A Transgenic Model Reveals Important Roles for the NF-κB Alternative Pathway (p100/p52) in Mammary Development and Links to Tumorigenesis*

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Linda Connelly†, Cheryl Robinson-Benion†, Melissa Chont†, Leshana Saint-Jean†, Haijing Li†, Vasiliy V. Polosukhin†, Timothy S. Blackwell‡§, and Fiona E. Yull††

From the †Department of Cancer Biology, Vanderbilt University, Nashville, Tennessee 37232 and the ‡Division of Allergy, Pulmonary and Critical Care Medicine, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-2650

A regulated pattern of nuclear factor κB (NF-κB) activation is essential for normal development of the mammary gland. An increase in NF-κB activity has been implicated in breast cancer. We have generated a novel transgenic mouse model to investigate the role of the alternative NF-κB pathway in ductal development and identify possible mediators of tumorigenesis downstream of p100/p52. By overexpressing the NF-κB p100/p52 subunit in mammary epithelium using the β-lactoglobulin milk protein promoter, we found that transgene expression resulted in increased overall NF-κB activity during late pregnancy. During pregnancy, p100/p52 expression resulted in delayed ductal development with impaired secondary branching and increased levels of Cyclin D1, matrix metalloproteinase-2 (MMP-2), matrix metalloproteinase-9 (MMP-9), and cyclo-oxygenase-2 (COX-2) in the mammary gland. After multiple pregnancies the p100 transgenics exhibited a ductal thickening accompanied by small hyperplastic foci. In tumors from mice expressing the polyoma middle T oncoprotein (PyVT) in the mammary gland, increased levels of p100/p52 were present at the time of tumor development. These results show that increased p100/p52 disrupts normal ductal development and provides insight into the mechanism by which this may contribute to human breast cancer.

The development of the murine mammary gland is a complex process. A simple ductal structure forms during embryogenesis through signaling between the epithelium and surrounding mesenchyme. The cyclic production of hormones promotes further ductal growth and branching during puberty. The majority of mammary gland proliferation is observed in pregnancy, with ductal branching and the formation of secondary lobular alveolar units. Following lactation and the weaning of pups there is a re-modeling of the epithelium termed involution, and the gland returns to the simpler structure observed in virgin animals (1–3).

The transcription factor nuclear factor κB (NF-κB)² is involved in the process of mammary development. NF-κB is a dimer formed from a multisubunit family consisting of p65 (Rel A), Rel B, c-Rel, p105/p50 (NF-κB1), and p100/p52 (NF-κB2; Ref. 4). The activation of NF-κB is regulated by the binding of inhibitory proteins termed IκBs (5). The C-terminals of the p105 and p100 subunits share homology with IκB (6, 7). These inhibitory subunits are phosphorylated in response to stimuli by an upstream IκB kinase complex (IKK). This contains two kinases, IKKα and IKKβ, and a regulatory subunit, IKKγ (NEMO; Refs. 8–10). In the canonical NF-κB pathway, a dimer composed of the p65 and p50 subunits is held in the cytosol through binding of the inhibitor IκBα. The phosphorylation of IκBα by IKKβ acts as a signal for the ubiquitination and degradation of the inhibitor (11). This allows p50/p65 to translocate to the nucleus and bind to the promoter of target genes that are involved in a diverse range of processes including inflammation, cell growth, and apoptosis (4, 12). More recently an alternative pathway of NF-κB subunit activation has been identified involving p100/p52. p100 acts as an inhibitor of p52 in a similar manner to IκB. In the alternative pathway p100 is phosphorylated by IKKα. This results in cleavage and truncation of the protein producing p52 which then translocates to the nucleus (13). As p52 lacks a transactivation domain it regulates transcription through the formation of heterodimers with partners including Rel B, which contains a C-terminal transcriptional activation domain (14).

