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Citation for published version:

Digital Object Identifier (DOI):
10.1101/gad.277756.116

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
Genes & Development

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RESEARCH COMMUNICATION

The adrenal capsule is a signaling center controlling cell renewal and zonation through Rspo3

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Adrenal glands are zonated endocrine organs that are essential in controlling body homeostasis. How zonation is induced and maintained and how renewal of the adrenal cortex is ensured remain a mystery. Here we show that capsular Rspo3 signals to the underlying steroidogenic compartment to induce β-catenin signaling and imprint glomerulosa cell fate. Deletion of Rspo3 leads to loss of SHH signaling and impaired organ growth. Importantly, Rspo3 function remains essential in adult life to ensure replenishment of lost cells and maintain the properties of the zona glomerulosa. Thus, the adrenal capsule acts as a central signaling center that ensures replacement of damaged cells and is required to maintain zonation throughout life.

Supplemental material is available for this article.

Received January 12, 2016; accepted in revised form May 16, 2016.

The permanent cortex is formed through recruitment of capsular cells in a process that involves SHH signaling [King et al. 2009]. By E17.5, steroidogenic cells have adopted specific expression profiles, with the outermost cell layers [zona glomerulosa [ZG]] producing enzymes that are required for mineralocorticoid production [e.g., CYP11B2], and deeper layers [zona fasciculata [ZF]] expressing genes involved in glucocorticoid synthesis [Cyp11b1]. In humans, but not rodents, a third layer [zona reticularis] can be distinguished that produces androgens and is located close to the medulla. Several lines of evidence suggest that β-catenin plays an important role in adrenal zonation and maintenance. Activation of the β-catenin pathway is restricted to the ZG [Kim et al. 2008; Walczak et al. 2014], and ectopic expression leads to the activation of ZG markers in ZF cells [Berthon et al. 2010]. Moreover, β-catenin seems to bind to and control the expression of Atr1, a gene specifically expressed within the ZG [Berthon et al. 2014]. Finally, stochastic deletion of β-catenin using a low-level expressing Sfi-Cre line leads to progressive loss of the adrenal cortex in adult life [Kim et al. 2008].

Results and Discussion

Recent lineage tracing studies have revealed that adrenal maintenance likely involves cell conversion of ZG cells into ZF cells [Freedman et al. 2013], with cells being displaced in a centripetal fashion. In our search for signaling molecules that may be involved in adrenal cell renewal and zonation, we identified members of the R-spondin gene family to be expressed from E12.5 onward within mesenchymal cells surrounding the forming adrenal [Supplemental Fig. S1]. Expression of Rspo1 and Rspo3 was maintained throughout development and well into adulthood [Fig. 1A; Supplemental Fig. S1A]. R-spondins are signaling molecules that bind to LGR receptors and positively modulate the β-catenin signaling pathway [de Lau et al. 2014]. As expected from previous studies, β-catenin was highly expressed in the ZG [Fig. 1B]. Moreover, immunofluorescence analysis revealed LGR5 to be enriched within ZG cells [Fig. 1B], which is consistent with an active Rspo/LGR5/β-catenin cascade.

The adrenal capsule consists of mesenchymal cells that are positive for the SHH mediator GLI1 [King et al. 2009] and COUPTFI [also known as NR2F2] [Wood et al. 2013] and surrounded by a thin layer of mesothelial lining expressing the Wilms tumor suppressor WT1 [Bandiera et al. 2013]. To determine whether Rspo1 and Rspo3 are expressed in the same cell population, we performed cell-sorting experiments on dissociated adult adrenals from Wt1-GFP animals [Hosen et al. 2007]. Quantitative PCR (qPCR) analysis revealed the majority of Rspo1 expression to be within the WT1+ population [Supplemental Fig. S1D], which is consistent with previously published data showing a direct regulation of this gene by this
transcription factor [Motamedi et al. 2014]. In contrast, Rspo3 appeared to be in a distinct cell population that does not express high levels of WT1. RNA-Scope analysis revealed Rspos3 to be expressed throughout the capsule (Fig. 1C), reminiscent of the expression pattern of Gli1 [King et al. 2009] and NR2F2/COUPTFII [Wood et al. 2013]. To test whether Rspos3-expressing cells may be identical to the Gli1-positive population, we used a Gli1-CreERT2 line in combination with a conditional allele for Gli1-CreERT2 (Rocha et al. 2015). Deletion was induced at E14.5 by tamoxifen induction, and samples were collected at E16.5 (Supplemental Fig. S1E). Strikingly, RNA expression analysis showed an almost 70% reduction after deletion, indicating that Gli1- and Rspos3-expressing cells are largely identical [Supplemental Fig. S1F].

