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Tumorigenesis and Neoplastic Progression

Overexpression of Activated Murine Notch1 and Notch3 in Transgenic Mice Blocks Mammary Gland Development and Induces Mammary Tumors

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The mouse mammary tumor virus (MMTV) provirus was found to target the *Notch1* gene, producing insertional mutations in mammary tumors of MMTV/neu transgenic (Tg) mice. In these mammary tumors, the *Notch1* gene is truncated upstream of the transmembrane domain, and the resulting Notch1 intracellular domain (Notch1^{intra}), deleted of most extracellular sequences, is overexpressed. Although Notch1^{intra} transforms mammary epithelial cells *in vitro*, its role in mammary gland tumor formation *in vivo* was not studied. Therefore, we generated MMTV/Notch1^{intra} Tg mice that overexpress murine Notch1^{intra} in the mammary glands. We observed that MMTV/Notch1^{intra} Tg females were unable to feed their pups because of impaired ductal and lobuloalveolar mammary gland development. This was associated with decreased proliferation of ductal and alveolar epithelial cells during rapid expansion at puberty and in early pregnancy, as well as decreased production of β-casein. Notch1^{intra} repressed expression of the β-casein gene promoter, as assessed *in vitro* with a β-casein/luciferase reporter construct. The MMTV/Notch1^{intra} Tg females developed mammary gland tumors, confirming the oncogenic potential of Notch1^{intra} *in vivo*. Furthermore, MMTV/Notch3^{intra} Tg mice exhibited a very similar phenotype. Thus, these Tg mice represent novel models for studying the role of Notch1 or Notch3 in the development and transformation of the mammary gland. *(Am J Pathol 2006, 168:973–990; DOI: 10.2353/ajpath.2006.050416)*

The mammalian *Notch* genes are the homologues of the *Drosophila Notch*, *Caenorhabditis elegans lin-12* and *glp-1* and *Xenopus Xotch* genes. The mammalian *Notch1* belongs to the family of *Notch* genes (*Notch1* to *Notch4*) that play critical roles in cell fate determination in many developmental systems.[1,2] *Notch1* encodes a large 330-kd transmembrane protein. Its extracellular domain harbors 36 EGF-repeats, 3 Notch-Lin12 repeats, and two conserved cysteines (C^{1652} and C^{1685}) close to the transmembrane domain. The intracellular Notch1 domain (Notch1^{intra}) contains the domain I (RAM 23), six CDC10/ankyrin repeats, and OPA and PEST sequences at the C-terminus. Truncated versions of Notch1 protein, containing only the intracellular part of the receptor (Notch1^{intra}), behave as constitutively active gain-of-function mutants in *Drosophila*,[3,4] in *C. elegans*,[5] in *Xenopus* embryos,[6,7] and in mammalian cells.[8–10] The *Notch3* gene has been detected only in mammals. Its structure is similar to that of *Notch1*, although its intracellular domain is shorter and lacks the OPA region.[11] In addition, several deletions, insertions, and substitutions of amino acid residues distinguish Notch1^{intra} from Notch3^{intra}. Moreover, its intracellular domain is a poor activator of HES-1 and HES-5, in contrast to that of Notch1.[12] The Notch4 protein intracellular domain is significantly shorter than that of Notch1 at the C-terminus.[13] A number of deletions, insertions, and substitutions of amino acid residues also differ between the Notch1^{intra} and Notch4^{intra} domains.

To date, two members of the murine *Notch* gene family, *Notch1* and *Notch4*, have been implicated in mammary gland development and neoplasia. The *Notch4/int3* gene was initially identified as a common

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provirus integration site in mammary tumors of mice infected with mouse mammary tumor virus (MMTV).[14] Itwas also found to be truncated, through a similar mechanism, by an intracisternal type A DNA provirus in a mouse mammary tumor.[15] Both MMTV and intracisternal type A provirus integrations generated truncated Notch4^{intra} domain that was overexpressed in mammary tumors. Expression of this same *Notch4^{intra}* in transgenic (Tg) mice under the regulatory sequences of the MMTV LTR also led to mammary tumor formation and impaired mammary ductal and secretory lobule development.[16,17] The same gene expressed in Tg mice under the regulation of the whey acidic protein gene promoter (which is active only in the secretory mammary epithelial cells), induced mammary tumors and inhibited growth and differentiation of the secretory lobules without affecting ductal growth.[18] In mouse mammary epithelial cells *in vitro*, activated *Notch4^{intra}* had a similar effect as *in vivo* and induced transformation of HC11 cells[19] and inhibited the branching morphogenesis induced by HGF and transforming growth factor-β2 in TAC-2 cells.[20] Together, these experiments indicated that the activated form of *Notch4* (*Notch4^{intra}*) has the capacity to transform mammary epithelial cells and to block the growth of epithelial ductal cells and the secretory lobules of the mammary glands.

Similarly, our group also observed MMTV provirus insertional mutagenesis of another *Notch* family member, *Notch1*, in MMTV/neu Tg mice infected with MMTV.[21] Truncation of the *Notch1* gene by the MMTV provirus occurred in a region just upstream of the exons coding for the transmembrane domain and resulted in the activation of the remaining truncated intracellular *Notch1* domain. The *Notch1^{intra}* domain was found to have transforming ability in HC11 mammary epithelial cells *in vitro*. This was the first indication that the *Notch1* gene could play an important role in mammary gland tumorigenesis. However, more direct evidence of the oncogenic potential of the truncated murine *Notch1^{intra}* sequences was lacking. In addition, it remains unknown whether the significant amino acid sequence differences between the intracellular domain of the members of the murine Notch family would affect their oncogenic potential. However, a recent report showed that Tg mice expressing human *Notch1^{intra}* in the mammary glands developed pregnancy/lactation-dependent mammary tumors.[22]

We have now directly assessed the oncogenic potential of murine *Notch1^{intra}* and *Notch3^{intra}* *in vivo* by generating Tg mice expressing murine *Notch1^{intra}* or *Notch3^{intra}* in mammary epithelial cells under the regulation of the MMTV LTR. These regulatory elements can direct expression of the transgene in mammary glands, at all of the different developmental stages as well as in male reproductive organs.[23–31] Our results show that murine *Notch1^{intra}* and *Notch3^{intra}*, in contrast to human *Notch1^{intra}*, affect the development and the cellular differentiation of the mammary glands, as well as induce tumors of the mammary glands, which are not pregnancy/lactation-dependent.

Materials and Methods

Generation of Tg Mice

The MMTV/Notch1^{intra} transgene was constructed by ligating the *Notch1* mutant A sequences (2.8 kbp)[21] to the MMTV LTR from the MMTV*neu*T construct (2.3 kbp)[32] through the *Notch1^{intra} Ssp*I restriction site, thus spanning nucleotides 5187 to 8064 of *Notch1*. The MMTV/Notch3^{intra} transgene was constructed by ligating the polymerase chain reaction-amplified *Notch3^{intra}* DNA fragment spanning nucleotides 5008 to 7189. One-cell (C3H $\times$ C56BL/6) F2 embryos were collected, microinjected, and transferred into pseudopregnant CD1 females as described before.[32] The resulting founder mice were analyzed for the presence of the transgene by Southern analysis of tail DNA with a *Notch1* (probe K) or SV40-specific probe.[21] *Notch1* probe K covers the entire intracellular region, from the *Bam*HI (nucleotide 4204) to *Eco*RI (nucleotide 8064). Tg mice were bred on CD1 background for at least 6 generations and for 10 to 15 generations for most experiments presented. The phenotypes described here have been observed throughout a period of 4 and 3 years, respectively, for *Notch1^{intra}* and *Notch3^{intra}* Tg mice.

Tissue Analysis

For mammary gland development study, groups of three Tg and three non-Tg mice were sacrificed at the age of 3, 5, 7, and 12 weeks, at days 6.5 and 18.5 of pregnancy, and at day 4 postnatally (involution). Mammary glands 1, 2, 3, 6, 7, and 8 were removed and frozen with liquid nitrogen for RNA extraction. Gland 9 (right inguinal) was dissected carefully, spread on a clean slide, and processed for whole mounts. Mice were then perfused with 4% paraformaldehyde and gland 4 (left inguinal), and a piece of intestine was postfixed by 1 hour immersion in fixative disection and embedded in paraffin using a Shandon tissue infiltrator (Hypercenter XP, Pittsburgh, PA), sectioned at 5 μm, and stained with hematoxylin and eosin (H&E), as described previously[33] or processed for terminal dUTP nick-end labeling (TUNEL) or immunohistochemistry (IHC). For mammary tumorigenesis studies, tumors were excised, immersed in paraformaldehyde, embedded in paraffin blocks, and sections (5 μm) were obtained for further staining or *in situ* hybridization.

Whole Mount Preparations of Mammary Glands

Mammary glands were processed as described,[34] according to the protocol in Biology of the Mammary Gland (*http://mammary.nih.gov*). The glands were spread on glass slides, fixed in Carnoy's fixative (six parts 100% ethanol, three parts chloroform, one part glacial acetic acid) for 4 hours at room temperature, washed in 70% ethanol for 15 minutes, changes gradually to distilled water, rinsed in distilled water for 5 minutes, and stained overnight in carmine alum [1 g of carmine (C1022; Sigma, Toronto, ON, Canada)] and 2.5 g of aluminum potassium

sulfate (A7167, Sigma) dissolved in 500 ml of H_2O. Tissues were dehydrated in an increasing ethanol series (75%, 95%, and 100% for 15 minutes each) followed by xylene (1 hour) and mounted with Permount (Fisher, Ottawa, ON, Canada) and coverslip.

Immunohistochemistry (IHC)

IHC was performed essentially as described.[35,36] After dewaxing, sections were treated with 0.3% hydrogen peroxide in phosphate-buffered saline (PBS), then blocked with 1% gelatin in PBS for 3 minutes at room temperature before incubation with rabbit polyclonal antibody to β-casein (1:1600) for 1 hour at room temperature. This antibody was kindly provided by Dr. Ernst Reichmann (Ludwig Institute for Cancer Research, Lausanne, Switzerland). This was followed by treatment with Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA), as described by the manufacturer. Finally, the positive signal was visualized with 4,4-diaminobenzidine tetrahydrochloride (DAB).

Cell Proliferation

Bromodeoxyuridine (BrdU) (Sigma) was administered by intraperitoneal injection at 200 μg/g mouse weight. Mice were sacrificed by anesthesia with Avertin 90 minutes after BrdU administration and rinsed by intracardiac PBS perfusion and fixed with 4% paraformaldehyde, as described.[33] BrdU-positive cells on tissue sections were detected with the BrdU *in Situ* Detection Kit II (BD Biosciences, Toronto, ON, Canada). The technique was essentially that recommended by the manufacturer. Mammary gland tissue analyzed was included in paraffin blocks together with several other tissues including small intestine and inguinal lymph nodes. These internal positive controls (intestinal epithelial cells in deep crypts and clusters of lymph node follicular cells were BrdU-positive) were checked before analysis and used to exclude a false BrdU signal.

TUNEL Assay

The detection of DNA fragmentation in cells undergoing apoptosis was made by using the TUNEL assay *in situ* on 5-μm paraffin sections from the same blocks used for BrdU incorporation. The technique was similar as before[33] except that Biotin 11-dUTP (Roche, Laval, QC, Canada) and streptavidin-horseradish peroxidase (DAKO, Carpinteria, CA) and DAB substrates were used. To exclude a false TUNEL signal, the internal positive controls (apical intestinal epithelial cells and clusters of lymph node follicular cells were TUNEL-positive) were checked also before analysis.

Northern Blot and Reverse Transcriptase-Polymerase Chain Reaction Analysis

Total RNAs were isolated from different tissues with the Trizol reagent (Invitrogen, Toronto, ON, Canada), and 15 μg was electrophoresed on 1% formaldehyde-agarose gels, transferred by the Northern blot procedure to Hybond-N membranes (Amersham Co., Baie d'Urfé, QC, Canada) and hybridized with ^{32}P-labeled probes as described previously.[21] The *Notch1* probe K was previously described.[21] The *Hes1* probe represents a 396-bp fragment at the 5′ end of the cDNA, starting at the first ATG (+1). The U5 LTR probe represents a 210-bp *Sac*I-*Bam*HI fragment of the MMTV LTR (Figure 1). Probes were prepared by the random primer method, as described previously.[21] Reverse transcriptase-polymerase chain reaction analysis was performed as described previously.[37]

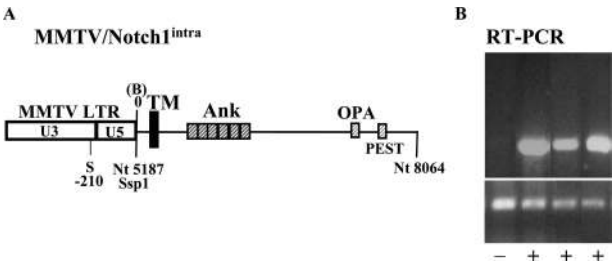


Figure 1. Structure of the MMTV/Notch1^{intra} transgene. **A:** The cDNA encoding the intracellular domain of murine *Notch1* was ligated to the MMTV LTR promoter (**open box**). The 2.8-kbp *Notch1^{intra}* cDNA includes the transmembrane domain (**black bar**), the six ankyrin (Ank) repeats (**strippled boxes**), and the OPA/PEST sequences (**striped bars**). S, *Sac*I; B, *Bam*HI. **B:** Reverse transcriptase-polymerase chain reaction analysis of transgene RNA expression in mammary glands of pregnant (18.5 days) Tg (+) and control non-Tg (−) mice (F47400).

