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A knowledge of the molecular signaling pathways which, when perturbed, result in mammary cell transformation would provide vital information in the fight against breast cancer. The NF-κB family of transcription factors are a major component of pathways regulating cell proliferation, differentiation and death. We are attempting to determine their role in the development of normal and transformed mammary epithelium. Our hypothesis is that expression of specific NF-κB factors is necessary for the proper differentiation and maturation of mammary epithelium and that altered expression of κB factors can effect transformation of mammary epithelium. We have created lines of transgenic mice which overexpress a family member suspected to be involved in carcinogenesis (NFκB2) or a dominant inhibitor of NF-κB activity (IκBα–ΔN) in the mammary gland. These murine models are now being analyzed to determine how proliferation, differentiation and death are changed in response to altered κB activity.

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Frank E. Tull 8/31/00

PI - Signature Date

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# INTRODUCTION

The nuclear factor-kappaB, NF-κB, family of transcription factors play roles in normal functions such as cellular proliferation, differentiation, migration, and programmed cell death (1, 2). They are also implicated in the pathogenesis of cancer, particularly in cells of the immune system (3). However, little is known concerning the precise mechanisms by which this occurs. Our research has two major goals. The first is to characterize the role of the transcription factor complex in normal mammary development. The second is to investigate the reported correlation between the presence of an unusual isoform of NF-κB, NFκB2, and breast adenocarcinoma (4). Our strategy has been to generate novel transgenic models. In the first model, the activity of NF-κB is blocked in mammary epithelial cells by the expression of a dominant inhibitor (IκBΔN) to determine the effects of disturbing normal signalling pathways. In the second model, NFκB2 (p100) is over-expressed in mammary epithelial cells to determine whether this protein can induce cellular transformation. If this were the case we would have generated a novel murine model of mammary tumorigenesis. In both cases we have used the promoter of the major whey protein of ruminants, β-lactoglobulin (BLG) to target transgene expression. We have generated four lines of transgenic mice targeting IκBΔN to the mammary gland, designated MAN for mammary -αΔN. We have also produced four lines of mice targeting overexpression of NFκB2 (p100), designated BIN for BLG-inhibitor. The lines are currently being characterized.

## BODY

The Rel/NF-κB family of transcription factors represents a distinct subset of nuclear transactivating factors whose activity is inducible via control of its nuclear localization. These enhancer binding proteins are sequestered in the cytoplasm by inhibitory molecules, termed IκBs. Upon stimulation the IκB is degraded, and the κB factors are released to enter the nucleus and associate with their cognate DNA binding sites initiating gene transcription (5). Authentic NF-κB is a heterodimer consisting of a 50 kD polypeptide (NFKB1=p50) (6) and a 65 kD polypeptide (RelA=p65)(7) whose primary sequence reveals that the amino-terminal halves of both p50 and p65 are highly homologous to that of the *rel* protein (defining the Rel homology domain, RHD). The RHD encompasses the DNA-binding, dimerization, IκB-binding, basal transcription factor binding, and nuclear localizing domains for these proteins. The carboxy terminal domains of RelA(p65) and cRel contain strong transcriptional transactivation regions (8). In contrast, p50 homodimers bind DNA but are thought to block transcription. In vertebrates, other genes also encode factors which participate in the NF-κB complex and bind to κB enhancer elements: NFκB2 (p52 and its precursor p100)(9), relb (RelB), and v-rel.

IκBα forms a complex with heterodimeric NF–κB and inhibits the DNA binding activity (10). Following stimulation with a wide variety of distinct agents, the IκB-α protein is phosphorylated on serine residues in the amino-terminus targeting it for destruction by the ubiquitinization/proteosome (26S) degradation pathway, allowing the translocation of NF-κB to the nucleus (10,11,12). A mutation in the IκB-α which removes these serine residues creates an IκB-α protein (termed IκBΔN) behaves as a transdominant inhibitor (13). This has provided an effective means by which to "capture" NF-κB in a cytoplasmic-bound state, thereby preventing its transactivating capabilities (14).