We next used genetic analysis to address whether R-spondins may play a direct role in adrenal formation and homeostasis. Deletion of Rspos1 was compatible with survival [Chassot et al. 2008; Tomizuka et al. 2008] and had no significant effect on adrenal development, zonation, or tissue maintenance (Supplemental Fig. S2). Rspos3-deficient embryos die early in development due to defects in placenta formation [Kazanskaya et al. 2008]. We therefore resorted to a tamoxifen-inducible approach [Fig. 1D] combining the Rspos3lox allele [Rocha et al. 2015] with the ubiquitously expressed cCAG-CreERT2 driver strain [Hayashi and McMahon 2002]. Induced deletion of Rspos3 at E11.5 resulted in smaller adrenals and thinning of the adrenal cortex at E16.5 [Fig. 1E]. Indeed, quantitation of SF1-positive cells revealed a 2.5-fold decrease of the adrenal steroidogenic compartment in the absence of Rspos3 [Fig. 1F,G].

The dramatic reduction of SF1+ cells in Rspos3 knockout animals indicated an important function for this gene in the formation of the adult adrenal cortex. Previous studies have demonstrated a key role for SHH signaling in the recruitment of capsular Gli1+ cells to the steroidogenic lineage [Ching and Vilain 2009; King et al. 2009; Huang et al. 2010], and Shh has been suggested to be regulated by canonical β-catenin signaling in the adrenal system [Drelon et al. 2015] and several other developmental systems [Iwatsuki et al. 2007; Ahn et al. 2010]. To test whether this pathway is affected in Rspos3 mutants, we carried out qPCR, immunohistochemistry, and in situ hybridization [ISH] analysis on E16.5 tissues. Deletion resulted in a near-complete loss of the β-catenin target Axin2 [Lastig et al. 2002], thus indicating a requirement of Rspos3 for canonical signaling [Fig. 1H]. Shh was prominently expressed in steroidogenic cells juxtaposing the capsule of control animals but became almost undetectable in Rspos3 mutant animals [Fig. 1H,I]. Consistent with the loss of SHH signaling, expression of its direct target, Gli1, also disappeared from capsular cells after Rspos3 deletion [Fig. 1I]. Wnt4, a gene that has been shown to be directly regulated by β-catenin in the kidney [Park et al. 2012], was no longer expressed in the mutant cortex [Fig. 1I].

Taken together, these data suggest a double-paracrine mechanism in adrenal cortex development, in which Rspos3 is released from the capsule to induce β-catenin signaling within juxtaposed steroidogenic cells, leading to the activation of Shh. In turn, SHH signals back to capsular cells, where it activates Gli1 and other downstream targets to recruit cells to the steroidogenic lineage. Loss of Shh signaling leads to lack of cell recruitment and, as a consequence, a severely reduced adrenal cortex.