Microscopic Analysis and in Situ *Hybridization*

Organs to be assessed were fixed with paraformaldehyde or periodate-polylysine-paraformaldehyde fixative, embedded in paraffin, and processed for *in situ* hybridization, as previously described.[21] The *Notch1^{intra}* riboprobe used represents a 279-nucleotide-long Bru36I fragment from the pMN7 construct.[37] This fragment covers the intracellular C-terminal region of *Notch1* from nucleotides 5357 to 5636. Tissues from non-Tg control animals hybridized with sense and anti-sense probes, as well as tissues from Tg mice hybridized with a sense probe, failed to exhibit any specific signal. For histopathological analysis, sections were stained with H&E.

Image Analysis

Quantification of mammary gland characteristics was performed on digital images of whole mount tissue, of H&E-stained sections and of TUNEL and IHC sections of inguinal mammary glands, by using Northern Eclipse 6.0 software (Empix Imaging, Mississauga, ON, Canada). The sections were scanned and color images were captured by using Zeiss Axiophot supported with Northern Eclipse. On whole mount tissue, penetration length nipple to front edge, diameter of primary and secondary branches, the number and diameter of terminal end buds (TEBs) were measured in virgin females of different ages. In addition, the length of tertiary branches and the density of alveoli from tertiary branches were counted in 12-week-old virgin females and during early (6.5 days) preg-

nancy. On H&E-stained sections, the percentage area occupied by fat, by alveoli, and by secretory vesicles was estimated in late (18.5 days) pregnancy and at 4 days of involution, by randomly choosing 10 images at ×10 and ×20 from each section. For evaluation of BrdU- and TUNEL-positive cells, the percentages of positive cells of the mammary epithelium were counted along with negative cells, by randomly choosing 10 images at ×40 from each section. At least 6000 cells were counted for each structure such as TEBs, ducts, and alveoli. For quantitation of β-casein production during pregnancy (18.5 days), nine images (×20) from each section were randomly taken from each inguinal mammary gland section with β-casein IHC staining visualized by DAB (without counterstaining). In total, 677 alveoli in Tg and 644 alveoli in non-Tg tissues were quantitated by measuring the dark brown area (μm^2) in each alveolus with Northern Eclipse software.

β-Casein/Luciferase Reporter Assays

The prolactin receptor (PRLR) and the β-casein-luciferase constructs (containing 2.8 kbp of sequences upstream of the rat *β-casein* gene) were given by Dr. J. M. Boutin, Hôtel-Dieu Hospital, Montréal, QC, Canada. The Notch1^{intra} construct has previously been described.[21] Notch3^{intra} was constructed similarly in pcDNA3 vector. The dominant-negative human RBP-Jκ construct[38] was provided by Dr. Aly Karsan, BC Cancer Research Centre, Vancouver, BC, Canada. Transfection was performed as described earlier.[39,40] Briefly, the human 293T cells were grown in Dulbecco's modified Eagle's medium (Life Technologies, Inc., Grand Island, NY) containing 10% fetal calf serum. Two days before transfection, cells were passaged at a density of 2.5×10^5/well, and transfected by using the calcium phosphate technique with a mixture of DNA constructs: the PRLR cDNA (0.2 μg), Notch1^{intra} or Notch3^{intra} (0.8 μg), β-galactosidase (0.005 or 0.5 μg), and β-casein reporter gene construct (0.5 μg). Transiently transfected 293T cells were induced with insulin (5 μg/ml), dexamethasone (1 μmol/L), and oPRL (5 μg/ml) for 48 hours. Cells were harvested and the β-galactosidase activity was measured for normalization of luciferase activity. All experiments were repeated a minimum of three times.

Statistical Analysis

All data from Tg and non-Tg groups were tabulated as means ± SEMs. These include the number and diameter (μm) of TEBs, the length of penetration (mm), the diameter (μm) of primary, secondary, and tertiary branches, the length (μm) of tertiary branches, the density (number/mm) of alveoli from tertiary branches, the area occupied by β-casein-positivity in each alveolus or fat area on surface area or vesicles (%) relative to that occupied by alveoli (%), the percentage of TUNEL- and BrdU-positive cells. Comparison between Tg and non-Tg groups was performed by using an impaired two-tailed Student's *t*-test. Differences were considered significant if P was <0.05.

Results

Construction and Phenotypes of the MMTV/Notch1^{intra} Tg Mice

The biological role of mutated truncated murine *Notch1^{intra}* in mammary gland development and tumorigenesis was assessed in Tg mice expressing this gene in mammary epithelial cells under the regulatory sequences of the MMTV LTR (MMTV/Notch1^{intra}) (Figure 1A). Four MMTV/Notch1^{intra} Tg founders (F) were generated (F47400, F39580, F45347, and F51256). With the exception of one female founder 39580 that had a sterile male as offspring, the remaining founders transmitted the transgene to their progeny in a Mendelian ratio. Southern blot analysis showed that the structure of the Tg was grossly intact (data not shown). Mice were bred as heterozygotes with CD1 mice, for at least 6 generations and for 10 to 15 generations for most studies. Mice from two of the four Tg lines (F45347, F51256) did not show any abnormal phenotype, expressed low levels of the transgene, and were not studied further (data not shown). Tg males from lines expressing the transgene (Figure 1B) were sterile (to be reported separately). Female Tg founder mice F47400 and F39580 were fertile and expressed the transgene (see below and data not shown). They were mated successfully and carried their pups to term, but were unable to feed them. The pups died within 48 to 72 hours with no visible milk in their stomach. In view of this failure of lactation, these Tg lines were thus maintained by foster nursing to non-Tg CD1 mothers.

Impaired Ductal Growth and Ductal Dysplasia in the MMTV/Notch1^{intra} Tg Mammary Glands

The defective milk production observed in MMTV/Notch1^{intra} Tg females was indicative of an impaired development of the mammary glands. We therefore first assessed the growth of the mammary gland ductal tree in these Tg mice. In normal mice, the ductal system does not develop until puberty (5 to 6 weeks). During puberty (5 to 8 weeks) a progressive outgrowth of the primary ducts with secondary and tertiary branching fills the subcutaneous fat pad. This process is driven by the growth of a highly proliferative cell mass, designated the TEBs, which eventually disappear in mature virgin animals (12 weeks), once arborization is completed.

Whole mount and histological analysis of the mammary glands of prepubescent Tg mice (3 weeks) showed that the ducts appear normal, as compared to those from control non-Tg littermates (data not shown), suggesting that overexpression of Notch1^{intra} did not affect the precursor cells giving rise to the primitive ductal tree. A similar whole mount analysis performed in Tg mice at puberty (5, 7, and 12 weeks) revealed no significant differences between Tg and non-Tg females. The struc-

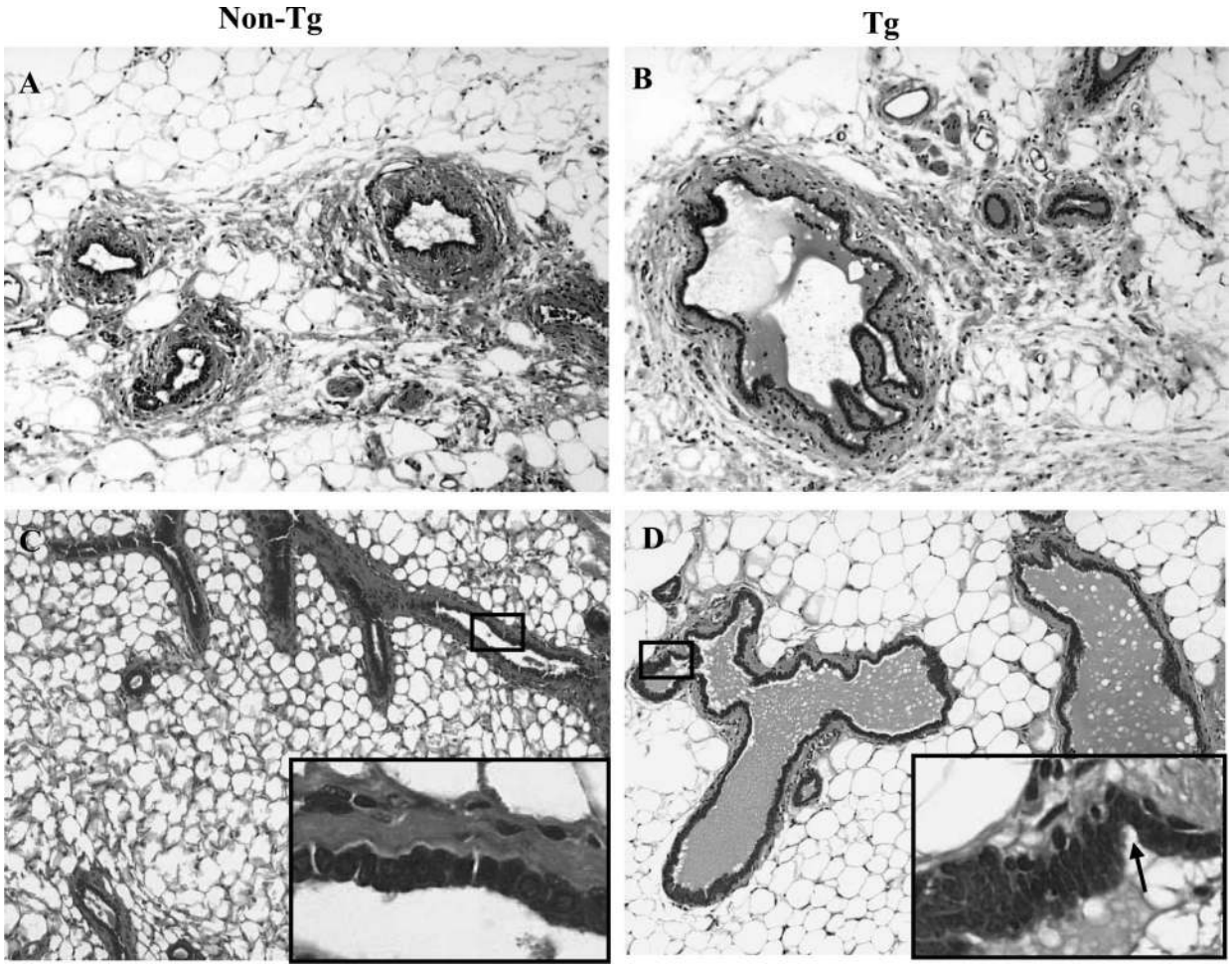


Figure 2. Histological evaluation of mammary glands from virgin MMTV/Notch1^{intra} Tg mice. Mammary glands from young (12 weeks) (**A**, **B**) and older (10 months old) (**C**, **D**) virgin non-Tg (**A**, **C**), and Tg (**B**, **D**) mice. Note the dysplasia and the hyperplastic zones of epithelial cells and the enlarged ducts in the mammary gland of an older Tg mouse. **Insets** (**C**, **D**) show the areas delineated by a **square** at higher magnification. Note at the **left** of the **arrow** in **D** a multilayer epithelium adjacent to apparently normal epithelium at the **right** of the **arrow**. Original magnifications: ×10; ×40 (**insets** in **C**, **D**).

tures evaluated include penetration length, diameter of primary and secondary branches, the number and diameter of TEBs, the length of tertiary branches, and density of alveoli from tertiary branches (data not shown). However, in some Tg mice, some ducts were dilated and show small foci of hyperplasia (Figure 2B). Older (6 to 12 months) virgin Tg mice were found to have enlarged primary ducts and reduced secondary and tertiary branches (data not shown). The paucity and enlargement of the Tg ducts in older Tg mice was confirmed on histological sections. Moreover, in these older Tg mice, dysplasia of the ducts was evident (Figure 2D).

During gestation, additional ductal branching occurred both in Tg and non-Tg mice. However, the ductal arborization in early (6.5 days) (Figure 3B) or late (18.5 days) (Figure 3F) pregnancy, including length of tertiary branches (Figure 3C) and density of alveoli from tertiary branches (Figure 3D) were much less pronounced in Tg than in non-Tg mice. In older pregnant Tg mice, the ducts became enlarged, distorted, and dysplasic, having two to four layers of epithelial ductal cells, as compared to a single layer in non-Tg mice (data not shown).