NF-κB activity in epithelial cells is essential for normal mammary development particularly during pregnancy and involution. NF-κB activity peaks during late pregnancy, declines rapidly at the onset of lactation, then rises during involution (15, 16). The essential role of NF-κB in pregnancy is apparent in mouse models where signaling is disrupted. Mice that lack receptor activator of NF-κB ligand (RANKL) fail to form lobular alveolar structures and do not produce milk (17). A similar impairment of epithelial cell proliferation accompanied by a lactation defect is observed in a mouse model where IKKα signaling was disrupted (18). A transient delay in mammary ductal branching was observed during early pregnancy (day 7.5) when the classical pathway of NF-κB activation was inhibited by an

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² The abbreviations used are: NF-κB, nuclear factor κB; IKK, IκB kinase complex; BLG, β-lactoglobulin; NGL, NF-κB-GFP-Luciferase; GFP, green fluorescent protein; MMP, matrix metalloproteinase; COX, cyclo-oxygenase; PyVT, polyoma middle T oncoprotein.

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epithelial specific dominant-negative inhibitor of IκBα. This effect was overcome during later in pregnancy (day 14.5) when the alternative pathway, namely p52, was activated (19). The opposite effect is observed when hyperactivation of NF-κB occurs. In mice that have been transplanted with mammary tissue from IκBα-deficient donors there is an increase in lateral ductal branching and pervasive intraductal hyperplasia (20). The previous studies have demonstrated that NF-κB can regulate ductal growth and is therefore important for normal mammary development. Although p52 has been identified as one of the NF-κB subunits involved in this effect, there have been no murine models generated that directly study the alternative signaling pathway in mammary gland development.

The aberrant activation of NF-κB has also been observed in human breast tumor cell lines and carcinogen-induced rat mammary tumors (21–23). In Western blots from several breast cancer cell lines high expression of p100/p52 was observed, while normal human epithelial cells had low levels of expression. In the same study, 18 primary breast tumor samples out of 24 displayed significant expression of the p100/p52 protein (24). In a later study, the overexpression of p100/p52 in human carcinomas of the breast was also observed. It was demonstrated that there is an up-regulation of p52 which is specific to tumor cells and not observed in invading stromal fibroblasts (25).

Although it has been detected at high levels in tumors, the role of p100/p52 has not been elucidated. Furthermore, previous studies have not directly studied the role of p100/p52 during mammary development. In the current study we have created a novel transgenic model where p100/p52 is up-regulated during pregnancy and lactation. This model has been used to investigate the effects of p100/p52 signaling during the proliferative phase of pregnancy. The results obtained provide novel information about the function of the alternative NF-κB pathway in mammary development. Moreover, this model provides insight into the mechanism by which overexpression of p100/p52 contributes to tumorigenesis.

**EXPERIMENTAL PROCEDURES**

**Transgenic Mice**—A 3.0-kb HindIII/BamHI human NF-κB p100 fragment was isolated from plasmid pBS100 and the ends filled in prior to blunt end ligation into the EcoR5 cloning site of plasmid pBJ41 (obtained from Dr. John Clark). This places the coding region for human p100 downstream of the sheep β-lactoglobulin (BLG) promoter, and the resulting plasmid was named pBLG-p100. A SalI/XbaI microinjection fragment was filled in prior to blunt end ligation into the EcoR5 cloning site of p100 transgene and termed NF-κB reporter males termed NF-κB-GFP-Luciferase (NGL). These mice have been described previously and were also on a B6D2 strain background (26). Tail biopsies from double transgenics were genotyped by PCR as above for the p100 transgene and by luciferase assay (Promega, Madison, WI) for the NGL transgene. These bi-transgenic mice were termed p100 transgenics/NGL. Polyclonal middle T oncoprotein (PyVT) transgenic mice were originally developed by Bill Muller and colleagues and are now commercially available from Jackson Laboratories. All animal experiments were performed in accordance with the regulations of the Vanderbilt University Institutional Animal Care and Use Committee.

**Whole Mount Analyses**—Number 4 inguinal mammary glands were fixed in 10% formalin overnight at 4 °C. The glands were washed in acetone and equilibrated to 95% ethanol before staining in Mayer’s hematoxylin solution (Sigma) for 3 h. The glands were de-stained in tap water and further de-stained in 50% ethanol acidified with hydrochloric acid at a 0.05 m final concentration. The glands were then dehydrated in a graded ethanol series followed by xylene and stored in methyl salicylate. Images were taken using a Zeiss Stemi 1000C microscope and QImaging’s Micropublisher 3.3 camera and software.

