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The Rel/NF-κB family of transcription factors has been implicated in such diverse cellular processes as proliferation, differentiation, and apoptosis. These processes occur during mammary gland morphogenesis, and we previously demonstrated that NF-κB factors are expressed and active in the mammary gland during pregnancy and involution, when the epithelium is proliferating and undergoing apoptosis, respectively. Moreover, NF-κB activity is upregulated in breast cancer derived cell lines and primary adenocarcinomas. To address the function of NF-κB activity during early post-natal morphogenesis, the development of *iκbα*-deficient mammary epithelium in a wild-type stromal environment was examined. Removal of this major inhibitor resulted in elevated NF-κB transcriptional activity in the mammary gland. The *iκbα*-deficient mammary epithelium displayed disorganization and pervasive intraductal hyperplasia, with a 2 to 3 fold increase in epithelial cell number and proliferation in virgin animals. No change in the level of apoptosis in *iκbα*-deficient mammary epithelium, relative to controls, was detected. The extracellular matrix adjacent to *iκbα*-deficient epithelium was greatly reduced, and epithelial adherins junctions were disrupted. These data demonstrate that NF-κB positively regulates proliferation of the mammary epithelium, and suggest that NF-κB may contribute to mammary epithelial tumorigenesis.

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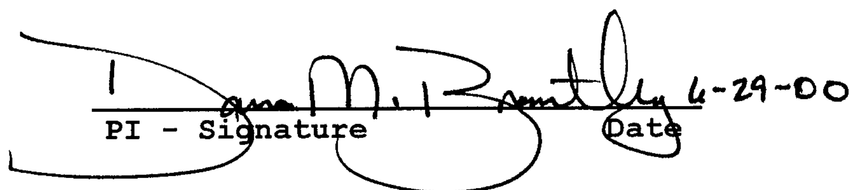

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Introduction

Cell growth and differentiation are fundamental to the development and function of all tissues and organs. Misregulation of growth and differentiation is a major contributor to neoplasia and tumorigenesis. Transcription factors are critical mediators of these cellular processes, as these proteins bind to regulatory sequences within genes and enhance or repress their expression. The Rel/Nuclear Factor-kappa B (NF-κB) family of ubiquitously expressed transcription factors has been shown to regulate proliferation, differentiation, and cell death in several different cell types, and is characterized by its unique and rapid activation mechanism. NF-κB complexes are normally sequestered in the cytoplasm of resting cells by association with specific inhibitory molecules, called IκBs (Verma et al., 1995; May et al., 1998; Ghosh, May and Kopp, 1998). Upon receipt of a growth factor, cytokine, or pathogenic signal, the IκB molecule is phosphorylated by specific kinases, called IKKs. This phosphorylation event triggers pr oteolytic degradation of the inhibitor, which releases the NF-κB complex to translocate to the nucleus to modulate gene transcription (Karin 1999; Zandi and Karin, 1999).

NF-κB regulates proliferation, differentiation, and cell death in lymphoid cells of the immune system, fetal liver cells, bone, skin, and embryonic limb and lung *in vivo* (Beg et al., 1995a; Kötegen et al., 1995; Sha et al., 1995; Weih et al., 1995; Boothby et al., 1997; Esslinger et al., 1997; Attar et al., 1998; Schwarz et al., 1997; Li et al., 1999; Franzoso et al., 1997; Beg et al., 1995b; Klement et al., 1996; Seitz et al., 1998; Hu et al., 1999; Takeda et al., 1999; Bushdid et al., 1998; Kanagae et al., 1998; Muraoka et al., in press). All of these diverse cellular events also occur in the mammary glands of female mammals during and after each cycle of pregnancy. The epithelial cells within the mammary gland normally proliferate during pregnancy and specific cells at the end of each duct begin to differentiate into milk-producing, secretory alveolar cells near the end of pregnancy (Medina, 1996). Following cessation of nursing, the mammary epithelium undergoes an involution process that is mediated by apoptosis (Furth, 1999). As the epithelial cells are the targets of neoplastic transformation in breast cancer, and as NF-κB expression and activity is elevated in several breast cancer-derived epithelial cell lines and in primary tumors (Dejardin et al., 1995; Nakashatri et al., 1997; Sovak et al., 1997; Sovak et al., 1999; Cogswell et al., 2000), the expression and function of NF-κB factors and inhibitors in the murine mammary gland over the course of post-natal morphogenesis was investigated. Analysis of the expression and activity patterns of NF-κB in the developing mouse mammary gland demonstrated that NF-κB factors and inhibitors were expressed in the mammary epithelial cells during post-natal mammary gland morphogenesis. Moreover, NF-κB activity in the mammary gland correlated with epithelial proliferation during pregnancy and epithelial apoptosis during involution (Brantley et al., in press). These data suggest that NF-κB functions to promote growth and/or differentiation of the mammary gland epithelium during pregnancy, as well as apoptosis during involution.

In order to address the function of NF-κB in the mammary epithelium, mammary epithelial morphogenesis in mice lacking a major inhibitor of NF-κB, IκBα, was examined. As these mice exhibit perinatal lethality approximately 9 days after birth, mammary tissue from neonatal mice was isolated and transplanted into wild-type recipient mice stroma in which the endogenous epithelium had been surgically removed (DeOme et al., 1959; Medina, 1996). Donor tissue from *iκbα*-deficient exhibited elevated NF-κB transcriptional activity. Mammary epithelium lacking *iκbα* displayed an increase in lateral ductal branching and pervasive intraductal hyperplasia. A 2 to 3 fold increase in epithelial cell number and proliferation was observed in *iκbα*-deficient mammary epithelium relative to controls. No alteration in the level of apoptosis in *iκbα*-deficient mammary epithelium relative to controls was observed. Extracellular matrix adjacent to *iκbα*-deficient mammary epithelium was greatly reduced, and altered adherins junctions were observed. These data demonstrate that NF-κB positively regulates mammary epithelial proliferation *in vivo*, and suggests that NF-κB may contribute to mammary epithelial tumorigenesis.

Body

Experimental Methods

Materials and methods for immunoblot, immunohistochemistry, electrophoretic mobility shift assay, and quantification of luciferase reporter activity in transgenic mammary tissue are detailed in the first manuscript appended to this report (Brantley et al., in press). Materials and methods for mammary gland transplantation and analysis of *iκbα*-deficient mammary epithelium are detailed in the second manuscript appended to this report (Brantley et al., submitted).

Results and Discussion

NF-κB and IκBα are Expressed in the Mammary Epithelium. NF-κB DNA-binding and *In Vivo* Transcriptional Activity are Maximal During Pregnancy and Involution

Analysis of the normal expression patterns of NF-κB/IκBα over the course of post-natal development (Technical Objectives: Tasks 1 and 2) has been completed. The data have been incorporated into a manuscript that is in press (*Mechanisms of Developmentt*) and this manuscript has been appended to this report. Immunoblot and immunohistochemical analyses demonstrated that p50 and RelA subunits of NF-κB, as well as the major inhibitor IκBα, were expressed in the mammary gland, with higher level expression in the epithelium (Figures 1, 2, and 3; appended manuscript number one). Expression levels of p50 and RelA appeared to be slightly elevated during pregnancy and involution as compared to levels seen during lactation, and nuclear p50 and RelA were detected during pregnancy and involution. These data suggest that NF-κB is likely active during pregnancy and involution, but not during lactation given elevated expression of IκBα.

To assess the DNA-binding and transcriptional activity of NF-κB over the course of post-natal mammary gland development, electrophoretic mobility shift assays (EMSAs) on mammary nuclear extracts and quanitfication of reporter activity in transgenic mice (HLLs; Blackwell et al., in press) expressing luciferase under the control of the NF-κB responsive human immunodeficiency virus long terminal repeat (HIV-LTR) were performed (Technical Objectives: Tasks 4 and 7). These data have also been incorporated into the appended manuscript (number one). EMSAs demonstrated that NF-κB complexes containing p50 and RelA were present in mammary nuclear extracts collected from virgin, pregnant, and involuting animals (Figure 4, appended manuscript number one). Virtually no NF-κB DNA-binding was observed in nuclear extracts collected from lactating animals, as predicted based on elevated expression of IκBα at this stage of development. Quantification of NF-κB-driven luciferase activity in mammary gland extracts collected from HLL transgenic mice confirmed maximal NF-κB transcriptional activity during pregnancy and involution (Figure 5, appended manuscript). Consistent with DNA-binding activity, reporter activity in samples from lactating animals droped to the level of non-transgenic controls. These data demonstrate that NF-κB expression and activity are maximal in the mammary gland during pregnancy and involution, but not during lactation, suggesting that NF-κB may regulate proliferation and differentiation during pregnancy, as well as apoptosis during involution.

Task 3 under technical objectives (analysis of NF-κB expression in normal and transformed human breast tissue samples) is ongoing, with sample acquisition underway. For progress on Tasks 5, 6, and 8 (involving analysis of transgenic animals expressing the transdominant inhibitor of NF-κB (IκB-αΔN) and NFKB2 under the control of the sheep betalactoglobulin promoter), please refer to the final report for grant number DAMD17-97-7266, as this work is being completed in collaboration with grantee Dr. Fiona E. Yull, Research Assistant Professor also in the laboratory of Dr. Lawrence D. Kerr.

Analysis of *iκbα*-Deficient Mammary Epithelium Demonstrates that Elevated NF-κB Activity Enhances Lateral Ductal Branching, Induces Epithelial Hyperplasia, and Results in Disorganization in the Mammary Epithelium of Virgin Animals

In addition to studies outlined in the Statement of Work, studies utilizing *iκbα*-deficient mice recently generated in Dr. Kerr's laboratory have been pursued to determine how removal of this major inhibitor, and thus elevated NF-κB activity, affects post-natal mammary gland morphogenesis. These mice, like similar IκBα deficient mice generated by other investigators (Beg et al., 1995b; Klement et al., 1996), exhibit skin and immunological defects and die approximately 9 days after birth (Chen et al., in press). As perinatal lethality precludes analysis of the mature mammary gland, mammary tissue transplantation technology was used to permit growth and development of IκBα deficient mammary glands in a wild-type host. IκBα deficient 6 day old female animals, identified based on runting and scaly skin phenotype and later confirmed by genomic Southern, or normal female littermates were sacrificed and a portion of each number 4 inguinal mammary gland located between the adjacent nipple and the mammary lymph node was isolated. This region contains the epithelium (Medina, 1996). The tissue harboring IκBα deficient or normal epithelium was then implanted into the number 4 mammary fat pad that has been surgically cleared of endogenous epithelium within recipient female animals (DeOme et al., 1959; Medina, 1996). This strategy has been used successfully in analysis of mammary epithelial defects in C/EBPβ deficient animals,which also die perinatally (Robinson et al., 1998; Seagroves et al., 1998). These data have been incorporated into a manuscript that has been submitted to *Molecular Biology of the Cell*, and this manuscript has been appended to this report (appended manuscript number 2).

