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Expression of *Notch* receptors, ligands and target genes during development of the mouse mammary gland

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Abstract

Notch genes play a critical role in mammary gland growth, development and tumorigenesis. In the present study we have quantitatively determined the levels and mRNA expression patterns of the *Notch* receptor genes, their ligands and target genes in the postnatal mouse mammary gland. The steady state levels of *Notch3* mRNA are the highest among receptor genes, *Jagged1* and *Dll3* mRNA levels are the highest among ligand genes and *Hey2* mRNA levels are highest among expressed *Hes/Hey* target genes analyzed during different stages of postnatal mammary gland development. Using an immunohistochemical approach with antibodies specific for each Notch receptor, we show that Notch proteins are temporally regulated in mammary epithelial cells during normal mammary gland development in the FVB/N mouse. The loss of ovarian hormones is associated with changes in the levels of *Notch* receptor mRNAs (*Notch2* higher and *Notch3* lower) and ligand mRNAs (*Dll1* and *Dll4* are higher, whereas *Dll3* and *Jagged1* are lower) in the mammary gland of ovariectomized mice compared to intact mice. These data define expression of the *Notch* ligand/receptor system throughout development of the mouse mammary gland and help set the stage for genetic analysis of *Notch* in this context.

Keywords

Notch; Notch ligand; RT-PCR; mouse mammary gland; mammary cleared fat pad

1. Introduction

Mammary gland development is governed by a variety of signaling pathways involved in cell fate specification and differentiation. The mouse gland is a powerful model for the study of mammary epithelial growth, morphogenesis, differentiation and neoplastic transformation (Daniel and Smith, 1999). The majority of growth and development takes place after birth and progresses through defined stages, including puberty and pregnancy leading to lactation and subsequently involution. At birth, female mice possess rudimentary mammary glands, each composed of a simple system of branching ducts opening at the nipple. At approximately 3 weeks of age, the ducts begin to grow rapidly due to intense mitotic activity within terminal end buds (TEBs). The end buds contain undifferentiated stem cells or cap

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cells that give rise to the epithelial cells (Smith and Boulanger, 2003). The epithelial ducts invade, elongate rapidly and branch. By 10 weeks of age in nulliparous females, the fat pad is filled with a highly organized ductal structure, and the end buds have disappeared. During pregnancy, secretory alveolar lobules develop from the branching duct structure until they completely fill the fatty stroma. At the end of lactation massive apoptosis occurs leading to mammary gland involution (Daniel and Smith, 1999).

The mesodermally derived fatty stroma of the mammary gland is an understudied but important structure. These cells regulate growth, patterning and differentiation of ventral ectodermally derived mammary epithelial cells. In mice, white adipose tissue appears to be required for normal mammary development. Transplantation of mammary epithelium into other types of organ stroma does not support mammary epithelial growth, or results in abnormal growth (Parmar and Cunha, 2004; Wiseman and Werb, 2002). Stromal cells appear to influence epithelial cell behavior through secretion of growth factors and/or by altering the composition of the extracellular matrix in which mammary epithelial cells reside.

The *Notch* gene family encodes four related transmembrane receptors that interact with five membrane-bound ligands encoded by the *Delta/Jagged* gene families (reviewed in Callahan and Egan, 2004). Ligand binding stimulates signaling by first inducing proteolytic cleavage of Notch receptors, followed by nuclear translocation of the Notch intracellular domain (ICD) (Kopan and Ilagan, 2009). The Notch-ICD enters the nucleus and serves as a transcriptional activator, although it does not possess DNA binding activity. Rather, Notch-ICD associates with the transcription factor Rbpj (CBF1), the primary transcriptional mediator of canonical *Notch* signaling. The Notch-ICD/Rbpj complex trans-activates promoters containing Rbpj binding sites, such as those that control expression of *Hes* and *Hey* bHLH-family transcriptional repressors (Kato et al., 1996; Kopan and Ilagan, 2009). Conditional knockout of the *Rbpj* gene in mammary progenitors disrupts cell fate specification and differentiation during pregnancy (Buono et al, 2006). In addition, Notch activation can directly stimulate luminal cell fate specification in purified progenitor cells (Raouf et al., 2008 and Boras et al., 2008). Interestingly, these latter studies also included analysis of *Notch* pathway gene expression on sorted populations of mammary epithelial cells from non-pregnant humans and mice, respectively. However, little is known about *Notch* receptor, ligand and target gene expression in the developing mammary gland during puberty, pregnancy, and involution as well as the effect of estrogen on the expression of the *Notch* pathway. The aim of the present study was to determine timing and levels of mRNA expression of the different *Notch* receptors, their ligands and their canonical target genes during different stages of mammary gland development in FVB/N inbred mice. In addition, we have examined the pattern of Notch receptors and *Hey2* expression in mouse mammary gland by immunohistochemistry using well-characterized Notch-specific antibodies.

2. Materials and Methods

2.1. Quantitative PCR and RT-PCR

Total RNA from two independent pools of mammary tissue was collected from the number 4 inguinal mammary gland (Brill et al., 2008). Each pool was from five FVB/N females, at indicated developmental stages and extracted as previously described (Gallahan et al., 1996). Briefly, RNA was prepared using Trizol reagent (Invitrogen, Carlsbad, CA) followed by treatment with RQ1 DNase-I (Promega, Madison, Wisconsin), according to the manufacturer's recommendations. Also, nine-week old FVB/N female mice, from our colony, underwent bilateral ovariectomy as previously described (Raafat et al., 1999). Mammary tissue was collected one week after ovariectomy and total RNA was prepared and subjected to DNase-I treatment as described above. This study was approved by the

