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# R-spondin1 is required for normal epithelial morphogenesis during mammary gland development.

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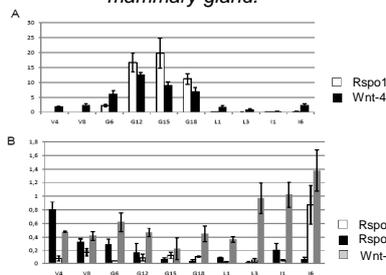
**INTRODUCTION:** The mammary gland is an organ that undergoes an important development cycles of epithelial tissues after birth under control of female reproductive hormones, through signaling pathway as Wnt/ $\beta$ -catenin pathway.

R-spondin ( Rspo) family genes were recently isolated in mouse (Kamata et al., 2004), human (Kim et al., 2005). The physiological functions of the Rspo in various developmental processes, as mouse apical ectodermal ridge (Nam et al., 2007), placental (Aoki et al., 2007) and ovarian (Tomizuka et al., 2008; Chassot et al., 2008) developments were reported. All members of the Rspo family directly activate the canonical Wnt signaling pathway by binding to the Wnt co-receptor LRP6 (Nam et al., 2006; Wei et al., 2007; Kim et al., 2008). Their gene expression analyses in mouse embryogenesis revealed a different expression pattern for each transcript (Nam et al., 2007).

To address the function of Rspo1, some knockout mice have been generated (Tomizuka et al., 2008; Chassot et al., 2008) . In this study, we have analyzed the expression pattern of the 4 Rspo genes in the mammary gland and explored the specific function of Rspo1 in this organ using Rspo1-/- mice.

## RESULTS:

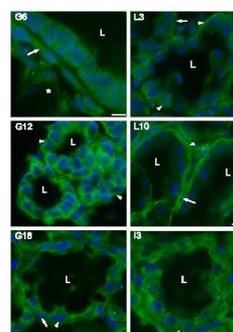
### 1/ Expression profiles of Rspo genes in mouse mammary gland.



Quantitative RT-PCR analysis of Rspo1, Wnt-4, Rspo3, Rspo4 and Wnt-1 mRNA of mammary gland at different physiological stages are presented. For each gene the expression was normalized to GAPDH gene expression. Virgin 4 weeks (V4), 8 weeks (V8), Gestation (G), Lactation (L) and Involution (I) time points (in days). Note that the scale in A is 20 fold greater than in B. The Rspo3 transcript was not detected.

The expression levels and patterns of the 4 members of Rspo family are different during the development and differentiation of the mouse mammary gland, suggesting that they have different roles. Rspo1 gene, which is the most expressed, has an expression pattern similar to that of the Wnt-4 gene.

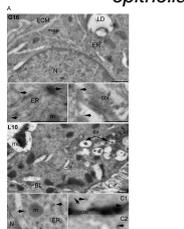
### 2/ Localisation of Rspo1 in mammary gland.



Immunodetection of Rspo1 (FITC) was performed on section of wild-type mouse mammary gland at different stages of differentiation. Nuclear detection was done by using DAPI. G: gestation, L: lactation, I: involution. Time points are in days. L: lumen, arrows: myoepithelial cells, arrowhead: basement membrane, \* : adipocyte. Bars: 10  $\mu$ m.

Rspo1 protein was detected in all mammary gland cell types. At gestation day-6 a diffuse widespread signal was found in the entire cytoplasm of ductal epithelial cells, myoepithelial cells and adipocytes. A progressive re-localization of Rspo1 was observed over the gestation period and lactation: the protein was more concentrated in the basolateral and basal membranes areas, as well as on basement membrane. At involution, the labelling was again found over the entire cytoplasm.

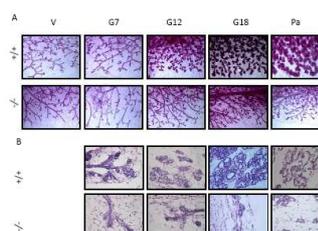
### 3/ Localization of Rspo1 in mammary epithelial cells.



A/ Immunoelectron microscopic images of Rspo1 in mammary epithelial cells of wild-type mouse during gestation day-16 (G16) or lactation day-10 (L10). Small images focus on areas including either the endoplasmic reticulum (ER) or the basal zone of the cells (right). Arrows indicate gold particles. BL: basolateral membrane, col: collagen, ECM: extracellular matrix, ER: endoplasmic reticulum, LD: lipid droplet, L: lumen, mep: myoepithelial cell, m: mitochondria, N: nucleus, sv: secretory vesicles, c: casenin micelle. Bar: 1  $\mu$ m. B/ Quantitation: gold particles were counted in the apical (grey), central cytoplasmic (white) and basal (black) part of the cells and their density per 100  $\mu$ m<sup>2</sup> was calculated.

At gestation day-16, Rspo1 labelling was found over the cytoplasm and the endoplasmic reticulum, as well as over the basal membranes of the cells. Semiquantitative analysis of the cellular distribution of gold particles further demonstrated the relocalization of Rspo1 between gestation and lactation, with a 4-fold increase of the density of gold particles in the basolateral area.

### 4/ Mammary gland development during gestation is affected in Rspo1 -/- mice.



A/ Whole mount analysis of transplanted mammary epithelium from wild-type (+/+), and Rspo1 -/- mice (-/-). Virgin (V, 11 weeks after transplantation), 7, 12 and 18 days of gestation (G), or at parturition (P8) (Magnification x 100). B/ Histological sections of the same samples are presented (Magnification x 400).

To address the function of Rspo1 during the normal mouse mammary gland development, we analyzed the incidence of its inactivation. Due to the fertility defect of the Rspo1 knockout mice, we performed Rspo1 -/- mammary gland epithelium transplantations in "cleared" fat pad nude mice. A defect of side-branching formation is observed in virgin animal and during gestation. This branching phenotype became associated with a deep defect of lobulo-alveolar development at the end of gestation and parturition.

**CONCLUSIONS:** The expression levels and patterns of the 4 Rspo genes are different during the development and differentiation of the mouse mammary gland, suggesting that they have different roles in its biology.

Mammary epithelial Rspo1 expression appears to be essential to the mammary gland development and especially to the side-branching development and lobulo-alveolar formation. The comparison with Wnt-4 knockout effect suggest that Rspo1 could be activated upstream of Wnt-4 and may turn on Wnt-4 alongside other signaling pathways.