Analysis of hematoxylin stained whole-mount preparations of reconstituted glands isolated from mature virgin animals revealed an increase in the number of lateral ductal branches and apparent hyperplasia in IκBα deficient epithelium compared to comparable regions in epithelium derived from control littermates or in epithelium from native host glands (Figure 1A and B; appended manuscript number 2). The IκBα deficient epithelium also appeared to be disorganized as several regions within the ducts lacked a central lumen, as seen in hematoxylin and eosin stained sections (Figure 1B and C; appended manuscript number 2). Those regions in which a lumen was formed displayed an increase in the number of epithelial cell layers lining the lumen, rather than being limited to a single luminal epithelial cell layer as seen in controls. Moreover, the IκBα deficient epithelium appeared to have defects in cell contacts and extracellular matrix contact, as IκBα deficient epithelium abnormally penetrated into the surrounding stroma rather than being tightly confined as in control tissue (Figure 1C; appended manuscript number 2). These defects are most likely due to elevated NF-κB activity in IκBα deficient epithelium, as the epithelium lacking expression of IκBα protein was derived from donor tissue that displayed increased NF-κB transcriptional activity (Figure 2; appended manuscript number 2).

In order to determine if the apparent increase in epithelial cell number was due to increased proliferation and/or decreased apoptosis within IκBα deficient epithelium, bromodeoxyuridine (BrdU) incorporation and terminal deoxytransferase mediated deoxyuridine triphosphate nick end labeling (TUNEL) assays, respectively. The percentage of BrdU incorporation was increased 2 to 3 fold in IκBα deficient epithelium relative to controls, indicating that the elevated NF-κB transcriptional activity results in increased proliferation in IκBα deficient epithelium (Figure 3; appended manuscript number 2). No change in the percentage of epithelial apoptosis was detected in IκBα deficient epithelium relative to controls as assessed by TUNEL assay (Figure 4; appended manuscript number 2). Given that the IκBα deficient epithelium was poorly confined and penetrated into the surrounding stroma, the expression of extracellular matrix (ECM) components was examined by trichrome staining. Compared to the abundant ECM adjacent to host epithelium and epithelium derived from wild-type littermates, a pronounced reduction in ECM expression was observed adjacent to IκBα deficient epithelium (Figure 4; appended manuscript number 2). This reduction appeared to be specific to the epithelium, as adjacent blood vessels within stroma

containing IκBα deficient epithelium displayed abundant ECM deposition. These data demonstrate that elevated NF-κB activity in IκBα deficient epithelium results in inceased lateral ductal branching, epithelial hyperplasia, and diruption of the normal mammary architecture. The data further suggest that elevated NF-κB activity in breast adenocarcinomas may directly contribute to tumorigenesis.

Conclusions

The data presented in this report demonstrate that (i) NF-κB/IκBα is present in native mammary tissue, with elevated expression in the epithelium; (ii) NF-κB factors p50 and RelA are the major family members present in mammary gland nuclei during pregnancy and involution; (iii) transcriptional activity of NF-κB is elevated during pregnancy and involution, and disappears during lactation. Data from IκBα deficient epithelium demonstrate that that elevated NF-κB activity in the absence of this major inhibitor functions to promote epithelial branching and proliferation. Given that elevated NF-κB activity has been associated with breast cancer, these ongoing studies on the function of NF-κB in mammary epithelial proliferation will likely contribute to understanding the pathways that lead to neoplasia and perhaps provide a new target for therapeutic intervention.

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Key Reseach Accomplishments

1. Completed first description of p50, RelA, and IκBα expression patterns in the murine mammary gland over the course of post-natal morphogenesis

2. Completed first description and quantification of NF-κB transcriptional activity in the murine mammary gland over the course of post-natal morphogenesis *in vivo*

3. Established an *in vivo* model system (gland-free fat pads reconstituted with IκBα deficient epithelium) for assessing the function of NF-κB in the murine mammary gland in early morphogenesis

4. Demonstrated that elevated NF-κB transcriptional activity within IκBα deficient epithelium results in elevated ductal branching and hyperplasia *in vivo*

Reportable Outcomes

1. Manuscript entitled "Dynamic Expression of NF-κB During Post-Natal Mammary Gland Morphogenesis" in press in *Mechanisms of Development*

2. Manuscript entitled "Epithelial Hyperplasia and Disrupted Epithelial Architecture in Murine Mammary Glands Reconstituted with IκBα Deficient Mammary Tissue" submitted to *Molecular Biology of the Cell*

3. Poster presentation at the Department of Defense Era of Hope Meeting, June, 2000

4. Support of salary and tuition expenses for doctoral candidate Dana M. Brantley

5. Support for completion of dissertation research for Dana M. Brantley, Ph.D. expected August, 2000

GENE EXPRESSION PATTERNS SECTION

Dynamic Expression and Activity of NF-κB During Post-Natal Mammary Gland Morphogenesis

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Key Words: NF-κB, p105, p50, RelA, IκBα, mammary, epithelium, proliferation, differentiation, involution, apoptosis, transgenic

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GENE EXPRESSION PATTERNS SECTION

Abstract

The Rel/NF-κB family of transcription factors has been implicated in such diverse cellular processes as proliferation, differentiation, and apoptosis. As each of these processes occurs during post-natal mammary gland morphogenesis, the expression and activity of NF-κB factors in the murine mammary gland were examined. Immunohistochemical and immunoblot analyses revealed expression of the p105/p50 and RelA subunits of NF-κB, as well as the major inhibitor, IκBα, in the mammary epithelium during pregnancy, lactation, and involution. Electrophoretic mobility shift assay (EMSA) demonstrated that DNA-binding complexes containing p50 and RelA were abundant during pregnancy and involution, but not during lactation. Activity of an NF-κB-dependent luciferase reporter in transgenic mice was highest during pregnancy, decreased to near undetectable levels during lactation, and was elevated during involution. This highly regulated pattern of activity was consistent with the modulated expression of p105/p50, RelA, and IκBα.

Text

The Rel/NF-κB family of ubiquitous, inducible transcription factors consists of multiprotein complexes specialized for rapid cellular responses to a wide variety of growth factor, cytokine, and pathogenic stimuli (Verma et al., 1995). Although NF-κB activity has been well characterized in lymphoid tissues as a mediator of inflammatory and immune responses, several cell culture and *in vivo* models have implicated NF-κB in fundamental cellular processes, including proliferation and apoptosis (Verma et al., 1995; Attar et al., 1997; Ghosh, May, and Kopp, 1998). Each of these processes are critical during the highly dynamic morphogenesis of the mammary gland (Medina, 1996). Moreover, several recent studies have demonstrated aberrant expression of NF-κB factors and enhanced DNA binding activity in breast cancer cell lines and primary tumors (Dejardin et al., 1995; Nakashatri et al., 1997; Sovak et al., 1997; Sovak et al., 1999; Cogswell et al., 2000). Given these observations, the expression of several NF-κB/IκB family members over the course of post-natal mammary gland development was investigated. These family members included p105/p50, which acts as an NF-κB inhibitor in the 105 kDa isoform and as a transcriptional cofactor in the 50 kDa isoform, RelA, one of the major transcriptional activators in the NF-κB family, and IκBα, a direct inhibitor of NF-κB. In addition, the DNA-binding activity and *in vivo* transcription mediated by NF-κB throughout post-natal development was assessed.

Immunohistochemistry revealed expression of the p105/p50 and RelA subunits of NF-κB and the direct inhibitor, IκBα, in the epithelium of virgin, pregnant, lactating, and involuting mammary glands (Figure 1). Expression was observed in both the epithelium and in the surrounding stroma, with a more intense and dynamic pattern within the epithelium. At 16.5 days of pregnancy, when the mammary epithelium is proliferating and beginning to express milk proteins, RelA expression appeared to be upregulated as compared to levels in the virgin epithelium (Figure 1B). IκBα expression appeared to be decreased at 3 days of involution, when the mammary epithelium is regressing by apoptosis, compared to 5.5 day fully differentiatied, lactating epithelium (Figure 1C). Specificity of the anti-p105/p50 antibody was confirmed by

probing sections from p105/p50-deficient virgin mammary glands. The ductal morphology and histology of p105/p50-deficient mammary glands appeared relatively normal, though a slight increase in lateral branching was observed in some samples (data not shown). Expression of p105/p50 was detected in wild-type virgin glands, while no staining was detected in glands collected from p105/p50-deficient virgin animals (Figure 1D). Specificity of anti-p105/p50 antisera, as well as antisera against RelA and IκBα, was also confirmed by peptide competition. The colorimetric signal in sections probed with antisera minus specific competitor was more intense than in control sections probed with primary antisera that was presorbed with specific competitor peptides, but not in sections probed with primary antisera that was presorbed with non-specific peptides (Figure 2). Higher magnification of p105/p50 and RelA stained sections revealed both nuclear and cytoplasmic localization of these subunits in epithelium during pregnancy and involution (Figure 3A).

In order to quantify p105/p50, RelA, and IκBα expression over the course of post-natal mammary gland morphogenesis, immunoblot analysis was performed on nuclear and cytoplasmic protein extracts from virgin, pregnant, lactating, and involuting mammary glands (Figure 3B). Enrichment of cytoplasmic and nuclear proteins within these extracts was confirmed by using antibodies directed against β-tubulin, a cytoplasmic protein, and SP1, a nuclear factor (Figure 3B). SP1 expression in nuclear extracts from involuting mammary samples was not observed, however, likely due to the cessation of much general transcription due to massive epithelial apoptosis during these stages. Moreover, the presence of intact proteins in these extracts and the relative uniformity of loading was confirmed by Ponceau S stain following transfer of SDS-PAGE fractionated proteins to polyvinylidinefluoride (PVDF) membranes (data not shown). IκBα functions as an inhibitor of NF-κB nuclear import primarily in the cytoplasm, and uniform IκBα expression was observed in cytoplasmic extracts from virgin, pregnant, and early lactating glands. Cytoplasmic expression of IκBα was observed to decrease in late lactation and involution. Cytoplasmic levels of latent p50 and RelA were also relatively uniform. Upon activation, NF-κB complexes are known to be transported to the nucleus, and an increase in nuclear RelA expression was observed

in pregnancy as compared to virgin samples, and again at 1 and 3 days involution as compared to 9.5 days lactation. Again, specificity of the p105/p50 antibody was confirmed by probing cytoplasmic extracts from wild-type virgin glands and from p105/p50-deficient glands with anti-p105/p50 antisera. Expression of p105/p50 was detected only in extracts from wild-type mammary glands, and uniform loading of proteins was confirmed by probing with an antibody against β-tubulin (Figure 3C). These data demonstrate a dynamic pattern of expression and intracellular localization for p105/p50, RelA, and IκBα in the mammary gland epithelium.