Finally, during the involution of the gland after the first pregnancy, a minority of Tg ducts remained larger and failed to regress as completely as those of non-Tg animals (Figure 4B). Histological observations confirmed these results (data not shown). This phenotype was even worse after a first pregnancy at older age (data not shown) and after the third pregnancy, in which very large Tg ducts were observed (Figure 4, C and F). In older Tg mammary tissues, a severe dysplasia, with larger and abnormally formed ducts, was observed and a severe hyperplasia of the ductal epithelial cells was apparent (data not shown). These results indicated that overexpression of murine Notch1^{intra} prevents a normal development of the mammary ducts and induced dysplasia and hyperplasia of the ductal tree, rendering it refractory to the involution process.

Impaired Alveolar Growth and Development in the MMTV/Notch1^{intra} Tg Mammary Glands

In normal mice, the lobules and their alveoli are formed at puberty and remain in a primitive form at puberty and in

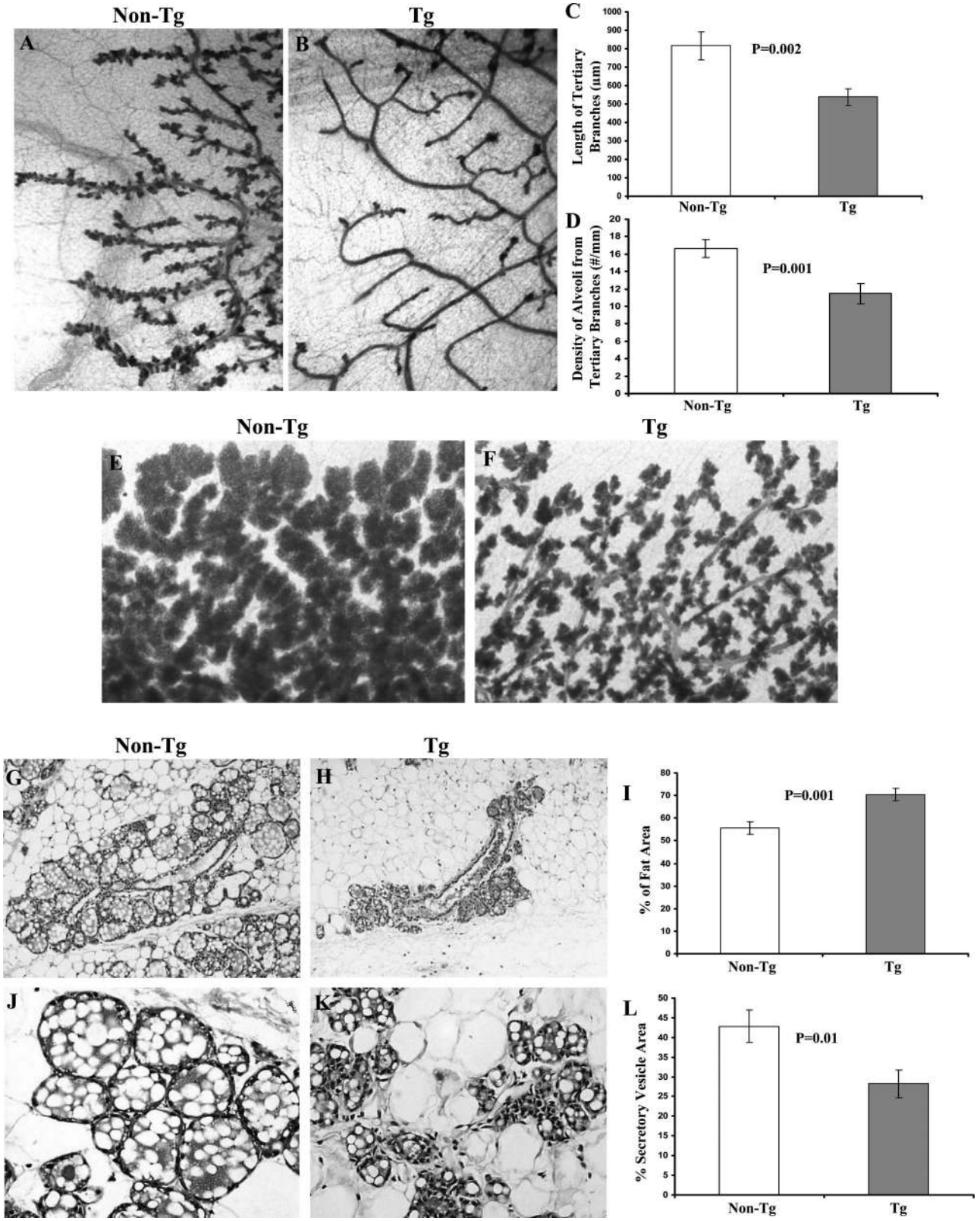


Figure 3. Whole mounts and histological analysis of mammary glands from pregnant MMTV/Notch1^{intra} Tg females. Inguinal mammary glands from Tg females and control non-Tg littermates (line 47400) were compared by whole mount (**A–F**) or histological (**G–L**) analysis. Mammary glands from early (6.5 days) (**A–D**) and late (18.5 days) (**E–L**) pregnancy. Length of tertiary branches (**C**), density of alveoli (number per mm) (**D**), and area occupied by fat (**I**) and by secretory vesicles (per alveoli) (**L**) were evaluated. Original magnifications: ×10 (**G**, **H**); ×40 (**J**, **K**).

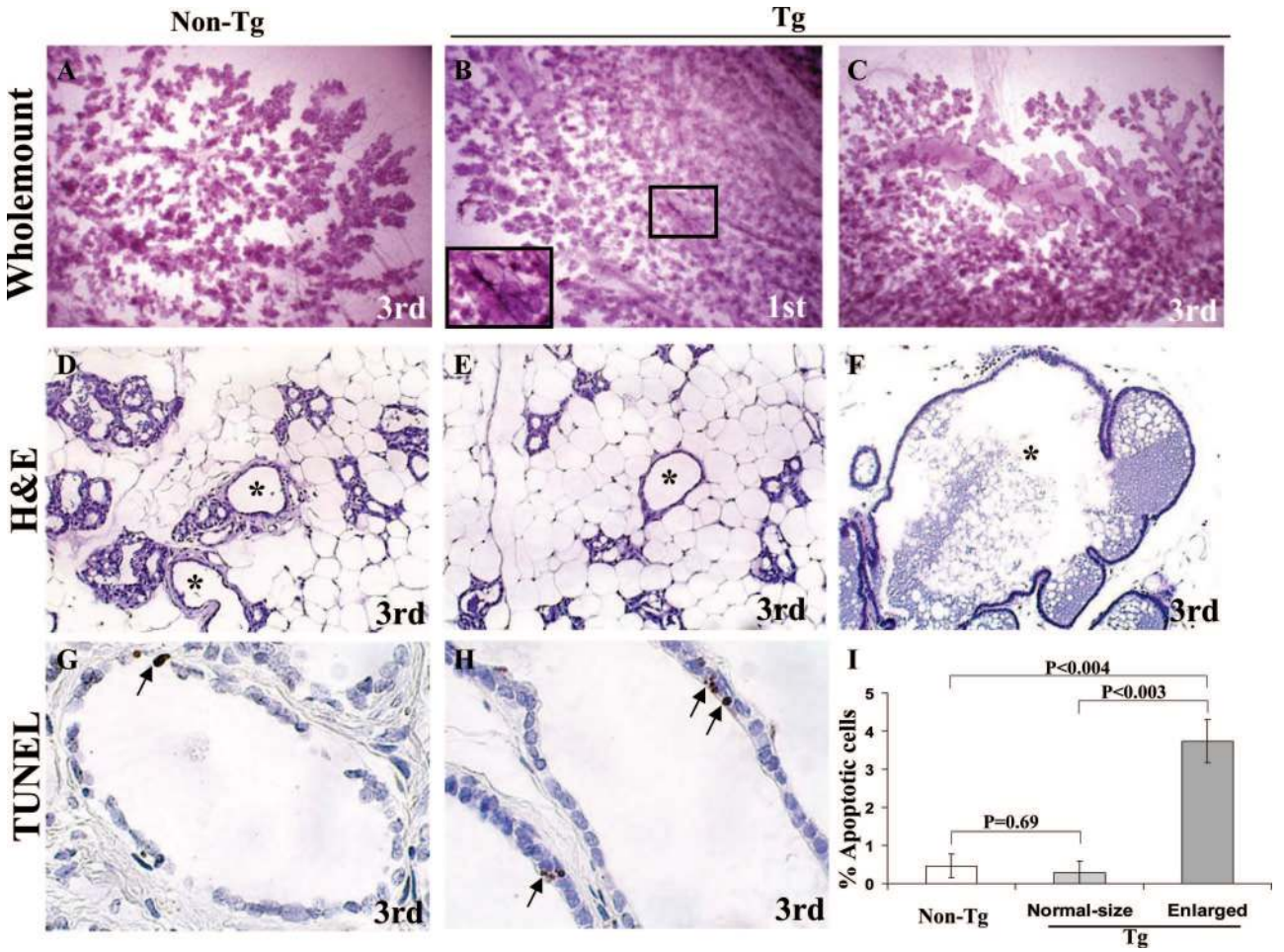


Figure 4. Whole mounts, histological, and TUNEL analysis of mammary ductal changes in MMTV/Notch1^{intra} Tg mice at 4-day involution. Inguinal mammary glands from female Tg (F47400) and their non-Tg littermates were compared by whole mount (**A–C**) and by histological (H&E staining) (**D–F**) and TUNEL (**G–I**) analyses. Mammary glands at 4-day involution after the first (**B**) or the third (**A**, **C**, **D–I**) pregnancy. Note that enlarged ducts were rarely observed in young Tg mice after the first pregnancy (**B**, *square*) but frequently after the third pregnancy (**C**, **F**, **H**). Enlarged Tg ducts are surrounded by less connective tissue (**F**, **H**) than non-Tg ones (**D**, **G**). **I:** TUNEL-positive epithelial cells in normal-size or enlarged Tg ducts as well as in non-Tg ducts were quantitated. Statistical analyses was performed with the Student's *t*-test. Symbols: **asterisks** in **D–F**: ductal lumen; **arrows** in **G** and **H**: TUNEL-positive cells. Original magnifications: ×10 (**D–F**); ×40 (**G**, **H**).

virgin mice. They start to grow significantly in early pregnancy and continue their growth and differentiation during pregnancy to form an epithelial rich glandular structure with mature alveoli.

Whole mount analysis showed that the lobules developed during pregnancy of Tg mice, but these lobules remained small and their numbers were severely reduced as compared to those of non-Tg littermates. This was observed both in early (6.5 days) (Figure 3B) and late (18.5 days) (Figure 3F) pregnant Tg females. In pregnant Tg mice, the lobulo-alveolar proliferation never resulted in the complete filling of the fat pad at parturition, as seen in the non-Tg mammary glands (Figure 3, A and B and E and F). On histological sections, underdeveloped small lobules with small alveoli could be observed (Figure 3, H and K). The Tg alveoli were also undifferentiated and their cells contained less fat droplets and a lower percentage area of secretory vesicles than in non-Tg mice (Figure 3, J–L). This resulted in a significant increase in interstitial fat area (Figure 3I). During involution, after weaning, after the first pregnancy the underdeveloped lobular/alveolar Tg structure regressed at a slower rate as compared to those of non-Tg mice, as evaluated in the percentage of fat area (Figure 3, G–I; regression of 35% in non-Tg versus 14% in Tg mice). Together, these results indicated that murine Notch1^{intra} overexpression in mammary glands not only affects ductal development, but also the lobulo-alveolar proliferation associated with pregnancy.

Notch1^{intra} Represses the Production of β-Casein in Vivo *and the Transcriptional Activity of the β-Casein Gene Promoter*

To determine whether this apparent lobulo-alveolar proliferation defect of the mammary glands of MMTV/Notch1^{intra} Tg mice was also reflected in the milk composition, the levels of β-casein, a major milk protein, were measured by IHC. Production was high during gestation of normal non-Tg mice as expected, but was much lower in Tg mammary glands (Figure 5, A and B). A similar decrease in β-casein protein levels was also documented in murine mammary

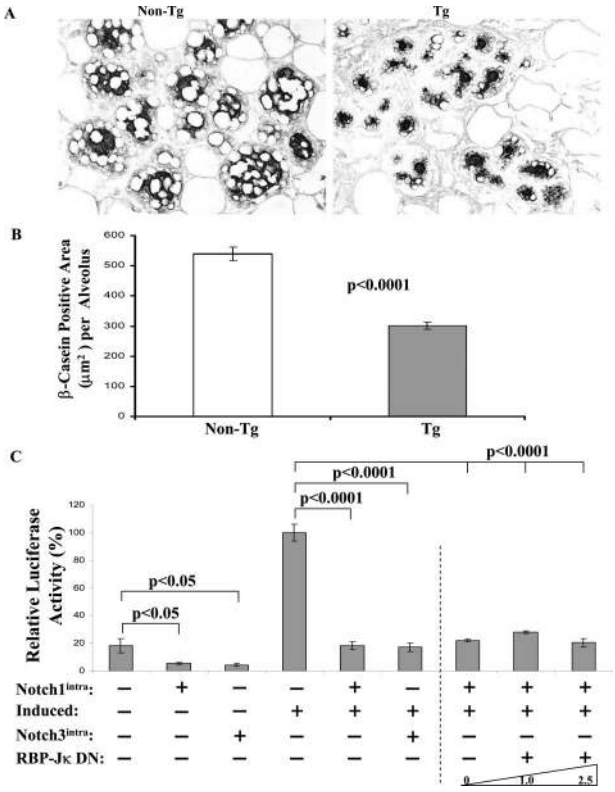


Figure 5. Expression of the milk β-casein protein in mammary glands of MMTV/Notch1^{intra} Tg mice. **A:** Mammary glands from pregnant (18 days) female mice were analyzed by IHC for expression of the milk β-casein protein, using rabbit anti-β-casein antibodies on tissue sections. **B:** Data were quantitated by image analysis ($P < 0.0001$). **C:** β-Casein/luciferase reporter assay in 293T cells in the presence (+) or absence (−) of Notch1^{intra}, Notch3^{intra}, or RBP-Jκ dominant-negative mutant (1.0 and 2.5 μg). The **dotted line** indicates that these experiments were done separately. Original magnifications, ×20.

epithelial HC11 cells overexpressing Notch1^{intra} relative to control HC11 cells (data not shown).