Institutional Ethics Committee for Laboratory Animals use in Experimental Research. Mice were kept under standard laboratory conditions according to guidelines of the National Cancer Institute. DNase treated RNA was subjected to PCR analysis to ensure successful DNA degradation. The quality and quantity of RNA was measured by Agilent bioanalyzer-2100 (Agilent Technologies, CA, USA) according to the manufacture's instructions, with a cut-off value of 1.5. Mammary gland cDNA synthesis was performed using SuperscriptII reverse transcriptase (Invitrogen, Carlsbad, Ca, USA) with 1 µg of DNase-I treated total RNA used as template in a 20-µl-reaction volume. For mammary gland quantitative gene expression analysis 1 µl cDNA was subjected to PCR amplification using TaqMan Universal PCR Master Mix Reagents from Applied Biosystems (Foster City, CA, USA). qPCR primers were obtained from Applied Biosystems. For each gene, a standard curve was created using a specific cDNA clone containing the region to be amplified. Quantitative-PCR (qPCR) efficiency was calculated from the slope of the standard curve. The cut-off value of the slope was -3.58, which corresponds to 90% efficiency. This approach insures equal efficiency among qPCR runs, thereby allowing comparison of gene-specific expression during development as well as comparison among different genes. Standard curve slope and amplification plots were analyzed using MxPro PCR software (Stratagene). The relative abundance of mammary gland target mRNA was calculated as the ratio of the copy number of target mRNA normalized to the copy number of *Gapdh* mRNA, a housekeeping gene in mammary tissue. Reactions were run in duplicates and repeated at least three times in optical 96-well plates with optical caps using the MX3000 multiplex quantitative PCR system (Stratagene). We used the recommended MX3000P optimized cycling program: 95°C, 10 min (1 cycle); 40 cycles of 95°C, 15 s; 60°C, 1 min.

2.2 Notch1 and Notch2 null HC11 cells

HC11, a clonal mouse mammary epithelial cell line derived from spontaneously immortalized COMMA-1D cells (Ball et al., 1988) were grown in RPMI-1640 supplemented with 10% fetal calf serum (FCS), 10 ng/ml EGF (recombinant human, Sigma) and 6 µg/ml insulin (Sigma). All culture media were supplemented with penicillin and streptomycin. HC11 cells were transfected with control-shRNA plasmid (Santa Cruz, SC-108060), *Notch1*-shRNA plasmid (Santa Cruz, SC-36096-SH), or *Notch2*-shRNA plasmid (Santa Cruz, SC-40136-SH) using attractene transfection reagent (Qiagen, 301004) according to the manufacturer's recommendations. For stable cell lines, transfected cells were selected by adding 5µg/ml puromycin (Sigma, P9620) to the medium. The medium was changed every 48 h.

2.3 Notch3 and Notch4 knockout mice

Notch3 mutant mice were derived from the PST033 ES cell line, which was obtained in a gene trap screen for targeting into genes coding for transmembrane proteins (Mitchell KJ et al., 2001; Arboleda-Velasquez JF et al., 2008). The specific allele disrupts Notch3 at a site just downstream of the exon coding for EGF repeat 21 (Xu K et al., 2010). For Notch4, mammary tissue was collected from 10 wk-old *Notch4/Int3* knockout mice (Krebs et al., 2000) and age matching FVB/N mice.

2.4 Immunohistochemistry and western blot analysis

HC11- *Notch1*-shRNA and HC11- *Notch2*-shRNA cells were grown on chamber slides and then fixed for 5 min in 4% paraformaldehyde, followed by brief washing in PBS (pH 7.4). After 60 min blocking in PBS-1% bovine serum albumin, cells were incubated for 1 h with anti-Notch1 or anti-Notch2 antibodies diluted in PBS-1% bovine serum albumin. After being washed twice with PBS and stained with the proper fluorochrome-coupled secondary

antibodies, samples were mounted for microscopic inspection in mounting medium (DAPI, Vector Lab. Inc.).

Immunohistochemical analysis of Notch receptors in paraffin embedded sections of mammary gland tissue, was carried out using the ABC method according to the manufacturer's recommendations (Vector Laboratories Inc., Burlingame, CA, USA). Primary antibody incubation was carried out overnight at 4°C, followed by incubation with secondary antibodies. The Hey2 antibody (Millipore, Billerica, Ma) was diluted 1000-fold and anti-rabbit secondary antibody (PK-6101, Vector Laboratories, Inc., Burlingame, CA, USA) was diluted according to the manufacturer's recommendations. Since active Notch signaling requires the localization of the Notch receptor ICD and its migration to the nucleus, we focused our discussion on the cells showing nuclear staining. Tissue sections were counterstained with hematoxylin. Staining was considered strictly nuclear if the nucleus is positive and the cytoplasm is negative and was considered cytoplasmic only if the nucleus is negative and the staining is strictly cytoplasmic. Labeling index was determined in at least a total of 3000 cells for each target protein.

Ten-week old FVB/N female mice, from our colony, underwent bilateral ovariectomy as previously described (Raafat et al., 1999). Mammary tissue was collected from intact females and one week ovariectomized female mice. Protein extracts were subjected to electrophoretic separation on polyacrylamide gels, after blotting, western analysis was performed using Notch2 antibody (Cell Signaling, 2420). The blots were stripped with Restore Western Blot Stripping Buffer (Pierce, Rockford, IL) according to the manufacturer's instructions and blotted using Notch3 antibody (Santa Cruz SC-5593) followed by β -actin antibody (Sigma, A3854)

Statistics—Quantitative values are represented as the mean of at least three experiments. All *in vivo* experiments were repeated at least twice and at least five mice were used in each experiment. All data are expressed as the mean $\pm$ SEM.

3. Results

3.1. Expression of Notch receptors

To compare steady state mRNA levels for the four different *Notch* receptors, five *Dll* and *Jagged* ligands as well as *Hes/Hey* target genes, mRNA was harvested from two independent pools of mammary tissue at different stages of development. The levels of these mRNAs were normalized to the levels of glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) mRNA, and reported as the ratio of mRNA copy number for the gene of interest to mRNA copy number for *Gapdh* (Figure 1). To validate this approach we examined levels of the epithelial cell marker *Keratin-8* (*Krt8*) mRNA. The relative levels of *Krt8* mRNA increased through mammary gland development to the first day of lactation and then decreased during involution. By day 14 of involution *Krt8* mRNA levels have significantly decreased and are most similar to levels in the 10 wk old mouse mammary gland. These results are well correlated with the epithelial content of the mouse mammary gland (Daniel and Smith, 1999), validating use of the quantitative approach for gene expression analysis.