Electrophoretic mobility shift assays were performed on mammary gland nuclear extracts to assess the pattern of NF-κB DNA-binding activity during pregnancy, lactation, and involution. An oligonucleotide bearing an NF-κB consensus element from the human immunodeficiency virus-long terminal repeat (HIV-LTR) was used to detect nuclear NF-κB in these extracts. This enhancer binds a broad range of NF-κB homodimeric and heterodimeric complexes (Kretzshmar et al., 1992; Liu et al., 1992; Doerre et al., 1993). Two protein complexes capable of binding to this NF-κB consensus element were observed in mammary gland nuclear extracts. The relatively strong complex (Complex A) migrated with a higher mobility than the weaker complex (Complex B; Figure 4A). Specificity of the protein:DNA complex was demonstrated by the addition of excess unlabeled competitor oligonucleotides to 16.5 day pregnant, 5.5 day lactation, and 3 day post-wean samples (Figure 4A, lanes 3, 7, and 11). DNA binding activity was highest during pregnancy, decreased substantially through lactation, and then increased following cessation of nursing, as recently described by others (Clarkson and Watson, 1999). To provide a more detailed characterization of these DNA-binding complexes, antibodies recognizing specific family members were used for supershifts to identify specific NF-κB family members. In nuclear extracts prepared from mammary glands at 16.5 dpc, a supershift was produced by an antibody against p50 (Figure 4A, lane 5). RelA is was also also supershifted in nuclear extracts prepared from mammary glands at 16.5 dpc (Figure 4A, lane 6). RelA and p50 supershifts were also detected in nuclear extracts prepared from mammary glands at 3 dpw (Figure 4A, lanes 13 and 14). The specificity of the p105/p50 antibody used in supershift experiments was confirmed by incubating the antibody with

nuclear extracts from virgin p105/p50-deficient mammary glands. Nuclear extracts from p105/p50-deficient mice exhibited a decrease in the level of DNA-binding relative to extracts from wild-type mammary glands, although DNA-binding was still present (Figure 4B, lane 1 versus lane 5). No p50 supershift was observed in nuclear extracts prepared from virgin p105/p50-deficient mammary glands, although the anti-p50 antisera was able to produce a supershift in nuclear extracts prepared from wild-type virgin mammary glands (Figure 4B, lane 3 versus lane 7). No supershift was detected with the RelA antisera in p105/p50-deficient glands, though the more slowly migrating DNA-binding complex in p105/p50-deficient extracts appears to co-migrate with the more slowly migrating DNA-binding complex in wild-type virgins (Figure 4B, lane 4 versus lane 8). It is possible that this complex contains RelA but that the levels are below the detection limits in this assay (Figure 4B, lane 8). These data demonstrate the dynamic pattern DNA-binding activity of nuclear p50 and RelA containing complexes in the mammary gland during post-natal morphogenesis.

In order to assess the transactivational function of NF-κB *in vivo*, the activity of an NF-κB responsive reporter was monitored in the mammary glands of transgenic mice at various developmental stages. Transgenic mice bearing the human immunodeficiency virus long terminal repeat (HIV-LTR) driving the *Photinus* luciferase cDNA, termed HLLs, have been used to quantify both constitutive and induced NF-κB activity (Blackwell et al., in press). Moderate reporter activity was detected in virgin glands, compared to background levels in non-transgenic control glands (Figure 6). Luciferase activity increased consistently through mid-pregnancy (10.5 to 16.5 dpc), and then decreased in late pregnancy (18.5 dpc, Figure 5). NF-κB activity levels decreased dramatically between 3.5 and 5.5 days of lactation, matching the levels measured in non-transgenic control glands. When pups were removed after 9.5 days of lactation, to induce epithelial involution, an increase in NF-κB reporter activity was observed.

Immunohistochemistry and immunoblot analyses revealed specific expression of p105/p50, RelA, and IκBα members in the developing mammary epithelium. Nuclear p50 and RelA expression was upregulated during pregnancy and involution. In addition, EMSA and the activity

of an NF-κB inducible reporter in transgenic mice revealed elevated NF-κB activity during pregnancy and involution post-weaning. DNA-binding and transcriptional activity were dramatically reduced during lactation. These highly regulated and dynamic expression and activity patterns indicate that further studies are imperative to determine the specific functions of NF-κB in mammary epithelial development during pregnancy and involution.

Materials and Methods

Immunohistochemistry: Paraformaldehyde-fixed, paraffin-embedded sections (5-7 μm) from number 3 thoracic glands of virgin, 16.5 day pregnant, 5.5 day lactating, and 3 day involuting (after forced wean at 9.5 days lactation) B6D2 mice were probed with primary antisera (rabbit anti-IκBα, SC-371, 1 μg/ml; rabbit anti-p105/p50, SC-114, 2 μg/ml; rabbit anti-RelA, SC-109, 5 μg/ml; Santa Cruz Biotechnology). For peptide competition assays, primary antisera were incubated with a 5 fold excess of specific peptides as per supplied protocol (Santa Cruz) prior to immunohistochemistry, or with a 5 fold excess of nonspecific peptides directed against p52 (SC-298P, Santa Cruz) as a negative control. Specific immunoreaction was detected using the Vectastain Elite ABC kit (Vector Laboratories) and 3,3'-Diaminobenzidine tetrahydrochloride (DAB) horseradish peroxidase substrate (Zymed Laboratories) according to manufacturers' protocols. Sections were counterstained with hematoxylin prior to dehydration, mounting, and photodocumentation.

Immunoblot: Nuclear and cytoplasmic extracts were prepared using number 4 inguinal mammary glands collected from virgin, 10.5 and 16.5 day pregnant, 5.5 and 9.5 day lactating, and 1,3 and 5 day post forced wean animals as described by Baldwin et al. (1991). Whole cell extracts from the FL5.12 pro-B cell line were used as a positive control for NF-κB/IκB expression, as progenitor B-cells are known to express these factors (Grumont and Genrondakis, 1994). Protein concentration was determined using a Lowry assay (Bio-Rad Protein Assay Kit). Protein extracts (15 μg or 30 μg) were fractionated on 8 to 12% SDS-polyacrylamide gels and transferred to polyvinylidinefluoride (PVDF) membranes (Immobilon). The membranes were probed with primary antisera (rabbit anti-IκBα SC-371, 1 μg/ml; rabbit anti-p105/p50, NR1157, 1:1000, generously provided by Dr. Nancy Rice, National Cancer Institute ; rabbit anti-RelA, SC-109, 2 μg/ml; rabbit anti-SP1, SC-59, 1 μg/ml; mouse anti-β-tubulin, N-357, 1:1000, Amersham) according to manufacturer's protocol (Santa Cruz Biotechnology). The specificity of the NR1157 antisera for immunoblot was confirmed previously by comparing expression in wild-type murine

thymic cell extracts versus extracts from p105/p50 deficient mice (Pereira et al., 1996). The membranes were then incubated with a horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antisera (Southern Biotechnology Associates) diluted 1:3000. Specific proteins were visualized using Renaissance chemiluminescence reagents according to the manufacturer's instructions (NEN Life Sciences Products).

Electrophoretic Mobility Shift Assays (EMSAs): Conditions for nuclear extraction and NF-κB EMSA have been detailed elsewhere (Baldwin et al., 1991). Oligonucleotides encoding an NF-κB consensus element from the HIV-LTR were used as probes (Stratagene Gelshift Assay Kit; plus strand 5'- GAT CGA GGG GAC TTT CCC TAG C- 3'). Briefly, 10 μg of nuclear extracts from number 4 inguinal mammary glands of virgin, 10.5 and 16.5 day pregnant, 5.5 and 9.5 day lactating, and 1, 3, and 5 days involuting B6D2 mice were incubated in binding buffer containing 1 μg poly(dI-dC) (Boehringher-Mannheim) and, where appropriate, 2 μg specific antiserum (anti-RelA, SC-109X, anti-p50, SC-114X; Santa Cruz Biotechnology). Probe (30,000 cpm) was added to each sample, and the samples were incubated for 25 minutes at ambient temperature. Samples were fractionated on a 4% polyacrylamide gel in 0.5X TBE for 2 hours at 150V. The gel was dried and exposed to autoradiographic film (Kodak) for 1-2 days at -80°C.

p50 Deficient Mice: Mice deficient for p105/p50 were obtained from the Jackson Laboratory (B6,129P - Nfkb1[tm1Bal]). The genotype was confirmed by polymerase chain reaction (PCR) analysis as per supplier's protocol using the following primers: common primer 5' - GCA AAC CTG GGA ATA CTT CAT GTG ACT AAG - 3', wild-type primer 5' - ATA GGC AAG GTC AGA ATG CAC CAG AAG TCC - 3', and null primer 5' - AAA TGT GTC AGT TTC ATA GCC TGA AGA ACG - 3'.

Generation of HIV-LTR-luciferase (HLL) transgenic animals and luciferase assay: The HIV-LTR-luciferase transgene consists of a 680 bp HIV-LTR XhoI/HindIII fragment from an HIV-LTR lacZ plasmid derived from the pU3R-III HIV-LTR-CAT plasmid (Sodroski et al., 1985; Nabel et al., 1987) driving luciferase from the pGL2-Basic vector (Promega). Transgenic animals were generated by pronuclear injection (Vanderbilt University Cancer Center Transgenic Core Facility),

and two transgenic founders were identified and used to generate two expressing lines (20 and 27). Presence of the luciferase transgene was assessed by Southern blot and/or PCR analysis of genomic DNA from tail biopsy, and confirmed by constitutive luciferase activity in the brain (Blackwell et al., in press). Basal expression of the luciferase reporter, as well as inducible expression upon treatment with bacterial lipopolysaccharide (LPS) was used to validate these mice as a model system for NF-κB-mediated transcriptional activity *in vivo* (Blackwell et al., in press).

Total cell extracts were prepared in passive lysis buffer (Analytical Bioluminescence) from number 4 inguinal mammary glands of HLL +/- transgenic mice and wild-type littermates at the following stages: virgin; 10.5, 12.5, 14.5, 16.5, and 18.5 days pregnancy; 1.5, 3.5, 5.5, 7.5, and 9.5 days lactation; 1, 3, and 5 days involution. Luciferase activity was measured according to the manufacturer's instructions (Analytical Bioluminescence). Protein concentrations in the extracts were determined by Lowry assay (Bio-Rad). All values are presented as relative light units (RLUs)/μg protein.