**Immunohistochemistry**—Number 4 inguinal mammary glands were fixed in 10% formalin overnight at 4 °C. The glands were then dehydrated in a graded ethanol series followed by xylene and embedded in paraffin. 5-μm sections were prepared with one section from each sample stained with hematoxylin and eosin (Vanderbilt University Medical Center Immunohistochemistry Core Laboratory). Morphometry was performed using Image-Pro Express (Media Cybernetics, Inc., Silver Spring, MD). Glands were de-waxed and reconstituted through a series of xylens and a graded ethanol series. Endogenous peroxidase activity was blocked by incubation in 0.6% hydrogen peroxide in methanol for 30 min. Antigen retrieval was performed by incubating in 10 mM sodium citrate for 5 min at full power followed by 9 min at 50% power in a 700-watt microwave. After cooling to room temperature, tissues were incubated in blocking solution (10 mM Tris-HCl, pH 7.4, 0.1 mM MgCl2, 0.5% Tween 20, 1% bovine serum albumin, 5% goat serum) for 1 h at room temperature. Tissues were incubated with primary antibody (p100/p52 (Santa Cruz Biotechnology, Santa Cruz, CA) or GFP (Clontech, Mountain View, CA)) diluted in blocking solution overnight at 4 °C in a humidified chamber followed by washing in TBST (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20). Tissues were incubated with secondary antibody (anti-rabbit IgG biotinylated antibody; Vector Laboratories, Burlingame, CA) diluted in blocking solution for 1 h at room temperature in a humidified chamber. Specific immunoreaction was detected using the Vectastain Elite ABC kit (Vector Laboratories) and Diaminobenzadine (Sigma). Sections were counterstained with hematoxylin before

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dehydration and mounting in Permount (Fisher Scientific). Images were captured on a Zeiss Axioplan 2 microscope, QImaging camera, and Improvision’s OpenLab 4.0.2 software.

**Tissue Luciferase Assay**—Whole mammary glands were snap-frozen in liquid nitrogen then ground in a glass homogenizer in Lysis Buffer (Promega). The extracts were then centrifuged (13,793 × g, 4 °C, 15 min) and the supernatants retained for subsequent analysis. Luciferase activity was measured in a Monolight 3010 Luminometer (Analytical Luminescence Laboratory) after adding 100 μl of freshly reconstituted luciferin to 20 μl of mammary tissue homogenate. Results were expressed as relative light units normalized for protein content, which was measured by protein assay (Bio-Rad).

**SDS-PAGE and Western Blot Analysis**—Whole mammary glands were snap-frozen in liquid nitrogen then ground in a glass homogenizer in whole-cell homogenization buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 8 mM EGTA, and Mini-EDTA free protease inhibitor mixture tablet; Roche Applied Science). The extracts were then centrifuged (13,793 × g, 4 °C, 15 min), and the supernatants retained for subsequent analysis. For nuclear extracts, tissues were homogenized in cold lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EGTA, 0.1 mM EDTA, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride). Samples were transferred to a 1.5-ml tube, and 50 μl of 10% Nonidet P-40 was added. Tubes were centrifuged (150 × g, 5 min, 4 °C), and the resulting nuclear pellets were washed with lysis buffer by centrifugation as before. Cold nuclei extraction buffer (10 mM HEPES, pH 7.9, 420 mM NaCl, 1.5 mM MgCl₂, 0.1 mM EGTA, 0.1 mM EDTA, 25% glycerol, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride) was added to each tube followed by centrifugation (13,793 × g, 10 min, 4 °C). The resulting supernatants were then retained as nuclear extracts. Protein concentrations were determined by protein assay (Bio-Rad). Equal volumes of protein were subjected to 7.5% or 10% SDS-PAGE under reducing conditions. The proteins were transferred to nitrocellulose membranes with a mini Trans-blot cell (Bio-Rad). The membranes were then incubated with shaking in 5% milk in wash buffer (phosphate-buffered saline, 0.1% Tween 20) for 1 h at room temperature. The membrane was rinsed in wash buffer before incubation overnight at 4 °C with gentle shaking, with primary antibody (anti-p100/p52, p65, Rel-B, Bcl-2, Bcl-XL, and actin (Santa Cruz Biotechnology), anti-cyclin D1 (Cell Signaling Technology, Danvers, MA), anti-A1 (R&D Systems, Minneapolis, MN), anti-COX-2 (Cayman Chemicals, Ann Arbor, MI)) diluted in 1% milk in wash buffer. The membrane was washed six times (5 min/wash) and then incubated, with gentle shaking for 1 h at room temperature, with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Santa Cruz Biotechnology) diluted in 1% milk in wash buffer. The membrane was washed as described previously, and proteins were visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce). Bands were quantified by densitometry (NIH Image).

