

Nuclear Factor- κ B (NF- κ B) Regulates Proliferation and Branching in Mouse Mammary Epithelium

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The nuclear factor- κ B (NF- κ B) family of transcription factors has been shown to regulate proliferation in several cell types. Although recent studies have demonstrated aberrant expression or activity of NF- κ B in human breast cancer cell lines and tumors, little is known regarding the precise role of NF- κ B in normal proliferation and development of the mammary epithelium. We investigated the function of NF- κ B during murine early postnatal mammary gland development by observing the consequences of increased NF- κ B activity in mouse mammary epithelium lacking the gene encoding I κ B α , a major inhibitor of NF- κ B. Mammary tissue containing epithelium from inhibitor κ B α (I κ B α)-deficient female donors was transplanted into the gland-free mammary stroma of wild-type mice, resulting in an increase in lateral ductal branching and pervasive intraductal hyperplasia. A two- to threefold increase in epithelial cell number was observed in I κ B α -deficient epithelium compared with controls. Epithelial cell proliferation was strikingly increased in I κ B α -deficient epithelium, and no alteration in apoptosis was detected. The extracellular matrix adjacent to I κ B α -deficient epithelium was reduced. Consistent with *in vivo* data, a fourfold increase in epithelial branching was also observed in purified I κ B α -deficient primary epithelial cells in three-dimensional culture. These data demonstrate that NF- κ B positively regulates mammary epithelial proliferation, branching, and functions in maintenance of normal epithelial architecture during early postnatal development.

INTRODUCTION

The mammary gland is an organ designed to deliver nourishment and passive immunity to infant mammals. It consists of an epithelium that synthesizes and secretes lipid and milk proteins, as well as a fatty stroma that provides support and local growth regulatory cues to the epithelium (reviewed by Medina, 1996). Although the mammary gland rudiment is established during embryogenesis, the majority of mammary gland development occurs postnatally. During puberty, the epithelium proliferates and branches in response to hormonal signals, eventually extending throughout the entire stroma. More extensive growth and differentiation of the epithelium occurs during each round of

pregnancy. The distal tips of each epithelial branch proliferate and differentiate into lobuloalveoli, which synthesize and secrete milk during lactation. Upon cessation of nursing, the majority of the epithelium undergoes apoptosis in a process called involution (reviewed by Furth, 1999). After involution, the epithelium remains relatively quiescent until the next pregnancy, when the morphogenetic cycle is repeated.

The nuclear factor- κ B (NF- κ B) family of transcription factors regulates growth, differentiation, and apoptosis in several tissues, including lymphocytes, embryonic limb, lung and liver, skin, and bone (Beg *et al.*, 1995; Klement *et al.*, 1996; Boothby *et al.*, 1997; Franzoso *et al.*, 1997; Bushdid *et al.*, 1998; Kanegae *et al.*, 1998; Seitz *et al.*, 1998; Bendall *et al.*, 1999; Hu *et al.*, 1999; Li *et al.*, 1999a,b,c; Takeda *et al.*, 1999; Tanaka *et al.*, 1999; Chen *et al.*, 2000a; Muraoka *et al.*, 2000; Rudolph *et al.*, 2000). In unstimulated cells, NF- κ B dimeric complexes are sequestered in the cytoplasm by association

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with members of a family of specific inhibitors of κB ($\text{I}\kappa\text{Bs}$). Upon receipt of extracellular stimulators, such as growth factors, cytokines, or pathogenic agents, $\text{I}\kappa\text{B}$ is phosphorylated on evolutionarily conserved amino-terminal serine residues by specific $\text{I}\kappa\text{B}$ kinases. This phosphorylation event leads to polyubiquitination and proteasome-mediated degradation of $\text{I}\kappa\text{B}$. Proteolytic degradation of $\text{I}\kappa\text{B}$ liberates the $\text{NF-}\kappa\text{B}$ dimer, allowing the active complex to translocate to the nucleus, bind specific DNA regulatory elements, and mediate changes in the expression of downstream target genes (reviewed by Verma *et al.*, 1995; Ghosh *et al.*, 1998; May and Ghosh, 1998).

The $\text{NF-}\kappa\text{B}$ family members p50 and RelA, as well as the $\text{I}\kappa\text{B}$ factors p105 and $\text{I}\kappa\text{B}\alpha$, are expressed in the murine mammary epithelium over the course of normal postnatal morphogenesis (Brantley *et al.*, 2000; Clarkson *et al.*, 2000). Moreover, maximal $\text{NF-}\kappa\text{B}$ activity is detected in the mammary gland during pregnancy, when the epithelium is proliferating, and also during involution, when the epithelium is undergoing apoptosis. These data suggest that $\text{NF-}\kappa\text{B}$ may regulate proliferation, apoptosis, or both during normal postnatal mammary epithelial morphogenesis. To ascertain the function of elevated $\text{NF-}\kappa\text{B}$ in the mammary epithelium during early postnatal morphogenesis, we have examined the morphology and development of mouse mammary epithelium lacking the gene encoding a major inhibitor of $\text{NF-}\kappa\text{B}$, $\text{I}\kappa\text{B}\alpha$. Because $\text{I}\kappa\text{B}\alpha$ -deficient mice die before the majority of mammary epithelial development occurs, a transplantation approach was taken. Mammary tissue was transplanted from $\text{I}\kappa\text{B}\alpha$ -deficient neonatal mice into wild-type, gland-free mammary fat pads to circumvent neonatal lethality and to permit postnatal development of the mammary epithelium. We demonstrate that $\text{I}\kappa\text{B}\alpha$ -deficient mammary epithelium is hyperplastic, displays increased lateral ductal branching, and contains decreased levels of extracellular matrix in virgin animals, suggesting that $\text{NF-}\kappa\text{B}$ modulates proliferation, branching, and normal structural development of the mammary epithelium during early postnatal morphogenesis.

MATERIALS AND METHODS

Mouse Strains

Generation and characterization of $\text{I}\kappa\text{B}\alpha$ -deficient mice was described by Chen *et al.* (2000a). The phenotype of these mice is consistent with independently derived $\text{I}\kappa\text{B}\alpha$ -deficient mice (Beg *et al.*, 1995; Klement *et al.*, 1996). The genotype of $\text{I}\kappa\text{B}\alpha$ -deficient neonatal mice and littermates was confirmed by Southern analysis of genomic DNA isolated from tail biopsies. Genomic DNA (10 μg) was digested with *Bam*HI and probed with a 1300-bp *Xba*I/*Nde*I fragment encompassing sequences from the 5'-flanking region of the *ikba* gene present within the *Bam*HI restriction site boundaries of the null allele (Chen *et al.*, 2000a). Wild-type, heterozygous, or $\text{I}\kappa\text{B}\alpha$ -deficient mice were housed in microisolators under identical conditions. Mice heterozygous for $\text{I}\kappa\text{B}\alpha$ were bred with homozygous HLL mice, a transgenic mouse model harboring a luciferase transgene under the regulation of the $\text{NF-}\kappa\text{B}$ -responsive human immunodeficiency virus long terminal repeat (HIV-LTR; Blackwell *et al.*, 2000; Brantley *et al.*, 2000). Mice that were *ikba*^{+/-}, and positive for the HLL transgene were intercrossed to generate *ikba*^{+/+}, *ikba*^{+/-}, and *ikba*^{-/-} mice that were either positive or negative for HLL. Again, the genotype of these animals was confirmed by Southern analysis for *ikba* wild-type and null alleles, as well as the *hll* transgene, by using genomic DNA from tail biopsy.