Notch1^{intra} has been shown to behave as a transcriptional factor.[41] To determine whether the repression of the *β-casein* gene expression by Notch1^{intra} was transcriptional, we used a luciferase reporter gene expressed under the regulation of the rat β-casein promoter region (2.8 kbp) (β-casein/luc). The analysis was performed in 293T cells co-transfected with the rat prolactin cDNA and β-casein/luc DNA constructs. Notch1^{intra} was able to repress the β-casein-dependent luciferase expression (Figure 5C), suggesting that the Notch1^{intra}-mediated repression occurs at the level of the *β-casein* gene promoter itself. However, although this promoter contains two CSL binding sites, the inhibitory action of Notch1^{intra} on this promoter was not relieved by expression of a dominant-negative mutant of RBP-Jκ/CBF1 (Figure 5C), suggesting that this repression is CBF1-independent. Moreover, Hes1 was not significantly enhanced in mammary glands from pregnant (18.5 days) Tg females (data not shown), suggesting that the development of the mammary phenotypes in MMTV/Notch1^{intra} Tg mice may be Hes1-independent.

Decreased Proliferation of Ductal and Alveolar Epithelial Cells of MMTV/Notch^{intra} Tg Mammary Glands during Rapid Expansion at Puberty and in Early Pregnancy

The dysplasia observed in the ductal epithelial cells suggested that murine Notch1^{intra} was affecting proliferation of these cells. To determine whether cells of the Tg mammary gland proliferated at a different rate than in non-Tg mice, proliferation was directly measured by counting the percentage of cells stained with anti-BrdU antibodies after a BrdU-labeling period *in vivo* (Figure 6, A–H). This analysis revealed that proliferation of ducts was significantly reduced in young (5 weeks), but not in older (12 weeks) Tg virgin females (Figure 6G), whereas the proliferation of TEBs in young (5 weeks) virgin Tg mice was not significantly affected (Figure 6G). During pregnancy, the proliferation of ducts was reduced early (6.5 days), but enhanced significantly later (18.5 days) (Figure 6, B and G). A similar analysis of alveoli revealed a significant decrease in incorporation of BrdU in Tg females during early pregnancy (Figure 6, D and G), but paradoxically an increase in late pregnancy (Figure 6G). During involution (4 days) after the first pregnancy, there was a trend for increased proliferation in Tg ducts or lobules as compared to non-Tg ones (Figure 6G and data not shown), but this did not reach a statistical significance, even after a second experiment (data not shown). However, at 4-day involution after the third pregnancy, proliferation of the epithelial cells of the enlarged Tg ducts (but not those of the normal-size Tg ducts) was significantly increased, as compared to those from the non-Tg ducts (Figure 6, E, F, and H). In contrast, proliferation of Tg alveolar epithelial cells remained unchanged relative to that of non-Tg mice at this stage (data not shown).

These data indicate that murine Notch1^{intra} impairs proliferation of ducts and alveoli, at a time they expanded significantly (early virgin for ducts and early pregnancy for both ducts and alveoli). Subsequently, as pregnancy progresses, Notch1^{intra} appears to have an opposite effect, stimulating proliferation of both tubular and alveolar cells, consistent with its oncogenic potential (see below).

Apoptosis of Ductal and Alveolar Epithelial Cells of Mammary Glands Remains Unchanged in MMTV/Notch1^{intra} Tg Mice

In normal mice, apoptosis is mainly responsible for the regression of the ductal tree and of the lobulo-alveolar structure that occurs during involution. The fact that, during involution, the Tg ducts remained large and dysplastic and that Tg alveoli poorly regressed, suggested that apoptosis may be impaired and/or delayed. We measured the levels of apoptosis in mammary glands of Tg mice and of their non-Tg littermates at the same stage of gland development using the TUNEL assay. The percentage of TUNEL-positive cells in ducts and TEBs of virgin (5 weeks), in alveoli during pregnancy (18.5 days), and during involution of mammary glands after the first preg-

nancy was not statistically different for Tg and non-Tg females (data not shown). However, at 4-day involution after the third pregnancy, the percentage of apoptotic ductal epithelial cells was significantly increased in the Tg enlarged ducts, but not in the normal-size Tg ducts, as compared to the non-Tg ones (Figure 4, G–I). These results indicated that murine Notch1^{intra} does not appear to affect the apoptotic process at different stages of the mammary gland development at an early age, but definitively has a major proapoptotic effect during involution of the gland after successive pregnancies.

Mammary Tumorigenesis in MMTV/Notch1^{intra} Tg Mice

The histological examination of mammary glands from medium-age Tg females, revealed the presence of focal cell proliferation arising next to the normal mammary epithelial tissue (Figure 7D) (data not shown). These lesions thus suggested a premalignant condition. As these Tg females from lines 47400 ($n = 25$) and 39580 ($n = 1$) aged and after a few months of breeding, they developed rapidly growing, solid tumors by 7 to 10 months of age (Figure 7A). Three of four mammary tumors (line 47400) injected in nude mice were able to grow (data not shown), confirming their malignant nature. However, there was no evidence of metastases in lungs and liver. These data indicated that mammary tumors developed in Notch1^{intra}-expressing mammary glands of Tg mice after one or a few pregnancies.

To determine whether these tumors were pregnancy-dependent, as reported for other mouse mammary tumors, a group of virgin Tg females were allowed to age and were observed for the development of mammary tumors. The proportion (50%) of these virgin Tg mice that develop mammary tumors within 12 months was comparable to that of parous Tg females (Figure 7B), indicating that mammary tumors induced by overexpression of murine Notch1^{intra} can be totally pregnancy/lactation-independent.

Northern blot analysis on RNA extracted from these mammary tumors showed that the Tg was expressed at high levels, at the expected 3 kb size (Figure 7C). Moreover, *in situ* hybridization with a Notch1-specific riboprobe revealed that the proliferating mammary epithelial cells in the tumors expressed much higher level of the transgene than the adjacent morphologically nonneoplastic mammary glands (Figure 7D).

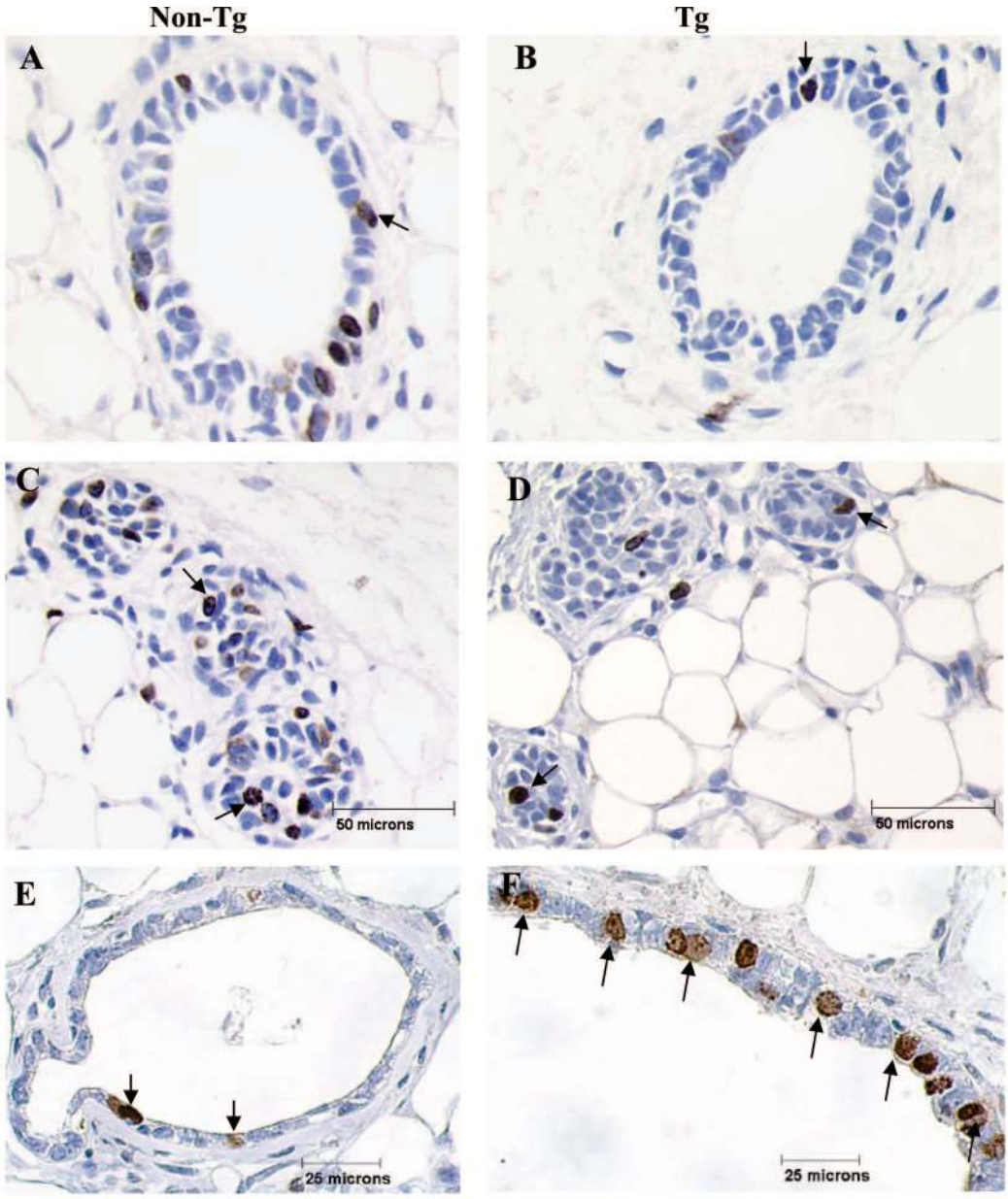
Histological examination of the mammary gland lesions showed that they were of epithelial origin and ranged from hyperplasia to invasive carcinoma (Figure 8). The hyperplastic changes included an increased number of ducts and intraductal epithelial proliferation of various pattern and intensity. In some ducts, the epithelial cells formed tufts and papillary fronds projecting into the lumen (Figure 8A). The epithelial proliferation was often more florid filling and distending the ducts with residual lumina being reduced to narrow spaces (Figure 8, B and C). Foci of atypical hyperplasia and of carcinoma *in situ* were present (data not shown). Poorly (Figure 8E) to moderately (Figure 8D) differentiated invasive carcinomas were observed. The moderately differentiated carcinomas presented gland-like differentiation and secretion (Figure 8D). Marked nuclear polymorphism and numerous mitosis were observed (Figure 8E). Areas of necrosis were often found within the tumors (Figure 8F). Together, these data indicated that Notch1^{intra} behaves as an oncogene when overexpressed in mammary glands of Tg mice. However, exophthalmia (caused by Harderian gland hyperplasia) and salivary, submandibular, or nasal mucosal/submucosal gland hyperplasia were not observed (data not shown), in contrast to that reported for MMTV/Notch4^{intra} Tg mice.[16]