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Figure Legends

Figure 1: NF-κB and IκBα are expressed in the mammary gland epithelium over the course of post-natal morphogenesis. Histological sections were prepared from virgin, 16.5 day pregnant (16.5 dpc), 5.5 day lactating (5.5 lac), and 3 days post-wean involuting (3 dpw) number 3 thoracic glands. These sections were probed with primary antisera for p105/p50 (A) , RelA (B), or IκBα (C). Epithelial expression of RelA increased at 16.5 days of pregnancy (B), whereas expression of IκBα decreased at 3 days post-wean involution. Histological sections from wild-type and p105/p50-deficient mammary glands were probed with antisera against p105/p50 to confirm the specificity of the antisera. Expression of p105/p50 was detected in sections from wild-type virgin animals but not in sections from p105/p50-deficient virgin animals (D). Data presented are a representation of results from 3 to 6 independent samples per developmental stage. Scale bar = 50 μm.

Figure 2: Specific expression of NF-κB and IκBα in the mammary gland. Histological sections were prepared from number 3 thoracic glands collected from 3 days post-wean involuting (3 dpw) mice as both NF-κB factors and IκBα are expressed at detectable levels during this stage of development. These sections were probed with primary antisera for p105/p50 (A), RelA (B), or IκBα (C), with or without 5 fold excess of specific competitor peptides or nonspecific peptides against p52. The specific peptides abrogated the signal (left hand panel versus middle), whereas the nonspecific peptides did not (left hand panel versus right), demonstrating the specificity of these antisera for NF-κB factors and IκBα. Data presented are a representation of results from 3 independent samples. Scale bar = 50 μm.

Figure 3: NF-κB factors are localized to both the nuclei and cytoplasm of mammary epithelium during pregnancy and involution. Higher magnification of 16.5 dpc and 3 dpw sections probed for p105/p50 and RelA expression revealed nuclear localization in several epithelial cells (A, arrowhead) in addition to the cytoplasmic localization in adjacent cells. Scale bar = 10 μm. Cytoplasmic and nuclear protein extracts (15 μg) from mammary glands or FL5.12 pro-B cells as a positive control were subjected to immunoblot analysis for expression of IκBα, p105/p50, and RelA (B) or for β-tubulin and SP1 as cytoplasmic and nuclear markers respectively. IκBα expression in the cytoplasm was uniform until 9.5 days lactation (9.5 lac), when levels decreased continuing through 1, 3 and 5 days involution (1,3, 5 dpw). Expression of p50 and RelA was relatively uniform in the cytoplasm. Nuclear RelA expression increased in pregnancy and involution. The specificity of the p105/p50 antisera was confirmed by immunoblot analysis probing cytoplasmic extracts (30 μg) from wild-type or p105/p50-deficient virgin mammary glands (C). Expression of p105/p50 was detected in cytoplasmic extracts prepared from wild-type mammary glands, but not in extracts prepared from p105/p50-deficient glands. Antisera against β-tubulin was used to confirm that equal quantities of protein were loaded for each sample (C). Data presented are a representation of results from 3 to 6 independent samples per developmental stage.

Figure 4: DNA binding activity of NF-κB in mammary gland nuclear extracts is highest during pregnancy and involution. Nuclear extracts were prepared from virgin, pregnant (10.5 dpc and 16.5 dpc), lactating (5.5 lac and 9.5 lac), and involuting (1 dpw, 3 dpw, and 5 dpw) number 4 inguinal glands. Nuclear extracts (10 μg) were incubated with an end-labeled oligonucleotide harboring an NF-κB enhancer element from the HIV-LTR. Two protein complexes capable of binding this oligonucleotide were present in virgin, pregnant, and involuting mammary samples, a strong complex (Complex A) migrating below a relatively weaker complex (Complex B; A). The specificity of these complexes was confirmed by competition with excess, unlabeled oligonucleotide (A; lanes 3, 7, and 11). Supershifts for p50 and RelA confirmed the presence of these NF-κB components in the complex (A; lanes 5, 6, 13, and 14). In order to confirm the specificity of the antisera used to supershift p50, nuclear extracts prepared from virgin p105/p50-deficient mice were probed with anti-p50 antisera. DNA-binding in p105/p50-deficient nuclear extracts (10 μg) was apparent, although reduced compared to that in wild-type nuclear extracts (B; lane 1 versus lane 5). The antisera used to detect p50 in EMSA experiments was specific, as a supershift was detected in wild-type but not p105/p50-deficient nuclear extracts (B; lane 3 versus lane 7). Data presented are a representation of 3 to 6 independent samples per developmental stage.

Figure 5: *In vivo* transcriptional activity of NF-κB in the mammary glands of transgenic reporter mice is highest during pregnancy and involution. Protein extracts were prepared from virgin, pregnant (10.5 dpc, 12.5 dpc, 14.5 dpc, 16.5 dpc, and 18.5 dpc), lactating (1.5 lac, 3.5 lac, 5.5 lac, 7.5 lac, and 9.5 lac), and involuting (1 dpw, 3 dpw, and 5 dpw) number 4 inguinal mammary glands from transgenic mice expressing luciferase under the regulation of the HIV-LTR or from non-transgenic animals at 10.5 days of pregnancy. Luciferase activity (relative light units, RLUs) was assessed in 20 μl of protein extract. Data were normalized based on protein concentration and are the average of 3 independent samples per developmental stage with standard error of the mean.

Figure 1

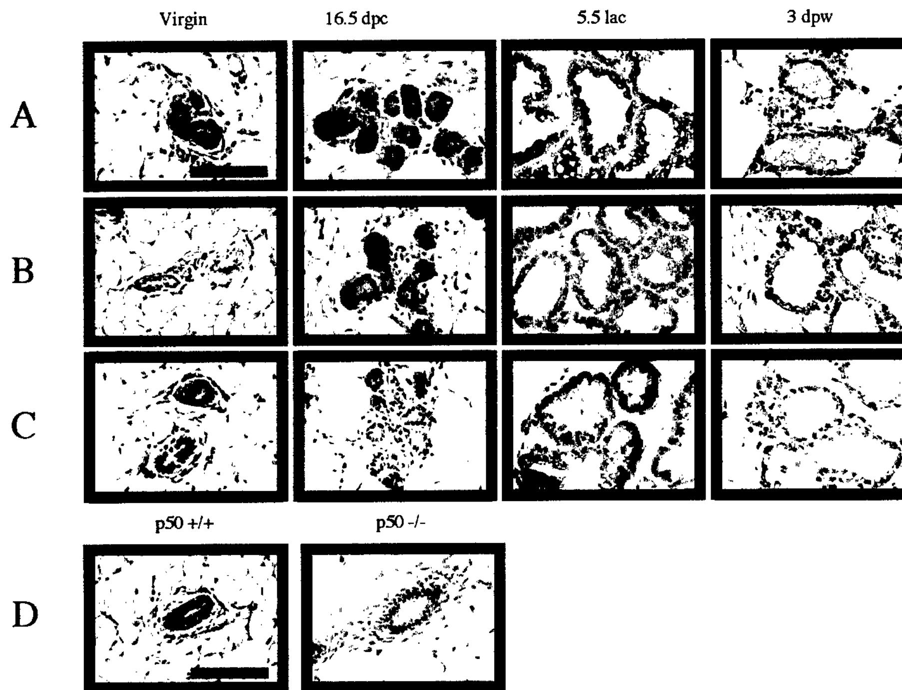

Figure 2

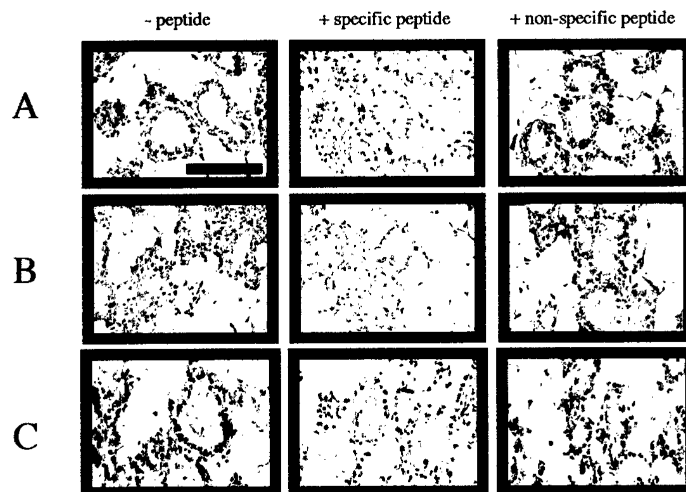

Figure 3

A

p105/p50

RelA

16.5 dpc

3 dpw

B

IκBα

p105

p50

RelA

β-tubulin

SP1

Cytoplasm

p50

RelA

β-tubulin

SP1

Nucleus

C

p50 -/-

p105

p50

β-tubulin

Figure 4

A

Complex B

Complex A

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

comp - - + - - - + - - - + - - - - -

α-RelA - - - - - + - - - - - - - + - -

α-p50 - - - - + - - - - - - - + - - -

B

p50 -/-

1 2 3 4 5 6 7 8 9

comp - + - - - + - - -

α-RelA - - - + - - - + -

α-p50 - - + - - - + - -

Figure 5

Relative Light Units (RLUs)/ng protein

25000

20000

15000

10000

5000

0

virgin

10.5 dpc

12.5 dpc

14.5 dpc

16.5 dpc

18.5 dpc

1.5 lac

3.5 lac

5.5 lac

7.5 lac

9.5 lac

1 dpw

3 dpw

5 dpw

non tg

Developmental Stage

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Epithelial Hyperplasia and Disrupted Epithelial Architecture in Murine Mammary Glands Reconstituted with IκBα Deficient Mammary Tissue

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Running Title: IκBα deficiency in mammary epithelium

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Abstract

The Nuclear Factor-kappa B (NF-κB) family of transcription factors has been shown to regulate proliferation in several cell types. Recent studies have demonstrated aberrant expression and/or activity of NF-κB in breast cancer cell lines and in primary breast adenocarcinomas. Given these observations, and that NF-κB and Inhibitors of κB (IκBs) are expressed in normal mouse mammary epithelium, we investigated the function of NF-κB in the proliferation and post-natal development of the murine mammary epithelium. Mammary tissue containing epithelium from *iκbα*-deficient female donors was transplanted into the gland-free mammary stroma of wild-type mice to permit analysis of post-natal development of the mammary epithelium under conditions of elevated NF-κB activity. Mammary glands reconstituted with *iκbα*-deficient tissue displayed an increase in lateral ductal branching and diffuse hyperplasia. A 2 to 3 fold increase in epithelial cell number and proliferation was observed in *iκbα*-deficient epithelium compared to wild-type or heterozygous tissue, or to endogenous host epithelium in intact glands. A reduction extracellular matrix was observed adjacent to *iκbα*-deficient epithelium. No alteration in apoptosis was detected in *iκbα* deficient epithelium. An increase in epithelial branching was also observed in purified *iκbα*–deficient primary epithelial cells compared to wild-type epithelial cells in three-dimensional culture. These data demonstrate that NF-κB positively regulates mammary epithelial proliferation and maintenance of normal epithelial architecture, suggesting that elevated NF-κB activity in the mammary epithelium may contribute to breast cancer.