Tissue Luciferase Assay

Luciferase activity in the mammary tissue extracts prepared from all 10 neonatal whole mammary glands was quantified as previously described (Brantley *et al.*, 2000), and protein concentrations in the extracts were determined by Lowry assay (Bio-Rad, Richmond, CA). All values are presented as relative light units (RLUs)/ μg protein.

Mammary Tissue Transplantation

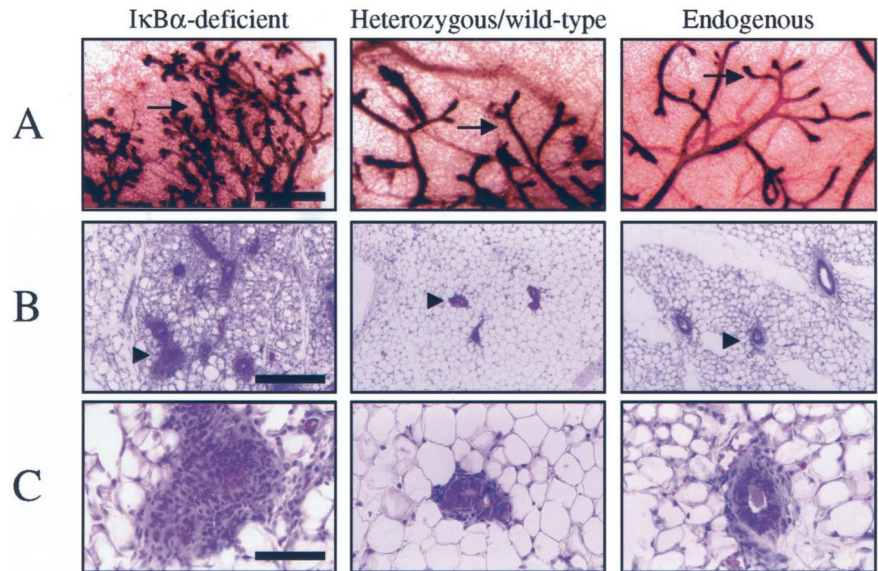
Isolation and transplantation of neonatal mammary epithelial tissue has been described previously (DeOme *et al.*, 1959; Seagroves *et al.*, 1998; Robinson *et al.*, 1999). Briefly, number 4 inguinal mammary donor tissue located between the nipple region and lymph node, the region that contains the epithelial rudiment, was surgically removed from 6-d-old female wild-type, heterozygous, or $\text{I}\kappa\text{B}\alpha$ -deficient neonatal mice and stored in DMEM (Mediatech, Herndon, VA) on ice before transplantation. The epithelium within the number 4 inguinal mammary glands of 3-wk-old virgin female C57Bl/6J recipient mice was surgically cleared by removing the portion of mammary tissue between the nipple region and the lymph node, the region in which all endogenous epithelium is contained at this stage of development. Neonatal donor tissue containing the epithelial rudiment ($\sim 2 \times 2$ mm) was then implanted into an incision in the center of the remaining surgically cleared mammary fat pad. The mammary glands were analyzed 6 to 8 wk after transplantation. A portion of these samples was used for secondary rounds of mammary tissue reconstitution of wild-type cleared mammary stroma. These secondary recipient glands were collected 6 to 8 wk later, as well as the number 3 thoracic glands harboring unmanipulated host epithelium.

Histological Analyses

Whole-mount hematoxylin staining of reconstituted mammary glands, as well as native glands from host animals, was performed as described previously (Seagroves *et al.*, 1998). Briefly, number 4 inguinal mammary glands were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.2, overnight at 4°C. The glands were washed in acetone, equilibrated into 95% ethanol, and stained in Mayer's hematoxylin solution (VWR Scientific, West Chester, PA) overnight at room temperature, light protected. The following day, the glands were destained in tap water and then further destained 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 xylenes, equilibrated into methyl salicylate (Sigma, St. Louis, MO), and photodocumented (Zeiss Stemi SV 11).

For analysis of the subcellular architecture, expression of matrix proteins, and expression of $\text{I}\kappa\text{B}\alpha$ and RelA proteins, mammary glands were embedded in paraffin and 7- μm sections prepared. Hematoxylin and eosin staining was performed as described previously (Seagroves *et al.*, 1998). Trichrome staining for visualization of the extracellular matrix was performed by the Vanderbilt University Skin Disease Research Center. Reconstituted glands were subjected to immunohistochemistry by using anti- $\text{I}\kappa\text{B}\alpha$ and anti-RelA antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) as previously described (Brantley *et al.*, 2000). Specific immunoreaction was detected using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) and 3',3'-diaminobenzidine tetrahydrochloride horseradish peroxidase substrate (Zymed Laboratories, South San Francisco, CA) according to manufacturer's protocols. Sections were counterstained with hematoxylin before dehydration, mounting in Permount (Fisher Scientific, Pittsburgh, PA), and photomicroscopy (Olympus BX60). Mammary glands from 20 to 25 primary and secondary recipients from three to four primary donors per condition were analyzed.

Figure 1. Mammary glands harboring $\text{I}\kappa\text{B}\alpha$ -deficient epithelium display increased lateral branching, pervasive intraductal hyperplasia, and abnormal epithelial morphology. (A) Whole-mount hematoxylin staining of mammary glands revealed that epithelial tissue derived from $\text{I}\kappa\text{B}\alpha$ -deficient donors displayed an increase in the number of lateral ductal branches. Arrows indicate epithelium. Representative samples are shown and all samples are age-matched. Bar, 2 mm. (B) Hematoxylin and eosin-stained sections of glands reconstituted with $\text{I}\kappa\text{B}\alpha$ -deficient, heterozygous/wild-type epithelium, and intact endogenous host glands revealed that $\text{I}\kappa\text{B}\alpha$ -deficient epithelium displayed pervasive intraductal hyperplasia. Samples are shown at low (B) and high (C) magnification. Arrowheads denote epithelium. Bar, 500 μm (B) and 50 μm (C). Data are a representation of 20 to 25 independent glands per genotype.