The levels of *Notch1*, *Notch2* and *Notch3* mRNA in the mammary gland increased as the mammary gland developed from 5 weeks of age through early pregnancy (Figure 2). *Notch1-3* mRNAs levels decreased in the mammary gland of late pregnant mice. Expression of *Notch* receptors is at its lowest levels in the apoptotic stage (day1 involution) and in the quiescent mammary gland (day 14 involution), respectively. Quantification of *Notch* receptor mRNA expression in the mouse mammary gland during development revealed that

Notch3 expression is most abundant, whereas *Notch4* mRNA was the lowest. Bouras et al (2008) also reported low levels of *Notch4* expression as compared to *Notch1*, -2 and -3.

3.2. Expression of Notch ligands

Expression levels for *Notch* ligand mRNA varied dramatically with developmental stage and gene (Figure 3). Of the ligands, levels of *Jagged1* and *Dll3* mRNA were highest throughout mammary development. However, unlike *Dll3*, *Jagged1* expression decreased markedly during late pregnancy after reaching peak expression in early pregnancy. Both *Dll3* and *Jagged1* decreased markedly during lactation in comparison to the levels of the early and late pregnancy levels. *Dll1*, *Dll4* and *Jagged2* mRNAs were at very low levels in mammary glands of nulliparous and pregnant mice. Higher levels of *Dll1* mRNA, but not *Dll4* mRNA, were detected in early involuting mammary glands. The highest levels of *Jagged 2* mRNA were detected in mammary glands of pregnant mice.

3.3. Expression of Notch target genes

Canonical *Notch* signaling is mediated through interaction of Notch-ICD with Rbpj; the primary transcriptional mediator of *Notch* signaling. Mammary expression of *Rbpj* mRNA (Figure 4) peaked at 5 days of pregnancy. Activation of *Notch* target genes should represent a reliable readout of Notch/Rbpj signaling activity. Known targets of *Notch* signaling are members of *Hes/Hey* gene families that encode transcriptional repressors (Iso et al., 2001). We analyzed expression of *Hes1*, *Hes5*, *Hey1*, and *Hey2* mRNA in the mammary gland during postnatal development (Figure 4). *Hes1* mRNA levels were high in mammary glands of 10 week old nulliparous and day 5 pregnant mice. These levels began to drop during late pregnancy and then rose again at day 1 and 14 of involution, where *Hes1* mRNA was at its highest levels. *Hes5* mRNA was least abundant of the four *Notch* target genes analyzed, and was expressed at a constant level throughout postnatal mammary development. The highest levels of *Hey1* mRNA were detected in mammary glands of pregnant mice and early involuting mammary glands. Expression of this gene then decreased through lactation and late involution, with the lowest levels occurring in mammary gland at involution day 14. High levels of *Hey2* mRNA were observed in mammary glands of 5 and 10 week old nulliparous mice. These levels did not change during pregnancy, but decreased during lactation and then increased again during involution. Levels of *Hey2* mRNA, at any given point during postnatal development of the mammary gland, were at least two orders of magnitude higher than *Hey1*.

3.4. Specificity of Notch receptor antibodies for immunohistochemistry

The high degree of homology among Notch receptors (Raafat and Callahan; 2001) makes it important to confirm specificity of Notch antibodies. Three Notch1 antibodies were tested, Cell Signaling (3268), Cell Signaling (4147) and Epitomics (1935-1). In addition, three Notch2 antibodies were tested, abcam (ab52302), Santa Cruz (SC117423) and Cell Signaling (2420). Since deletion of *Notch1* or *Notch2* is embryonic lethal (Conlon et al., 1995; Hamada et al., 1999), we developed two HC11 stable cell lines where *Notch1* (HC11-*Notch1*-shRNA) or *Notch2* (HC11-*Notch2*- shRNA) expression was blocked. Real time qRT-PCR analysis of RNA from HC11-*Notch1*-shRNA and HC11-*Notch2*-shRNA cells showed that these lines have reduced RNA levels of *Notch1* (Figure 5Aa) and *Notch2* (Figure 5Ab), respectively, compared to wild type and control transfected HC11 cell lines. We used these lines to screen several Notch1 and Notch2 primary antibodies at several dilutions. The Cell Signaling (4147) Notch1 antibody showed the most specific staining at 1000X dilution (Figure 5B, a, b and c). For Notch2, ab-cam (ab52302) antibody showed the most specific signal at 2000X dilution (Figure 5B, d, e and f). Fluorescence signal was observed in control transfected HC11 cells (Figure 5B, b and e) but not in HC11-*Notch1*-shRNA and HC11-*Notch2*-shRNA stained with anti-Notch1 and anti-Notch2 antibodies,

respectively (figure 5B, c and f). We interpreted these staining patterns as confirming specificity of 4147 and ab52303 antibodies to detect only Notch1 and Notch2 respectively.

To confirm specificity of Notch3 and Notch4 antibodies we used sections of mammary tissue from *Notch3* and *Notch4* knockout mice (Krebs et al., 2000) as negative tissue for each receptor. Sections of mammary tissue from female FVB/N mice were used as positive tissue for Notch3 and Notch4. Three different Notch3 primary antibodies were tested, abcam (ab23426), Santa Cruz (M-134:SC-5593) and Santa-Cruz, (M-20:SC-7424). Also, three different Notch4 primary antibodies were tested: Santa Cruz (SC-8646) and (SC-8644) and Millipore (07-189). Once again, several dilutions of these primary antibodies were tested. The Santa-Cruz M-134 antibody showed the most specific Notch3 staining at 1000X dilution (Figure 5C, compare a and b). No Notch3 staining was observed in *Notch3* knockout mammary tissue (Figure 5C, b), whereas mammary sections from 10wk old FVB mice showed a high degree of positivity (Figure 5C, a). The Millipore (07-189) antibody showed the most specific Notch4 staining at 1200X dilution. No Notch4 staining was observed in *Notch4* knockout mammary sections (Figure 5C, d) but positive-nuclear staining was observed in the ducts, and also in the myoepithelial cells in mammary glands of 10wk old wild type FVB mice (Figure 5C, c).