Zonation of the adrenal cortex into ZG and ZF commences at around E16.5 in mice and has been suggested to depend on β-catenin signaling. Ubiquitous deletion of Rspos3 at E11.5 results in heart failure [D Silva, AS Rocha, J Ryler, C Basboga, H Morrison, KD Wagner, and A Schedl, in prep.], thus prohibiting the analysis of later time points. We therefore resorted to the capsule-expressed Gli1-CreERT2 strain and induced deletion at E13.5, which resulted in a lack of Rspos3 expression at E18.5 [Fig. 2A,B]. Immunofluorescent analysis revealed loss of the ZG-specific marker DAB2 [Fig. 2C]. In contrast, expression of the ZF-specific markers AKR1B7 and CYP11B1 persisted and now extended throughout the remaining cortex up to the capsule. BrdU labeling experiments confirmed an almost complete loss of proliferation in the adrenal cortex, whereas dividing cells persisted in other tissues [Supplemental Fig. S3]. Nr2f2 expression persisted in the capsule [Fig. 2C], although the number of cells also appeared to be somewhat reduced.

**Figure 1.** Rspos3 is expressed in the adrenal capsule and is required for adrenal growth and the onset of zonation. [A] In situ hybridization [ISH] analysis reveals Rspos1 and Rspos3 in the capsule surrounding the adrenal cortex. [B] Immunostaining showing the LGR5 receptor to be enriched in ZG cells that also show strong staining for β-catenin. [C] RNA-Scope analysis on adult adrenals. [D] Schematic outline of the experimental strategy used. Rspos3 was deleted by injecting tamoxifen into pregnant mothers at E11.5 and collecting embryos at E16.5. [E] Macroscopic analysis [panels i,iii] and hematoxylin and eosin [H&E] staining [panels iii,iv] demonstrate a dramatic reduction of adrenal size in the absence of Rspos3 expression. [CAG] cCAG-CreERT2. [F] Immunofluorescent analysis shows a reduction of adrenal cortex [SF1-positive cells, green] but not medullary [TH, light blue cells]. [G] Quantification of SF1-positive cells confirms dramatic loss of the steroidogenic compartment. nwt = 4; nko = 4; P < 8 × 10−4. Student’s t-test. [H] Quantitative PCR (qPCR) analysis of RNA extracted from E16.5 adrenals. nwt = 3; nko = 4; Axin2, P = 9 × 10−4; Shh, P = 0.02. [I] ISH analysis shows complete loss of Wnt4, Shh, and Gli1 expression.
To test whether continuous Rspo3 signaling was required to maintain adrenal homeostasis, we induced Cre recombination (cCAG-CreERT2 driven) in adult mice (12 wk) and sacrificed animals at various time points after induction [Fig. 3A]. ISH confirmed near-complete loss of Rspo3 expression in the adrenal capsule [Fig. 3B; Supplemental Fig. S4B]. Adrenal weight in Rspo3 mutants progressively decreased over time and dropped to 50% of that in control animals 6 wk after induction [Fig. 3C]. Histological analyses confirmed a severely reduced adrenal cortex, while the medulla appeared unaffected [Fig. 3D]. High-power views revealed loss of the typical ZG organization in arches. Instead, the remaining cortical cells showed the columnar arrangement reminiscent of the ZF. To test whether mutant adrenals showed reduced proliferation, we performed long-term BrdU tracing experiments. In control animals, a large number of SF1/BrdU double-positive cells were detected, indicating that they underwent cell division during the labeling period [Fig. 3E]. In contrast, BrdU-labeled cells were dramatically reduced after Rspo3 deletion, and SF1/BrdU double-positive cells were almost absent.

To confirm the loss of ZG differentiation, we next analyzed a range of molecular markers using ISH and immunostaining. Wnt4, Shh, Gli1 [Fig. 4A], DAB2, and CYP11B2 [Fig. 4B,C] were dramatically reduced in mutant mice. Similar to development, the ZF marker AKR1B7 now extended up to the capsule [Fig. 4B]. qPCR assays confirmed these findings with no alteration in ZF markers and an almost complete loss of ZG marker genes [Fig. 4B].