Proliferation and Apoptosis Assays

For proliferation assays, a sterile solution containing 10 mg/ml bromodeoxyuridine (BrdU; Sigma) in PBS was injected intraperitoneally (100 μl /10 g of body weight). After 4 h, the mice were sacrificed, mammary glands were collected, fixed, and 7- μm sections prepared. BrdU incorporation was visualized by immunohistochemistry by using a BrdU detection kit (Zymed Laboratories) according to manufacturer's protocol, and nuclei were counterstained with hematoxylin. For quantification, 10 random fields per section at 40 \times magnification were documented by photomicroscopy, and the percentage of BrdU-positive epithelial cell nuclei relative to the total number of epithelial cell nuclei was calculated. The averages of 12 to 14 independent secondary reconstitution samples derived from three to four primary donor animals per genotype were quantified. The total number of nuclei was also quantified in each of these samples.

For apoptosis assays, mammary glands were collected, fixed, and 7- μm sections prepared as described. Fragmented DNA was labeled with a digoxigenin-conjugated UTP by using terminal deoxynucleotidyl transferase (Intergen, Purchase, NY). Positive nuclei were visualized by immunohistochemistry with an ApopTag labeling and detection kit (Intergen) according to manufacturer's protocol, and nuclei were counterstained with methyl green. For quantification, 10 random fields per section were documented by photomicroscopy, and the percentage of terminal deoxynucleotide UTP nick-end labeling (TUNEL)-positive epithelial cell nuclei relative to the total number of epithelial cell nuclei was calculated. The averages of six independent primary or secondary reconstitution samples per genotype were determined.

Three-dimensional Mammary Epithelial Cell Culture

Primary mammary epithelial cells were isolated from mammary glands reconstituted with heterozygous/wild-type or $\text{I}\kappa\text{B}\alpha$ -deficient mammary tissue and embedded in reconstituted matrix (Matrigel, growth factor-reduced; Becton Dickinson, Franklin Lakes, NJ). Briefly, the reconstituted glands were surgically removed, the lymph nodes excised, and the glands minced and digested in DMEM:F12 (Cellgro) supplemented with collagenase A (1.5 mg/ml; Roche Molecular Biochemicals, Indianapolis, IN) and hyaluronidase (100 U/ml; Sigma) at 37 $^{\circ}\text{C}$, shaking. The primary cells were then washed in PBS supplemented with 10% fetal bovine serum (Hy-

clone Laboratories, Logan, UT) and embedded into growth factor-reduced Matrigel as per supplier's instructions. The three-dimensional cultures were maintained in DMEM:F12 supplemented with L-glutamine, antibiotics (Mediatech), and 10% fetal bovine serum, and the cultures were monitored over several days to observe *in vitro* tubulogenesis. The cultures were photodocumented and the number of lateral branches in 6 to 10 fields per culture quantified.

RESULTS

$\text{I}\kappa\text{B}\alpha$ -deficient Mammary Epithelium Displays Increased Ductal Branching and a Disorganized Epithelial Architecture

To ascertain the function of NF- κ B in mammary epithelial development, mammary tissue lacking the gene encoding a major inhibitor of NF- κ B, $\text{I}\kappa\text{B}\alpha$, was examined. Because $\text{I}\kappa\text{B}\alpha$ -deficient mice die \sim 9 d after birth (Beg *et al.*, 1995; Klement *et al.*, 1996; Chen *et al.*, 2000a), postnatal development of mammary epithelium was enabled by transplanting epithelium-containing mammary tissue from neonatal $\text{I}\kappa\text{B}\alpha$ -deficient female mice or heterozygous/wild-type littermates ($n = 3$ –4 primary donors/genotype) into juvenile wild-type mouse mammary fat pads in which the endogenous epithelium was surgically removed (DeOme *et al.*, 1959; Medina, 1996). Six to eight weeks after the initial transplantation, reconstituted mammary glands were isolated and used as donor tissue for a second round of reconstitution into several wild-type recipients ($n = 6$ –8 recipients/original donor/genotype) to reduce the level of contaminating $\text{I}\kappa\text{B}\alpha$ -deficient stroma introduced in the primary recipients and to provide more samples for analysis. These glands were then collected and analyzed 6 to 8 wk after transplantation. The epithelia of the reconstituted glands were stained in whole-mount with hematoxylin to visualize the gross morphology of the gland. Glands reconstituted with $\text{I}\kappa\text{B}\alpha$ -deficient epithelium contained ductal branches that permeated the fat pad to the same degree and in a pattern similar to that observed in heterozygous/wild-type epithelium, as well as