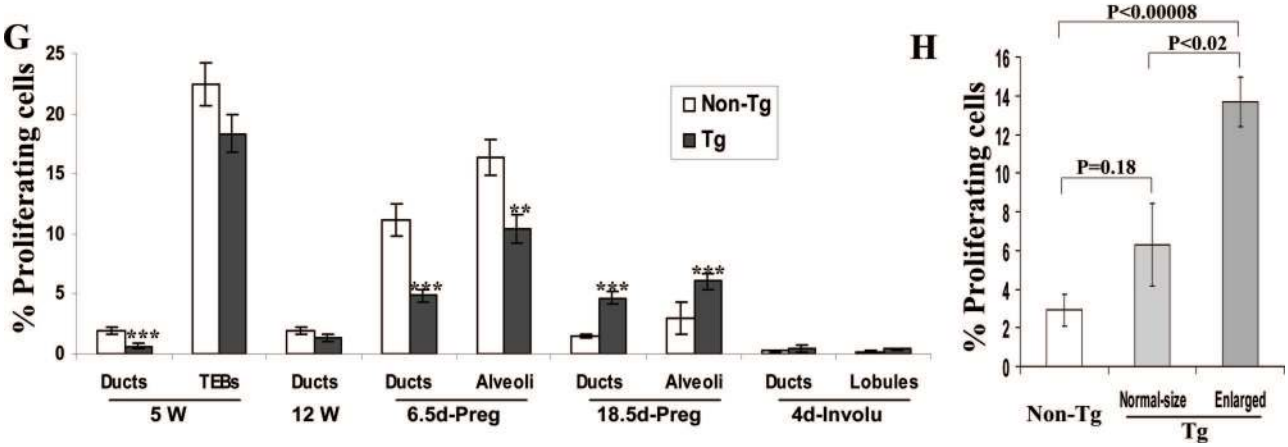
Impaired Mammary Gland Development and Mammary Tumorigenesis in MMTV/Notch3^{intra} Tg Mice

Because overexpression of truncated murine Notch4^{intra}[16,18] or murine Notch1^{intra} (Figures 3 and 8) were found to prevent mammary gland development and to induce mammary tumors in Tg mice, we investigated whether expression of another member of the murine Notch gene family, Notch3, will also induce similar developmental and oncogenic phenotypes in mammary glands. Notch3 has been reported to be overexpressed in human breast tumors.[42] In addition, Notch3^{intra} has already been reported to induce T-cell leukemia when expressed in T cells of Tg mice.[43,44]

The MMTV/Notch3^{intra} DNA having a structure very similar to that of the MMTV/Notch1^{intra}, was constructed (Figure 9A). Eight Tg founders (F85824, F85825, F85826, F85827, F85834, F85835, F85836, F85837) were generated. Among these, three founders (F85827, F85834, F85835) did not give rise to progeny and were considered sterile. One female founder (F85836) was found dead before a line could be established. Among the mice from other founders from which lines could be established, only one (F85824) expressed the transgene in mammary glands, while the three others (F85825, F85826, F85837) did not. In fact, expression of the Notch3^{intra} transgene in the F85824 founder was much higher than that of Notch1^{intra} (F47400), when measured with a probe (U5 LTR) common to both transcripts (Figure 9B). Tg mice from this latter founder (F85824) were expanded. Expression of Hes1 in mammary glands of pregnant MMTV/Notch3^{intra} Tg mice was much higher than in non-Tg mice (Figure 9C). This contrasted with the absence of detectable induction of Hes1 in mammary glands of MMTV/Notch1^{intra} Tg mice (see above).

The clinical phenotypes of these MMTV/Notch3^{intra} Tg mice were very similar to those of MMTV/Notch1^{intra} Tg mice. The Tg males were sterile and the Tg female mice could not feed their pups and did not produce milk. Moreover, these Tg females also developed mammary tumors at high frequency, but after a long latency (Figure 9D). Further analysis of the mammary glands at the different stages (virgin, pregnancy at 6.5 and 18.5 days, and involution) showed similar abnormalities in MMTV/Notch3^{intra} Tg mice as those described above for MMTV/Notch1^{intra} Tg mice: those include enlarged primary


Non-Tg
Tg
A
B
C
D
E
F
50 microns
50 microns
25 microns
25 microns


G
% Proliferating cells
25
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Non-Tg
Tg
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Ducts
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Ducts
Alveoli
Ducts
Alveoli
Ducts
Lobules
5 W
12 W
6.5d-Preg
18.5d-Preg
4d-Involu
H
P<0.00008
P<0.02
P=0.18
% Proliferating cells
16
14
12
10
8
6
4
2
0
Non-Tg
Normal-size
Enlarged
Tg

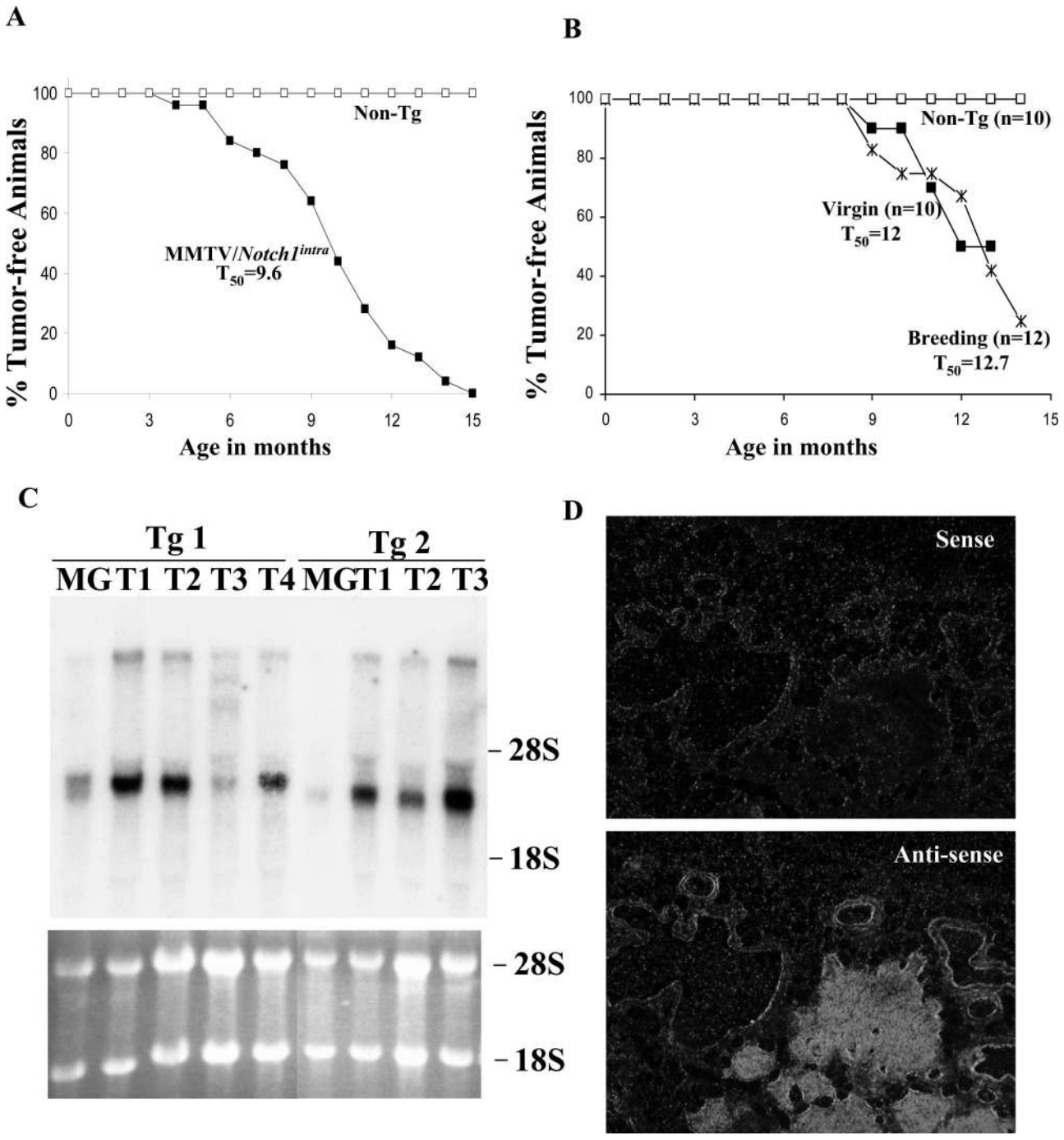


Figure 7. Incidence of tumor formation and transgene RNA expression in mammary tumors from MMTV/Notch1^{intra} Tg females. **A:** Tg females (n = 25) (line 47400) (**filled squares**) and control non-Tg female littermates (n = 25) (**open squares**) bred on a mixed BALB/c-CD1 background and allowed to have multiple pregnancies were observed for the appearance of mammary tumors for up to 15 months. **B:** In a second experiment, virgin (**filled squares**) and breeding (**asterisks**) Tg females bred on the CD1 background for at least eight generations were studied and compared to non-Tg females (**open squares**). T_{50}: Time required for 50% of Tg mice to develop large mammary tumors requiring the sacrifice of the diseased mice. **C:** Northern blot analysis. Total RNA extracted from distinct tumors (T) and from lactating mammary glands (MG) from two MMTV/Notch1^{intra} Tg mice (line 47400) were hybridized with a ^{32}P-labeled Notch1 probe K. A Notch1-specific RNA species of the expected size (3 kb) is observed. Ethidium bromide of the agarose gel is presented as control for the loading of RNAs. **D:** *In situ* hybridization of sections of mammary tumors from a Tg female (line 47400) were hybridized with ^{35}S-labeled Notch1^{intra} anti-sense and control sense riboprobes. Note that very high levels of expression of the Notch1 Tg is observed with the anti-sense probe in the neoplastic tissue. Original magnifications, ×10.

Figure 6. Proliferation of epithelial cells from mammary glands of MMTV/Notch1^{intra} Tg mice. Proliferation was assayed by BrdU incorporation after intraperitoneal inoculation of non-Tg (**A**, **C**, **E**) and Tg (**B**, **D**, **F**) mice. BrdU-positive cells (some shown with **arrows**) were detected by IHC with anti-BrdU antibodies. Ducts (**A**, **B**) and alveoli (**C**, **D**) were evaluated independently during early pregnancy (6.5 days). The same experiment was also performed in virgin and late pregnancy and during 4-day involution after the first pregnancy (**G**), as well as during involution (4 days) after the third pregnancy (**E**, **F**, **H**). Statistical analysis was performed with Student's t-test. $^{**}P < 0.003$, $^{***}P < 0.0005$. Original magnifications, ×40.

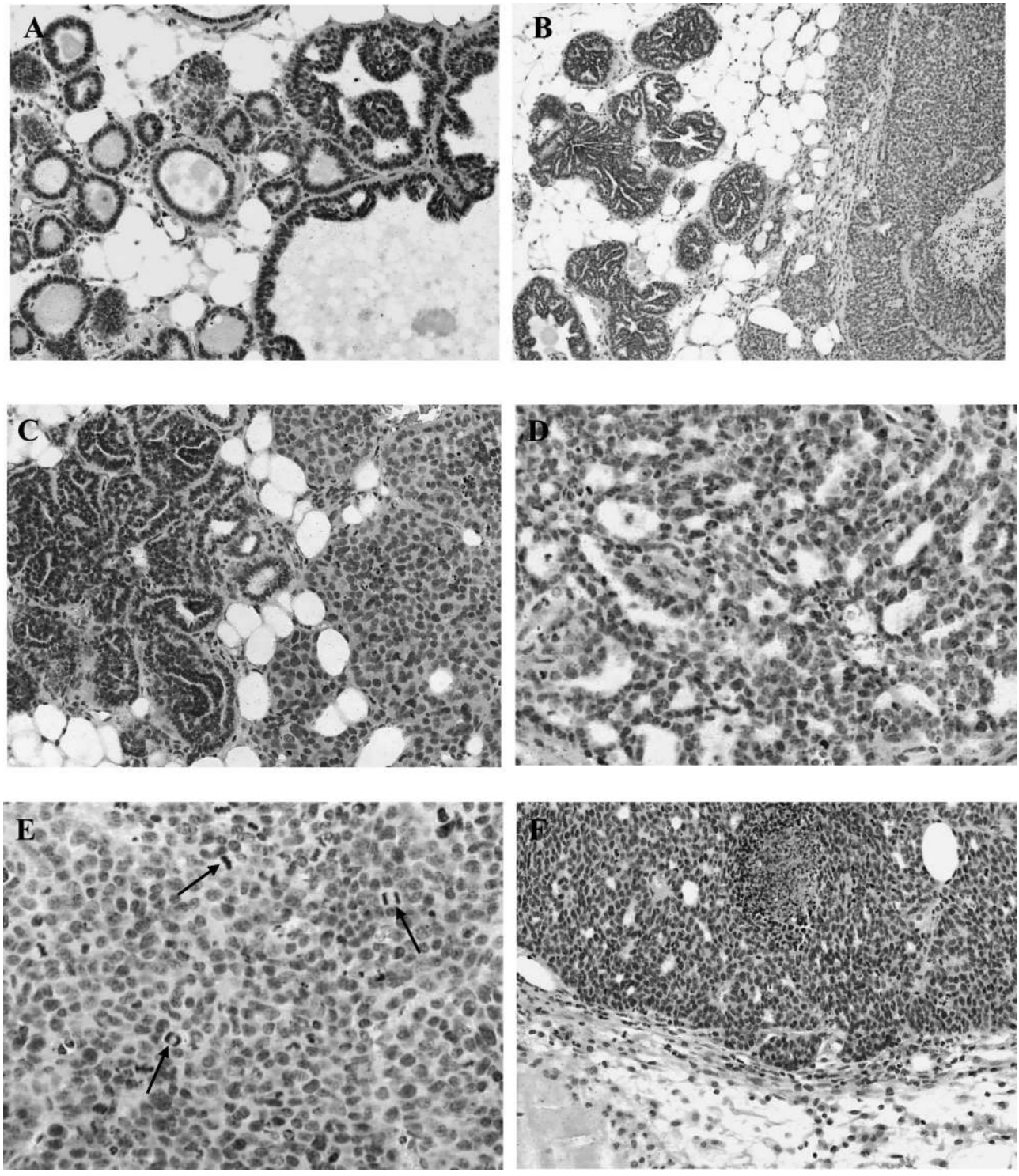


Figure 8. Histopathology of mammary lesions from MMTV/Notch1^{intra} Tg females. Representative sections of various types of mammary gland lesions from MMTV/Notch1^{intra} Tg females (lines 47400 and 39580) are shown. **A:** Epithelial hyperplastic changes with increased number of ducts (**left**) and formation of tufts and papillary excrescences protruding into the lumina (**right**). **B:** Intraductal epithelial hyperplasia with lumina induced to slit-like spaces (**left**) and adjacent invasive carcinoma (**right**). **C:** Higher magnification of ductal hyperplastic changes (**left**) composed of epithelial cells with small regular nuclei contrasting with adjacent invasive carcinoma in which the nuclei are enlarged and hyperchromatic. **D:** Moderately differentiated carcinoma forming glandular structures with open lumina. **E:** Poorly differentiated carcinoma showing a solid growth pattern and numerous mitoses (**arrows**). **F:** Focus of necrosis (**top middle third**) in a carcinoma. H&E stain. Original magnifications, ×20.