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 and milk, as well as 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 post-natally. During puberty, the epithelium proliferates and branches in response to hormone signals, permeating 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). Following involution, the epithelium remains quiescent until the next pregnancy, when the morphogenetic cycle is repeated.

The processes of proliferation, differentiation, and apoptosis are tightly controlled by complex molecular pathways. Local growth factor signals synthesized within the stroma cooperate with systemic hormones to regulate proliferation and differentiation during puberty and pregnancy (Medina, 1996). Often, these molecular pathways result in the activation of transcription factors that bind DNA in the nucleus and initiate changes in gene expression (Hunter, 1997). These signaling events that regulate normal morphogenesis of the mammary gland epithelium are often misregulated in the formation and progression of breast cancer in humans (Hunter, 1997; Daniel and Smith, 1999). Therefore, understanding how these molecular pathways operate will enhance our understanding of breast cancer and lead to the identification of new targets for therapeutic intervention.

The Nuclear Factor-kappa B (NF-κB) family of transcription factors regulate growth and/or differentiation in several tissues, including lymphocytes, embryonic limb and lung, skin, and bone (Boothby *et al.*, 1997; Bendall *et al.*, 1999; Bushdid *et al.*, 1998; Kanegae *et al.*, 1998; Muraoka *et al.*, in press; Beg *et al.*, 1995; Klement *et al.*, 1996; Chen *et al.*, submitted; Seitz *et al.*, 1998; Hu

et al., 1999; Takeda *et al.*, 1999; Franzoso *et al.*, 1997). The precise response elicited by NF-κB depends, in part, on the signals that stimulate the activity of NF-κB. Inactive NF-κB dimeric complexes are sequestered in the cytoplasm of unstimulated cells by association with members of a family of specific Inhibitors of kappa B (IκBs). Upon receipt of extracellular stimulators, such as growth factors, cytokines, or pathogenic agents, the IκB is phosphorylated on conserved amino-terminal serine residues by specific IκB kinases (IKKs). This phosphorylation event leads to polyubiquitination and proteosome-mediated degradation of the IκB. Proteolytic degradation of the IκB liberates the NF-κB 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; May and Ghosh, 1998; Ghosh, May and Kopp, 1998).

Mice that are deficient in *iκbα* have served as an excellent model system in which to observe the effects of elevated levels of NF-κB *in vivo*. Increased levels of NF-κB have been observed in various tissues, including B- and T-lymphocytes, skin, liver, and spleen (Beg *et al.*, 1995; Klement *et al.*, 1996; Chen *et al.*, in press). The *iκbα*-deficient mice display severe inflammation and skin disease, and loss of *iκbα* results in lethality at approximately post-natal day 9. Mice lacking *iκbα* show an increase in the proliferation lymphocytes and keratinocytes, suggesting that NF-κB activity is involved in the regulation of proliferation within these cell types, and possibly others. NF-κB has also been shown to be involved in the development of various organ systems that, like the mammary gland, rely on paracrine signaling pathways. These include the limb, in which signals from the apical ectodermal ridge (AER) stimulate NF-κB activity within the underlying limb mesenchyme and controls the proliferation and outgrowth of limb mesenchyme (Bushdid *et al.*, 1998). This suggests that NF-κB, when activitated by the appropriate factors, can function to enhance cell proliferation.

Previously, we demonstrated that the NF-κB family members p50 and RelA, as well as the IκB factors p105 and IκBα, were expressed in the murine mammary epithelium over the course of normal post-natal morphogenesis (Brantley *et al.*, in press). Moreover, we found that NF-κB activity was maximal in the mammary gland during pregnancy, when the epithelium is

proliferating, and during involution, when the epithelium is undergoing apoptosis. These activity data suggest that NF-κB may regulate proliferation and/or apoptosis during normal post-natal mammary epithelial morphogenesis. In addition, several recent studies demonstrated that NF-κB factors were aberrantly expressed and/or activated in cell lines derived from human breast tumors, as well as in primary human breast adenocarcinomas (Dejardin *et al.*, 1995; Nakashatri *et al.*, 1997; Sovak *et al.*, 1997; Sovak *et al.*, 1999; Cogswell *et al.*, 2000). These observations suggest that misregulation of NF-κB may contribute to alterations in proliferation of the mammary epithelium.

In order to ascertain the function of NF-κB in the mammary epithelium, we have examined the morphology and development of mouse mammary epithelium lacking the gene encoding a major inhibitor of NF-κB, IκBα. We demonstrate that *iκbα*-deficient mammary epithelium is hyperplastic, displays increased lateral ductal branching, and contains decreased levels of extracellular matrix deposition. Moreover, the increased branching and tubulogenesis was observed in three-dimensional epithelial cell cultures derived from mammary glands reconstituted with *iκbα*-deficient epithelium relative to wild-type epithelial cell cultures, suggesting that some of the defects in *iκbα*-deficient epithelium are intrinsic to epithelial cells. These data demonstrate that NF-κB modulates normal proliferation of the mammary epithelium during early post-natal morphogenesis, and suggest that NF-κB might contribute to abberant proliferation during tumorigenesis.

Materials and Methods

Mouse strains: Generation of *iκbα*-deficient mice was described by Chen *et al.* (in press). The phenotype of these mice is consistent with independently derived *iκbα* deficient mice (Beg *et al.*, 1995; Klement *et al.*, 1996). The genotype of *iκbα*-null neonatal mice and littermates was confirmed by Southern analysis of genomic DNA isolated from tail biopsies and digested with BamHI using a 1300 bp XbaI/NdeI fragment encompassing sequences from the 5' flanking region of the *iκbα* gene that was within the BamHI restriction site boundaries of the null allele (Chen *et al.*, in press). Wild-type (C57B/6J; Jackson Laboratory), *iκbα* +/-, and *iκbα* -/- mice were housed in microisolators under identical conditions. Heterozygous *iκbα* mice were bred with HLL mice, a transgenic mouse line harboring a luciferase transgene under the regulation of the NF-κB responsive human immunodeficiency virus long terminal repeat (HIV-LTR; Blackwell *et al.*, in press; Brantley *et al.*, in press). Heterozygous *iκbα* mice which were HLL +/- were intercrossed to generate *iκbα* +/+, *iκbα* +/-, and *iκbα* -/- that were either HLL +/- or HLL -/-. Again, the genotype of these animals was confirmed by Southern analysis for both *iκbα* and the HLL transgene using genomic DNA from tail biopsy. Luciferase activity in the mammary tissue extracts prepared from all ten neonatal whole mammary glands was assessed as previously described (Brantley *et al.*, in press), and protein concentrations in the extracts was determined by Lowry assay (Bio-Rad). 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; Robinson *et al.*, 1998; Seagroves *et al.*, 1998). Briefly, number 4 inguinal mammary donor tissue located between the nipple region and lymph node, the region which contains the epithelial rudiment, was surgically removed from 6 day old female *iκbα* +/+, +/-, or -/- neonatal mice and stored in DMEM on ice prior to transplantation. The epithelium within the number 4 inguinal mammary glands of 3 week old virgin female C57Bl/6J recipient mice were 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. The neonatal tissue 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 weeks following 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 weeks 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) overnight at room temperature, light protected. The following day, the glands were destained in tap water, then further destained in 50% ethanol acidified with hydrochloric acid at a 1M final concentration. The glands were then dehydrated in a graded ethanol series followed by xylenes, equilibrated into methyl salicylate (Sigma), and photodocumented (Zeiss Stemi SV 11).

For analysis of the subcellular architecture and expression of matrix proteins and IκBα, 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 using an anti-IκBα antibody (Santa Cruz Biotechnology) as previously described (Brantley *et al.*, in press). Specific immunoreaction was detected using the Vectastain Elite ABC kit (Vector Laboratories) and 3',3'-Diaminobenzidine tetrahydrochloride (DAB) horseradish peroxidase substrate (Zymed Laboratories) according to manufacturers' protocols. Sections were counterstained with hematoxylin prior to dehydration, mounting in Permount (Fisher Scientific), and photomicroscopy (Olympus BX60).

Proliferation and Apoptosis Assays: For proliferation assays, a sterile solution containing 10 mg/mL BrdU (Sigma) in PBS was injected intraperitoneally (100 μl/gm body weight). After 4

hours, the mice were sacrificed, mammary glands were collected, fixed, and sections (7 μm). BrdU incorporation was visualized by immunohistochemistry 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 40X 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 average of 12 to 14 independent secondary reconstitution samples derived from 4 primary donor animals per condition were quantified. The total number of nuclei was also quantified in each of these samples.

For apoptosis assays, mammary glands were collected, fixed, and sections prepared as described (7 μm). Fragmented DNA was labeled with a digoxigenin-conjugated UTP using terminal deoxytransferase (TdT; Intergin). Positive nuclei were visualized by immunohistochemistry using an ApopTag labeling and detection kit (Intergin Company) 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 ApopTag positive epithelial cell nuclei relative to the total number of epithelial cell nuclei was calculated. The average of 6 independent primary or secondary reconstitution samples per condition were quantified.

Results

Wild-type Mouse Mammary Glands Reconstituted with *iκbα*-Deficient Mammary Tissue Displayed an Increase in Ductal Branching and a Disorganized Epithelial Architecture

In order to ascertain the function of NF-κB in mammary epithelial development, mammary tissue lacking the gene encoding the major inhibitor of NF-κB, IκBα, was examined. Because *iκbα*-deficient mice die approximately 9 days after birth (Beg *et al.*, 1995; Klement *et al.*, 1996; Chen *et al.*, in press), post-natal development of mammary epithelium was enabled by transplanting epithelium-containing mammary tissue from neonatal *iκbα*-null female mice or wild-type/heterozygous littermates 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, reconsituted mammary glands were isolated and used as donor tissue for a second round of reconsitution into several wild-type recipients. These glands were then collected and analyzed 6 to 8 weeks 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 *iκbα*-deficient epithelium contained ductal branches that permeated the entire fat pad in a pattern similar to that observed in *iκbα* +/- or +/+ epithelium as well as in native host epithelium. 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 there were numerous secondary and teritiary lateral branches in the *iκbα*-deficient epithelium that were not observed in control glands (Figure 1A). In addition, the epithelium appeared to be hyperplastic, a phenotype that was confirmed by examining the cellular architecture in histological sections (Figure 1B and C). Compared to 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 luminae were absent in *iκbα*-deficient epithelium or were surrounded by multiple epithelial cell layers rather than a single layer as seen in wild-type/heterozygous epithelium or host epithelium (Figure 1B and C). Higher magnification revealed that the *iκbα*-deficient epithelial structure appeared to be disorganized, with peripheral epithelial

cells penetrating into the stroma rather than remaining confined by the extracellular matrix as in wild-type or host epithelium (Figure 1C).