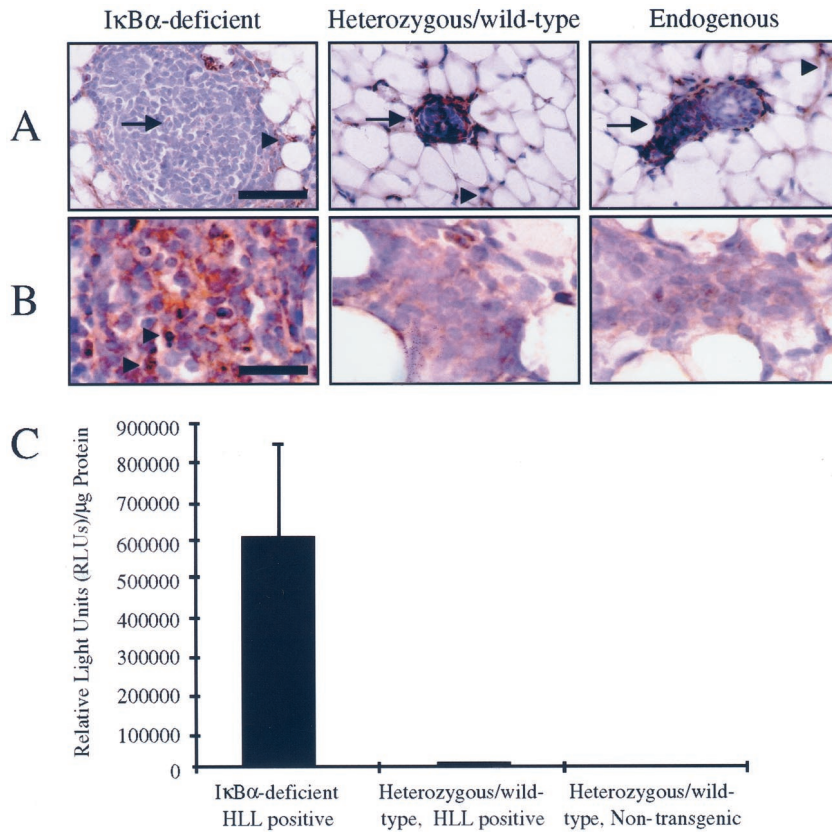


Figure 2. Lack of IκBα protein expression and enhanced transcriptional activity in IκBα-deficient mammary tissue. (A) Mammary glands reconstituted with IκBα-deficient tissue, heterozygous/wild-type tissue, or native host glands were subjected to immunohistochemistry for detection and localization of IκBα protein. Arrow indicates epithelium, arrowhead indicates surrounding host stroma. Samples shown are representative of IκBα expression for each genotype. Bar, 50 μm. (B) Mammary glands reconstituted with IκBα-deficient tissue, heterozygous/wild-type tissue, or native host glands were subjected to immunohistochemistry for detection and localization of RelA protein. Arrowhead indicates positive nuclear RelA expression. Bar, 10 μm. (C) Mammary glands from neonatal IκBα-deficient or heterozygous/wild-type littermates harboring a NF-κB-responsive luciferase reporter transgene (HLL) were analyzed for luciferase activity as a measure of in vivo NF-κB activity. An approximate 50-fold increase in luciferase activity was observed in IκBα-deficient, HLL-positive glands relative to controls. Data are a representation of three independent samples per genotype. Error bars represent SE of the mean, $p < 0.03$, χ^2 analysis.

in native host epithelium (Figure 1A). However, an overall increase in the number of lateral ductal branches was observed in IκBα-deficient epithelium. These lateral branches emanated from a larger ductal network, and displayed numerous tertiary lateral branches in the IκBα-deficient epithelium that were not observed in control glands. In addition, the epithelium displayed pervasive intraductal hyperplasia, a phenotype that was confirmed by examining the cellular architecture in histological sections (Figure 1, B and C). Compared with control glands, mammary glands harboring IκBα-deficient epithelium presented a much higher epithelial-to-stromal cell ratio (Figure 1B). In many regions, the central ductal lumina were absent in IκBα-deficient epithelium or were surrounded by multiple epithelial cell layers rather than a single layer as seen in heterozygous/wild-type epithelium or host epithelium (Figure 1, B and C). Higher magnification revealed that the IκBα-deficient epithelial structure appeared to be disorganized (Figure 1C).

To confirm that IκBα protein was absent in the epithelium in mammary glands reconstituted with IκBα-deficient tissue, immunohistochemical analysis was performed. IκBα was not detected in mammary epithelium derived from null animals, although IκBα was present in the surrounding host stroma (Figure 2A). Consistent with previous studies, expression of IκBα was observed in mammary epithelium derived from heterozygous/wild-type littermates and also in host mammary epithelium from unmanipulated glands (Figure 2A; Brantley *et al.*, 2000). These data suggest that loss of IκBα in the mammary is responsible for increased ductal

branching and alteration of the histological structure of the epithelium derived from *ikbα*-defective donors.

To assess expression levels and localization of RelA, a major transactivator within the NF-κB family that is present in mammary epithelium, immunohistochemical analysis was performed (Brantley *et al.*, 2000). Compared with mammary glands reconstituted with heterozygous/wild-type epithelium or endogenous epithelium, glands reconstituted with IκBα-deficient epithelium displayed an increase in expression and nuclear localization of RelA (Figure 2B). These data confirm that loss of IκBα in the mammary epithelium results in increased nuclear import of the transactivator RelA.

To determine whether the morphological alterations of the IκBα-deficient epithelium are due to elevated NF-κB activity, *ikbα*^{+/-} mice were mated to HLL mice, a transgenic mouse model expressing a luciferase reporter transgene under the regulation of the NF-κB-responsive HIV-LTR. Because the HIV-LTR contains two NF-κB enhancer elements that bind a broad range of homodimeric and heterodimeric NF-κB complexes (Kretzschmar *et al.*, 1992; Liu *et al.*, 1992; Doerre *et al.*, 1993), HLL transgenic animals have been used as a model to quantify both constitutive and induced NF-κB activity in vivo in several organ systems, including the mammary gland (Blackwell *et al.*, 2000; Brantley *et al.*, 2000; Hao *et al.*, 2000). Mice heterozygous for the targeted *ikbα* allele and hemizygous for the transgene were intercrossed to generate *ikbα*^{-/-}, *ikbα*^{+/-}, or *ikbα*^{+/+} offspring that also harbored the HLL transgene. Quantification of luciferase

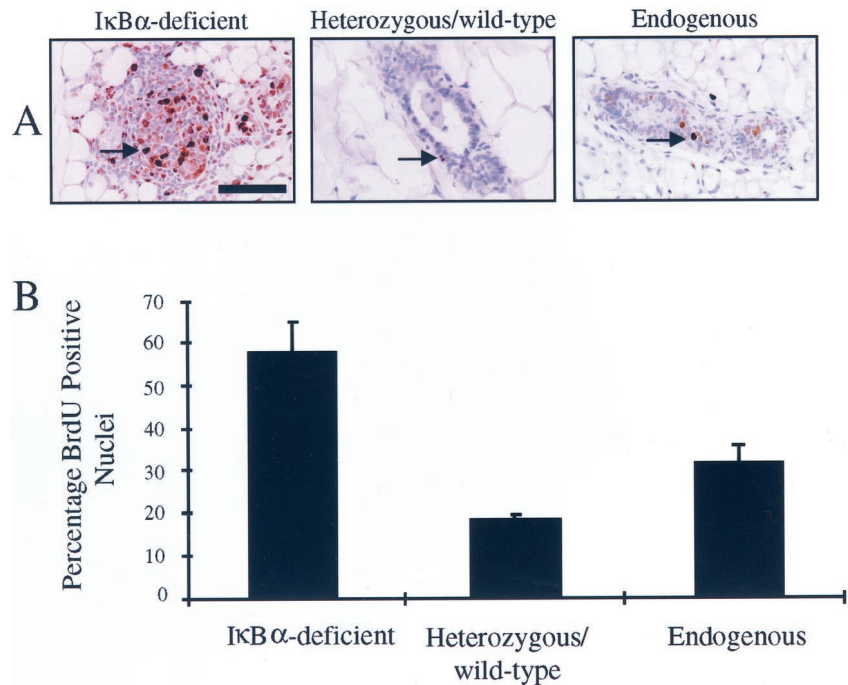


Figure 3. Mammary glands harboring I κ B α -deficient epithelium display increased epithelial cell proliferation. (A) Immunohistochemical analysis of BrdU incorporation into glands reconstituted with I κ B α -deficient tissue or with heterozygous/wild-type tissue, or endogenous host epithelium revealed hyperplasia in I κ B α -deficient epithelium. Arrows indicate BrdU-positive nuclei. Bar, 50 μ m. (B) Percentage of BrdU-positive nuclei was calculated according to the following formula: no. of positive nuclei/no. of total nuclei per 40 \times field. Values presented are the mean of a total of 10 individual fields from each section analyzed. A two- to threefold increase in the percentage of BrdU incorporation was observed in I κ B α -deficient epithelium. Data are a representation of 12 to 14 independent secondary reconstitution samples derived from three to four primary donor animals per genotype. Error bars represent SE of the mean, $p < 0.0004$, χ^2 analysis.