3.5 The distribution of Notch receptors and Hey2 expressing cells in the mouse mammary gland during postnatal development

At 5 weeks of age, Notch-positive cells were observed in terminal end buds (Fig. 6A, ad). Notch1-positive end bud cells were localized to the body cell layer. The cap cell layer was negative for Notch1 (Fig. 6A, a). Notch3-positive cells were detected throughout the cap cells layer and many body cells were positive for Notch3 staining as well (Fig 6A, c). A few cap and body cells stained for Notch4 (Fig 6A, d). In addition, Notch1, -2, and -3 positive cells were detected in basal and luminal layers of the more mature ducts. Notch2-positive cells showed cytoplasmic staining. The percentage of Notch1 and Notch3 positive cells (3% and 32% respectively) in mammary epithelium of 5 wk- old mice was higher than the percentage of Notch2 and Notch4 positive cells (1% each) (Figure 6B).

In the mammary glands of 10 wk-old virgin mice, the ductal tree had grown to the limits of the mammary fat pad. Notch2 staining was mainly cytoplasmic (Figure 6A, f). Notch3 showed nuclear staining in both the basal and luminal cells of the ducts (Figure 6A, g). Interestingly, Notch4 showed a strong myoepithelial staining and low nuclear staining agreeing with the low mRNA levels of this receptor (Figure 6A, h). Notch1 showed weak nuclear staining (Figure 6A, e). The percentage of Notch1, Notch2 and Notch4 positive cells remained low (1%, 3% and 1% respectively) in 10 wk mammary sections, whereas, 20% of ductal cells expressed Notch3 (Figure 6B).

In early pregnancy, Notch receptor staining was observed in alveolar buds (Figure 6A. i-l). Notch1 and 2 showed cytoplasmic and nuclear staining (Figure. 6A, i and j). The percentage of Notch1, Notch2 and Notch4 nuclear positive cells remained low (2, 1 and 0.5 % respectively) (Figure 6B). The percentage of Notch3-positive cells decreased significantly to 12%, compared to levels detected in the nulliparous female gland (Figure 6B).

In the lactating mammary gland, Notch2 showed nuclear staining (Figure 6A, n). Notably, the percentage of Notch2-positive cells was significantly higher in lactating mice (16%), compared with virgin (1% in 5 week old mice and 3% in 10 week old mice) and pregnant mice (1%)(Figure 6B). In contrast, the percentage of Notch3-positive cells in glands of lactating mice decreased significantly to 10%, compared to the glands from 5 week old (32%) and 10 week old (20%) mice (Figure 6B). The FVB lactating mammary gland (Figure

6A, p), was almost devoid of Notch4 positive cells. In contrast 5% of cells were Notch1-positive in the lactating gland (Figure 6B).

To examine *Notch* signaling, immunohistochemical analysis of Hey2 was also performed on sections of mammary tissue corresponding to the stages tested for Notch expression (Figure 6A, q to t). Intense Hey2 nuclear staining was observed in TEB cap cells and basal cells of ducts in 5 wk old mammary glands (Figure 6A, q). Nuclear staining was also observed in basal and luminal cells of mammary ducts from 10 wk-old mice (Figure 6A, r). In the early pregnant mouse mammary gland (Figure 6A, s), Hey2 was detected in luminal epithelial cells of newly formed and proliferating alveoli. In the lactating mammary gland (Figure 6A, t), Hey2 was detected in basal epithelial cells of secretory alveoli. Hey2 staining decreased as mammary gland development progressed through pregnancy and lactation (Figure 6B) and was at its lowest level during involution (data not shown).

3.6. Expression of the Notch receptors and ligands in the mammary glands of normal and ovariectomized mice

Postnatal mammary gland development and function are highly dependent upon ovarian hormones; estrogen and progesterone (Fendrick et al., 1998). Rizzo et al (2008) reported on cross-talk between Notch and estrogen receptors in breast cancer. To determine whether estrogen affects *Notch* receptor and ligand mRNA expression we measured their levels in mammary glands of FVB/N mice that were ovariectomized at 9 weeks of age. Ovariectomy positively affected expression of *Notch2* and negatively affected *Notch3*, whereas *Notch1* and *Notch-4* mRNA levels were unaffected by ovariectomy (Figure 7A). The levels of *Notch2* mRNA were increased by 10 fold, whereas the levels of *Notch3* mRNA were down regulated by 15 fold in mammary glands from ovariectomized mice. Western blot analysis of mammary protein extracts, confirmed the effect of ovariectomy on Notch 2 and Notch3 (Figure 7B). Similarly, *Dll1* and *Dll4* mRNA levels were higher (50 and 18 fold, respectively) whereas *Dll3* and *Jagged1* levels were lower (90 and 2 fold, respectively) in mammary glands of ovariectomized mice (figure 7C). In contrast, *Jagged2* mRNA levels were unaffected by ovariectomy.

Sequence analysis of 4500 nucleotides 5' to the ATG start codon of mouse *Notch* receptors and their ligands showed that only *Jagged1* does not contain an estrogen response element (ERE) within the tested region. In contrast, all four *Notch* receptor genes as well as genes coding for *Dll3*, *Dll4* and *Jagged2* had imperfect ERE binding sites (Table 1). *Dll1* had a near perfect ERE (GGTCAN⁵TGACC). Analysis of intron-1 also showed an imperfect ERE in *Notch1*, *Notch2*, *Notch3*, *Notch4*, *Dll4* and *Jagged1* genes.

