lines		
Human: 293T cell lines		N/A
Experimental Models: Organisms/Strai	ns	
Mouse: ICR mouse	B&K universal	N/A
	(Shanghai)	
Oligonucleotides		
Sequence of Areg-shRNA is GAAACGATACTTCAGGGAATA	This paper	N/A
qPCR: Axin2-F,	This paper	N/A
AGCCTAAAGGTCTTATGTGGCTA		
	This paper	N/A
	(con	tinued on next column)

(continued)		
qPCR:Axin2-R, ACCTACGTGATAAGGATTGACT		
qPCR: Wnt4-F,	This paper	N/A
qPCR: Wnt4-R,	This paper	N/A
GCACTGAGTCCATCACCT qPCR: Wnt5a-F,	This paper	N/A
CGCTAGAGAAAGGGAACGAATC	This paper	NZA
TTACAGGCTACATCTGCCAGGTT		14/21
qPCR: Wnt7b-F, CTTCACCTATGCCATCACGG	This paper	N/A
qPCR: Wnt7b-R, TGGTTGTAGTAGCCTTGCTTCT	This paper	N/A
qPCR: Rspo1-F,	This paper	N/A
qPCR: Rspo1-R,	This paper	N/A
qPCR: Esr-F,	This paper	N/A
qPCR: Esr-R,	This paper	N/A
qPCR: Pgr-F,	This paper	N/A
qPCR: Pgr-R,	This paper	N/A
qPCR: Areg-F,	This paper	N/A
qPCR: Areg-R,	This paper	N/A
qPCR: Sca1-F,	This paper	N/A
qPCR: Sca1-R,	This paper	N/A
qPCR: Rankl-F,	This paper	N/A
qPCR: Rankl-R, CCACAATGTGTTGCAGTTCC Recombinant DNA	This paper	N/A
Software and Algorithms RefGene	Pruitt et al., Nucleic	http://genome.
Illumina HiSeq 2000	Acids Res, 2007 CAS-MPG Partner Institute for Computational Biology Omics Core, Shanghai Kim et al., Nat Methods, 2015	ucsc.edu/ N/A
HISAT2 (v 2.1.0)		https://ccb.jhu.ed u/software/hisat2/ index.shtml
()ther		

3. Results

3.1. Identify upstream regulators for Rspo1 expression

To identify upstream regulator for the expression of Rspo1, we performed two distinct transcriptome analyses. First, we isolated ER⁺ (Lin⁻, CD24⁺, CD29^{low}, Sca1⁺) and ER⁻ (Lin⁻, CD24⁺, CD29^{low}, Sca1⁻) mammary luminal cells (Sleeman et al., 2007), and looked for genes that are differentially expressed in ER⁺ luminal cells through RNAseq analysis (Fig. 1a). Among the genes that are differentially expressed in ER⁺ cells, we aimed to identify secreted factors whose expression is positively regulated by hormones. Thus, we performed another RNAseq using cultured luminal cells (Lin⁻, CD24⁺, CD29^{low}) in the presence or absence of Estrogen (17β-estradiol, E2) and Progesterone (Pg) treatment (Fig. 1b). By comparative analysis, we identified 294 genes that are highly expressed in ER⁺ cells *in vivo*, and can be upregulated by hormonal stimulation (Fig. 1c, Fig. S1). Among these genes, we focused on the ones C. Cai et al.



Fig. 1. Areg mediates the hormonal influence to upregulate Rspo1 expression.

(a) Primary mammary cells in 8-week-old female mice were separated into basal (Lin-, CD34⁺, CD29hi), luminal (Lin-, CD24, CD29lo), and stromal (Lin-, CD24⁻) populations. Luminal cells were further separated into Sca1+ (ER+) and Sca1- (ER-) populations.

(b) Isolated luminal cells were cultured in Matrigel in the presence or absence of E2 and Pg for 6 days.

(c) RNAseq analysis of freshly sorted samples in (a) identifying 1377 genes that are upregulated in ER + luminal cells compared to ER-luminal cells. RNAseq analysis of cultured cells samples in (b) identifying 866 genes are upregulated upon E2+Pg treatment. 294 genes are overlapped between the two analyses.