activity in neonatal mammary gland extracts collected from these mice revealed an ~ 50 -fold increase in luciferase reporter activity in I κ B α -deficient mice compared with wild-type/heterozygous littermates or nontransgenic animals (I κ B α -deficient, HLL-positive $596,849 \pm 238,716$ RLU/ μ g protein; heterozygous/wild-type, HLL-positive $11,906 \pm 2,454$ RLU/ μ g protein; heterozygous/wild-type, HLL negative $3,402 \pm 1,964$ RLU/ μ g protein, $p > 0.03$, χ^2 analysis; Figure 2C). The level of luciferase activity in heterozygous/wild-type, HLL-positive mammary extracts are consistent with previously published levels of luciferase activity in virgin HLL mammary glands (Brantley *et al.*, 2000). Therefore, loss of I κ B α results in elevated activity of NF- κ B, suggesting that morphological abnormalities of I κ B α -deficient mammary epithelium are due to increased NF- κ B activity.

I κ B α -deficient Mammary Epithelium Displays Increased Epithelial Proliferation, but no Change in Apoptosis

The observed increase in the epithelial content in mammary glands harboring I κ B α -deficient epithelium may reflect an increase in the rate of epithelial proliferation and/or a decrease in the level of epithelial apoptosis. We tested both of these possibilities by quantifying BrdU incorporation and TUNEL assays, respectively. To assess the levels of proliferation in I κ B α -deficient epithelium versus heterozygous/wild-type or host epithelium, reconstituted virgin animals were labeled with the thymidine analog BrdU. Based on immunohistochemical analysis, the number of BrdU-positive nuclei in I κ B α -deficient mammary epithelium appeared to be greater than that in mammary glands reconstituted with heterozygous/wild-type tissue or in native mammary epithelium (Figure 3A). To quantify this increase, the per-

centage of BrdU-positive nuclei relative to the total number of nuclei was calculated. A two- to threefold increase in the percentage of BrdU-positive epithelial nuclei was observed in I κ B α -deficient epithelium versus control epithelium (I κ B α -deficient: $57 \pm 7\%$; heterozygous/wild-type: $18 \pm 1\%$; endogenous $30 \pm 5\%$, $p < 0.0004$, χ^2 analysis; Figure 3B). Because glands reconstituted with I κ B α -deficient epithelium appear to contain a greater number of epithelial cells than age-matched controls, epithelial cell density was also quantified. Consistent with the higher level of proliferation in I κ B α -deficient epithelium, there was a striking increase in the number epithelial cells per 40 \times field (I κ B α -deficient: 276 ± 50 cells/field; heterozygous/wild-type: 93 ± 12 cells/field; endogenous: 105 ± 16 cells/field, $p < 0.0004$, χ^2 analysis). These data demonstrate that mammary epithelium lacking I κ B α is indeed hyperplastic in virgin animals.

To assess the level of apoptosis in I κ B α -deficient mammary epithelium, TUNEL analysis was performed on sections prepared from reconstituted or control virgin glands. TUNEL-positive nuclei were detected by immunohistochemistry. Based on immunohistochemical analysis, the percentage of TUNEL-positive nuclei in I κ B α -deficient mammary epithelium was similar to the percentage in mammary glands reconstituted with heterozygous/wild-type tissue or in native mammary epithelium (Figure 4A). This was confirmed by quantification of the levels of apoptosis in the epithelium. As predicted based on immunohistochemical analysis, no statistically significant difference in the percentage of TUNEL-positive nuclei relative to the total number of nuclei was detected between I κ B α -deficient mammary epithelium and epithelium derived from heterozygous/wild-type donors or native host epithelium (I κ B α -deficient: $2.6 \pm 0.13\%$; heterozygous/wild-type: $3.8 \pm 0.42\%$; endogenous: $2.7 \pm 0.30\%$, $p > 0.7$, χ^2 analysis; Figure 4B). These data

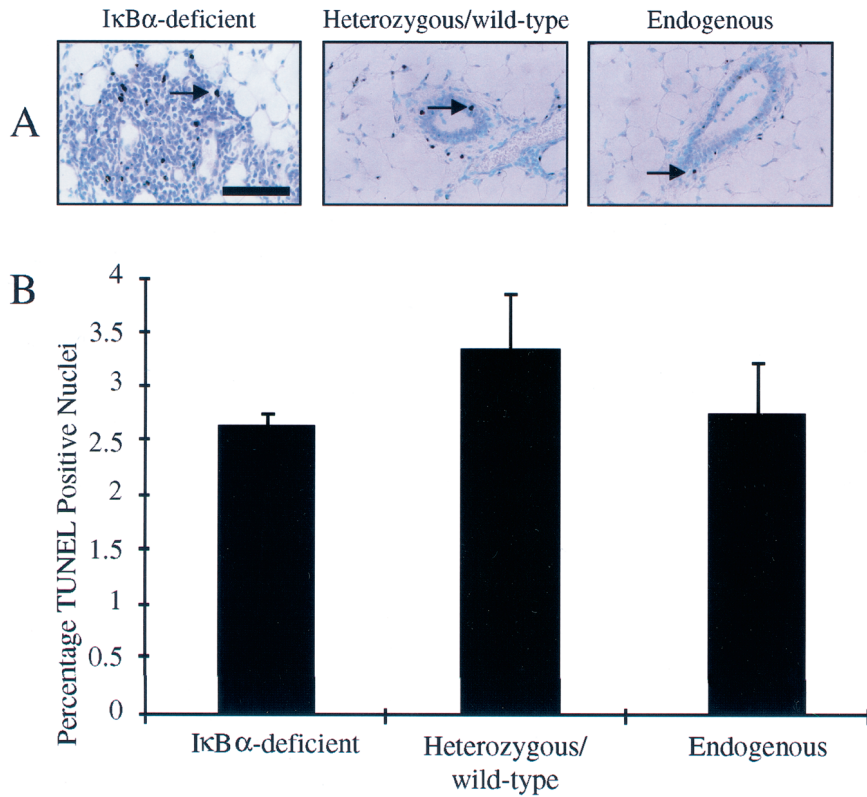


Figure 4. Mammary glands harboring IκBα-deficient epithelium display no significant alteration in epithelial apoptosis. (A) Immunohistochemical detection of TUNEL-positive nuclei in sections from mammary glands containing IκBα-deficient epithelium, heterozygous/wild-type epithelium, or endogenous host epithelium revealed no apparent difference in the level of apoptosis. Arrows indicate TUNEL-positive nuclei. Bar, 50 μm. (B) Percentage of TUNEL-positive nuclei was calculated according to the following formula: no. of positive nuclei/no. of total nuclei per 40×. Values presented are the mean of a total of 10 individual fields from each section analyzed. No difference in the percentage of TUNEL-positive nuclei was observed in IκBα-deficient epithelium. Data are a representation of six independent samples per genotype. Error bars represent SE of the mean, $p < 0.7$, χ^2 analysis.

suggest that, although proliferation and cell density are increased, IκBα-deficiency in the mammary epithelium does not affect apoptosis in virgin animals.