**Zymography**—Equal volumes of protein were loaded onto a 10% SDS-PAGE gel containing 0.25% porcine gelatin (Sigma). After electrophoresis, the gels were washed twice for 15 min each in 2.5% Triton X-100 and then incubated overnight at 37 °C in substrate buffer (50 mM Tris-HCl, pH 7.6, and 10 mM CaCl₂) in the absence or presence of 10 mM EDTA. The gels were stained with Brilliant Blue R stain (Sigma) for 2 h. The gels were de-stained briefly in 50% methanol, 10% acetic acid. Areas of gelatinolytic activity appeared cleared against the blue background of the blue-stained undigested gelatin.

**Data Analysis**—Statistical analyses were performed using Graph Pad Prism (GraphPad Software Inc., San Diego, CA). Densitometric analyses were performed using NIH image. All data are plotted graphically as mean ± standard error. A Student’s t test was used to assess differences between experimental conditions. A probability (p) value of <0.05 was taken as an appropriate level of significance.

**RESULTS**

**Generation and Characterization of p100/p52 Transgenic Mice**—To determine the role of p100/p52 in mammary gland development, we developed several independent lines of transgenic mice in which p100/p52 is expressed in mammary epithelial cells. The human p100/p52 protein was targeted to the mammary epithelium using the β-lactoglobulin promoter (Fig. 1A). Three founder lines were generated which show equivalent transgene expression (data not shown) and representatives from all lines were used for further experiments. Expression was undetectable in extracts prepared from spleen, liver, kidney, lung, and brain suggesting that this promoter successfully targets the mammary epithelium as was predicted (data not shown). There was no detectable p100 expression (endogenous or transgenic) in virgin mice. A low level of p100 expression was observed in wild type mice at day 12 (P12) and day 14 (P14) of pregnancy and absent at all other stages. The pattern of p100/p52 expression observed in the transgens was consistent with expression from a milk protein promoter: present during pregnancy, peaking during mid lactation (day 9; L9), and falling during involution (Fig. 1B). There was no change in corresponding levels of Rel B. The epithelial specific overexpression of the transgene was confirmed and localized by immunohistochemistry for p100/p52 in tissue sections at P16 (Fig. 1C). To determine whether cytosolic expression resulted in the nuclear translocation of p52, nuclear extracts were prepared from mammary glands at P16. At P16, p52 protein was detected in nuclear extracts from mammary glands from the p100 transgenic compared with an absence of p52 in the wild type controls (Fig. 1D). There was no change in corresponding levels of Rel B or p65.

The effect of p100/p52 overexpression in the mammary gland on NF-κB activity was investigated using an NF-κB reporter transgenic termed NGL. This transgenic mouse contains four tandem copies of the NF-κB binding element from the 5’ human immunodeficiency virus long terminal repeat placed upstream of the herpes simplex virus minimal thymidine kinase promoter driving expression of an enhanced GFP-luciferase fusion protein. This reporter allows the detection of NF-κB activity in specific tissues and cells by luciferase assay or immunohistochemistry. Mammary glands were harvested at P16, and NF-κB activity was measured by luciferase assay of mammary gland extracts (Fig. 1E). In mice that were negative for the p100 transgene but positive for NGL there was a base-
line level of NF-κB activity. However, there was a significant increase in luciferase activity in the p100 transgenics indicating that the transgene was elevating NF-κB activity. There was no luciferase activity present in wild type or p100 transgenic mice. The increase in NF-κB activity in ductal epithelium of p100/NGL transgenics compared with wild type/NGL mice was confirmed by immunohistochemistry for GFP in tissue sections from wild type/NGL and p100/NGL transgenics (Fig. 1). Not only did this confirm the increased expression of the reporter, hence NF-κB activity, but confirmed localized expression within the ductal epithelium.