To exclude secondary effects that might have been caused by systemic deletion of Rspo3, we next performed tissue-specific deletion experiments using the Gli1-CreERT2 strain [Supplemental Fig. S4A]. qPCR analysis revealed a reduction of Rspo3 expression to ~30% of wild-type levels, which is somewhat lower than what we observed using the ubiquitously expressed cCAG-CreERT2 line [Supplemental Fig. S4B]. The less efficient deletion is likely to be due to lower levels of expression from the Gli1 promoter. Axin2 expression levels closely followed those of Rspo3 [Supplemental Fig. S4A]. Importantly, Gli1-CreERT2-specific deletion also caused a reduction of the adrenal cortex and the loss of ZG markers [Supplemental Fig. S4C,D], indicating that, indeed, zonation depends on capsular expression of Rspo3.

The striking loss of ZG markers could be caused by either apoptosis of ZG cells, lack of cell renewal, or a continuous requirement for Rspo3 signaling to specify ZG cells. To distinguish between these alternatives, we analyzed...
ingly, mice survived into adulthood (>12 mo) with no overt defects detectable. Adrenals in Wnt4 knockout mice were only slightly smaller [Supplemental Fig. S5B], demonstrating that this gene does not play a major role in cell renewal. Molecular analysis revealed a significant reduction of the ZG markers Axin2 and At1b but only a mild reduction of Rspo3, Shh, and Gli1 expression that did not reach statistical significance [Supplemental Fig. S5C,D]. Thus, Wnt4 appears to act as a local relay signal that reinforces β-catenin activity in the ZG but does not have a major influence on organ renewal.

A recent study suggested Rspo3 to bind to LGR5 and induce noncanonical signaling in adrenal cortical cells. This signaling appeared to inhibit aldosterone production and cell renewal and induce apoptosis [Shaikh et al. 2015]. These findings are in stark contrast to the data presented in this study. We presume the discrepancy to be caused by the in vitro setting of the previously reported study, which does not take into account the complex interactions of cells in an in vivo environment. Our data clearly demonstrate a canonical role for Rspo3 signaling within the adrenal cortex, where it induces well-established direct downstream targets of β-catenin [Axin2 and Dab2] and maintains organ size in the adult organism. This role is consistent with the generally accepted function of Rspo/LGR5 signaling in controlling the activation of the stem cell pool, and we can now add the adrenal cortex as an additional organ in which this pathway is used.

Even more surprising than the involvement of Rspo3 in controlling organ renewal is its absolute requirement for maintaining adrenal zonation. The rapid loss of zonation markers after Rspo3 deletion in the adult demonstrates that it acts as an instructive signal to direct samples 1 d after completion of the tamoxifen deletion protocol [Fig. 5], in which lack of cell renewal can be excluded. Knockout adrenals at this early time point showed only a very mild reduction in size that did not reach statistical significance [Fig. 3C]. Caspase3 analysis identified only a very mild reduction in size that did not reach statistical significance (Fig. 3C). Caspase3 analysis identified only a very mild reduction in size that did not reach statistical significance (Fig. 3C). Caspase3 analysis identified only a very mild reduction in size that did not reach statistical significance (Fig. 3C). Caspase3 analysis identified only a very mild reduction in size that did not reach statistical significance (Fig. 3C). Caspase3 analysis identified only a very mild reduction in size that did not reach statistical significance (Fig. 3C). Caspase3 analysis identified only a very mild reduction in size that did not reach statistical significance (Fig. 3C). Caspase3 analysis identified only a very mild reduction in size that did not reach statistical significance (Fig. 3C). Caspase3 analysis identified only a very mild reduction in size that did not reach statistical significance (Fig. 3C). Caspase3 analysis identified only a very mild reduction in size that did not reach statistical significance (Fig. 3C). Caspase3 analysis identified only a very mild reduction in size that did not reach statistical significance (Fig. 3C). Caspase3 analysis identified only a very mild reduction in size that did not reach statistical significance (Fig. 3C). Caspase3 analysis identified only a very mild reduction in size that did not reach statistical significance (Fig. 3C). Caspase3 analysis identified only a very mild reduction in size that did not reach statistical significance (Fig. 3C). Caspase3 analysis identified only a very mild reduction in size that did not reach statistical significance (Fig. 3C).