IκBα-deficient Mammary Epithelium Displays Decreased Adjacent Extracellular Matrix

The epithelial architecture in mammary glands reconstituted with IκBα-deficient mammary tissue appeared disorganized and poorly confined by the extracellular matrix normally surrounding the ducts (Figure 1C). To examine this defect more closely, sections from mammary glands harboring

IκBα-deficient mammary epithelium or heterozygous/wild-type epithelium and sections from host glands were subjected to trichrome staining to visualize extracellular matrix (ECM) proteins (Figure 5). Although trace amounts of ECM are detected, staining of ECM proteins (shown in blue) was strikingly reduced adjacent to IκBα-deficient donor epithelium, compared with heterozygous/wild-type donor epithelium and intact host epithelium (Figure 5, A and B). ECM expression surrounding the vascular endothelium of the reconstituted glands remained intact. Because the vasculature of the reconstituted gland is host-derived, it might be

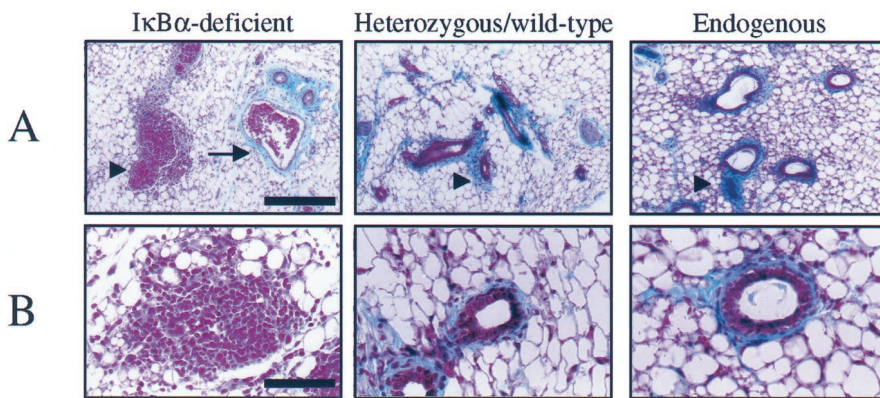
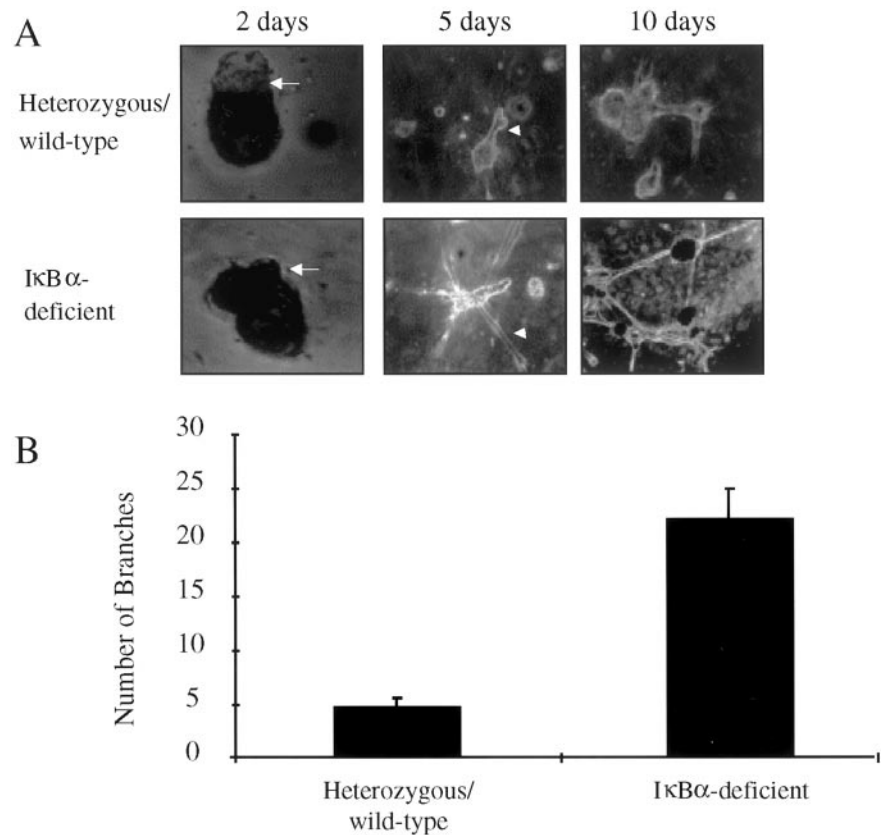


Figure 5. Mammary glands harboring IκBα-deficient epithelium display a reduction in ECM. (A) Trichrome staining was used to visualize the ECM adjacent to IκBα-deficient epithelium, heterozygous/wild-type epithelium, or endogenous host epithelium. The ECM adjacent to IκBα-deficient epithelium was much reduced relative to the ECM in controls. Arrowheads indicate ECM stained in blue. This reduction was specifically associated with the epithelium, because ECM adjacent to blood vessels in IκBα-deficient samples was intact. Arrow indicates blood vessels. Bar, 100 μm. (B) Higher magnification further illustrates the reduction in ECM adjacent to IκBα-deficient epithelium relative to controls. Bar, 50 μm. Data are a representation of five independent samples per genotype.