NFκB2 was originally identified via its involvement in a lymphoma-associated chromosomal translocation and is expressed as a 3.2 kilobase mRNA encoding a 100 kDa protein (15). Following proteolytic cleavage to the mature p52 kDa protein, NFκB2-p52 can heterodimerize with all known κB factors and activate the transcription of genes containing κB-enhancer elements. The NFκB2-p100 precursor has activity on its own; p100 behaves as an IκB

molecule by sequestering NF-κB complexes in the cytoplasm. In one lymphoid-tumor case reported, overexpressed NFκB2 proteins resulted in abnormal transcriptional regulatory properties (16) suggesting that alteration in NFκB2-regulated gene expression may be causal for transformation. These results suggest that overexpression of either the precursor molecule, p100, or its processed fragment, p52, might alter the normal cellular NF-κB complexes and disregulate cellular homeostasis.

In order to target expression of our transgenes to mammary epithelial cells we have used the promoter of the major whey protein of ruminants, β-lactoglobulin (BLG). Despite the fact that rodents do not have an endogenous BLG gene transgenic mice carrying BLG constructs express specifically and abundantly in the murine mammary gland in an appropriate temporal pattern (17,18). Transgenes using 5' and 3' BLG flanking sequences can target expression of heterologous genes in an appropriate developmental pattern to the mammary (19,20,21). In the previous annual report we had succeeded in generating four lines of transgenic mice targeting IκBΔN to the mammary gland, designated MAN for mammary -αΔN. We had also produced four lines of mice targeting overexpression of NFκB2 (p100), designated BIN for BLG-inhibitor. This report details further characterization of these lines.

## Results:

Specific aim 1 (Task 1) for this proposal was to define the expression pattern of NF-κB factors in normal murine mammary gland development. This aim was addressed in collaboration with Dana Brantley (awarded a pre-doctoral fellowship - Award Number DAMD17-97-1-7017) and has led to publication of a manuscript (22). See relevant final report for details.

Specific aim 2 proposed the generation and characterization of two types of transgenics targeting the transdominant inhibitor IκBΔN or NFκB2 expression to the murine mammary gland. Four lines representing each type of transgenic have been generated (Tasks 2- 5). All lines have been backcrossed five generations onto an FVB strain background for future carcinogenesis studies. A repository of samples has been collected representing a range of developmental stages throughout pregnancy, lactation and regression (Tasks 6-7). These include; virgin, 10.5 and 16.5 dpc (days post coitem, ie. pregnant), 1.5 and 9.5 lactating and 1, 3, 5 and 40 days and 5 weeks post forced wean (at day 10 of lactation) and greater than 1 year. At each stage one gland is removed for histological processing, one is processed in TriZol reagent for RNA extraction, one is flash frozen for isolation of cytoplasmic and nuclear proteins for EMSA, and one gland is preserved for whole mount gland preparation. The particular gland collected for each type of analysis remains constant. Collection of these stages represents a major time commitment. Transgenic animals are identified by tail biopsy and PCR. Female transgenics are set up in timed matings and day post coitem noted by detection of a vaginal plug. Then animals are sacrificed on the relevant day of pregnancy, lactation etc. Breeding studies to generate BLG-ΔN/HLL:MAN and BLG-ΔN/HLL:BIN double transgenics are well underway and preliminary analysis completed (Tasks 8-9). The cross breeding to generate BLG-NFκB2/p53-/- lines has not been started. The p53-/- carcinogenesis model mice are on the FVB strain background and it was decided that it would be prudent to backcross the MAN and BIN transgenics onto the same genetic background before commencing these studies. Such backcrosses were not included in the original Statement of Work and require considerable time investment.

To summarize in more detail progress to date:

Primary characterization of expression has been completed by Northern blot analysis. Expression of IκBΔN is detected in all four separately derived MAN transgenic lines ie. MAN8, 13, 18 and 19. Expression of p100 has also been detected in three out of four separately derived

NFκB2 (p100) lines ie. BIN10, 16 and 18. Offspring from the fourth founder animal, representing the BIN6 line, whilst carrying the transgene have either extremely low expression or a "silent transgene" (see prev. reports). We have elected to continue sample collection and backcrossing with the BIN6 line as it has proved to be a difficult line to propagate suggestive of some trangene effect.

Northern analysis included investigation of β-casein expression. Although the transgenics appear capable of nursing litters we were interested in whether transgene expression alters milk protein production. We have analysed total RNA samples representing 16.5 dpc, 1.5 days lactation and 9.5 days lactation in all four IκBΔN expressing (MAN) lines and in all four BIN lines. In all lines tested and at all stages, the expression of either transgene does not appear to have a significant effect on β-casein expression.