Figure 4. Loss of Rspo3 abolishes adrenal zonation. ISH (A), immunofluorescent (B), and immunohistochemical (C) analyses reveal loss of all ZG-specific markers analyzed. Note the persistence of pockets of DAB2 expression [white arrowhead in B] that are likely associated with incomplete deletion of Rspo3. (B) NR2F2 capsular cells appear to be largely unaffected. (D) qPCR analysis confirms the dramatic reduction of expression of the β-catenin target Axin2 and a near loss of ZG markers Cyp11b2 and Shh as well as the capsular marker Gli1. nwt = 5; nwko = 5. Rspo3, P = 8 × 10⁻³; Axin2, P = 1 × 10⁻³; Cyp11b2, P = 3 × 10⁻³; Shh, P = 2 × 10⁻³; Gli1, P = 5 × 10⁻³, Student’s t-test.

Figure 5. Rspo3 deletion leads to a rapid loss of ZG identity. (A) Experimental design. (B) Caspase staining revealed some apoptotic cells close to the medulla, whereas the ZG was devoid even after deletion of Rspo3. (C) ISH and immunostaining demonstrated a rapid loss of ZG marker gene expression. Note the complete loss of DAB2 in a portion of subcapsular cells [white arrow] upon Rspo3 deletion. (D) Model describing the central role of Rspo3 in establishing adrenal zonation and ensuring cell renewal. Large red arrows symbolize the centripetal movement of cells during cell renewal. (Meso) Mesothelial lining; (C) capsule.
steroidogenic cells toward the ZG phenotype. The adrenal capsule is the ideal location to provide this stimulus. Since R-spondins appear to be tightly bound to the extracellular matrix (Kim et al. 2005), diffusion is limited, and only several cell layers are instructed to express ZG markers. Activation of Wnt4 within the ZG cells is likely to serve as a local reinforcement signal, as loss of Wnt4 also leads to a reduction of ZG markers [Supplemental Fig. S5; Heikkila et al. 2002].

Apart from the adrenal cortex, hepatocytes also display metabolic zonation along the liver lobule that is set up by a gradient of canonical β-catenin signaling [Benhamouche et al. 2006]. We showed recently that, in a fashion similar to the adrenal, liver zonation strictly depends on RSPO3 that is released from endothelial cells located in the central vein (Rocha et al. 2015). Thus, our evidence suggests that the liver and adrenal cortex have adopted the same signaling system to establish organ-specific zonation despite the fact that both organs are derived from distinct embryological origins.

In conclusion, our data establish a genetic network in which RSPO3 induces Wnt4 and Shh expression within steroidogenic cells that are in close contact with the capsule [Fig. 5D]. Shh in turn signals back to recruit capsular cells to form the adrenal cortex, at least during development. The fact that Shh knockout mice show normal adrenal zonation [Huang et al. 2010] while RSPO3 deletion affects ZG identity indicates that RSPO3 must also act through additional, Shh-independent pathways. The dramatic thinning of the entire steroidogenic compartment further supports the model of a ZG-driven adrenal cortex renewal, a concept that has been put forward by Freedman et al. [2013]. Thus, our study identifies the adrenal capsule as a crucial signaling center that, through the production of RSPO3, not only ensures organ renewal but also provides an instructive signal that is continuously required for proper zonation.

Materials and methods

Mouse work

All animal work was conducted according to national and international guidelines and was approved by the local ethics committee (PEA-NCV-2013-88). The Rspo1 (Chassot et al. 2008), Rspo3lox (Rocha et al. 2015), Wt1-GFP (Hosen et al. 2007), Gli1-CreERT2 (Ahn and Joyner 2004), cCAG-CreERT2 (Hayashi and McMahon 2002), Sf1-Cre (Bingham et al. 2006), and Wnt4Flox (Shan et al. 2009) lines have been described previously. Cre activation during development was obtained by a single tamoxifen injection of Wt1-CreERT2 mice to drive Rspo1, Rspo3, and Shh expression in the adrenal cortex [Heikkila et al. 2002]. 