Figure 6. Mammary epithelial cells from $\text{I}\kappa\text{B}\alpha$ -deficient glands display increased branching and tubulogenesis in three-dimensional culture. (A) Primary mouse mammary epithelial cells were isolated from glands reconstituted with $\text{I}\kappa\text{B}\alpha$ -deficient tissue or with heterozygous/wild-type tissue and were maintained in three-dimensional Matrigel cultures. Cultures were assessed for tubulogenesis, branching, and outgrowth at 2, 5, and 10 d of culture and were photodocumented accordingly. Photographs shown are representative samples. Photographs taken on day 2 are 400 \times , whereas those taken on days 5 and 10 are 200 \times . Arrows indicate primordial tubules, arrowheads indicate branches. An apparent increase in the rate of branching and in the number/complexity of branching was observed in cultures containing $\text{I}\kappa\text{B}\alpha$ -deficient epithelial cells. (B) Number of branches in $\text{I}\kappa\text{B}\alpha$ -deficient epithelial cultures and in control cultures after 10 d was determined. A fourfold increase in the number of branches was observed in $\text{I}\kappa\text{B}\alpha$ -deficient epithelial cultures. Values presented are the mean of three to six independent colonies per genotype. Data are a representation of 6 to 10 independent samples per genotype. Error bars represent SE of the mean, $p < 0.003$, χ^2 analysis.



expected that vessel ECM expression is unaffected. This strongly supports the notion that alteration in ECM expression is specifically due to $\text{I}\kappa\text{B}\alpha$ deficiency within the mammary epithelium, particularly because contaminating $\text{I}\kappa\text{B}\alpha$ -deficient stroma cotransplanted with the epithelial rudiment is greatly reduced by transplantation into secondary recipients after initial transplantation from neonatal donors. Furthermore, these data suggest that $\text{I}\kappa\text{B}\alpha$ /NF- κ B plays a pivotal role in the proper structural development of the mammary gland.

Increased Branching in $\text{I}\kappa\text{B}\alpha$ -deficient Epithelium Is Intrinsic to the Epithelium

To determine whether the branching and proliferation defects are intrinsic to $\text{I}\kappa\text{B}\alpha$ -deficient epithelium, purified epithelial cells from mammary glands reconstituted with $\text{I}\kappa\text{B}\alpha$ -deficient tissue or with heterozygous/wild-type tissue were embedded within Matrigel, an artificial basement membrane. These purified epithelial cells were maintained in three-dimensional culture and monitored for growth and tubulogenesis. After 2 d in culture, both heterozygous/wild-type and $\text{I}\kappa\text{B}\alpha$ -deficient cells formed epithelial protuberances that represent primordial tubules (Figure 6A). Although both heterozygous/wild-type and $\text{I}\kappa\text{B}\alpha$ -deficient cells formed tubule branches by 5 d in culture, $\text{I}\kappa\text{B}\alpha$ -deficient cells formed a greater number of these branches. The branches composed of $\text{I}\kappa\text{B}\alpha$ -deficient cells were of greater length than controls. These observations were even more

apparent after 10 d in culture, with an increase in the number of branches and in the structural complexity of this interconnecting tubule network. To quantify the increase in branching within $\text{I}\kappa\text{B}\alpha$ -deficient cultures, the number of branches in heterozygous/wild-type cultures and in $\text{I}\kappa\text{B}\alpha$ -deficient cultures was determined. A fourfold increase in the number of branches in $\text{I}\kappa\text{B}\alpha$ -deficient cultures relative to wild-type cultures was observed after 10 d in culture ($\text{I}\kappa\text{B}\alpha$ -deficient: 22.5 ± 2.9 branches/focal plane; heterozygous/wild-type: 5.2 ± 0.58 branches/focal plane, $p < 0.003$, χ^2 analysis; Figure 6B). These data suggest that $\text{I}\kappa\text{B}\alpha$ deficiency results in increased branching outgrowth of the mammary epithelium, and that these effects are intrinsic to epithelial cells.

DISCUSSION

Post-natal morphogenesis of the mammary gland epithelium is tightly regulated by signal transduction cascades involving the activation of transcription factors that modulate changes in gene expression. By analyzing $\text{I}\kappa\text{B}\alpha$ -deficient mammary tissue that lacks a major endogenous inhibitor of NF- κ B transcriptional activity, we have been able to determine the consequences of elevated NF- κ B activity on the growth and morphogenesis of mammary epithelium. Here, we provide novel, *in vivo* evidence that hyperactivation of NF- κ B within the mammary epithelium perturbs normal growth and development of this organ. In previous studies,

mice lacking $I\kappa B\alpha$ exhibited elevated levels of NF- κ B activity in several tissues, including B and T lymphocytes and skin (Beg *et al.*, 1995; Klement *et al.*, 1996; Chen *et al.*, 2000a). We show here that the absence of $I\kappa B\alpha$ protein also causes a remarkable elevation in NF- κ B activity in mammary tissue, as well as elevated expression and nuclear localization of RelA. This is consistent with the known expression of NF- κ B factors p50 and RelA in virgin mammary tissue (Brantley *et al.*, 2000; Clarkson *et al.*, 2000). In wild-type virgin mammary glands, NF- κ B activity is low, even though p50 and RelA are expressed. This suggests that inhibition of NF- κ B is accomplished by one of the family of $I\kappa$ Bs, likely $I\kappa B\alpha$, which is also expressed in wild-type virgin mammary tissue.

Increased epithelial growth of $I\kappa B\alpha$ -deficient epithelium was clearly evident by the number of epithelial cells present in the glands reconstituted with $I\kappa B\alpha$ -deficient tissue, and BrdU labeling confirmed a significant increase in epithelial cell proliferation. This increase in proliferation may contribute to the altered morphology of the $I\kappa B\alpha$ -deficient epithelium. For example, the increase in branching of the $I\kappa B\alpha$ -deficient epithelium may be a direct result of increased proliferation, and the reduced levels of ECM expression may be due to heightened proliferation in the $I\kappa B\alpha$ -deficient epithelium. Correlations between levels of proliferation and branching morphogenesis have been observed in other branching organ systems, including lung, another organ that uses NF- κ B to establish epithelial branching patterns through epithelial–mesenchymal interactions (Serra *et al.*, 1994; Muraoka *et al.*, 2000). Alternatively, the reduction of ECM may be a direct effect of increased NF- κ B activity. This hypothesis is also plausible, because several biochemical and *in vivo* studies have demonstrated that NF- κ B can regulate the expression of ECM-degrading matrix metalloproteinases enzymes, such as MMP-1, MMP-3, MMP-9, and urokinase-type plasminogen activator (Hansen *et al.*, 1992; Mohan *et al.*, 1998; Bond *et al.*, 1999; Wang *et al.*, 1999; Yan *et al.*, 2001). Increased expression of ECM-degrading enzymes and subsequent loss of ECM components could then permit increased branching and proliferation as a secondary effect in $I\kappa B\alpha$ -deficient mammary epithelium. Precedence for this hypothesis comes from the observation that restoration of proper cell–ECM contacts in malignant breast cancer cells was found to restore normal tissue architecture and growth in three-dimensional culture, as well as reduced tumor formation *in vivo*, suggesting that loss of cell–ECM contacts are permissive for abnormal growth and malignancy (Weaver *et al.*, 1997).