In order to determine whether transgene expression perturbs normal NF-κB activity, we have analyzed the binding activity of protein extracts by electrophoretic mobility shift assay (EMSA) using a probe derived from the HIV-LTR that represents a consensus binding site for NF-κB. We have investigated binding activity at the 16.5 dpc and 9.5 lactating points. The four p100 (BIN) lines have so far shown no significant differences at the 16.5 dpc stage. However, analysis of the 9.5 lactation stage detects intriguing differences (see previous report). In non-transgenic animals there is barely detectable binding activity during lactation (22). In transgenics a strong shifted complex is present. This complex can be competed for by competitor oligos. Supershift experiments are being attempted using anti-p52, anti-p65 and anti-p50. Whilst no supershifts indicating the presence of p52 or p65 are detected we have been experiencing some technical difficulties with these antibodies such that further investigation is underway to confirm this result. It appears as though the p50 protein represents a large component of the transgenic complex. Preliminary analysis of protein extracts representing the 9.5 day lactation stage of IκBΔN (MAN) transgenics has detected no significant alteration in NF-κB binding activity with no retarded complexes in transgenics or controls. However, EMSA of protein samples from the 16.5 dpc stage show subtle changes. At this stage of pregnancy in a non-transgenic animal there is strong NF-κB binding activity (22). Formation of this complex is inhibited by unlabelled oligonucleotide. There appears to be a decrease in the retardation of the complex (or a decrease in the presence of the upper band) in separately derived transgenic lines MAN8 and MAN19. This is interesting in the light of our observation of a decrease in cyclinD1 RNA expression (as determined by RPA analysis) in these lines.

One of the possible predicted effects of perturbation of NF-κB activity would be to alter proliferation. Intraperitoneal injections of BrdU were administered to animals at day 16.5 of pregnancy. Mice were sacrificed 4 hours later, mammary glands fixed overnight in 4% paraformaldeyde, dehydrated and paraffin embedded sections prepared. Sections were processed using a BrdU staining kit (Zymed) to investigate proliferation of the alveolar epithelial cells (see previous report). Proliferating cells were detected in all samples tested with no obvious qualitative differences in proliferation between transgenics and non-transgenic controls. However, some animals exhibited histological differences including a relative decrease in the proportion of alveolar cells populating the "web-like" stroma, a less uniform morphology and an increase in the number of ductal structures. A limited number of samples have been tested. We plan to expand this set and carry out more quantitative measurements by counting relative numbers of proliferating cells. Particularly in light of the results from the work supported by pre-doctoral fellowship - Award Number DAMD17-97-1-7017 in this laboratory which suggest that in the absence of a major inhibitor of NF-κB activity the developing mammary gland becomes hyperplastic.

A continuing concern is the potential for between animal variation (especially as the original mice are B6/D2 (ie.a mixture of both the C57Bl6 and DBA mouse strains). At this stage we are still unable to confirm whether there may be a measure of mosaicism in the expression of the

transgenes. We attempted to addess this issue using immunohistochemistry of collected paraffin sections, however, our attempts were unsuccessful due to technical difficulties with the available commercial p52 antibody. We immunized rabbits in an effort to produce a COOH-terminal IκB-α antibody which would be effective for both immunohistochemical and western analyses of the IκBΔN (MAN) transgenics. A second paired set of immunizations were to produce N- and COOH-terminal antibodies to the p100 protein. These would enable us to effect immunohistochemical and western studies on the BIN lines and to distinguish between the unprocessed p100 form of the protein and the truncated p52 protein. The terminal bleeds of the rabbits have been completed. An N-terminal antibody (antigen designed and injected with the original set) has proved to be very useful for other studies in the laboratory. However, this antibody cannot be used to recognize the N-terminally deleted form of IκBΔN used in our transgenics. The COOH-terminal antibody has not proved to be useful as it cross reacts with endogenous mouse MAD3. The p100 sera remain to be tested.