In order to confirm that IκBα protein was absent in donor tissue from and in mammary glands reconstituted with *iκbα*-deficient tissue, immunohistochemical analysis was performed. Immunohistochemical analysis for the expression of IκBα protein in reconstituted mammary glands revealed absence of IκBα in mammary epithelium derived from null animals, although IκBα was present in the surrounding host stroma (Figure 2A). As expected, expression of IκBα was observed in mammary epithelium derived from *iκbα* +/- or +/+ littermates and also in host mammary epithelium from unmanipulated glands (Figure 2A). These data confirm that mammary epithelium derived from *iκbα*-deficient donors did not express IκBα.

To assess the level of NF-κB transcriptional activity in the presence or absence of IκBα, *iκbα* +/- mice were mated to HLL mice, a transgenic mouse line expressing a luciferase reporter transgene under the regulation of the NF-κB responsive human immunodeficiency virus-long terminal repeat (HIV-LTR). Because the HIV-LTR contains two NF-κB enhancer elements which bind a broad range of homodimeric and heterodimeric NF-κB complexes (Kretzshmar *et al.*, 1992; Liu *et al.*, 1992; Doerre *et al.*, 1993), HLL transgenic animals have been used to quantify both constitutive and induced NF-κB activity *in vivo*, as well as to assess the level of *in vivo* transcriptional activity in the mammary gland over the course of post-natal morphogenesis (Blackwell *et al.*, in press; Brantley *et al.*, in press). Mice heterozygous for the *iκbα* null allele and hemizygous for the transgene were intercrossed to generate *iκbα* -/-, +/-, or +/+ offspring that were also HLL+. Quantification of luciferase activity in neonatal mammary gland extracts collected from these mice revealed an approximately 15 fold increase in NF-κB reporter activity in *iκbα*-deficient mice as compared with wild-type/heterozygous littermates or non-transgenic animals (Figure 2B). These data suggest that loss of IκBα in the mammary epithelium results in increased NF-κB activity.

Wild-type Mouse Mammary Glands Reconstituted with *iκbα*-Deficient Mammary Tissue Displayed an Increase in Epithelial Proliferation, but no Change in Apoptosis

To assess the levels of proliferation in *iκbα*-null epithelium versus wild-type/heterozygous or host epithelium, reconstituted virgin animals were labeled with the thymidine analog BrdU to quantify the number of epithelial cells in the process of DNA replication. BrdU incorporation into the nuclei of proliferating cells was detected by immunohistochemistry. Based on immunohistochemical analysis, the number of BrdU positive nuclei in *iκbα*-deficient mammary epithelium appeared to be greater than in mammary glands reconstituted with wild-type/heterozygous tissue or in native mammary epithelium (Figure 3A). To quantify this apparent increase, the percentage of BrdU positive nuclei relative to the total number of nuclei was calculated. A 2 to 3 fold increase in the percentage of BrdU positive epithelial nuclei was observed in *iκbα*-deficient epithelium versus control epithelium (Figure 3B). In addition, the increase in cell density was also determined by counting the number of epithelial nuclei in reconstituted and control mammary glands. Again, a 2 to 3 fold increase in epithelial cell number was observed in glands harboring *iκbα*-deficient epithelium relative to wild-type/heterozygous host epithelium (data not shown). These data demonstrate that mammary epithelium lacking *iκbα* is indeed hyperplastic in virgin animals.

In order to assess the level of apoptosis in *iκbα* deficient mammary epithelium, TUNEL analysis was performed. Sections prepared from reconstituted or control virgin glands were treated with TdT in an enzymatic reaction that labels 3'-OH nicks in the DNA of cells undergoing apoptosis with a digoxigenin-tagged nucleotide. TUNEL positive nuclei were detected by immunohistochemistry. Based on immunohistochemical analysis, the number of TUNEL positive nuclei in *iκbα*-deficient mammary epithelium appeared to be similar to the number in mammary glands reconstituted with wild-type/heterozygous tissue or in native mammary epithelium (Figure 4A). To quantify the levels of apoptosis in the epithelium, the percentage of TUNEL positive nuclei relative to the total number of nuclei was calculated. As predicted based on immunohistochemical analysis, no statistically significant difference in the number of TUNEL

positive nuclei was detected between *iκbα*-deficient mammary epithelium and epithelium derived from wild-type/heterozygous donors or native host epithelium (Figure 4B). These data suggest that *iκbα*-deficiency in the mammary epithelium does not alter apoptosis in virgin animals.

Wild-type Mouse Mammary Glands Reconstituted with *iκbα*-Deficient Mammary Tissue Displayed a Decrease in Extracellular Matrix Adjacent to the Epithelium

The epithelial architecture in mammary glands reconstituted with *iκbα*-deficient mammary tissue appeared to be disorganized and poorly confined by the extracellular matrix normally surrounding the ducts based on histological analysis (Figure 1C). In order to more closely examine this defect, sections harboring *iκbα*-deficient mammary epithelium or wild-type/heterozygous epithelium and sections from host glands were subjected to trichrome staining to visualize extracellular matrix proteins. Extracellular matrix (ECM) adjacent to *iκbα*-deficient donor epithelium was strikingly reduced, as compared to the amount of ECM adjacent to wild-type/heterozygous donor epithelium and intact host epithelium (Figure 5A and B). Interestingly, this reduction was restricted to the mammary epithelium, as ECM deposition adjacent to vascular endothelium remained intact, even within mammary glands reconstituted with *iκbα*-deficient epithelium (Figure 5A). These data demonstrate that ECM deposition associated with *iκbα*-deficient epithelium is perturbed, and suggest that *iκbα*-deficiency in the mammary epithelium affects the ability to establish the ECM.

Discussion

Morphogenesis of the mammary gland epithelium, both during puberty and during each cycle of pregnancy, lactation, and involution, is tightly regulated by signal transduction cascades involving the activation of transcription factors, which modulate changes in gene expression. By analyzing *iκbα*-deficient mammary tissue that lacks a major endogous inhibitor of NF-κB transcriptional activity, we have been able to determine the consequences of elevated NF-κB activity on the growth and morphogenesis of mammary epithelium. Here, we provide novel, *in vivo* evidence that hyperactivation of NF-κB within the mammary epihtleium causes a hyperproliferative phenotype in conjunction with alterations in basement membrane integrity and cell-cell and cell-matrix contacts. In previous studies, mice lacking *iκbα* exhibited elevated levels of NF-κB activity in several tissues, including B and T lymphocytes and skin (Beg *et al.*, 1995; Klement *et al.*, 1996; Chen *et al.*, in press). These mice displayed severe inflammation and skin disease, and died approximately 9 days after birth. As the perinatal lethality precludes any analysis of the mammary gland in mature animals, we have utilized mammary transplantation technology to investigate the morphology and development of the mammary epithelium. Classical studies have demonstrated that mammary donor tissue containing epithelium can be used to reconstitute recipient mammary stroma in which the endogenous epithelium has been surgically removed (DeOme *et al.*, 1959; Medina, 1996). Using this approach, we isolated neonatal mammary tissue containing epithelium from *iκbα*-deficient or wild-type/heterozygous littermates and transplanted this tissue into gland-free mammary stroma of wild-type recipient mice. As expected, IκBα protein was absent and NF-κB transcriptional activity was elevated in *iκbα*–deficient mammary donor tissue. Glands reconstituted with *iκbα*-deficient tissue displayed an increase in lateral ductal branching and diffuse epithelial hyperplasia in virgin animals, with a 2 to 3 fold increase in both epithelial cell number BrdU incorporation as compared to glands harboring mammary epithelium from normal littermates or as compared to unmanipulated host epithelium. In addition, a reduction in the extracellular matrix adjacent to *iκbα*-null mammary epithelium was observed. No alteration in the level of apoptosis, as assessed TUNEL analysis, was observed in *iκbα*-deficient epithelium versus

normal epithelium. These data demonstrate that NF-κB regulates both proliferation and the maintenance of the normal architecture of the mammary epithelium. Given the profound impact of increased NF-κB activity on proliferation and architecture of the mammary epithelium, it is possible that NF-κB may play a direct role in the development or progression of breast tumorigenesis. Increasing evidence of NF-κB overexpression and elevated activity in various human breast cancer cell lines and primary tumors supports the powerful implications of the results presented herein.

The Function of NF-κB in Mammary Epithelial Proliferation and in Maintenance of Normal Epithelial Architecture

Deficiency in *iκbα* causes hyperactivation of NF-κB in many tissues, including the mammary epithelium as shown here (Figure 2). This 15 fold increase in NF-κB activity resulted in severe mammary epithelial hyperplasia. These data are consistent with the established role of NF-κB in promoting keratinocyte proliferation in mice (Klement *et al.*, 1996; Chen *et al.*, in press). Several *in vivo* studies have provided support for the function of NF-κB in regulating proliferation. Previous revealed that *iκbα*-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). Targeted disruption of the gene encoding IKKα causing downregulation of NF-κB transcriptional activity perturbed normal proliferation and differentiation of the epidermis as well (Hu *et al.*, 1999; Li *et al.*, 1999; Takeda *et al.*, 1999). Targeted disruption of one or more NF-κB family members, as well as expression of a transdominant inhibitory isoform of IκBα that blocks NF-κB in transgenic mice, perturbed the proliferation and function of B and T lymphocytes within the immune system (Kontegen *et al.*, 1995; Sha *et al.*, 1995; Weih *et al.*, 1995; Horwitz *et al.*, 1997; Grossman *et al.*, 1999; Boothby *et al.*, 1997; Bendall *et al.*, 1999). Moreover, delivery of retroviruses or adenoviruses harboring the transdominant inhibitory IκBα to embryonic chick limb and lung blocked proliferation in both of these tissues (Bushdid *et al.*, 1998; Kanegae *et al.*, 1998; Muraoka *et al.*, in press). These data provide precedence for positive regulation of epithelial proliferation by NF-κB.

Loss of *iκbα* in the mammary epithelium resulted in an increase in lateral ductal branching, loss of extracellular matrix components, and altered expression of E-cadherin as a marker of adherens junctions. Epithelial morphogenesis and lateral ductal branching of the mammary epithelium during puberty is regulated by interactions between the epithelium and the surrounding stroma (reviewed by Cunha and Hom, 1996; Robinson, Karpf, and Kratochwil, 1999). NF-κB family members have been shown to modulate epithelial-mesenchymal interactions in embryonic limb and lung, suggesting that it may play a similar role in the regulation of growth and branching within the mammary epithelium. NF-κB expression is induced in embryonic limb mesenchyme by signals derived from a specialized epithelial structure called the apical ectodermal ridge (AER) that is adjacent to the limb mesenchyme. Disrupting NF-κB activity in embryonic limb mesenchyme impaired limb outgrowth and resulted in abberant morphology of the AER (Bushdid *et al.*, 1998). Similar experiments in embryonic lung demonstrated that activity of NF-κB in lung mesenchyme is necessary for proper budding and growth of the adjacent lung epithelium (Muroaka *et al.*, in press). It is possible that NF-κB 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.