4. Discussion

Earlier work demonstrating involvement of *Notch* signaling in mammary development and tumorigenesis (Jhapan et al., 1992; Gallahan et al. 1996; Hu et al. 2006; Buono et al., 2006) led us to investigate expression of *Notch* receptors, ligands and target genes at multiple developmental stages. The mammary gland contains a mixture of cell populations that move geographically within the gland during postnatal development. At birth, female mice possess rudimentary mammary glands composed of a simple system of branched ducts opening at the nipple. Normal mammary development can be divided into several stages that differ in morphology, function, and hormonal responsiveness.

Relative to *Notch3* mRNA levels, only low steady state levels of other *Notch* receptors mRNAs were detected at different stages of postnatal mammary development. These levels are a function of cell type complexity in this tissue, with a mixture of expressing and non-expressing cells noted for each gene. In addition, absolute expression levels within

expressing cells are likely controlled in response to physiological and developmental status of the mouse. To address this point, we validated the specificity of several antibody preparations against mouse *Notch* receptors by demonstrating the absence of staining if expression of a specific *Notch* receptor was inhibited or disrupted (Figure 5B and C). Whereas, Notch receptor specific staining was observed in wild type HC11 cells, FVB/N mammary tissue or *Notch* transgenic mammary tumor tissue. Notch receptors and Notch target gene expression was observed in mammary epithelial cells at all developmental stages analyzed.

In the peripubertal mammary gland, TEBs that drive ductal morphogenesis, are comprised of an outer cap cell layer enriched in mammary stem cells and inner body cells fated to the luminal compartment (Daniel et al., 1987). High levels of Notch3 and Hey2 expression were detected in TEB cap cells, which are the most highly proliferative population at this stage (Fendrick et al., 1998). The cap cell layer was devoid of Notch1 and Notch2 suggesting that Notch 3 and Notch4-positive cells constitute the major pool of proliferating cells at this developmental stage. Proliferation of cap cells gives rise to a multilayered internal mass of body -cells below the cap (Hovey et al., 2002). Formation of the ductal lumen requires apoptotic cell death within the body cell layer (Hovey et al., 2002; Mailloux et al., 2007). Notch activity affects proliferation and apoptotic programs (Artavanis-Tsakonas et al., 1999). Data from two groups indicate that *Notch* signaling blocks apoptosis during thymocyte maturation (Jehn et al., 1999; Deftos et al., 1998). It is possible that Notch expression in end bud body cells, protects these cells from apoptosis. As a result mammary ductal luminal cells are Notch-positive. Alternatively, Notch signaling could be involved in inducing their death (Miller and Cagan, 1998). Expression of Notch by cells in the TEBs also suggests a role for Notch signaling in regulating proliferation during peripuberty. Stem cell units have been identified in TEBs and ducts of the mouse mammary gland (Kenney et al., 2001).

Deletion of *Notch* signaling in a mouse mammary stem cell (MaSC) enriched population, resulted in increased stem cell activity *in vivo*, as well as formation of aberrant end buds, indicating a role for endogenous Notch signaling in limiting MaSC expansion (Bouras et al., 2008).

Notch positive cells are evenly distributed in mammary ducts of post-pubertal mice. Inhibition of Notch signaling in both basal and luminal cells of the postnatal mouse mammary gland can lead to an imbalance in the proportion of basal and luminal cells resulting in basal cell expansion (Buono et al., 2006). This suggests that Notch can regulate epithelial cell fate and may impact stem cell activity. Notch signaling also targets luminal progenitor cells for expansion, leading to hyperplasia and tumor development (Bouras et al., 2008). These data indicate that signaling by one or more members of the Notch family of receptors promotes luminal cell fate commitment. Mammary alveolar units originate from alveolar luminal epithelial cells that line the ducts. Targeted disruption of Notch expression or Notch signaling in the mouse mammary gland during pregnancy has also revealed that Notch signaling regulates alveolar cell maintenance (Buono et al., 2006; Raafat et al., 2009). Interestingly, the mammary gland appears grossly normal in *Notch3* and *Notch4* knockout mice, suggesting that *Notch1*, *Notch2* or multiple *Notch* genes function redundantly to control cell fate specification, proliferation and differentiation in this tissue.

In the normal mammary gland, the percentage of Notch1 and Notch3 expressing cells was lowest during early pregnancy and lactation (Figure 6B). It is interesting, therefore, that transgenic mice overexpressing activated *Notch1* or *Notch3* were unable to feed their pups because of impaired ductal and lobulo-alveolar mammary gland development. In addition the β -casein gene promoter was repressed in these mice (Hu et al., 2006). Thus it is possible

that the reduced number of cells expressing Notch1 and Notch3 in the mammary glands of pregnant and lactating mice is important for normal lobular alveolar development and lactation. Low expression levels and positive cell numbers of Notch4 in the mammary gland during development, suggest that the Notch4 levels are very low per cell. Like *Notch1* and *Notch3* transgenic mice, Notch4 transgenic mice expressing the intracellular domain of Notch4 were also incapable of lactation due a block in mammary lobular-alveolar development. This suggests that in addition to the number of cells expressing a particular activated Notch receptor, expression levels per cell is also important in determining the consequence of Notch signaling. Consistent with this hypothesis is the observation that in *Drosophila* severity of a Notch phenotype is influenced by the level of Notch ICD in the nucleus (Artavanis-Tsakonas et al, 1999). Of the ligands, levels of *Jagged1* and *Dll3* mRNA were highest throughout mammary development. *DLL3* has been found to play a Notch-inhibitory role (Ladi et al., 2005). *Jagged1* mRNA levels decreased markedly during late pregnancy after reaching peak expression in early pregnancy. The other ligands were expressed at lower levels throughout postnatal mammary gland development. In one study of human mammary tissue, using mRNA *in situ* hybridization, *Jagged1* expression was detected in myoepithelium and high levels of *Notch3* mRNA in adjacent luminal cells (Reedijk et al, 2005). In another study, *Dll4*, *Jagged1*, *Jagged2*, *Notch1* and *Notch3* mRNAs were detected by RT-PCR in tissue from reduction mammoplasty (expression of *Dll1*, *Dll3*, *Notch2* and *Notch4* mRNAs could not be detected) (Stylianou et al, 2006). In this study, *Notch3* mRNA levels were higher than those for *Notch1*, while *Jagged1* mRNA levels were higher than *Jagged2*, which is consistent with our data in the developing mouse mammary gland. This similarity is likely due to a conservation of *Notch* gene function during mammary gland development.