Mammary epithelial transformation and neoplastic progression, like most cancers, involves cooperative changes in more than one oncogenic or tumor-suppressor pathway. Eventually, it is our intention to cross selected BIN and MAN lines with mice expressing the mouse mammary tumor virus (MMTV) driven-activated *neu* transgene, which reproducibly develop clonal mammary tumors, to determine whether transgene expression can either inhibit or cooperate in producing mammary tumorigenesis. Furthermore, it would be interesting to breed BLG-NFκB2/p100 expressing mice with mice expressing the most common p53 mutation ($Arg^{172}$-His) in human breast cancers to explore the ability of NFκB2 to act as a dominant oncogene in the absence of the well characterized tumor suppressor, p53. These interbreedings would explore the cooperative nature between these NF-κB components and known mammary carcinogenic signal transduction and genetic pathways in the hopes of better understanding the mechanistic interactions of the oncogenic pathways in human mammary tumorigenesis. The lines of mice described above exist on an FVB strain background whereas our lines were generated on a B6/D2 background. In order to avoid the potential difficulties of interpreting data on a background consisting of a genetic mix of three strains we have backcrossed our lines onto the FVB strain. We have achieved at least 5th generation backcross and are characterizing these animals for transgenic phenotype.

We have generated double transgenics between our mammary lines and reporter lines generated in this lab designated HLL (23). The HIV-1 LTR has been extensively characterized as a NF-κB responsive promoter (24). These lines carry the NF-κB responsive human immunodeficiency virus-1 long terminal repeats, HIV-LTR, promoter fused to luciferase which enables direct analysis of the expression of an *in vivo* NF-κB reporter. This provides an extremely valuable system to determine whether expression of IκBΔN is blocking NF-κB activity *in vivo*. and to determine whether the overexpression of NFκB2 results in an active transcription factor or whether the full length protein (p100) is behaving as an inhibitor of other NF-κB complexes and thus, preventing transcriptional activation. Our results so far are as follows:

Preliminary analysis of double transgenic animals for luciferase expression at 16.5dpc stated as relative light units per μg total protein (rlu/μg).

IκBα–ΔN (MAN) transgenics have relatively decreased NF-κB activity.

| | |
|---|---|
| HLL | 1106 ± 288 rlu/μg |
| HLL/MAN | 537 ± 268 rlu/μg |

p100-NFκB2 (BIN) transgenics have relatively increased NF-κB activity.

| | |
|---|---|
| HLL | 24 ± 8 rlu/μg |
| HLL/BIN10 | 118 ± 79 rlu/μg |
| HLL/BIN16 | 73 ± 8 rlu/μg |

[The difference in control single HLL transgenic background between the MAN study and the BIN study is the result of use of a different protocol and type of luminometer with decreased background levels of luciferase activity]

The FVB strain background is believed to be more susceptible to tumor formation than the current B6/D2 strain background. In addition very limited effects of leaky transgene expression would be expected in virgin animals (as the promoter used normally directs expression of a milk protein). Thus we were surprised on examining haematoxylin and eosin stained sections from older, virgin BIN transgenic animals to note some morphological differences relative to control glands ( see previous report). This may be an indication of future more profound effects on older animals on the FVB strain background after a couple of rounds of pregnancy. We are collecting samples from older FVB transgenic virgins and have set up a small pilot study of control and BIN transgenics on a continuous mating schedule.

## Key Research Accomplishments

1) Generation of four lines of transgenic mice expressing a dominant inhibitor of NFκB (IκBΔN).

2) Generation of four lines of mice carrying a mammary specific NFκB2 (p100) transgene.

3) Characterization of above lines confirming mammary specific transgene expression.

4) Transgene expression has no profound effect on milk production.

5) Backcross of 8 lines to 5th generation onto the FVB strain background for future carcinogenesis studies.

6) Putative generation of antibodies to the COOH terminal of IκBα and both termini of NFκB2.

## Reportable Outcomes

1) Manuscript:
Brantley, D.M., Yull, F.E., Muraoka, R.S., Hicks, D., Cook, C.M. and Kerr, L.D. Dynamic expression and activity of NF-κB during post-natal mammary gland morphogenesis. (In Press, Mechanisms of Development).

Abstracts:
Yull, F.E., Brantley, D.M., Seetharaman, R., Bradberry, J. and Kerr, L.D. Transgenic models to study the role of NF-κB in mammary morphogenesis and neoplasia. Era of Hope, Department of Defense Breast Cancer Research Program Meeting, Atlanta (2000).

Brantley, D.M., Chen, C-L., Yull, F.E. and Kerr, L.D. Nuclear factor-kappa B (NF-κB) factors in mammary epithelial development and proliferation. Era of Hope, Department of Defense Breast Cancer Research Program Meeting, Atlanta (2000).

Yull, F.E., Brantley, D.M., and Kerr, L.D. Transgenic models to investigate the role of NF-κB in normal and transformed mammary epithelium. Society for Developmental Biology SE Regional Meeting, Emory University, (1998).