(d) Heat map of the overlapping list among which 294 genes were upregulated and 227 genes were down regulated. Secreted factors among the upregulated, e.g. *Wnt4*, *Wnt7b* and *Areg* are listed.

(e) qPCR analysis confirming the upregulation of *Esr1*, *Pgr*, *Wnt4*, *Wnt7b* and *Areg* in freshly sorted ER + luminal cells and in cultured luminal cells upon E2+Pg treatment.

(f) qPCR analysis verifying that Wnt5a expression is upregulated in ER + luminal cells, but remained unchanged in culture with E2+Pg.

(g) qPCR analysis confirming that *Rankl* is drastically upregulated in culture in the presence of hormones, but is not differentially expressed in luminal cells in virgin mammary glands; and that *Rspo1* is upregulated in the presence of hormones, but has higher expression level in ER-luminal cells.

(e-g) mRNA expression was normalized to GAPDH. Data are presented as mean \pm s.d. from three independent experiments.

encoding secreted factors, including *Wnt4*, *Wnt7b* and *Amphiregulin* (*Areg*) (Fig. 1d). Areg is a member of epidermal growth factor (EGF) family and has been associated with mammary development and breast cancer (Shoyab et al., 1988; Ciarloni et al., 2007). Quantitative real-time PCR (qPCR) analyses were carried out to validate the RNAseq results. By measuring the expression of estrogen receptor *Esr1* and progesterone receptor *Pgr*, we confirmed that both hormone receptors were expressed in higher level in ER⁺ (Sca1⁺) cells and were up-regulated in the presence of hormones, validating the accuracy of cell isolation and RNAseq

analyses (Fig. 1e). Next, we examined the expression of *Wnt4*, *Wnt7b* and *Areg*, and validated their specific expressions in ER^+ cells, and their expressions were elevated in culture in response to hormones (Fig. 1e). It is noteworthy that Wnt5a showed enriched expression in ER^+ cells, yet hormone stimulation in culture did not alter its expression (Fig. 1f). A recent study suggests that Rank can modulate Rspo1 level rendering a Wnt agonist effect after hormone stimulation (Joshi et al., 2015). Therefore, we also investigated the possible involvement of Rank and Rank ligand (Rankl). The expression of *Rankl* was significantly elevated

by hormone in culture, while its expression in sorted primary populations was very low and has no disenable difference between ER^+ and ER^- luminal cells (Fig. 1g). This is in fact in accordance with the prevailing role of Rankl during pregnancy when hormones are drastically elevated, but is barely required for mammary development and maintenance before pregnancy (Fata et al., 2000). qPCR analysis also validated our previous observation on the expression of *Rspo1*; which is restricted in ER^- (Sca1⁻) luminal cells *in vivo* and is elevated in culture after hormonal treatment (Fig. 1g).

3.2. Areg from ER⁺ cells upregulates Rspo1 expression in ER⁻ cells

Next, we investigated which of the above candidates are able to regulate Rspo1 expression. Isolated luminal cells were cultured in Matrigel in the presence of one of the above secreted factors (Fig. 2a). E2 and Pg were used in culture as a positive control for stimulating Rspo1 expression. We found that Areg can significantly induce *Rspo1* expression, while Wnt4, Wnt7b cannot (Fig. 2b). In addition, we examined the influence of Wnt3a, Wnt5a or Rankl, and found none of them can



Fig. 2. Areg from ER + luminal cells upregulates Rspo1 expression in ER-cells

(a) Luminal cells were FACS-isolated and cultured in Matrigel as illustrated. (b) qPCR analysis of 7 day cultured cells after various treatments indicating that Rspo1 level is increased in the presence of Areg. E2+Pg were used as a positive control for Rspo1 induction. mRNA expression was normalized to GAPDH. Data are presented as mean \pm s.d. from three independent experiments.

(c) Western blot analysis and quantification indicating that Rspo1 protein level is increased in the presence of either E2+Pg or Areg. Actin serves as a loading control. Quantification data are pooled from three independent experiments.