p100/p52 Expression Leads to a Delay in Mammary Development with a Reduction in Ductal Branching during Pregnancy—To study the effects of p100/p52 overexpression on mammary development, whole mount analyses were performed on glands from P12, P16, and L1 wild type and p100 transgenic mice. Littermates were used as controls. As would be expected from the lack of transgene expression, there was no difference in ductal development in virgin animals (Fig. 2A). An equivalent level of ducts and branching was observed at P12 in wild type and p100 transgenics. However, while primary ductal branching appeared unchanged there was a reduction in secondary branching in the p100 transgenic glands as compared with wild type at P16 (Fig. 2A). As p100 transgenic females were able to lactate and maintain the same litter sizes as wild type females, whole mounts from lactating mice were prepared and analyzed. During day 1 of lactation (L1), there was no observable difference in filling of the glands by epithelium between wild type and p100 transgenic mice (Fig. 2A) indicating that the delay in secondary branching was a transient effect. The reduction in branching at P16 was confirmed by H and E staining of tissue sections and morphometric analyses (Fig. 2, B and C). The wild type mice had 20.1 ± 1.1% of the gland filled with ductal epithelium while the p100 transgenics had 13.7 ± 1.5% ductal epithelium.

Cyclin D1 Expression Is Increased in p100 Transgenics at P16—To investigate the mechanism for the delay in secondary ductal branching, cell proliferation and apoptosis were analyzed. No significant difference in levels of proliferating cell nuclear antigen or terminal deoxynucleotidyltransferase nick-end labeling staining was detected between wild type and transgenics tissues (data not shown). To further investigate proliferation differences levels of Cyclin D1 were analyzed. There was an increase in protein levels of Cyclin D1 in mammary gland extracts from p100 transgenics as compared with wild type (Fig. 3A). The increased Cyclin D1 may represent the initiation of a signal to attain normal levels of gland filling and lactation, which has yet to have a significant effect on proliferation. As NF-κB can also regulate multiple proteins involved in apoptosis, we investigated expression of a number of potential NF-κB downstream target genes that could be involved in apoptosis. There was no significant change in the...
expression of the anti-apoptotic proteins Bcl-2, Bcl-X$_L$, and A1 as assessed by Western analysis (Fig. 3B).

**MMP-2, MMP-9, and COX-2 Are Increased in p100 Transgenic Mice**—The regulation of MMP activity is known to be important in epithelial cell branching and normal mammary gland development (27, 28). The expression and activity of MMPs is also associated with mammary tumors and human breast cancer where they contribute to invasion and metastasis (29, 30). In addition, the expression of MMP-9 has been demonstrated to require activation of NF-$\kappa$B (31). MMP-2 activity can also be modulated by NF-$\kappa$B by the up-regulation of the activating protein MT1-MMP (32). Therefore we investigated levels of both MMPs using gelatin zymography. The results show an increase in the levels of MMP-2 and MMP-9 in mammary glands of the p100 transgenics as compared with wild type mice (Fig. 4, A and B). No bands were detected in control gels incubated in the presence of EDTA (data not shown).

The increased expression of MMPs has been associated with increased expression of COX-2 in breast cancer (33, 34). COX-2 is frequently overexpressed in human breast cancer (35), and its expression can be regulated by NF-$\kappa$B (36, 37). Therefore, we performed Western analyses using mammary protein extracts from transgenics and littermate controls. COX-2 protein was expressed in the mammary glands of pregnant p100 transgenics, while it was barely detectable in the mammary glands of wild type mice (Fig. 4C).