2) Accumulation of major repository of tissue, RNA, protein, whole mount and paraffin section samples representing multiple stages of mammary development from wild-type and 8 transgenic lines. Samples collected from both the original mixed C57Bl6/DBA animals and 5th generation backcross to the FVB strain.

3) The IκBΔN transgenics (MAN) represent a novel model which will be exploited to investigate the roles of NFκB in normal mammary development. These will permit examination of the role of NFκB in pregnancy (proliferation/differentiation), lactation (secretion) and post-weaning (apoptosis).

4) The NFκB2 transgenics (BIN) represent a novel model which will be exploited to investigate the role of NF-κB in carcinogenesis.

## CONCLUSIONS

Our hypothesis was that the nuclear expression of specific NF-κB factors is necessary for the proper differentiation and maturation of mammary epithelium and that alteration in the expression of κB factors can effect transformation of mammary epithelium. We have established lines of transgenic mice in which NF-κB levels are perturbed by overexpression of an NF-κB family member correlated with human mammary carcinogenesis (NFκB2) or by expression of a dominant negative factor (IκBα–ΔN), inhibitory towards nuclear NF-κB activity, in the mammary gland. These transgenics are designated BIN and MAN respectively. Analysis of the transgenic lines is continuing. Indications are that the transgenes are able to perturb NF-κB activity at different stages but further studies are required to determine the nature of these alterations. No profound effect on the ability of either transgene to alter milk protein production has been detected. Suspected effects on proliferation are proving difficult to confirm.

We have backcrossed our animals onto the FVB strain to alleviate any potential difficulties associated with variation due to the original C57Bl6/DBA mixed genetic background and are continuing characterizations on the FVB genetic background. The FVB backcrosses are also required for tumorigenesis studies which we hope to secure funding to continue. We have also attempted to generate antibodies to determine whether the transgenes are expressed consistently across entire glands or whether some degree of mosaic expression may be present.

We have utilized transgenic lines generated in this laboratory in which the NF-κB responsive HIV-LTR directs expression of luciferase, enabling direct *in vivo* analysis of NF-κB activity. These transgenic lines have been crossed with the mammary transgenics. Results suggest that expression of the dominant inhibitor of NF-κB, IκB-αΔN, blocks κB activity*in vivo*. Overexpression of NFκB2 appears to result in an active transcription complex during pregnancy. Initial histological studies also suggest that expression of the transgenes can result in aberrant morphogenesis of the mammary gland during pregnancy.

At this time no clear indications of transformation are apparent in the BIN lines. This may be due, in part to genetic susceptibility. Representative lines have been backcrossed five generations onto the FVB genetic background, known to be susceptible to tumorigenesis for further studies. These are scheduled to include generation of MMTV-neu/BIN double transgenics to investigate the potential for co-operative effects.

At this stage indications are that the transgenes differentially affect NFκB complex formation and activity. More information is necessary before it can be determined whether disregulation of NF-κB complex formation results in altered regulation of gene products leading to unscheduled growth, misregulated differentiation, altered rates of apoptosis, excess angiogenesis or invasiveness.

In summary, in relation to the original statement of work, samples collected for technical objective 1 have been included in the results reported for award #DAMD17-97-1-7017. Tasks 2-7 of technical objective 2 have been completed. Task 8 has been completed for the breeding studies to generate BLG-ΔN/HLL animals and characterization as per Tasks 9 is underway.

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# Appendices

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GENE EXPRESSION PATTERNS SECTION

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

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## 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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## ure Legends

ure 1: NF-κB and IκBα are expressed in the mammary gland epithelium over the course of t-natal morphogenesis. Histological sections were prepared from virgin, 16.5 day pregnant .5 dpc), 5.5 day lactating (5.5 lac), and 3 days post-wean involuting (3 dpw) number 3 thoracic ids. These sections were probed with primary antisera for p105/p50 (A) , RelA (B), or IκBα . Epithelial expression of RelA increased at 16.5 days of pregnancy (B), whereas expression κBα decreased at 3 days post-wean involution. Histological sections from wild-type and 5/p50-deficient mammary glands were probed with antisera against p105/p50 to confirm the ificity of the antisera. Expression of p105/p50 was detected in sections from wild-type virgin nals but not in sections from p105/p50-deficient virgin animals (D). Data presented are a esentation of results from 3 to 6 independent samples per developmental stage. Scale bar = 50

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.

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

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

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.

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