(d) In situ hybridization of Areg (in pink) and Esr1 (in cyan) indicating their colocalization in the same cell. Nuclei were counterstained with hematoxylin (in purple). Scale bar, 20 µm

(e) Quantification of Areg and Esr1 mRNA co-localization in all luminal cells.

(f) Quantification of Areg + cells in total Esr1-expressing cells.

(g) In situ hybridization of Areg (in pink) and Rspo1 (in cyan) indicating neighboring distribution. Nuclei were counterstained with hematoxylin (in purple). Scale bar, 20 μ m

(h) Quantification of Areg+ and Rsp01+ as neighboring luminal cells indicate that 77.8% of Rsp01+ cells are suited next to Areg + luminal cells.

(i) qPCR analysis indicating the efficacy of Areg shRNA (sh-Areg). Scramble shRNA (Sc) used as control.

(j) Cultured luminal cells were infected by either scramble control or sh-Areg lentiviruses. qPCR analysis of cultured luminal cells indicated that induction of *Rspo1* by E2+Pg is counteracted by knockdown of *Areg*. While addition of Areg protein could rescue *Rspo1* induction in sh-Areg condition (red bars). Data are presented as mean \pm s.d from three independent experiments. Student's *t*-test: ns, not significant. (e, f, h) 34 independent views were used for quantification. Data are presented as mean \pm s.d.

stimulate *Rspo1* expression (Fig. 2b). The effect of Areg in Rspo1 induction was confirmed by Western blot analysis (Fig. 2c). Next, we performed double-color RNA in situ to analyze the expression pattern of *Areg* and *Esr1*. Quantification of in situ staining showed that among total luminal cells, 58.6% have co-localization of *Areg* and *Esr1* mRNA expression (Fig. 2d and e), while in all *Esr1*-expressing cells, 76.2% also express *Areg* (Fig. 2f). These results indicated that *Areg* and *Esr1* are expressed in same population, which is consistent with qPCR results showed in Fig. 1. In addition, we observed that *Areg* and *Rspo1* are always positioned in neighboring luminal cells (Fig. 2g and h), consistent with the qPCR data that *Areg* is expressed in ER⁺ cells and *Rspo1* is in ER⁻ cells. These data support the notion that Areg is a paracrine upstream regulator for Rspo1 expression.

To investigate whether the upregulation of Rspo1 by hormones is dependent on Areg, we knocked down Areg by shRNA. The knockdown efficiency was validated by qPCR analysis (Fig. 2i). In luminal cell culture, consisting a mixture of ER^+ (Areg-expressing) and ER^- (Rspo1-expressing) luminal cells, we found that knockdown of *Areg* completely abolished the *Rspo1* expression induced by hormones, and this can be rescued by the addition of Areg protein (Fig. 2j). Together, our data suggest that Areg emanated from ER^+ cells act as a paracrine factor, mediating hormonal control over Rspo1 expression in ER^- cells.

3.3. Induction of Rspo1 by estrogen is dependent on Egfr

Areg, as a member of EGF family, is known to associate with the EGF receptor (Egfr) (Harris et al., 2003). To examine the role of Egfr in this

context, we first evaluated the expression of Egfr in luminal subpopulations. We found that *Egfr* mRNA is predominantly expressed in Sca1⁻ (ER⁻) luminal cells that also produce Rspo1 (Fig. 3a). Next, we suppressed Egfr function by using two inhibiting agents: Canertinib or Erlotinib. Western blot analysis indicated that both inhibitors significantly repress the Rspo1 upregulation induced by Areg or by E2+Pg in primary luminal cell culture (Fig. 3b). To further verify this *in vivo*, we adopted a previous described drug administration method (He et al., 2017; Zhang et al., 2012). In brief, Erlotinib was administered to mice at the dosage of 25 mg/kg animal body weight for 6 consecutive days (Fig. 3c). On day 7, mammary luminal cells (Lin⁻, CD24⁺, CD29^{low}) were isolated by FACS and Rspo1 expression was evaluated. qPCR analysis revealed a drastic decrease in *Rspo1* mRNA level after Erlotinib injections (Fig. 3d). Taken together, these data suggest that Egfr is required for the upregulation of Rspo1 by Areg (Fig. 3e).