ducts in older virgin Tg mice, reduced ductal Tg arborization, and decreased density of Tg alveoli in early pregnancy, reduced proliferation in ducts of virgin Tg mice and in ducts and in alveoli of 6.5-day pregnant Tg mice (data not shown), lower number of secretory vesicles (Figure 9, H and J), severe decrease of β-casein produc-

tion (Figure 9J), and significantly enhanced proliferation of alveoli during late pregnancy (Figure 9, L and M) relative to non-Tg mice (Figure 9K). In addition, Notch3^{intra} was as efficient as Notch1^{intra} in repressing the β-casein promoter (Figure 5C). However, in late (18.5 days) pregnancy, some phenotypes were clearly different from those observed in MMTV/Notch1^{intra} Tg mice. In particular, in contrast to the reduced lobulo-alveolar development of MMTV/Notch1^{intra} Tg mice, the Notch3^{intra}-expressing lobules and alveoli were significantly expanded (Figure 9F) as compared to those of non-Tg mice (Figure 9E). The proliferation of ductal epithelial cells was not enhanced, but rather showed a modest decrease (Figure 9M). The alveolar epithelial Tg cells were hyperplastic, showing an increased cell density with large, dark nuclei and paucity of cytoplasm (Figure 9H). Moreover, apoptosis was significantly enhanced in alveoli and ducts of these pregnant (18.5 days) Tg mice relative to that of non-Tg alveoli (Figure 9, N–P). Therefore, it appears that constitutive overexpression of murine Notch3^{intra}, like murine Notch1^{intra}, in mammary glands severely affects mammary gland development and leads to tumor formation.

Discussion

We previously reported the involvement of Notch1 in mammary tumor development by finding MMTV provirus insertional mutants of Notch1 (generating truncated Notch1^{intra}) and by showing that Notch1^{intra} exhibits transforming ability in HC11 mouse mammary epithelial cells *in vitro*.[21] To further investigate the tissue-specific oncogenic potential of activated Notch1^{intra} *in vivo* and to establish an *in vivo* system amenable to genetic studies, we extended these initial studies and we generated Tg mice expressing murine Notch1^{intra} or Notch3^{intra} under the regulation of the MMTV LTR. This viral promoter can direct expression of surrogate genes in mammary glands as well as in the male reproductive system. Our results show that Notch1^{intra} or Notch3^{intra} expression in these tissues has severe consequences.

Expression of Murine Notch1^{intra} or Notch3^{intra} Blocks the Development of Mammary Glands

One striking phenotype observed in these MMTV/Notch1^{intra} or MMTV/Notch3^{intra} Tg females is their inability to nurse and feed their pups. Whole mount and histological analysis of MMTV/Notch1^{intra} Tg mice showed inhibition of mammary gland development, affecting both ductal and alveolar growth. This developmental defect resulted in decreased β-casein milk protein levels and impaired milk production. The small amount of residual milk being produced in the glands may not be secreted either, because pups from Tg females had no visible milk in their stomach.

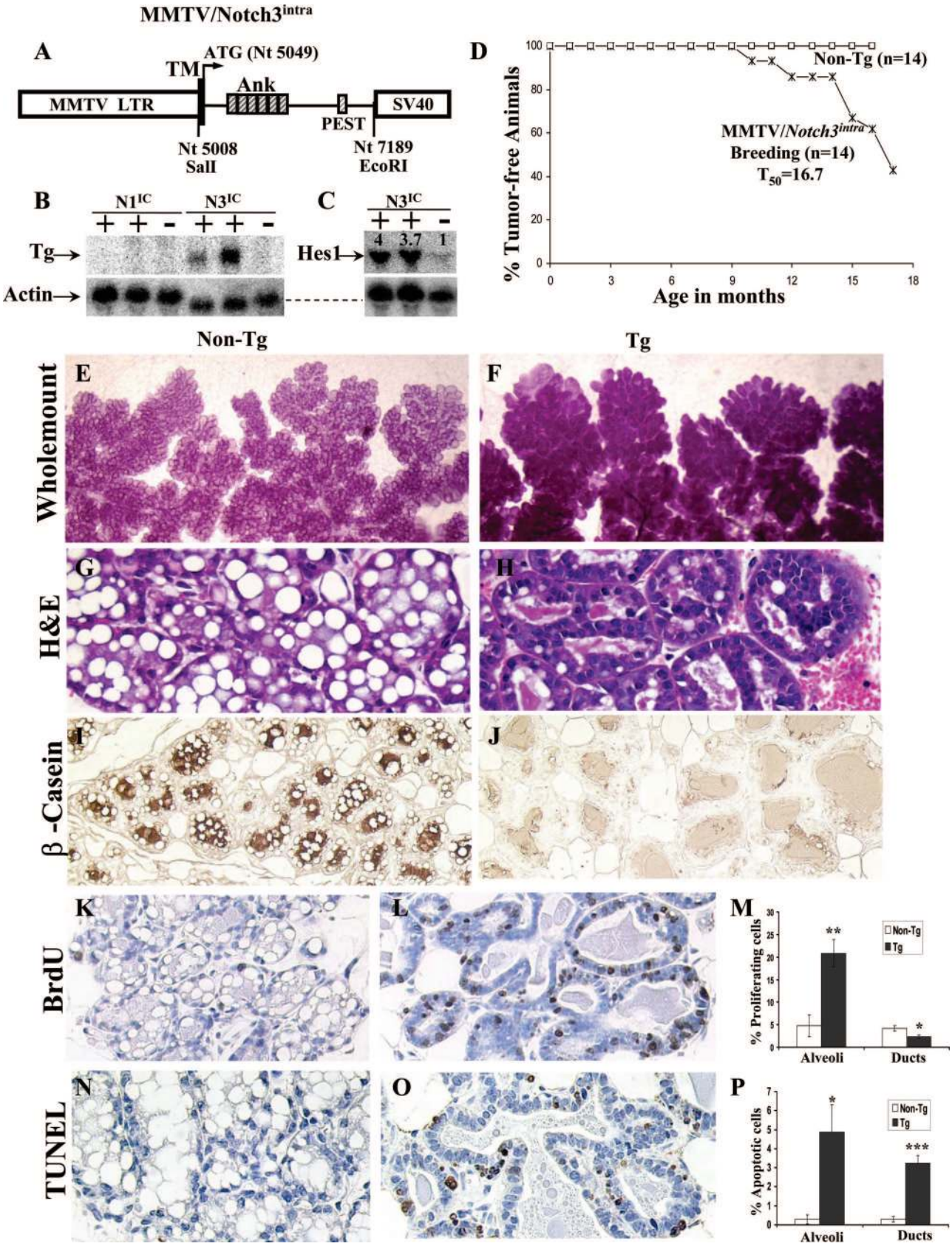
The role of Notch1 or Notch3 signaling in mammary gland development has not been studied extensively. The fact that Notch1^{intra} or Notch3^{intra} is capable of having such a profound effect on mammary gland development, suggests that the mammary epithelial cells express their downstream effectors. Gain-of-function mutants of Notch1, such as Notch1^{intra}, have previously been shown to affect mammalian cell fate determination and to prevent differentiation of other cells, namely myeloid cells,[45–47] CD34^{+} progenitor cells,[48] myoblasts,[10,49–51] neurons,[10] oligodendrocytes,[52] and T-cells.[53–56] Our results extend this effect of Notch1 or Notch3 to another tissue, the mammary gland.

A very similar phenotype of impaired mammary gland differentiation has previously been reported in Tg mice expressing murine Notch4^{intra} under the regulation of the whey acidic protein or MMTV promoter,[16,18] suggesting that overexpressed Notch1, Notch3, and Notch4 intracellular domains are likely to interact through the same effector(s) in mammary epithelial cells. Because Notch4^{intra} is shorter than Notch1^{intra} or Notch3^{intra}, only motifs shared by the three molecules are likely to be involved in such interactions. These results were not unexpected considering that these molecules share relatively a high degree of structural homology. However, they nevertheless remained intriguing in view of a recent report showing that Tg mice expressing human Notch1^{intra} do not exhibit impaired mammary gland development (see below).[22]

Defective mammary gland development has also been reported in other mutant mice, harboring overexpressed genes, such as β1,4 galactosyltransferase,[57] parathyroid hormone or parathyroid hormone-related protein,[58] whey acidic protein,[59] mutant p53,[60] neuregulin, and hepatocyte growth factor.[61] Also, impaired mammary gland differentiation has been reported in some gene-deficient mutant mice. This is notably the case for mice made defective for cyclin D1,[62] EGF and amphiregulin,[63] progesterone[64] and prolactin[65] receptors, activin/inhibin RB,[66] A-myb,[67] C/EBPβ,[68,69] CSF-1,[70] TIMP-1,[71] osteoprotegenin ligand/RANKL/TRANCE and RANK,[72] Id2,[73] or Stat5a[74] gene. Although some characteristics of these mutant mice are similar for some aspects to the Notch1^{intra} or Notch3^{intra} phenotype observed here, they nevertheless show distinct features,[62,72–74] making comparison between these phenotypes not easy and possibly hazardous. Future work may determine whether Notch1 or Notch3 signaling involves the transcriptional down-regulation of the expression of some of these molecules, as we have documented for β-casein. These pathways may not be identical in view of some differences observed between Notch1^{intra} and Notch3^{intra} Tg mice: hyperplasia and expansion of Notch3^{intra}-expressing alveoli versus reduced expansion of Notch1^{intra}-expressing alveoli.

Murine Notch1^{intra} and Notch3^{intra} Behave as Oncogenes in Mammary Epithelial Cells

We found that overexpression of Notch1^{intra} or Notch3^{intra} was not only affecting mammary gland development, but also induces mammary tumors at high frequency in Tg mice. Tumor types were diverse and represented the full


MMTV/Notch3intra
A
ATG (Nt 5049)
TM
Ank
MMTV LTR
SV40
PEST
Nt 5008
SalI
Nt 7189
EcoRI
B
N1IC
N3IC
Tg
Actin
C
N3IC
Hes1
4
3.7
1
D
Non-Tg (n=14)
MMTV/Notch3intra
Breeding (n=14)
T50=16.7
% Tumor-free Animals
Age in months
Non-Tg
Tg
Wholemount
H&E
β-Casein
BrdU
TUNEL
E
F
G
H
I
J
K
L
M
% Proliferating cells
Non-Tg
Tg
Alveoli
Ducts
N
O
P
% Apoptotic cells
Alveoli
Ducts

spectrum of differentiation from hyperplasia, *in situ* ductal carcinoma, glandular adenocarcinoma, to poorly differentiated adenocarcinoma. These results on the oncogenic function for Notch1^{intra} *in vivo* confirm and extend our previous data *in vitro* showing that Notch1^{intra} can transform HC11 mammary epithelial cells in culture.[21] Mammary tumor formation occurs in these Tg mice after a relatively long latency (4 to 15 months) and stochastically, suggesting that Notch1^{intra} or Notch3^{intra} is not sufficient by itself to promote transformation and requires the action of other oncogenes or the inactivation of tumor suppressor genes. The transforming ability of Notch1^{intra} or Notch3^{intra} documented here for mammary epithelial cells has previously been reported for T cells.[43,44,75] In addition, overexpression of Notch1 and Notch3 in human breast tumors has recently been reported to be associated with poor overall survival.[42] However, Notch1^{intra} may have an oncogenic potential more restricted than other oncogenes. In contrast to the activated Ha-Ras^{V12} or the truncated erbB2/neu or int3/Notch4^{intra} oncogenes that induce frequent hyperplasia of the Harderian glands, when expressed under the regulation of the same MMTV LTR,[16,30,32,76,77] we have not observed such a phenotype with Notch1^{intra} or Notch3^{intra}. MMTV/Notch1^{intra} did not develop either hyperplasia and adenocarcinoma of salivary glands and hyperplasia of mucosal/submucosal glands described in MMTV/Notch4^{intra} Tg mice.[16] It is possible however that levels of Tg expression in these tissues may explain some of these differences.