The effect of $I\kappa B\alpha$ deficiency on the morphology of the mammary epithelium is profound and appears to be intrinsic to the epithelium. Increased branching and tubulogenesis were observed in purified mammary epithelial cell cultures. Stromal contamination in these cultures is absent; therefore, the possibility of indirect effects of the mesenchyme is eliminated. This is a critical consideration, because the mammary epithelium relies heavily on the corresponding stroma for signaling and support. These data demonstrate that NF- κ B regulates both proliferation and the maintenance of the normal architecture of the mammary epithelium. Increasing evidence of NF- κ B overexpression and elevated activity in various human breast cancer cell lines and primary tumors supports the implications of the results presented in this study.

We have demonstrated that elevated levels of NF- κ B activity result in pervasive intraductal epithelial hyperplasia. Several studies have provided support for the function of NF- κ B in regulating proliferation *in vivo*. Previous studies revealed that $I\kappa B\alpha$ -deficient mice exhibited epidermal hyperplasia, with an increase in the number of proliferative keratinocytes relative to the number of differentiated keratinocytes (Klement *et al.*, 1996; Chen *et al.*, 2000b). Targeted disruption of the gene encoding $I\kappa$ B kinase α resulted in down-regulation of NF- κ B transcriptional activity and perturbed normal proliferation and differentiation of the epidermis (Hu *et al.*, 1999; Li *et al.*, 1999; Takeda *et al.*, 1999). Conversely, targeted disruption or inhibition of NF- κ B family members in knockout/transgenic mice perturbed the proliferation and function of B and T lymphocytes (Kontgen *et al.*, 1995; Sha *et al.*, 1995; Weih *et al.*, 1995; Boothby *et al.*, 1997; Horwitz *et al.*, 1997; Bendall *et al.*, 1999; Grossmann *et al.*, 1999). Moreover, inhibition of NF- κ B activity blocked growth within the developing chick limb (Bushdid *et al.*, 1998; Kanegae *et al.*, 1998). These data provide precedence for positive regulation of cell proliferation by NF- κ B.

Epithelial morphogenesis and lateral ductal branching of the mammary epithelium during puberty are regulated by interactions between the epithelium and the surrounding stroma (reviewed by Cunha and Hom, 1996; Robinson *et al.*, 1999). NF- κ B family members have been shown to modulate epithelial–mesenchymal interactions in embryonic limb and lung, suggesting that NF- κ B may play a similar role in the regulation of growth and branching within the mammary epithelium. This is particularly intriguing because elevated NF- κ B activity in the mammary epithelium results in increased branching and growth. NF- κ B expression is induced in embryonic limb mesenchyme by signals derived from a specialized epithelial structure called the apical ectodermal ridge that is adjacent to the limb mesenchyme. Disrupting NF- κ B activity in embryonic limb mesenchyme impaired limb outgrowth and resulted in aberrant morphology of the apical ectodermal ridge (Bushdid *et al.*, 1998). Similar experiments in embryonic lung demonstrated that activity of NF- κ B in lung mesenchyme is necessary for proper budding and growth of the adjacent lung epithelium (Muraoka *et al.*, 2000). Based on data derived from these model systems, it is possible that NF- κ B activity in the mammary epithelium is regulated by paracrine signals emanating from the adjacent stroma, or even neighboring epithelial cells, to control epithelial branching and proliferation during early mammary gland morphogenesis. In this scenario, loss of $I\kappa B\alpha$ would constitutively activate NF- κ B, mimicking the growth-activating signals from the stroma.

Loss of $I\kappa B\alpha$ in the mammary gland epithelium did not result in any change in the level of epithelial apoptosis in virgin animals, which is somewhat surprising given that several biochemical and *in vivo* studies have demonstrated that RelA expression and activity confer protection against apoptosis (Beg *et al.*, 1995; Beg and Baltimore, 1996; Liu *et al.*, 1996; Van Antwerp *et al.*, 1996; Wang *et al.*, 1996; Li *et al.*, 1999). Apoptosis of mammary epithelial cells during puberty is limited to small regions adjacent to terminal end buds during the process of lumen formation, and therefore it is possible any decrease in the level of apoptosis in $I\kappa B\alpha$ -deficient mammary epithelium may be below the limits of detection at this stage of development. It has been noted that

a large portion of the I κ B α -deficient mammary epithelium does not form lumina. The cause of this phenomenon is unknown, but based on results presented here, it is does not appear to be a result of a decrease in apoptosis at this stage of development. It will be interesting to ascertain the function of NF- κ B in mammary epithelial apoptosis *in vivo*, particularly during involution of the mammary epithelium. Very recently, nuclear RelA expression has been correlated with nonapoptotic epithelial cells within involuting mammary glands (Clarkson *et al.*, 2000). Moreover, activation of NF- κ B in a mammary cell culture model that mimics involution *in vitro* enhanced survival of these cells relative to controls. It is possible that NF- κ B functions to block apoptosis only during involution while functioning to promote proliferation during early morphogenesis, because apoptosis is not affected in virgin I κ B α -deficient mammary epithelium and proliferation is enhanced.

CONCLUSION

The NF- κ B family of transcription factors has been shown to regulate proliferation in several cell types. Here, we present the first *in vivo* evidence that NF- κ B activity regulates ductal branching and proliferation of the mammary epithelium and that increased activity disrupts the normal architecture of the epithelium. It would be of great interest to determine whether perturbations in NF- κ B activity contribute to the development of breast adenocarcinoma. Several recent studies have demonstrated that many NF- κ B family members are aberrantly expressed and/or activated in breast cancer cell lines and in primary tumors (Nakshatri *et al.*, 1997; Sovak *et al.*, 1997; Sovak *et al.*, 1999; Newton *et al.*, 1999). Amplification at the *relA* locus was observed in human breast adenocarcinomas (Matthew, *et al.*, 1993). In addition, overexpression and enhanced nuclear localization of p50, p52, c-Rel, and Bcl-3 have been reported in breast cancer cell lines and in human tumors (Dejardin *et al.*, 1995; Mukhopadhyay *et al.*, 1995; Cogswell *et al.*, 2000). In addition, the induction of NF- κ B activity may be an early event in chemical carcinogenesis, because NF- κ B activity was induced *in vivo* and in cell culture by 7,12-dimethylbenz[α]anthracene before neoplastic transformation (Kim *et al.*, 2000). Further investigation is required to delineate precisely the role NF- κ B plays in breast cancer and may lead to identification of new molecular targets for therapeutic interventions. We are currently monitoring mice reconstituted with I κ B α -deficient or heterozygous epithelium for tumor development. The data presented in this study demonstrate that NF- κ B plays a role in normal mammary epithelial proliferation during development, supporting a potential function for NF- κ B in the pathology of breast cancer.

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