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The canonical Notch/RBP-J signaling pathway controls the balance of cell lineages in mammary epithelium during pregnancy

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Abstract

Mammary alveoli are composed of luminal (secretory) and basal (myoepithelial) cells, which are descendants of a common stem cell. This study addressed the role of RBP-J-dependent Notch signaling in the formation, maintenance and cellular composition of alveoli during pregnancy. For this purpose, the genes encoding RBP-J, the shared transcriptional mediator of Notch receptors, and Pofut1, a fucosyltransferase required for the activity of Notch receptors, were deleted in mammary progenitor cells in the mouse using Cre-mediated recombination. Loss of RBP-J and Pofut1 led to an accumulation of basal cell clusters characterized by the presence of cytokeratins (K5) and K14 and smooth muscle actin (SMA) during pregnancy. Hormonal stimulation of mutant tissue induced the expression of the basal cell transcription factor p63 in luminal cells and excessive proliferation of basal cells. A transient enrichment of K6-positive luminal cells was observed upon hormonal treatment suggesting a temporary arrest at an immature stage prior to transdifferentiation and expansion as basal cells. Despite the extensive proliferation of RBP-J-null basal cells during pregnancy, hormonal withdrawal during involution resulted in complete remodeling and the restoration of normal tissue architecture. We propose that the Notch-RBP-J pathway regulates alveolar development during pregnancy by maintaining luminal cell fate and preventing uncontrolled basal cell proliferation.

Keywords: Notch; Myoepithelium; Basal cells; Pofut1; RBP-J; Mammary epithelium; Cytokeratins

Introduction

The Notch family of cell surface receptors controls the specification of a wide variety of cell types (Schweisguth, 2004; Tanigaki et al., 2003). Evidence for a link between Notch signaling and mammary tumorigenesis came from observations that integration of the mouse mammary tumor virus (MMTV) into an intron of the *Notch4 (int3)* gene leads to the formation of mammary tumors (Gallahan and Callahan, 1987). In this case, transcription initiated in the MMTV-LTR leads to hybrid transcripts that encode the constitutively active Notch4 Intra-Cellular-Domain (ICD) (Gallahan and Callahan, 1997). Moreover, expression of the Notch4 ICD under control of mammary-specific regulatory elements in transgenic mice confirmed that activation of Notch signaling leads to the establishment of mammary tumors (Gallahan et al., 1996; Jhappan et al., 1992; Kordon et al., 1995; Raafat et al., 2004). Expression of the Notch4 ICD under control of the *WAP* gene promoter resulted in an initial block of epithelial cell proliferation and differentiation during pregnancy (Gallahan et al., 1996) suggesting a genuine participation of Notch4 in normal development. However, mice from which the *Notch4* gene had been inactivated by homologous recombination were able to lactate (Krebs et al., 2000), suggesting that either Notch4 is not required for normal mammary development or that other Notch family members fill the void in its absence. Expression of Notch1 ICD under the control of MMTV-LTR induces tumors during lactation that regress after involution (Kiaris et al., 2004), identifying a potential role for another Notch receptor in mammary gland development.

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The functional unit in the mammary gland during lactation is the alveolus, which produces and secretes milk. An alveolus consists of two distinct cell types, the luminal or secretory cells and the basal or myoepithelial cells, both of which appear to originate from one common alveolar progenitor cell (Kordon and Smith, 1998; Smith and Boulanger, 2003). Development of mammary tissue occurs in distinct stages and is controlled by systemic hormones and local growth factors (Hennighausen and Robinson, 2001, 2005). The mammary anlage is established at around day 11.5 of fetal development and a rudimentary ductal tree develops prior to birth. Development of the extended ductal tree is driven by estrogen and progesterone during puberty. Formation of the alveolar compartment is controlled by the prolactin receptor (Ormandy et al., 1997), the kinase Jak2 (Shillingford et al., 2002), and the transcription factor Stat5 (Cui et al., 2004; Miyoshi et al., 2001).

The signals that determine the cell fate switch from the common progenitor to the basal and luminal compartments have not been identified. Since aberrant Notch signaling can disrupt the differentiation state of mammary epithelium, we postulated a role for this developmental cue in the lineage commitment of mammary progenitors. As it is not feasible to use mouse genetics to simultaneously inactivate all four Notch receptors, we addressed the role of Notch signaling using two approaches that address distinct aspects of the Notch signaling pathway. First, RBP-J, the common downstream partner of all Notch ICDs, was inactivated. Second, Pofut1, a fucosyltransferase essential for the activity of Notch receptors (Okajima and Irvine, 2002; Sasamura et al., 2003; Shi and Stanley, 2003), was inactivated. By inactivating Notch signaling using two distinct mechanisms, potential Notch-independent effects of RBP-J (Beres et al., 2006; MacKenzie et al., 2004) would be revealed. Since loss of the *Rbpsuh* (Oka et al., 1995) and *Pofut1* (Shi and Stanley, 2003) genes results in fetal death, we deleted the two genes specifically in mammary progenitor cells of the mouse using Cre-mediated recombination (Wagner et al., 1997, 2001). Using antibodies against specific basal and luminal proteins, it was possible to monitor on a single cell level molecular consequences of the absence of RBP-J and Pofut1. This approach also permitted an analysis of how basal and luminal cells respond to pregnancy hormones in the presence and absence of RBP-J and Pofut1.