**Multiple Pregnancy Results in Aberrant Ductal Structures in p100 Transgenic Mice**—The BLG promoter used to generate the transgenics is a milk protein promoter that is induced during pregnancy and lactation and turns off after involution. This results in a relatively short period of exposure to the increased expression of the transgene. As constitutive transgene expression does not occur in our model, we subjected mice to multiple pregnancies to investigate potential neoplastic effects of p100/p52 overexpression. Following three pregnancies, mammary glands were harvested for whole mount analyses. In wild type mice subjected to multiple pregnancies, the glands exhibited a simple ductal structure (Fig. 5A). However, in p100 transgenics an aberrant phenotype was observed including thickening of primary ducts and small areas of hyperplastic growth as indicated by white arrows (Fig. 5A). This abnormal phenotype is further demonstrated in H and E stained tissue sections at 10x (Fig. 5B) and $\times$40 (Fig. 5C) magnification where widened ducts and a loss of epithelial cell organization and ductal structure were observed.
Expression of p100/p52 in Mammary Glands from Mice Expressing the PyVT—To further examine the link between p100/p52 and tumorigenesis, the mammary glands from PyVT transgenic mice were studied. PyVT mice spontaneously develop mammary tumors in a pattern that models human disease (38). NF-κB is an important proliferative signal in development. It is possible that the expression of NF-κB p100/p52 observed in tumorigenesis represents a loss of regulation of a developmental signal resulting in aberrant proliferation. To determine whether p100/p52 overexpression was recapitulated during mammary tumorigenesis, protein extracts from PyVT mice were analyzed. At 4 weeks, when the mammary gland is still developing and tumors are not present, a similar low level of p100/p52 expression was observed in both wild type and PyVT mice (Fig. 6A). At 7 weeks old, the PyVT mice developed multiple tumors as compared with wild type and p100 transgenics (Fig. 6B). In glands from 7-week-old animals, expression of p100/p52 was elevated in the PyVT mice, where tumors are developing, but had fallen to very low levels in the wild type controls (Fig. 6C). This suggests that overexpression of p100/p52 in mammary tumors results from a failure to down-regulate a proliferative signal after development has occurred.

DISCUSSION

In the current study we have created a novel transgenic model in order to investigate the effects of overexpression of NF-κB p100/p52 in a proliferative phase of mammary gland development. This model also presents a novel tool with which to investigate the downstream effects of p100/p52 overexpression during tumorigenesis. p100/p52 overexpression in the mammary epithelium resulted in an increase in NF-κB activity. At P16 there was a transient delay in mammary gland development in the p100 transgenics, in particular a reduction in secondary ductal branching. This was overcome by the time lactation occurred. There was a reduction in the amount of epithelium in the p100 transgenics as compared with wild type at the P16 time point. However, approximately twice the expression of Cyclin D1 protein was detected in the mammary glands of the p100 transgenics. There were also increased levels of MMP-2, MMP-9, and COX-2. In multiparous p100 transgenics that had been exposed to elevated p100/p52 for a prolonged period, an abnormal pattern of mammary ductal branching was observed with enlarged ducts and small areas of hyperplasia. We investigated whether increased p100/p52 occurred during tumor development in PyVT mice. In 4-week-old wild type and PyVT mice there was a low level of p100 and p52 expression. This may be a signal for early post-natal mammary gland development and primary ductal formation as by 7 weeks of age p100/p52 is barely expressed in the wild type. However, in PyVT mice an increase in p100/p52 was apparent at 7 weeks when tumor development is observed.

In a previous study of p100/p52 overexpression in human breast cancer cell lines it was suggested that p100 acted in the cytosol to inhibit the classical NF-κB signaling pathway (24). In the current study p100 overexpression resulted in an increase in
nuclear p52 and the activation of NF-κB. Nuclear Rel B, a binding partner of p52, was observed at similar levels in p100 transgenics compared with wild type. There was no change in nuclear levels of p65, representing the NF-κB classical signaling pathway. It is interesting that changing the alternative NF-κB signaling pathway does not result in a corresponding change in p65 levels. This is suggestive that the classical and alternative pathways can be modulated independently and that the effects that we observe are not the result of changes in the classical signaling pathway. The difference between our observations and those of the previous study may be due to the presence of additional in vivo signals, which promote p100 processing that may not be present in isolated cells. These same in vivo signaling pathways may also be important within an intact tumor.