3.4. Upregulation of Areg and Rspo1 coincides at estrus phase

To reveal the physiological relevance of this regulatory axis, we investigated Areg-Egfr mediated Rspo1 upregulation during estrous cycle and pregnancy. The natural estrous cycle in mice can be divided into four stages, proestrus, estrus, metoestrus and dioestrus. Proestrus and estrus in rodent is comparable to the follicular phase of the human menstrual cycle, featuring increased estrogen level. Metoestrus and dioestrus in mice are similar to the luteal phase in humans, which is characterized by elevated levels of progesterone (Fata et al., 2001; Schedin et al., 2000; Walmer et al., 1992). To investigate the response of Rspo1 towards



Fig. 3. Induction of Rspo1 by estrogen is dependent on Areg and Egfr

(a) qPCR analysis of freshly sorted total luminal, Sca1+ luminal cell and Sca1-luminal cells indicating that *Egfr* is highly expressed in Sca1-(ER-) luminal cells. (b) Western blot analysis and quantification indicating that the increase of Rspo1 protein level by Areg or by E2+Pg is dependent on Egfr. Two individual Egfr inhibitor (Canertinib or Erlotinib) counteracted the elevation of Rspo1 induced by Areg or by E2+Pg. Quantification data is presented as means \pm s.d. from three independent experiments.

(c) Schematic illustration of intraperitoneal injection assay setup. Same volume control buffer injection was used as sham treatment. Erlotinib (25 mg/kg) or control buffer were intraperitoneal injected and check after 6 days.

(d) qPCR analysis indicating that Egfr inhibitor (Erlotinib) decreased the expression of *Rspo1 in vivo*. mRNA expression was normalized to GAPDH. Data are presented as mean \pm s.d from three independent experiments. (e) Schematic illustration showing upon E2+Pg stimulation, Areg emanating from ER + cells promotes *Rspo1* expression in ER-cells, and the action of E2+Pg is dependent on Areg and Egfr.

hormonal influences, we analyzed the expression of Areg and Rspo1 in the mammary gland across the estrous cycle. To properly stage the estrous cycle of the animal, histology of vaginal smear was examined (Fig. S2). qPCR analysis of luminal cells isolated from staged mammary glands indicated that *Areg* is upregulated in estrus phase (Fig. 4a). This was confirmed by Western blot analysis (Fig. 4b). We also found that *Rspo1* is upregulated at both estrus and dioestrus stages (Fig. 4c). Consistent patterns were observed by Western blot analysis (Fig. 4d). Thus, the endogenous expression elevation of Areg and Rspo1 coincides at estrus phase (Fig. 4g), supporting the notion that Areg mediates the hormonal effect on Rspo1 in the estrus phase. During pregnancy, *Areg* expression level declined as shown by qPCR analysis (Fig. 4e), which is



Fig. 4. Upregulation of Areg and Rspo1 coincides at estrus phase

(a) qPCR analysis indicating that Areg level is increased in estrus stage. mRNA expression was normalized to GAPDH.

(b) Western blot analysis and quantification indicating that Areg protein is elevated in estrus phase. Actin serves as a loading control.

(c) qPCR analysis of mammary cells indicating that Rspo1 expression is elevated in both estrus and dioestrus. mRNA expression was normalized to GAPDH.

(d) Western blot analysis and quantification indicating that Rspo1 protein expression is elevated in both estrus and dioestrus. Actin serves as a loading control. (e) qPCR analysis of luminal cells from virgin, pregnant day 12.5 and pregnant day 18.5, indicating highest *Areg* mRNA expression during virgin state. mRNA expression was normalized to GAPDH.

(f) qPCR analysis of luminal cells from virgin, pregnant day 12.5 and pregnant day 18.5, indicating robust upregulation of *Rspo1* expression during pregnancy. mRNA expression was normalized to GAPDH.

(g) Illustration of hormones, Rspo1 and Areg level fluctuation across estrous cycle.