Complete development of the mammary gland takes place after puberty, it depends on hormones and is closely regulated by the interaction of mammary epithelium with its stroma (Parmar and Cunha, 2004; Wiseman and Werb, 2002). Lower levels of *Notch* receptor and ligand mRNAs can also be detected in epithelium-divested (cleared) mammary fat pads (data not shown). Mammary gland development is highly dependent on ovarian hormonal stimuli. Mammary ductal elongation is directed by estrogen as well as by growth factors such as insulin-like growth factor-I and epidermal growth factor (Fendrick et al., 1998). The requirement for estrogen is supported by the absence of ductal elongation in estrogen receptor knockout mice (Bocchinfuso and Korach., 1997). Also, ovariectomy of mice at 2 months of age, retards mammary ductal and lobular alveolar development as compared to non-ovariectomized control mice (Nandi, 1958). Since Notch signaling is required for mammary lobular alveolar development (Buono et al., 2006), we tested whether ovariectomy would affect expression of *Notch* receptors and ligands. Ovariectomy positively affected levels of *Notch2*, *Dll1* and *Dll4* mRNA and negatively affected levels of *Notch3*, *Dll3* and *Jagged1* mRNAs. *Notch1*, *Notch4* and *Jagged2* mRNA levels were relatively unaffected by ovariectomy. The decrease in *Notch3* and increase in *Notch2* in the OVX mice mammary glands is notable but *Notch 3* levels are still the highest among the Notch receptors. However, the changes in ligand expression in the OVX mice do change the balances significantly compared to the intact mice. A primary consequence of ovariectomy is a loss of estrogen production, a known positive, or in some cases negative, regulator of gene transcription. Based on the effects of ovariectomy on transcription of some *Notch* receptor and ligand genes, we examined promoter regions for the presence of estrogen responsive elements (ERE). Although the EREs that were detected in *Notch* receptor and ligand genes were imperfect as compared to the perfect ERE palindrome (5'-GGTCANNNTGACC-3') (Lin et al., 2004), there is strong evidence that EREs can function in more loosely structured sequence motifs (Klein-Hitpass et al., 1986). For example, half-ERE palindromes (TGACC), that are more than 100 bp apart, can act synergistically to confer sensitivity to estrogen induction, either to a proximal promoter or to heterologous

promoters (Kato et al., 1992). Even a single, non-canonical ERE is capable of conferring transcriptional regulation by estrogen (Berry et al., 1989). Also, duplication of ERE half-palindromes in *Notch1*, *Notch2*, *Notch3*, *Dll3*, *Dll4* and *Jagged2* promoters provide a structural basis for estrogenic regulation of these genes. Down regulation of *Notch2*, *Dll1* and *Dll4* in the intact mammary gland compared to mammary glands from ovariectomized mice suggests that certain regulatory sequences and/or chromatin structures are playing a role in regulation of these genes (Muller et al., 2009, Strom et al., 2000). In addition estrogen receptor binding can lead to recruitment of coactivators or co-repressors (Heldring et al., 2007). In this regard, about half of ERE regulated genes are down regulated by estrogen (Carroll et al., 2006; Lin et al., 2007). However, induction of *Notch* receptor or ligand gene expression in mammary glands of ovariectomized mice may not translate into induction of *Notch* signaling, since in estrogen receptor positive breast cancer cell lines, estradiol induced *Notch1* expression but inhibited *Notch* signaling (Rizzo et al., 2008). Long and short-term ovariectomy affect both estrogens and progesterone and their receptors (Raafat et al., 1999). Currently we are investigating the relationship to progesterone receptor and its signaling pathways.

Expression levels of mRNA for multiple *Hes* and *Hey* *Notch* target genes were found to increase as the mammary gland develops from 5 weeks in the nulliparous mouse to day 5 in pregnant mice. This reflects the same pattern observed in expression of *Notch* receptor mRNAs in the mammary gland. Conditional ablation of *Rbpj* in the mouse mammary gland causes a profound cell fate switch when it occurs in the early pregnant or pubescent gland (Buono et al., 2006). However, when ablation of *Rbpj* occurs at day 15 of pregnancy, mammary gland development occurs normally (Buono et al., 2006, Raafat et al 2009). This is consistent with there being a window of time in which canonical *Notch* signaling can affect lobular alveolar development. The *Hes* and *Hey* gene families are the best-characterized canonical *Notch/Rbpj* target genes. Activation of *Notch* signaling up regulates *Hes* and *Hey* transcription (Iso et al., 2003; Raafat et al., 2009). In addition, *Hes* and *Hey* promoters contain *Rbpj* binding sites suggesting direct induction by *Notch* ICD/*RBPJ* complexes (Fischer and Gessler, 2003). Dimers of *Hes* and/or *Hey* proteins repress transcription of a variety of genes by interacting with transcriptional co-repressors or sequestering transcriptional activators (Iso et al., 2003, Shi and Harris, 2006). *Hes1* and *Hes5* double deficient mice phenocopy the neurogenic characteristics of *Notch1* null mice (Kageyama and Ohtsuka, 1999), whereas *Hey1* and *Hey2* double deficient mice replicate the angiogenic phenotype observed in the *Notch1* deficient mice (Fischer et al., 2004). Interestingly, the relative abundance of *Hes1*, *Hes5*, *Hey1* and *Hey2* mRNA levels varied over three orders of magnitude in the developing mammary gland, consistent with a complex pattern of expression and function of *Notch* receptors and their ligands. Adding complexity to this situation are reports of *Notch*-independent regulation of *Hes/Hey* gene transcription (Leimeister et al., 2000; Zavadil et al., 2004; Katoh and Katoh, 2007, and Doetzelhofer et al., 2009). In this regard, Doetzelhofer et al., 2009 have shown the *Fgf3* can upregulate *Hey2* independent of *Notch* signaling.