We observed a delay of the filling of the mammary gland with epithelium in the p100 transgenics. The transient nature of this effect is reminiscent of a recent study where modulation of classical NF-κB signaling with a dominant-negative IκBα also only transiently affected mammary gland development (19). In that study the gland development appeared to be rescued by the activation of the alternative signaling pathway at later stages of pregnancy. In the current study, a rescue of the phenotype was also observed, possibly due to an elevation of Cyclin D1 after several days of p52 activity.

In previous studies the inhibition of NF-κB signaling in the mammary gland resulted in a lack of formation of secretory alveoli and an inability to lactate (17, 18). Here we observe a transient reduction in branching accompanied by an increase in NF-κB activity. This conflicting effect may be due to the direct modulation of the alternative rather than the classical NF-κB signaling pathway and is evidence that there are distinct roles for multiple NF-κB subunits in the proliferating mammary gland.

Previous studies have shown that Cyclin D1 expression is modulated by NF-κB and is important for mammary gland development (39–42). In addition, Cyclin D1 expression via the murine mammary tumor virus promoter in the mammary gland causes hyperplasia which eventually results in the formation of adenocarcinomas (43). In the current study we observed an increase in Cyclin D1 expression in the presence of increased p100/p52. This increase in Cyclin D1 activity may be responsible for an accelerated rate of proliferation during the final days of pregnancy, resulting in fully filled glands for lactation. Extended exposure to increased levels of Cyclin D1 may also be responsible for the observed hyperplastic changes after multiple pregnancies.

MMP-2 plays a role in normal mammary gland development. Indeed, during ductal development, MMP-2−/− mice have an increase in lateral branching, specifically in secondary buds and branches arising from primary ducts (44). In the p100 transgenics, a transient reduction of secondary ductal branching is observed accompanied by an increase in MMP-2 activity. Therefore, despite the presence of proliferative signals, MMP-2 may be inhibiting lateral ductal branching. An increase was also observed in MMP-9 expression. Both MMP-2 and MMP-9 have been implicated in tumorigenesis (22) and may mediate hyperplastic effects of p100/p52 overexpression.

In a transgenic model in which COX-2 expression is induced in the mammary gland using the murine mammary tumor virus promoter, precocious lobuloalveolar development is observed. Furthermore, multiparous COX-2 transgenic females display an increased incidence of focal mammary gland hyperplasia, dysplasia, and transformation into metastatic tumors (45). An elevated level of COX-2 expression is also observed in human breast tumors and cell lines where resultant prostaglandin production regulates proliferation, apoptosis, angiogenesis, and enhances invasiveness (35). Our data reveal a correlation between elevated p100/p52 levels and those of COX-2; therefore, COX-2 represents another mediator that may be up-regulated by p100/p52 and contributes to the hyperplastic changes in the multiparous p100 transgenic females.

Extended exposure to p100/p52 with concomitant increased NF-κB activity resulted in an altered morphology in the mammary gland with thickened ducts and small areas of hyperplasia. This represents an interesting parallel with a previous study carried out by our group, in which constitutive activation of the classical NF-κB signaling in mammary glands from IκBα deficient mice resulted in ductal hyperplasia (20).

In summary, we have generated a novel transgenic model in which NF-κB p100/p52 is overexpressed in the mammary gland epithelium during a reproductive cycle. This model has been used to investigate phenotypic and signaling changes during mammary gland proliferation. The increase in p100/p52 results in aberrant ductal morphogenesis, which is accompanied by an increase in Cyclin D1, MMP-2 and -9, and COX-2 expression. p100/p52 is up-regulated during breast cancer, and we have
shown, in our mouse mammary model, that this correlates with the expression of four other tumor-related genes. Furthermore, we show that in a spontaneous murine tumor model there is also an increase in p100/p52 expression. This is the first direct in vivo demonstration of a link between activation of the alternative NF-κB family in the mammary gland and the up-regulation of tumor-promoting proteins.

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