(a-f) Quantification data are presented as mean \pm s.d from three independent experiments.

contrary to the elevated *Rspo1* expression detected in our hands (Fig. 4f) and in previous reports (Cai et al., 2014) (Chadi et al., 2009). Therefore, during pregnancy, Rspo1 upregulation is likely Areg independent.

4. Discussion

Concerted action of systemic hormones and local growth factors govern the development of the mammary gland. It has long been recognized that hormones control the level of local growth factors. Hormones exert their effect directly through the action of estrogen receptor signaling to induce the expression of local factors like, Wnt4, Wnt7B and Areg (Ciarloni et al., 2007; Hou et al., 2004; Miyakoshi et al., 2009), or indirectly through activation of Rspo1 expression in ER⁻ luminal cells (Cai et al., 2014). In this study, we identify Areg in ER⁺ cells as the intermediate paracrine factor for the hormonal regulation of Rspo1 expression in ER⁻ cells, and this paracrine signaling is Egfr dependent (Fig. 3e).

Rspo1 is known as the stem cell growth factor in many adult tissues with prominent biological and therapeutic significance. In the mammary gland, the level of Rspo1 is found to be elevated during dioestrus phase when progesterone level is high, which coincides with the occurrence of robust mammary stem cells activity (Asselin-Labat et al., 2010; Joshi et al., 2010). The increased Rspo1 expression at dioestrus is in line with its role in MaSC self-renewal and has been characterized in our previous study (Cai et al., 2014). In the current study, we detected an extra elevation in Rspo1 expression at estrus (summarized in Fig. 4g). This intriguing observation may suggest an additional role of Rspo1 in mammary development independent of stem cell regulation. During estrous cycles, estrogen production peaks at estrus phase, and estrogen-mediated proliferation is fundamental to mammary gland development and breast tumorigenesis (Fig. 4g). Estrogen promotes the expression of Areg (Shoyab et al., 1988; Martinez-Lacaci et al., 1995), and Areg has been reported to stimulate stromal cells secretion of niche factors promoting ductal growth (Sternlicht et al., 2005). How Areg is transported through basal cell layer and basement membrane remains obscure. In the current study, we identify the ER⁻ luminal cells as another cellular target of Areg, inducing the expression of Rspo1. Although decreased Rspo1 expression after in vivo injection of Egfr inhibitor Erlotinib could also involve the effect from stromal compartment (Fig. 3c), our in vitro assay clearly demonstrated a direct effect of luminal Egfr on Areg-mediated Rspo1 production (Fig. 3b). This represents a shorter range of Areg's action in regulating mammary development. The molecular mechanism on how Egfr upregulates Rspo1 transcription is yet to be determined, AREG overexpression is found in most ER α + primary breast tumors (Kenny and Bissell, 2007), and EGFR expression in breast cancer has been associated with poor prognosis and resistance to hortherapy (Johnston, 2006). Our study identifies mone the estrogen-Areg-Egfr-Rspo1 axis in normal mammary luminal cells, suggesting a mechanism by which Areg can influence the output of hormones.

In conclusion, our study revealed a novel Areg-Rspo1 regulatory axis in the mammary gland in response to hormonal stimulation. These findings provided new evidence for the orchestrated action of systemic hormones and local growth factors, which is important for a comprehensive understanding of mammary development and breast cancer prevention and treatment.

Author contribution

Y. A. Z. designed the experiments; C. C. performed cell sorting, primary cell culture, qPCR and Western blot analysis, RNA in situ; A. G. performed *in vivo* experiments and RNAseq related analysis; C. C. and M. W. performed RNAseq analysis; L. Y. supervised RNAseq analysis; C.C, Q. C. Y and Y. A. Z. analyzed the data; Q. C. Y and Y. A. Z. wrote the manuscript.

Declaration of competing interest

The authors declare no competing financial interest.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (31830056, 31861163006, 31625020, 31530045, 31661143043 to Y.A.Z.; 81873532 to Q.C.Y; 31671546 to C.C.), Chinese Academy of Sciences (XDB19000000 to Y.A.Z.), Shanghai Municipal Science and Technology Commission (17XD1404000 to Y.A.Z.).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ydbio.2019.10.006.

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