The Function of NF-κB in Mammary Epithelial Apoptosis

Loss of *iκbα* in the mammary gland epithelium did not result in any change in the level of epithelial apoptosis in virgin animals. This result was not unexpected, as the major transcriptional activator present in the mammary epithelium at this stage of development is RelA. Several biochemical and *in vivo* studies have demonstrated that RelA expression and activity confers 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 regions adjacent to terminal end buds, during the process of lumen formation. Although any decrease in the level of apoptosis in *iκbα*-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 *iκbα*–deficient mammary epithelium does not form luminae. The cause of this phenomenon is unkown, but based on results presented herein, it is unlikely to be a result of a decrease in apoptosis at this stage of development. It will be interesting to ascertain the function of NF-κB in mammary epithelial apoptosis *in vivo*, particularly during involution of the mammary epithelium. Very recently, nuclear RelA expression has been correlated with non-apoptotic epithelial cells within involuting mammary glands (Clarkson *et al.*, 2000). Moreover, blocking NF-κB activity in a mammary epithelial cell model of involution enhanced survival of these cells relative to controls. These data strongly suggest that NF-κB, particularly RelA, promotes cell survival within mammary epithelium during involution, consistent with the established function of RelA in protecting cells from apoptosis (Clarkson *et al.*, 2000).

Potential Mammary Growth Regulatory/Oncogenic Pathways in Which NF-κB May Function

The profound effect that increased NF-κB activity has on proliferation and epithelial architecture within the mammary epithelium implicates NF-κB as having oncogenic potential within the mammary gland. Several lines of evidence suggest possible molecular pathways in which NF-κB might operate to regulate mammary epithelial proliferation and tumorigenesis. For example, NF-κB may cooperate with CCAAT enhancer binding protein β (C/EBPβ) transcription factor in mammary epithelial proliferation and development. Studies involving transplantation of *c/ebpβ* deficient mammary tissue into a wild-type host mammary stroma demonstrated that C/EBPβ was required for proper epithelial proliferation during pregnancy (Robinson *et al.*, 1998; Seagroves *et al.*, 1998). C/EBPβ has been shown to physically associate and cooperate with RelA to activate gene expression (Stein and Yang, 1995). Physical association between RelA and the estrogen and progesterone nuclear hormone receptors, both of which promote mammary epithelial proliferation, has also been observed (Caldenhoven *et al.*, 1995; Unlap and Jope, 1995; Ray *et al.*, 1994; Kalkhoven *et al.*, 1996). Thus, NF-κB may cooperate with transcriptional coactivators in the mammary epithelium to regulate epithelial proliferation.

Many oncogenic signaling pathways are intiated at the cell membrane by the activation of

receptor tyrosine kinases that are known to modulate mammary epithelial proliferation. Several lines of evidence suggest that NF-κB expression or activity can be modulated by these RTK-dependent signaling pathways. For example, studies in avian embryos have demonstrated that NF-κB expression and activity may be induced by fibroblast growth factors (FGFs) and their receptors (Bushdid *et al.*, 1998). Members of the FGF family, including FGF-3 and FGF-4, have been shown to induce mammary epithelial proliferation and contribute to carcinogenesis *in vivo*. The genes encoding FGF-3 and FGF-4 are common integration sites for the mouse mammary tumor virus (MMTV), a naturally occuring retrovirus that induces mammary epithelial tumors (Asch *et al.*, 1996). Moreover, overexpression of FGF-3 in the mammary epithelium has been shown to induce diffuse hyperplasia followed by tumor formation in transgenic animals (Pattengale *et al.*, 1989; Ornitz, Moreadith, and Leder, 1991). More recently, it was reported that expression of a dominant negative FGF receptor isoform, FGFR-2(IIIb), in the mammary epithelium resulted in reduced lobuloalveolar development during pregnancy, supporting the positive effect of FGF signaling on mammary epithelial proliferation (Dickson, Creer, and Fantl, 2000). Given that NF-κB functions downstream of FGFs in embryonic limb, it is plausible that NF-κB might have a similar function in regulating FGF-induced proliferation within mammary epithelium.

It has also been reported that oncogenic erbB2, a receptor tyrosine kinase within the epidermal growth factor receptor (EGFR) family that is frequently amplified and/or activiated in human breast adenocarcinoma, induces NF-κB transcriptional activity in a ras/raf dependent manner (King *et al.*, 1985; Semba *et al.*, 1985; Coussens *et al.*, 1985; Bargmann *et al.*, 1986; Guy *et al.*, 1992; Galang *et al.*, 1996; Zhou *et al.*, 2000). Transgenic mice expressing either proto-oncogenic or oncogenic erbB2 in the mammary epithelium displayed epithelial hyperplasia followed by tumorigenesis (Guy *et al.*, 1992; Muller *et al.*, 1988). Although the precise role of NF-κB in erbB2 signaling is largely unkown, the data presented here suggest that NF-κB might function downstream erbB2 to regulate mammary epithelial proliferation and carcinogenesis.

Conclusions

The NF-κB family of transcription factors has been shown to regulate proliferation in several cell types. Several recent studies have demonstrated that many NF-κB family members are abberantly expressed and/or activated in breast cancer cell lines and in primary tumors (Nakashatri *et al.*, 1997; Sovak *et al.*, 1997). Amplification at the *relA* locus was observed in human breast adenocarcinomas (Motokura and Arnold, 1993). In addition, overexpression and enhanced nuclear localization of p50, p52, c-Rel, and Bcl-3 has been reported in breast cancer cell lines and/or in human tumors (Mukhopadhyay *et al.*, 1995; Dejardin *et al.*, 1995; Cogswell *et al.*, 2000). Here, we present the first *in vivo* evidence that NF-κB activity regulates proliferation of the mammary epithelium and maintains the normal architecture of the epithelium. Moreover, our data suggest that NF-κB activity may contribute to the development of breast adenocarcinoma. These data are consistent with the observation that the induction of NF-κB activity may be an early event in carcinogenesis, as NF-κB activity was induced *in vivo* and in cell culture by the chemical carcinogen 7,12-dimethylbenz[α]anthracene (DMBA) prior to neoplastic transformation (Kim *et al.*, 2000). Futher investigation is required to determine the role NF-κB plays in breast cancer, and may lead to identification of new molecular targets for therapeutic interventions.

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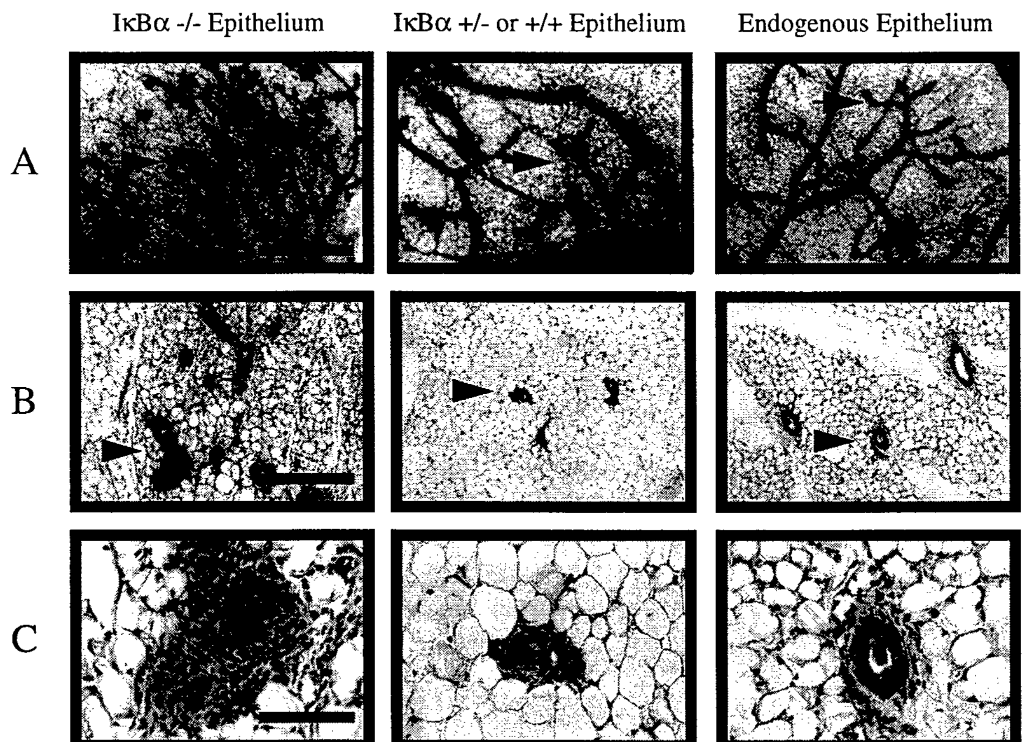
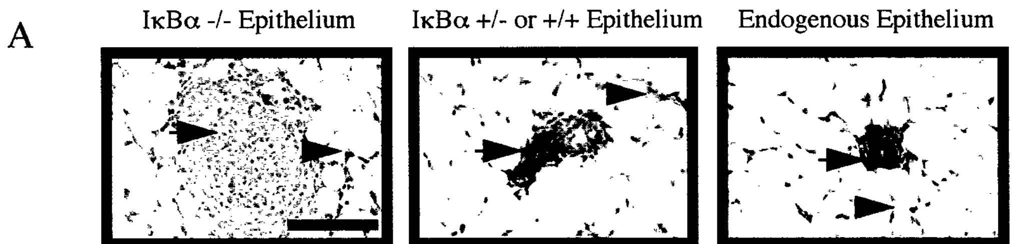


Figure 1: Mammary Glands Harboring *iκbα*-Deficient Epithelium Display Increased Lateral Branching, Diffuse Hyperplasia, and and Abnormal Epithelial Morphology. Whole-mount preparations of mammary glands revealed that epithelial tissue derived from *iκbα*-deficient (IκBα -/-) donors displayed an increase in the number of lateral ductal branches compared to epithelial tissue derived from wild-type/heterozygous (IκBα +/- or +/+) or endogenous host epithelium (A; arrows). Scale bar = 2 mm. Hematoxylin and eosin stained sections revealed that *iκbα*– deficient epithelium displayed diffuse hyperplasia, with an increased epithelial to stromal cell ratio relative to wild-type/ heterozygous or host epithelium (B; arrowhead marks epithelium). Note the reduction in the formation of central luminae in some ducts and the increased number of epithelial cells surrounding ducts that do form. Scale bar = 500 μm. Higher magnification revealed a disorganized structure in *iκbα*-deficient epithelium, with epithelial cells penetrating into the surrounding stroma rather than being confined by a well-defined basement membrane (C). Scale bar = 50 μm.