The mammary tumor phenotype induced by Notch1^{intra} or Notch3^{intra} Tg mice is very similar to the phenotype reported by Jhappan and colleagues[16] in MMTV/int3/Notch4^{intra} Tg mice. In both cases, the mammary tumors are diverse and at different stages of differentiation. However, one important difference could be noticed: whereas mammary tumors develop in both male and female MMTV/Notch4^{intra} Tg mice,[16] we did not observe mammary tumors in males of our MMTV/Notch1^{intra} or MMTV/Notch3^{intra} Tg mice. This may simply reflect the different background of the mouse used in both experiments. Alternatively, it could suggest that the molecular requirement for transformation of the male mammary glands may be more stringent and that the Notch1^{intra} or Notch3^{intra} protein is lacking elements, present in Notch4^{intra}, to meet these requirements.

In the Notch1^{intra} and Notch3^{intra} Tg mice studied here, the levels of expression of Notch3^{intra} were much higher than those of Notch1^{intra}. In addition, expression of Notch3^{intra} in mammary glands induced high expression of Hes1, whereas Hes1 was not induced in Notch1^{intra}-expressing mammary glands. Yet, the oncogenicity of Notch3^{intra} is not higher, but rather appears lower than that of Notch1^{intra}, suggesting intrinsic differences between these two molecules in their oncogenic potential. This may be reflected in the reported statistically stronger association of high-level Notch1 than Notch3 expression with overall survival in patients with breast cancer.[42] Intriguingly, Hes-1 was reported to be up-regulated in lactating Tg mice expressing human Notch1^{intra} in mammary glands.[22] Because Hes-1 levels were not measured in that study at the same stages of mammary gland development as we did (late pregnancy), direct comparison of these data remains difficult.

Is the Sequence Evolution of Notch1^{intra} Biologically Relevant?

Surprisingly, phenotypes of impaired mammary gland differentiation and of mammary tumor formation observed here in Tg mice expressing murine Notch1^{intra} contrast significantly with recent data obtained in similar Tg mice expressing human, instead of murine, Notch1 intracellular domain under the control of the same MMTV LTR promoter.[22] First, in these Tg mice, alveolar development did not appear to be compromised and absence of lactation was not observed, although some elongated ducts and reduced side branching could be documented in 5-week-old Tg mice. Second, increased ductal proliferation was observed in these young (5 weeks) virgin Tg females, in contrast to the decreased duct proliferation we observed at this stage (Figure 6G). Finally, the mammary tumor phenotype reported for the human Notch1^{intra}-expressing Tg mice[22] contrasts significantly with the one induced by murine Notch1^{intra}, Notch3^{intra} (this study), and Notch4^{intra}.[16,18] In Tg mice expressing any of these murine or human Notch genes, nonregressing mammary tumors were induced after a relatively long latency, suggesting common mechanisms. However, Tg mice expressing human Notch1^{intra} developed a novel phenotype of lactation-dependent regressing mammary tumors, not observed with the other murine Notch1^{intra}, Notch3^{intra}, and Notch4^{intra} transgenes, suggesting perturbation of a novel pathway by the human Notch1^{intra} molecule.

It is not clear why such distinct phenotypes were observed. This may be related to the genetic mouse background used [CD1 (this study) versus mixed C3H × C57BL/6 × FVB], although unlikely. It could also reflect different levels of expression of the transgene, although this is not very likely either in view of the fact that both groups of Tg mice (mouse versus human Notch1^{intra})

Figure 9. Analysis of MMTV/Notch3^{intra} Tg mice. **A:** Structure of the MMTV/Notch3^{intra} transgene construct. **B:** Tg RNA expression. Expression was measured on total RNA (20 μg) from mammary glands of pregnant (18.5 days) MMTV/Notch3^{intra} (N3^{IC}) or MMTV/Notch1^{intra} (N1^{IC}) Tg (+) and non-Tg (−) mice by Northern blot analysis using the ^{32}P-labeled U5 LTR probe specific to both transgenes. The membrane was washed and rehybridized with the actin probe (**bottom**). **C:** Hes1 expression. Expression was measured as described above in **B**, using the Hes1 probe. **D:** Incidence of tumor formation. Parous Tg females (**asterisks**) and control non-Tg female littermates (**open squares**) were observed for the appearance of mammary tumors for up to 16 months. **E–P:** Analysis of mammary glands from pregnant female mice at their first pregnancy (18.5 days). **E** and **F:** Whole mounts. Note the enhanced and much condensed lobules and alveoli in Tg mice. **G** and **H:** Histological sections of alveoli. Reduced number of secretory vesicles and hyperplasia are observed in Tg alveoli. **I** and **J:** β-Casein expression. IHC analysis was performed with rabbit anti-β-casein antibodies on tissue sections. **K–M:** Assessment of proliferation. Proliferation of alveolar epithelial cells was measured after BrdU incorporation as described in the legend to Figure 6, and quantitated (**M**). **N–P:** Assessment of apoptosis. TUNEL assay was used to measure the number of apoptotic alveolar epithelial cells and the percentage of apoptotic epithelial cells was quantitated (**P**). Statistical analysis in **M** and **P** was performed with the Student's *t*-test. $^{*}P < 0.05$, $^{**}P < 0.003$, $^{***}P < 0.0005$. Original magnifications: ×20 (**I–L**); ×40 (**G**, **H**, **N**, **O**).

developed nonregressing mammary tumors at high incidence. Rather, the different phenotype elicited by human and murine Notch1^{intra} may be directly related to their different amino acid sequences. These changes could prevent or favor the interaction of human Notch1^{intra} with key murine effector(s) required to elicit such a block of differentiation or such pregnancy/lactation-dependent tumor growth. Comparison of murine and human Notch1^{intra} amino acid residues in fact reveals complete identity of the transmembrane and the cdc1, -2, -5, and -6 ankyrin repeats and the PEST domain, very high identity of the cdc3 and -4 domains (with a single conserved amino acid difference in each), as well as of the RAM domain (a single amino acid difference, D1836S). However, the two molecules show significant differences in the amino acid residues of the region C-terminal to the last ankyrin repeat, including several conservative modifications and 18 nonconservative changes, 15 of which involve a proline. However, despite these differences, the human Notch1^{intra} construct still behaves as the murine Notch1^{intra} in the β-casein/luciferase reporter assay (data not shown).

This comparative analysis suggests that the region C-terminal to the last ankyrin repeat may be involved in the biological differences observed after expression of human or murine Notch1^{intra} in the mammary glands of Tg mice. It appears that this motif has significantly changed during evolution to a point of being incapable of interacting with murine effectors from the mammary glands, and possibly from other organs in a similar way as murine Notch1^{intra}.

In conclusion, the MMTV/*Not*ch1^{intra} and MMTV/Notch3^{intra} Tg mice represent novel animal models to study Notch1 or Notch3 signaling *in vivo*, especially in mammary epithelial cells, both in the context of differentiation and oncogenesis. This appears relevant in view of the recent observation that their high levels in some human breast tumors is associated with poor overall survival.[42]

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References

1. Artavanis-Tsakonas S, Rand MD, Lake RJ: Notch signaling: cell fate control and signal integration in development. Science 1999, 284:770–776
2. Greenwald I: LIN-12/Notch signaling: lessons from worms and flies. Genes Dev 1998, 12:1751–1762
3. Lieber T, Kidd S, Alcamo E, Corbin V, Young MW: Antineurogenic phenotypes induced by truncated Notch proteins indicate a role in signal transduction and may point to a novel function for Notch in nuclei. Genes Dev 1993, 7:1949–1965
4. Rebay I, Fehon RG, Artavanis-Tsakonas S: Specific truncations of Drosophila Notch define dominant activated and dominant negative forms of the receptor. Cell 1993, 74:319–329
5. Struhl G, Fitzgerald K, Greenwald I: Intrinsic activity of the Lin-12 and Notch intracellular domains in vivo. Cell 1993, 74:331–345
6. Coffman CR, Skoglund P, Harris WA, Kintner CR: Expression of an extracellular deletion of Xotch diverts cell fate in Xenopus embryos. Cell 1993, 73:659–671
7. Dorsky RI, Rapaport DH, Harris WA: Xotch inhibits cell differentiation in the Xenopus retina. Neuron 1995, 14:487–496
8. Kopan R, Weintraub H: Mouse Notch: expression in hair follicles correlates with cell fate determination. J Cell Biol 1993, 121:631–641
9. Kopan R, Nye JS, Weintraub H: The intracellular domain of mouse Notch: a constitutively activated repressor of myogenesis directed at the basic helix-loop-helix region of MyoD. Development 1994, 120:2385–2396
10. Nye JS, Kopan R, Axel R: An activated Notch suppresses neurogenesis and myogenesis but not gliogenesis in mammalian cells. Development 1994, 120:2421–2430
11. Lardelli M, Dahlstrand J, Lendahl U: The novel notch homologue mouse notch 3 lacks specific epidermal growth factor-repeats and is expressed in proliferating neuroepithelium. Mech Dev 1994, 46:123–136
12. Beatus P, Lundkvist J, Oberg C, Lendahl U: The notch 3 intracellular domain represses notch 1-mediated activation through Hairy/Enhancer of split (HES) promoters. Development 1999, 126:3925–3935
13. Uyttendaele H, Marazzi G, Wu G, Yan Q, Sassoon D, Kitajewski J: Notch4/int-3, a mammary proto-oncogene, is an endothelial cell-specific mammalian Notch gene. Development 1996, 122:2251–2259
14. Gallahan D, Callahan R: Mammary tumorigenesis in feral mice: identification of a new int locus in mouse mammary tumor virus (Czech II)-induced mammary tumors. J Virol 1987, 61:66–74
15. Lee JS, Haruna T, Ishimoto A, Honjo T, Yanagawa S: Intracisternal type A particle-mediated activation of the Notch4/int3 gene in a mouse mammary tumor: generation of truncated Notch4/int3 mRNAs by retroviral splicing events. J Virol 1999, 73:5166–5171
16. Jhappan C, Gallahan D, Stahle C, Chu E, Smith GH, Merlino G, Callahan R: Expression of an activated Notch-related int-3 transgene interferes with cell differentiation and induces neoplastic transformation in mammary and salivary glands. Genes Dev 1992, 6:345–355
17. Smith GH, Gallahan D, Diella F, Jhappan C, Merlino G, Callahan R: Constitutive expression of a truncated int3 gene in mouse mammary epithelium impairs differentiation and functional development. Cell Growth Differ 1995, 6:563–577
18. Gallahan D, Jhappan C, Robinson G, Hennighausen L, Sharp R, Kordon E, Callahan R, Merlino G, Smith GH: Expression of a truncated int3 gene in developing secretory mammary epithelium specifically retards lobular differentiation resulting in tumorigenesis. Cancer Res 1996, 56:1775–1785
19. Robbins J, Blondel BJ, Gallahan D, Callahan R: Mouse mammary tumor gene int-3: a member of the notch gene family transforms mammary epithelial cells. J Virol 1992, 66:2594–2599
20. Uyttendaele H, Soriano JV, Montesano R, Kitajewski J: Notch4 and Wnt-1 proteins function to regulate branching morphogenesis of mammary epithelial cells in an opposing fashion. Dev Biol (Orlando) 1998, 196:204–217
21. Dievart A, Beaulieu N, Jolicoeur P: Involvement of Notch1 in the development of mouse mammary tumors. Oncogene 1999, 18:5973–5981
22. Kiaris H, Politi K, Grimm LM, Szabolcs M, Fisher P, Efstratiadis A, Artavanis-Tsakonas S: Modulation of notch signaling elicits signature tumors and inhibits hras1-induced oncogenesis in the mouse mammary epithelium. Am J Pathol 2004, 165:695–705
23. Stewart TA, Hollingshead PG, Pitts SL: Multiple regulatory domains in the mouse mammary tumor virus long terminal repeat revealed by analysis of fusion genes in transgenic mice. Mol Cell Biol 1988, 8:473–479
24. Stewart TA, Pattengale PK, Leder P: Spontaneous mammary adenocarcinomas in transgenic mice that carry and express MTV/myc fusion genes. Cell 1984, 38:627–637
25. Henrard D, Ross SR: Endogenous mouse mammary tumor virus is expressed in several organs in addition to the lactating mammary gland. J Virol 1988, 62:3046–3049
26. Rollini P, Billotte J, Kolb E, Diggelmann H: Expression pattern of