One confounding problem affecting interpretation of *Notch* receptor and ligand mRNA expression is our inability to evaluate the impact of *Notch* signaling, or the lack of it, from the epithelium to the surrounding stroma, and how this might affect the level of *Notch* and ligand gene expression in the cleared fat pad. The functional significance of our *Notch* receptor, ligand and target gene expression data await development of mouse strains wherein individual or combinations of *Notch* receptor and ligand genes are conditionally ablated, or in which expression of a reporter gene is put under transcriptional control of a particular *Notch* gene or *Notch* ligand gene.

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Figure 1. Validation of normalized mammary qRT-PCR data

Total RNA was extracted from two separate pools (from 5 mice each) of the number 4 inguinal mammary glands. Mammary tissue was collected from different mammary developmental stages and RNA prepared from these tissues was used in qRT-PCR as described in Materials and Methods. Normalization of *Keratin 8* (*Krt8*) cDNA copy numbers to *Gapdh* shows a gradual increase in *Krt8* copy numbers as the mammary gland develops and reaches maximum copy number on the first day of lactation, after which it declines during involution. The change in ratio of *Krt8/Gapdh* copy numbers reflect changes in the mammary epithelial content during development and validates the use of *Gapdh* mRNA to normalize mammary qRT-PCR data. Each bar represents the *Krt8* mRNA to *Gapdh* mRNA copy number ratio; error bars represent SEM.

Figure 2. Relative levels of Notch receptor mRNAs in the mammary gland

Total RNA was extracted from two separate pools (from 5 mice each) of the number 4 inguinal mammary glands. Mammary tissue was collected from different mammary developmental stages and RNA prepared from these tissues was used in qRT-PCR as described in Materials and Methods. Expression of *Notch1*, *Notch2*, *Notch3* and *Notch4* mRNA was measured and normalized to expression of the housekeeping gene *Gapdh*. All *Notch* receptor mRNAs were expressed in the mammary gland. *Notch3* mRNA showed the highest level of expression in all developmental stages (note break in the Y-axis), followed by *Notch1* and -2. *Notch4* was the least abundant mRNA in the mammary gland. *Notch3* mRNA expression peaked during late pregnancy. Each bar represents the ratio of mRNA copy number for specific *Notch* genes to *Gapdh* mRNA; error bars represent SEM.

Figure 3. Relative levels of Notch ligand mRNAs in the mammary gland

Total RNA was extracted from two separate pools (from 5 mice each) of the number 4 inguinal mammary glands. Mammary tissue was collected from different mammary developmental stages and RNA prepared from these tissues was used in qRT-PCR as described in Materials and Methods. Notch ligands were expressed in all developmental stages of the mouse mammary gland. Among the ligands *Jagged1* showed the highest levels of mRNA. Its expression peaked at day 5 of pregnancy. At this stage, *Jagged1* expression was 3X higher than *Delta-like 3* (*Dll3*) and at least 12X higher than the other ligands. *Jagged1* mRNA levels were always higher than *Jagged2*. *Dll1* and *Dll4* mRNAs were barely detectable. Each bar represent the ratio of specific mRNA copy number normalized to *Gapdh* mRNA levels; error bars represent SEM.

Figure 4. Relative levels of *Notch* target gene mRNAs in the mouse mammary gland
Total RNA was extracted from two separate pools (from 5 mice each) of the number 4 inguinal mammary glands. Mammary tissue was collected from different mammary developmental stages and RNA prepared from these tissues was used in qRT-PCR as described in Materials and Methods. *Rbpj* and all of tested *Notch* target genes were expressed in the mammary gland. Expression levels of *Hey2* mRNA were highest (note the break in the Y-axis), followed by *Hes1* and *Hey1* mRNA, respectively, while *Hes5* mRNA levels were the least abundant at all tested developmental stages. Each bar represents the ratio of specific mRNA copy number normalized to *Gapdh* mRNA levels; error bars represent SEM.

Figure 5A

Figure 5B

Figure 5C

Figure 5. Notch receptors antibody specificity

(A) *Notch1* and *Notch2* HC11 null cells. HC11 cells were transfected with *Notch1*-shRNA (a) or *Notch2*-shRNA (b) or control (Crl) expression vectors. Each bar represent the ratio of specific mRNA copy number normalized to *Gapdh* mRNA levels. qRT-PCR analysis showed that levels of *Notch1* and *Notch2* mRNA were much lower in the HC11-*Notch1* shRNA and HC11-*Notch2* shRNA cells, respectively than in HC11 or HC11-Crl cells. **(B) *Notch1* and *Notch2* antibody specificity.** HC11 cells (a and d) were transfected with control-shRNA plasmid (b and e) and *Notch1*-shRNA plasmid (c) or *Notch2*-shRNA plasmid (f) and treated with Notch1 antibody (a, d and c) or Notch2 antibody (d, e and f). Green fluorescence staining was observed in HC11 cells (a and d) and in HC11 transfected with control shRNA (b and e). No or very low staining was observed with Cell Signaling (4147) Notch1 (c) and ab-cam (ab52302) Notch2 (f) antibody in the HC11-*Notch1*-shRNA and HC11-*Notch2*-shRNA cells respectively. The insets show the DAPI staining of cells. **(C) *Notch3* and *Notch4* antibody specificity.** Representative sections from 10-wk-old virgin FVB/N (a); or 10 wk old Notch3-Lac-Z knock-in (b) mouse mammary gland were treated with anti-Notch3 antibody (a and b) as described in Materials and Methods. Brown-stained Notch3-positive nuclei are indicated by arrows and Notch3-negative cells by arrowheads. No staining was detected with the anti-Notch3 antibody (abcam-ab23426) in Notch3-Lac-Z-Knock-in mammary gland (b), whereas Notch3-positive nuclear stained cells were detected in mammary glands from wild-type 10-wk-old virgin mice (a), confirming specificity of this antibody. In addition, sections of mammary tissue from a 10 wk old female FVB mice (c) and Notch4-knockout mammary gland tissue (d) were treated with anti-Notch4 antibody. No staining was detected with the anti-Notch4 antibody (Millipore, 07-189) in Notch4-knockout mammary gland (d), whereas Notch4-positive nuclear stained cells (arrows) were detected in mammary glands of 10-wk old mice, confirming the specificity of this antibody. Magnification 63X.