A



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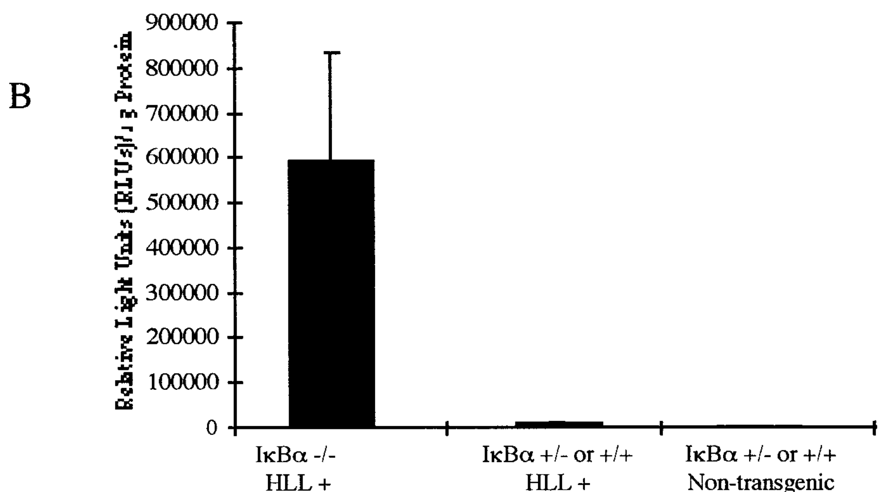
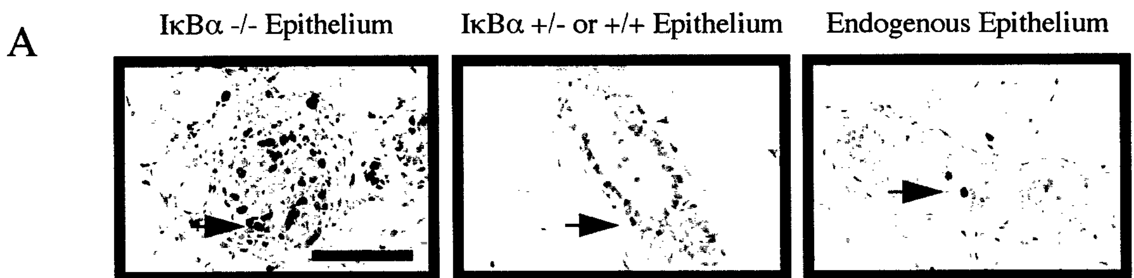


Figure 2: Lack of Epithelial IκBα Protein Expression and Enhanced Transcriptional Activity in *iκbα*-deficient Mammary Tissue. Mammary glands reconstituted with *iκbα*-deficient tissue (IκBα -/-), wild-type heterozygous tissue (IκBα +/- or +/+) or native host glands were subjected to immunohistochemistry to detect IκBα protein expression. Mammary epithelium derived from *iκbα*-deficient donors did not express IκBα in the epithelium (A, arrow), although expression was observed in the surrounding host stroma (A, arrowhead). Mammary epithelium derived from wild-type/heterozygous donors or native host mammary epithelium expressed IκBα protein in the epithelium (A, arrow) and in the stroma (A, arrowhead). Scale bar = 50 μm. Mammary glands from neonatal *iκbα*-deficient or wild-type/heterozygous littermates harboring an NF-κB responsive luciferase reporter transgene (HLL) were analyzed for luciferase activity as a measure of *in vivo* NF-κB transcriptional activity. A 15 fold increase in luciferase activity was observed in *iκbα*-deficient, HLL+ glands relative to controls.

A



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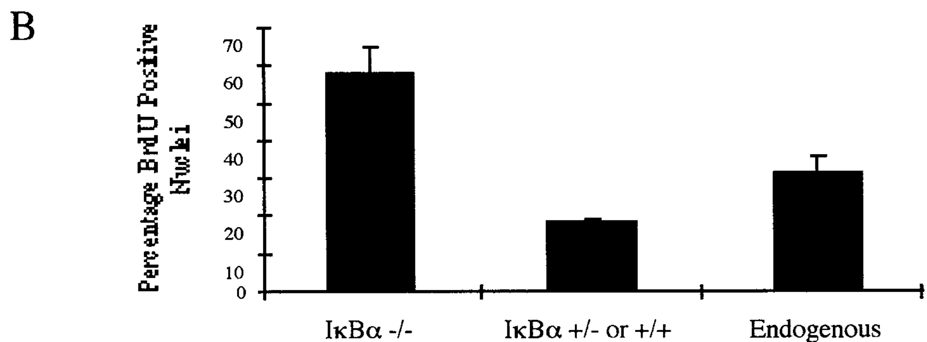

Figure 3: Mammary Glands Harboring *iκbα*-Deficient Epithelium Display Increased Epithelial Proliferation. Incorporation of BrdU was used measure proliferation in *ikba* -deficient mammary epithelium, wild-type/heterozygous (IκBα +/- or +/+) or endogous host epithelium. An apparent increase in the number of BrdU positive nuclei was observed in *ikba* deficient epithelium relatvie to controls (A; arrows indicate BrdU positive nuclei). Scale bar = 50 μm. To quantify this increase in proliferation, the percentage of BrdU positive nuclei relative to the total number of nuclei was calculated. A 2-3 fold increase in the percentage BrdU incorporation was observed in *ikba* deficient epithelium relative to controls (B).

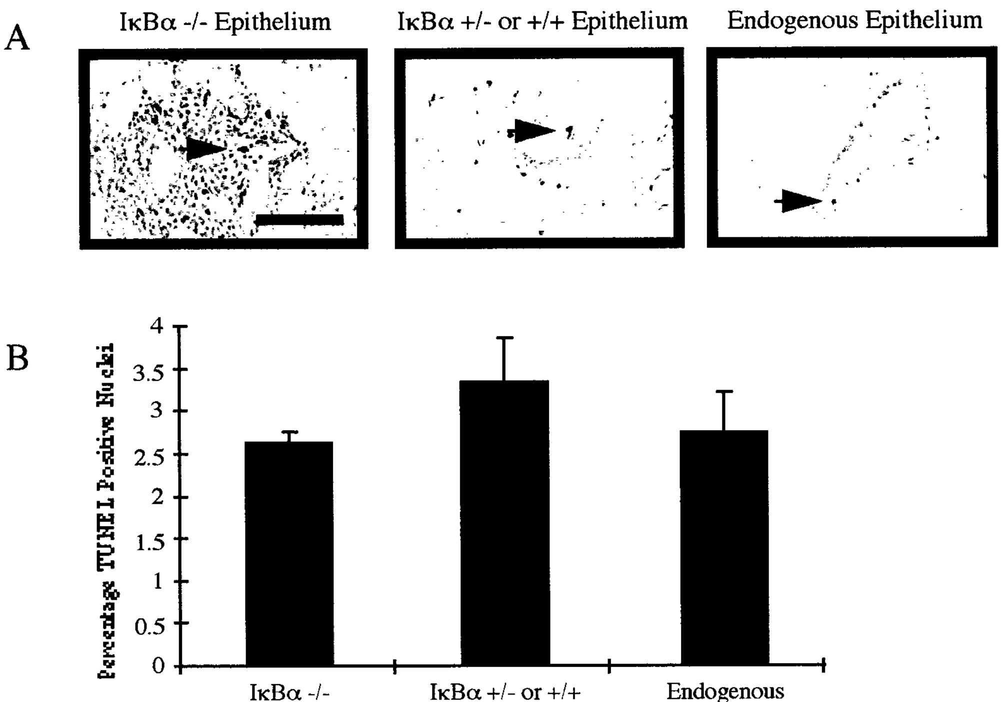

Figure 4: Mammary Glands Harboring *ikbα*-Deficient Epithelium Display No Alteration in Epithelial Apoptosis. TUNEL analysis was used to measure apoptosis in *ikba*-deficient mammary epithelium, wild-type/heterozygous (IkBa +/- or +/+) or endogous host epithelium. No apparent difference between the number of TUNEL positive nuclei was observed in *ikba*- deficient epithelium relatvie to controls (A; arrows indicate BrdU positive nuclei). Scale bar = 50 μm. To quantify the level of apoptosis, the percentage of TUNEL positive nuclei relative to the total number of nuclei was calculated. No difference in the percentage TUNEL postive nuclei was observed in *ikba*--deficient epithelium relative to controls (B).

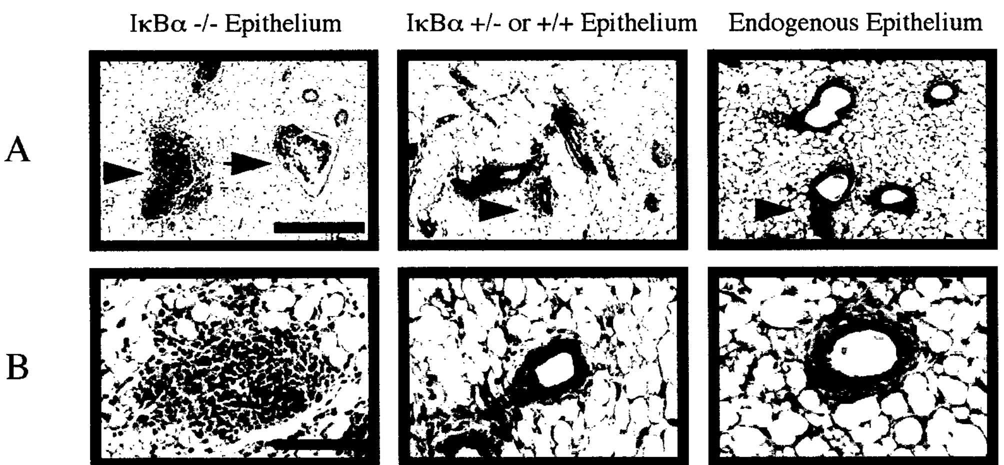

Figure 5: Mammary Glands Harboring *ikbα*-Deficient Epithelium Display A Reduction in Extracellular Matrix.
Trichrome staining was used to visualize the extracellular matrix (ECM) in *ikba*-deficient mammary epithelium, wild-type /heterozygous (IkBa +/- or +/+) or endogous host epithelium. The ECM adjacent to *ikbα*-deficient epithelium was much reduced(A; arrowheads indicate extracellular matrix stained in blue). This reduction was specifically associated with the epthelium, as ECM adjacent to blood vessels in *ikbα*-deficient samples was intact (A; arrow indicateds blood vessel). Scale bar = 100 μm. Higher magnification further illustrates the reduction in ECM adjacent to *ikbα* deficient epithelium relative to controls (B). Scale bar = 50 μM.

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