For proliferation assays, 1 mg/mL BrdU was added to drinking water, the cell suspension was filtered (40-µm filters), centrifuged for 2 min with antibiotic and DNase, and blocked in a PBS solution containing 10% normal donkey serum (Jackson Immunoresearch) and 3% BSA. All primary antibodies were applied overnight at 4°C at the concentrations listed in the antibody table in the Supplemental Material. Secondary antibodies were diluted 1:200 and applied at room temperature for 1 h. β-Catenin was detected using a biotinylated secondary antibody followed by streptavidin-Cy3 detection (Sigma-Aldrich).

For histological analysis, adrenal glands from embryos or adult mice were fixed overnight in 4% paraformaldehyde, progressively dehydrated, and embedded in paraffin. Seven-micrometer-thick sections were then stained with hematoxylin and eosin according to standard procedures.

For CYP11B2 and caspase3 staining, sections were unmasked, incubated for 30 min in H2O2 (30%), and blocked for 1 h in 5% PBS/BSA. Primary antibody was incubated in a PBS solution containing 1% normal donkey serum for 1 h at room temperature. Revelation was performed with a Novared kit (Vector Laboratories) for 4 min.

Azan staining

Histological sections at 5 µm were stained using the Azan trichrome kit (Diapath) according to the manufacturer’s instructions.

RNA ISH

Tissues were fixed overnight in 4% paraformaldehyde, progressively dehydrated, and paraffin-embedded. The Gli1 in situ probe was kindly provided by M. Studer (Institute of Biology Valrose, Université de Nice-Sophia Antipolis). Seven-micrometer-thick sections were hybridized with Rspo1, Rspo3, Wnt4, and Shh as well as Gli1 probes according to previously described protocols [Comai et al. 2010]. RNA-Sc Coupe analysis for Rspo3 was performed on adult sections according to the manufacturer’s instructions.

Real-time PCR analysis

RNA was extracted from E16.5 or adult adrenal glands using TRIzol reagent [Invitrogen] following the manufacturer’s instructions. Reverse transcription was performed using M-MLV reverse transcriptase [Invitrogen] in combination with random primers. The cDNA obtained was then used as template for qPCR analysis using the SYBR Green master kit (Roche) and a LightCycler 1.5 [Roche]. The expression levels were normalized for Hprt. Primers (see the primer table in the Supplemental Material) were designed on the Universal ProbeLibrary Web site [Roche].

Statistical analysis

Statistical significance was determined using two-tailed Student’s t-test. Error bars in all figures represent the standard error of mean, and P-values are indicated by asterisks as follows: P < 0.05 (*), P < 0.01 (**), and P < 0.001 (***)

For the developmental analysis [Fig. 1G], Sf1-positive cells were counted on every second section of four control and four knockout adrenals using Volocity 6.3 software. Statistical analysis was performed with the total of nuclei obtained for each adrenal using a two-tailed Student’s t-test.

Acknowledgments

We thank the staff of the animal facility for their dedication, and Agnès Loubat [cytometry platform, iBV] for FACs analysis. We are indebted to Hitoshi Okamoto [Riken Institute, Japan] for providing the Rspo3 conditional allele, and Celso Gomez-Sanchez [Jackson, MS] for the Cyp11B2 antibody. We are grateful to Pierre Val [Clermont Ferrand, France] for critically reading this manuscript, and Roberto Randiera [Cambridge, UK] for initial input into this project. This work was supported by grants from the Fondation du France, Association pour la Recherche sur le Cancer
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Genes Dev. published online June 16, 2016
Access the most recent version at doi:10.1101/gad.277756.116

Supplemental Material
http://genesdev.cshlp.org/content/suppl/2016/06/16/gad.277756.116.DC1.html

Published online June 16, 2016 in advance of the print journal.

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