Figure 6A

5 WK

10 wk

Notch1

Notch2

Notch3

Notch4

9 D prg.

Notch1

Notch2

Notch3

Notch4

1 D Lac.

Notch1

Notch2

Notch3

Notch4

Figure 6. Localization and quantitation of Notch receptors

(A) Immunoperoxidase localization of Notch receptors and Hey2 at different stages of mouse mammary gland development. Representative sections from IHC analysis of Notch receptors and Hey2 in FVB/N mammary glands during development: 5 week old nulliparous (5 wk); 10 week old nulliparous; (10 wk), 9 day pregnant; (9 D preg.) and one day lactation (1 D Lac.). The 5-week mammary gland shows strong nuclear staining of Notch3 and Hey2 in cap cells (arrowheads) of the end buds. Notch1, -2 and -3 and Hey 2 showed body cells (arrows) staining. Notch4 and Notch2 showed weak staining in the cap cells (arrowheads) and body cells (arrowheads). The 10 wk old mammary gland shows luminal staining (arrows) for Notch1, -2, -3 and Hey2, and basal staining (arrowheads) for Notch2, -3 and Hey2. The staining was cytoplasmic for Notch2 (open arrow) and mainly in the myoepithelial cells for Notch4 (arrowheads). The mammary gland from early pregnant mice shows nuclear (arrowheads) and cytoplasmic (arrows) staining for all the receptors and Hey2, with Notch4 showing the least number of positive cells. Notch1, 2, 3 and Hey 2 shows nuclear staining in the lactating mammary gland (arrowhead), while Notch4 staining was absent in this developmental stage. Magnification 63X. **(B) Quantitation of Notch receptors at different stages of mammary gland development.** Immunoperoxidase localization of Notch receptors was carried out using anti-Notch antibodies on mammary tissue sections from 5-, 10-wk-old virgin, 9 days pregnant and 1-day lactating (Lac.) mice as described in Materials and Methods. The values represent the mean $\pm$ SEM from three to five mice per group with a minimum of 1000 cells/mouse analyzed. The percentage of Notch1 and Notch4-positive cells remained the same in the tested developmental stages. Notch2 and Notch3 showed opposite patterns of staining. Notch2 levels peaked during lactation, whereas Notch3 levels decreased significantly during the same developmental stage.

Figure 7.

A: Relative levels of *Notch* receptors mRNAs in intact and Ovx mouse mammary gland. Total RNA was extracted from the number 4 inguinal mammary gland of 5 intact and ovariectomized mice. Total RNA was used to prepare cDNA that was subjected to qRT-PCR analysis, as described in the Materials and Methods. All *Notch* genes were expressed in mammary glands of intact and ovariectomized mice. *Notch* receptor expression patterns were the same in intact and ovariectomized mice. *Notch3* mRNA expression levels were the highest. *Notch2* was the next most abundant mRNA, while *Notch1* and *Notch4* mRNA were barely detectable. Each bar represents the ratio of mRNA copy number for the specific gene to *Gapdh* mRNA; error bars represent SEM. **Figure 7B: Effects of Ovx on *Notch2* and *Notch3*.** Protein extracts were prepared from the number 4 inguinal mammary gland of 3 intact and ovariectomized mice and were subjected to western blot analysis. *Notch2* protein levels increase (30%) in ovariectomized mice while *Notch3* levels decreased (25%) in ovariectomized mice. **Figure 7C: Relative levels of *Notch* ligands mRNAs in intact and OVX mouse mammary gland.** Total RNA was extracted from the number 4 inguinal complete mammary gland of 5 intact and ovariectomized mice. Total RNA was used to prepare cDNA that was subjected to qRT-PCR analysis, as described in Materials and Methods. All *Notch* ligand mRNAs were expressed in mammary glands of intact and ovariectomized mice, although different levels were noted for each. Levels of *Dll1* mRNA were the highest followed by *Dll4* and *Jagged1*, while *Jagged2* and *Dll3* mRNA were the least abundant in mammary glands from ovariectomized mice. Levels of *Dll3* and *Jagged1* are lower in the mammary gland of ovariectomized mice compared to intact mice. Each bar represent the ratio of mRNA copy number for a specific gene normalized to *Gapdh* mRNA; error bars represent SEM.

Analysis of estrogen response (GGTCAN³TGACC) element in mouse Notch receptors and their ligands

Table 1

Name	Accession #	seq	Promoter	Intron1
Notch1	NC-000068.6	GGTCA TGACC	-4033 -4985	425 1511
Notch2	NC_000069.5	GGTCA TGACC	-3284 -1752	3774 1525
Notch3	NC_000083.5	GGTCA TGACC	-3979 -3942	1223 1281
Notch4	NC_000083.5	GGTCA TGACC	-4253 -1901	45 none
Dll1	NC_000083.5	GGTCA TGACC	-4366 -4353	None None
Dll3	NC_000073.5	GGTCA TGACC	-3719 -3392	None None
Dll4	NC_000068.6	GGTCA TGACC	-2812 -4060	96 71
Jag1	NC_000068.6	GGTCA TGACC	None None	None 120
Jag2	NC_000078.5	GGTCA TGACC	-4513 -5216	None None