Materials and methods

Antibodies

Antigen	Antibody species	Provider	Product no.	Antigen retrieval (pH)	Dilution
Cre	Rabbit	Novagen	69050-3	9.0	1:200
E-cadherin	Mouse	BD Transduction Laboratories	610181	6.0	1:100
ERα	Rabbit	Santa Cruz	sc-542	6.0	1:100
Keratin 14	Sheep	The Binding Site	Discontinued	9.0	1:100
Keratin 14	Rabbit	Panomics	E2624	9.0	1:200
Keratin 18	Mouse	RDI (Research Diagnostics Inc)	RDI-PRO61028	9.0	1:100
Keratin 5	Rabbit	Covance	PRB-160P	9.0	1:1000
Keratin 6	Rabbit	Covance	PRB-169P	9.0	1:200
Keratin 8	Sheep	The Binding Site	Discontinued	9.0	1:100
PCNA	Mouse	DAKO	M 0879	9.0	1:200
Progesterone	Rabbit	DAKO	A0098	6.0	1:100
p63	Mouse	NeoMarkers	MS-1081-P	6.0	1:200
SMA	Mouse	Sigma	A 2547	9.0	1:1000
Stat-5a	Rabbit	Santa Cruz	sc-1081	9.0	1:100 O/N 4°C
Alexa Fluor® 488	Goat	Molecular Probes, Inc.	A-11001		1:400
Alexa Fluor® 488	Goat	Molecular Probes, Inc.	A-11008		1:400
Alexa Fluor® 488	Donkey	Molecular Probes, Inc.	A-11015		1:400
Alexa Fluor® 594	Goat	Molecular Probes, Inc.	A-11005		1:400
Alexa Fluor® 594	Goat	Molecular Probes, Inc.	A-11012		1:400
Alexa Fluor® 594	Donkey	Molecular Probes, Inc.	A-11016		1:400

Mouse breeding and genotyping

Mice which carry floxed *Rbpsuh* (Han et al., 2002) and *Pofut1* (Shi et al., 2005; Shi and Stanley, 2003) alleles were in a mixed 129/C57BL/6 background. Mice were generated that carried either two *Rbpsuh* or two *Pofut1* floxed alleles and the *MMTV-Cre* transgene (line A) (Wagner et al., 2001). The mice were treated according to the animal protocols approved by the Animal Care and Use Committee at NIH.

PCR analysis was used for determining the genotype of these mice. RBP-J wild-type intron was detected using primers: wt1: 5′-gTTcTTAAccTgTTggTcg-gAAcc-3′ and wt2: 5′-gcTTgAggcTTgATgTTcTgTATTgc-3′ (Han et al., 2002). For the detection of the floxed allele, primers from the neomycin cassette were used. neo1: 5′-AgAggcTATTcggcTATgAcTg-3′ and neo2: 5′-TTcgTccAgAT-cATccTgATc-3′ *Pofut1* wild-type and floxed alleles were detected by PCR using primers 644: 5′-AcccAcAggcTgTgcAgTcTTTg-3′ and 645: 5′-gggTcAccTT-cATgTAcAAgTgAgTg-3′ (95°C, 5 min; 35 cycles: 95°C, 1 min, 62°C, 1 min, 72°C, 1 min; 72°C, 7 min).

Primers for the Cre transgene are as follows: 5′-ggTTcTgATcTgAgcTcT-gAgTg-3′ binding in the MMTV long terminal repeat and 5′-cATcAcTcgTTg-cATcgAccgg-3′ binding in the Cre sequence.

Histology and immunohistochemistry

Mammary glands were fixed in 10% neutral buffered formalin overnight at 4°C. After fixation, tissues were placed in 70% ethanol, dehydrated and paraffin-embedded. For histology, sections were stained with hematoxylin and eosin (H&E). For immunostaining, paraffin sections (5 μm) were cleared in xylene and rehydrated through an alcohol series. Digital Decloaking Chamber (Biocare Medical; Walnut Creek, CA) was utilized for antigen retrieval. Sections were immersed in BORG*DECLOAKER* or Reveal 1× (heat-induced epitope-retrieval solution, 9.5 pH or 6.0 pH, respectively). SP1 (Set-Point1) was 125°C for 5 min and SP2 (Set-Point2) was at 90°C for 10 s. After carefully rinsing slides with running tap water, the sections were placed in PBS