mouse mammary tumor virus in transgenic mice carrying exogenous proviruses of different origins. J Virol 1992, 66:4580–4586
27. Mangues R, Seidman I, Pellicer A, Gordon JW: Tumorigenesis and male sterility in transgenic mice expressing a MMTV/N-ras oncogene. Oncogene 1990, 5:1491–1497
28. Matsui Y, Halter SA, Holt JT, Hogan BL, Coffey RJ: Development of mammary hyperplasia and neoplasia in MMTV-TGF alpha transgenic mice. Cell 1990, 61:1147–1155
29. Muller WJ, Lee FS, Dickson C, Peters G, Pattengale PK, Leder P: The int-2 gene product acts as an epithelial growth factor in transgenic mice. EMBO J 1990, 9:907–913
30. Sinn E, Muller W, Pattengale P, Tepler I, Wallace R, Leder P: Coexpression of MMTV/v-Ha-ras and MMTV/c-myc genes in transgenic mice: synergistic action of oncogenes in vivo. Cell 1987, 49:465–475
31. Tsukamoto AS, Grosschedl R, Guzman RC, Parslow T, Varmus HE: Expression of the int-1 gene in transgenic mice is associated with mammary gland hyperplasia and adenocarcinomas in male and female mice. Cell 1988, 55:619–625
32. Bouchard L, Lamarre L, Tremblay PJ, Jolicoeur P: Stochastic appearance of mammary tumors in transgenic mice carrying the MMTV/c-neu oncogene. Cell 1989, 57:931–936
33. Kay DG, Yue P, Hanna Z, Jothy S, Tremblay E, Jolicoeur P: Cardiac disease in transgenic mice expressing human immunodeficiency virus-1 Nef in cells of the immune system. Am J Pathol 2002, 161:321–335
34. Horseman ND, Zhao W, Montecino-Rodriguez E, Tanaka M, Nakashima K, Engle SJ, Smith F, Markoff E, Dorshkind K: Defective mammopoiesis, but normal hematopoiesis, in mice with a targeted disruption of the prolactin gene. EMBO J 1997, 16:6926–6935
35. Poudrier J, Weng X, Kay DG, Hanna Z, Jolicoeur P: The AIDS-like disease of CD4C/HIV Tg mice is associated with accumulation of immature CD11b^{hi} dendritic cells. J Virol 2003, 77:11733–11744
36. Radja F, Kay DG, Albrecht S, Jolicoeur P: Oligodendrocyte-specific expression of human immunodeficiency virus type 1 Nef in transgenic mice leads to vacuolar myelopathy and alters oligodendrocyte phenotype in vitro. J Virol 2003, 77:11745–11753
37. Girard L, Hanna Z, Beaulieu N, Hoemann CD, Simard C, Kozak CA, Jolicoeur P: Frequent provirus insertional mutagenesis of Notch1 in thymomas of MMTVD/myc transgenic mice suggests a collaboration of c-myc and Notch1 for oncogenesis. Genes Dev 1996, 10:1930–1944
38. MacKenzie F, Duriez P, Larrivee B, Chang L, Pollet I, Wong F, Yip C, Karsan A: Notch4-induced inhibition of endothelial sprouting requires the ankyrin repeats and involves signaling through RBP-Jkappa. Blood 2004, 104:1760–1768
39. Hoemann CD, Beaulieu N, Girard L, Rebai N, Jolicoeur P: Two distinct Notch1 mutant alleles are involved in the induction of T-cell leukemia in c-myc transgenic mice. Mol Cell Biol 2000, 20:3831–3842
40. Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, Cold Spring Harbor Laboratory, 1989
41. Kopan R: Notch: a membrane-bound transcription factor. J Cell Sci 2002, 115:1095–1097
42. Reedijk M, Odorcic S, Chang L, Zhang H, Miller N, McCready DR, Lockwood G, Egan SE: High-level coexpression of JAG1 and NOTCH1 is observed in human breast cancer and is associated with poor overall survival. Cancer Res 2005, 65:8530–8537
43. Bellavia D, Campese AF, Checquolo S, Balestri A, Biondi A, Cazzaniga G, Lendahl U, Fehling HJ, Hayday AC, Frati L, von Boehmer H, Gulino A, Screpanti I: Combined expression of pTalpha and Notch3 in T cell leukemia identifies the requirement of preTCR for leukemogenesis. Proc Natl Acad Sci USA 2002, 99:3788–3793
44. Bellavia D, Campese AF, Alesse E, Vacca A, Felli MP, Balestri A, Stoppacciaro A, Tiveron C, Tatangelo L, Giovarelli M, Gaetano C, Ruco L, Hoffman ES, Hayday AC, Lendahl U, Frati L, Gulino A, Screpanti I: Constitutive activation of NF-kappaB and T-cell leukemia/lymphoma in Notch3 transgenic mice. EMBO J 2000, 19:3337–3348
45. Milner LA, Bigas A, Kopan R, Brashem-Stein C, Bernstein ID, Martin DI: Inhibition of granulocytic differentiation by mNotch1. Proc Natl Acad Sci USA 1996, 93:13014–13019
46. Li L, Milner LA, Deng Y, Iwata M, Banta A, Graf L, Marcovina S, Friedman C, Trask BJ, Hood L, Torok-Storb B: The human homolog of rat Jagged1 expressed by marrow stroma inhibits differentiation of 32D cells through interaction with Notch1. Immunity 1998, 8:43–55
47. Bigas A, Martin DI, Milner LA: Notch1 and Notch2 inhibit myeloid differentiation in response to different cytokines. Mol Cell Biol 1998, 18:2324–2333
48. Carlesso N, Aster JC, Sklar J, Scadden DT: Notch1-induced delay of human hematopoietic progenitor cell differentiation is associated with altered cell cycle kinetics. Blood 1999, 93:838–848
49. Shawber C, Nofziger D, Hsieh JJ, Lindsell C, Bogler O, Hayward D, Weinmaster G: Notch signaling inhibits muscle cell differentiation through a CBF1-independent pathway. Development 1996, 122:3765–3773
50. Kopan R, Nye JS, Weintraub H: The intracellular domain of mouse notch: a constitutively activated repressor of myogenesis directed at the basic helix-loop-helix region of myod. Development 1994, 120:2385–2396
51. Nofziger D, Miyamoto A, Lyons KM, Weinmaster G: Notch signaling imposes two distinct blocks in the differentiation of C2C12 myoblasts. Development 1999, 126:1689–1702
52. Wang S, Sdrulla AD, diSibio G, Bush G, Nofziger D, Hicks C, Weinmaster G, Barres BA: Notch receptor activation inhibits oligodendrocyte differentiation. Neuron 1998, 21:63–75
53. Pui JC, Allman D, Xu L, DeRocco S, Karnell FG, Bakkour S, Lee JY, Kadesch T, Hardy RR, Aster JC, Pear WS: Notch1 expression in early lymphopoiesis influences B versus T lineage determination. Immunity 1999, 11:299–308
54. Radtke F, Wilson A, Stark G, Bauer M, van Meerwijk J, MacDonald HR, Aguet M: Deficient T cell fate specification in mice with an induced inactivation of Notch1. Immunity 1999, 10:547–558
55. Robey E, Chang D, Itano A, Cado D, Alexander H, Lans D, Weinmaster G, Salmon P: An activated form of Notch influences the choice between CD4 and CD8 T cell lineages. Cell 1996, 87:483–492
56. Washburn T, Schweighoffer E, Gridley T, Chang D, Fowlkes BJ, Cado D, Robey E: Notch activity influences the alphabeta versus gammadelta T cell lineage decision. Cell 1997, 88:833–843
57. Hathaway HJ, Shur BD: Mammary gland morphogenesis is inhibited in transgenic mice that overexpress cell surface beta1,4-galactosyltransferase. Development 1996, 122:2859–2872
58. Wysolmerski JJ, Philbrick WM, Dunbar ME, Lanske B, Kronenberg H, Karaplis A, Broadus AE: Rescue of the parathyroid hormone-related protein knockout mouse demonstrates that parathyroid hormone-related protein is essential for mammary gland development. Development 1998, 125:1285–1294
59. Hennighausen L, McKnight R, Burdon T, Baik M, Wall RJ, Smith GH: Whey acidic protein extrinsically expressed from the mouse mammary tumor virus long terminal repeat results in hyperplasia of the coagulation gland epithelium and impaired mammary development. Cell Growth Differ 1994, 5:607–613
60. Li B, Greenberg N, Stephens LC, Meyn R, Medina D, Rosen JM: Preferential overexpression of a 172Arg–>Leu mutant p53 in the mammary gland of transgenic mice results in altered lobuloalveolar development. Cell Growth Differ 1994, 5:711–721
61. Yang Y, Spitzer E, Meyer D, Sachs M, Niemann C, Hartmann G, Weidner KM, Birchmeier C, Birchmeier W: Sequential requirement of hepatocyte growth factor and neuregulin in the morphogenesis and differentiation of the mammary gland. J Cell Biol 1995, 131:215–226
62. Fantl V, Edwards PA, Steel JH, Vonderhaar BK, Dickson C: Impaired mammary gland development in Cyl-1(−/−) mice during pregnancy and lactation is epithelial cell autonomous. Dev Biol 1999, 212:1–11
63. Luetteke NC, Qiu TH, Fenton SE, Troyer KL, Riedel RF, Chang A, Lee DC: Targeted inactivation of the EGF and amphiregulin genes reveals distinct roles for EGF receptor ligands in mouse mammary gland development. Development 1999, 126:2739–2750
64. Lydon JP, DeMayo FJ, Funk CR, Mani SK, Hughes AR, Montgomery CA, Shyamala G, Conneely OM, O'Malley BW: Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. Genes Dev 1995, 9:2266–2278
65. Ormandy CJ, Camus A, Barra J, Damotte D, Lucas B, Buteau H, Edery M, Brousse N, Babinet C, Binart N, Kelly PA: Null mutation of the prolactin receptor gene produces multiple reproductive defects in the mouse. Genes Dev 1997, 11:167–178
66. Vassalli A, Matzuk MM, Gardner HA, Lee KF, Jaenisch R: Activin/inhibin beta B subunit gene disruption leads to defects in eyelid development and female reproduction. Genes Dev 1994, 8:414–427
67. Toscani A, Mettus RV, Coupland R, Simpkins H, Litvin J, Orth J, Hatton KS, Reddy EP: Arrest of spermatogenesis and defective

breast development in mice lacking A-myb. Nature 1997, 386:713–717
68. Robinson GW, Johnson PF, Hennighausen L, Sterneck E: The C/EB-Pbeta transcription factor regulates epithelial cell proliferation and differentiation in the mammary gland. Genes Dev 1998, 12:1907–1916
69. Seagroves TN, Krnacik S, Raught B, Gay J, Burgess-Beusse B, Darlington GJ, Rosen JM: C/EBPbeta, but not C/EBPalpha, is essential for ductal morphogenesis, lobuloalveolar proliferation, and functional differentiation in the mouse mammary gland. Genes Dev 1998, 12:1917–1928
70. Pollard JW, Hennighausen L: Colony stimulating factor 1 is required for mammary gland development during pregnancy. Proc Natl Acad Sci USA 1994, 91:9312–9316
71. Fata JE, Leco KJ, Moorehead RA, Martin DC, Khokha R: Timp-1 is important for epithelial proliferation and branching morphogenesis during mouse mammary development. Dev Biol 1999, 211:238–254
72. Fata JE, Kong YY, Li J, Sasaki T, Irie-Sasaki J, Moorehead RA, Elliott R, Scully S, Voura EB, Lacey DL, Boyle WJ, Khokha R, Penninger JM: The osteoclast differentiation factor osteoprotegerin-ligand is essential for mammary gland development. Cell 2000, 103:41–50
73. Mori S, Nishikawa SI, Yokota Y: Lactation defect in mice lacking the helix-loop-helix inhibitor Id2. EMBO J 2000, 19:5772–5781
74. Liu X, Robinson GW, Wagner KU, Garrett L, Wynshaw-Boris A, Hennighausen L: Stat5a is mandatory for adult mammary gland development and lactogenesis. Genes Dev 1997, 11:179–186
75. Ellisen LW, Bird J, West DC, Soreng AL, Reynolds TC, Smith SD, Sklar J: TAN-1, the human homolog of the Drosophila notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. Cell 1991, 66:649–661
76. Tremblay PJ, Pothier F, Hoang T, Tremblay G, Brownstein S, Liszaeur A, Jolicoeur P: Transgenic mice carrying the mouse mammary tumor virus ras fusion gene: distinct effects in various tissues. Mol Cell Biol 1989, 9:854–859
77. Muller WJ, Sinn E, Pattengale PK, Wallace R, Leder P: Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-neu oncogene. Cell 1988, 54:105–115