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Award Number: DAMD17-97-1-7266

TITLE: The Role of NF-kB in Normal and Transformed Mammary Epithelium

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REPORT DATE: August 1999

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPOR	ATES COVERED
	August 1999	Annual	(1 Aug 98 - 31 Jul 99)

4. TITLE AND SUBTITLE
The Role of NF-kB in Normal and Transformed Mammary Epithelium

5. FUNDING NUMBERS
DAMD17-97-1-7266

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

A knowledge of the molecular signalling pathways which when perturbed result in mammary cell transformation will provide us with information in the fight against human breast cancer. The NF-κB family of transcription factors are a major component of signal transduction pathways regulating cell proliferation, differentiation and death. We are investigating their role in the development of normal and transformed mammary epithelium. Our hypothesis is that expression of specific NF-κB factors is necessary for differentiation and maturation of mammary epithelium and that alteration in NF-κB factor expression can effect transformation. We have created lines of transgenic mice which perturb NF-κB levels by overexpression of the family member already suspected to be involved in carcinogenesis (NFκB2) and of a dominant inhibitor of NF-κB activity (IκBα-ΔN) in the mammary gland. Transgene expression of both types has been confirmed at various stages during mammary development. This expression alters the profile of NF-κB activity during pregnancy and lactation. The downstream effects of such alterations are being characterized.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer NF-κB, IκB, transcription factor, transgenics, adenocarcinoma	16
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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Frank E. Tull 8/27/99

PI - Signature Date

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INTRODUCTION

Breast cancer is a major cause of cancer mortality for women in the United States and represents the highest rate of new cancer cases for women (1). Knowledge of the molecular signalling pathways involved in the disease process may lead to novel therapeutic strategies. 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 (2,3,). They are also implicated in the pathogenesis of cancer, particularly in cells of the immune system (4). However, little is known concerning the precise mechanisms by which this occurs. This 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 correlation between the presence of an unusual isoform of NF-κB, NFκB2, and breast adenocarcinomas (5). These questions are being addressed by the generation and characterization of novel transgenic models. One strategy is to block the activity of NF-κB in a transgenic mammary gland and determine the effects of disturbing the normal signalling pathways. The other approach we are employing is to overexpress NFκB2 (p100) to determine whether this protein can induce cellular transformation. Should this prove to be the case we would have generated a novel murine model of mammary tumorigenesis. Our results to date are presented in this review.

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 (6). Authentic NF-κB is a heterodimer consisting of a 50 kD polypeptide (NFκB1=p50) (7) and a 65 kD polypeptide (RelA=p65)(8) 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 (9). 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)(10), relb (RelB), and v-rel.

IκBα forms a complex with heterodimeric NF–κB and inhibits the DNA binding activity (11). 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 (11,12,13). A mutation in the IκB-α which removes these serine residues creates an IκB-α protein (termed IκBΔN) behaves as a transdominant inhibitor (14). This has provided an effective means by which to "capture" NF-κB in a cytoplasmic-bound state, thereby preventing its transactivating capabilities (15).

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 (16). 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 (17) 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, B-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 (18,19). Transgenes using 5' and 3' BLG flanking sequences can target expression of heterologous genes in an appropriate developmental pattern to the mammary (20,21,22). 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 for this proposal was to define the expression pattern of NF-κB factors in normal murine mammary gland development. This aim is being addressed in collaboration with Dana Brantley (awarded a pre-doctoral fellowship - Award Number DAMD17-97-1-7017) and has led to submission of a manuscript (23). See relevant annual 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. A repository of samples has been collected representing a range of developmental stages throughout pregnancy, lactation and regression . 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 (as detailed in previous report). 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.

Primary characterization of expression is effected by Northern blot analysis. Results are consistent with those reported in the previous years report with expression of IκBΔN being 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".

Analysis at the level of total RNA has been extended to include 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. Figure 1a shows expression of the p100 transgene and β-casein RNA in mammary samples representing non-transgenic control and lines BIN6, 10, 16, and 18 at day 1.5 of lactation.To detect NFκB2 mRNA, probes of human origin are used that do not cross-react with the endogenous murine NFκB2. Figure 1b shows expression of the IκBΔN transgene and β-casein at 16.5 dpc in samples representing control animals and the four MAN 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 is perturbing normal NF-κB activity, we are analysing the binding activity of protein extracts by electrophoretic mobility shift assay (EMSA). We have started by looking at binding activity at the 16.5 dpc and 9.5 lactating points. The probe used is derived from the HIV-LTR and represents a consensus binding site for NF-κB. Results from the 16.5 dpc stage of the four p100 (BIN) lines have so far shown no significant differences (data not shown). However, analysis of the 9.5 lactation stage detects intriguing differences (Figure 2a). In non-transgenic animals there is barely detectable binding activity during lactation (23). In transgenics a strong shifted complex is present. This complex can be competed for by competitor oligos (+C). 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 (data not shown). However, EMSA of protein samples from the 16.5 dpc stage show subtle changes (Figure 2b). At this stage of pregnancy in a non-transgenic animal there is strong NF-κB binding activity (23). Formation of this complex is inhibited by unlabelled oligo (+C). 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 previous preliminary observation of a decrease in cyclinD1 RNA expression, particularly 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 (Figures 3+4). The staining of an intestinal sample is included as a control. 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.

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 have

attempted to addess this issue using immunohistochemistry of collected paraffin sections. So far our attempts have been unsuccessful due to technical difficulties with the available commercial p52 antibody. We have recently 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 have been carried out to produce both N-terminal 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.

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, BLG-NFκB2/p100 expressing mice will be interbred 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. All combined, these interbreedings will 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 are 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 been backcrossing our lines onto the FVB strain. For multiple lines we have achieved 5th generation backcross and are beginning to characterize these animals for transgenic phenotype.

We intend to cross our transgenic lines with reporter lines generated in this lab designated HLL (24). The HIV-1 LTR has been extensively characterized as a NF–κB responsive promoter (25). 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 will provide an extremely valuable system which will allow us to determine whether expression of IκBΔN is blocking NF-κB activity *in vivo*. Such crosses will also 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. An initial pilot study with a very limited number of animals appears promising. HLL transgenic animals at 16.5 days of pregnancy produce 1106 ± 288 relative light units/μg of protein. Mice carrying the MAN transgene in addition to the HLL transgene at the same stage produce 537 ± 268 rlu/μg protein. This relative decrease in measured luciferase activity suggests that the MAN transgene is able to inhibit NF-κB activity. Expansion of this study is underway.

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 (Figure 5). 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.

CONCLUSIONS

Analysis of the transgenic lines is in progress. 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. A degree of variation between animals is apparent, possibly exacerbated by the fact that our transgenics are on a B6/D2 background ie. a mixture between C57Bl6 and DBA strains. We have backcrossed our animals onto the FVB strain to alleviate any potential difficulties associated with such variation and are continuing characterizations on this genetic background. In addition FVB backcrosses were required for proposed tumorigenesis studies.

We are extremely interested in determining whether the transgenes are expressed consistently across entire glands or whether some degree of mosaic expression may be present and are generating antibodies for these studies.

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 2-4 of technical objective 2 have been completed and characterization as per Tasks 5-7 is underway. A small pilot study representing Task 8 has been completed.

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Appendices

Key Research Accomplishments

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

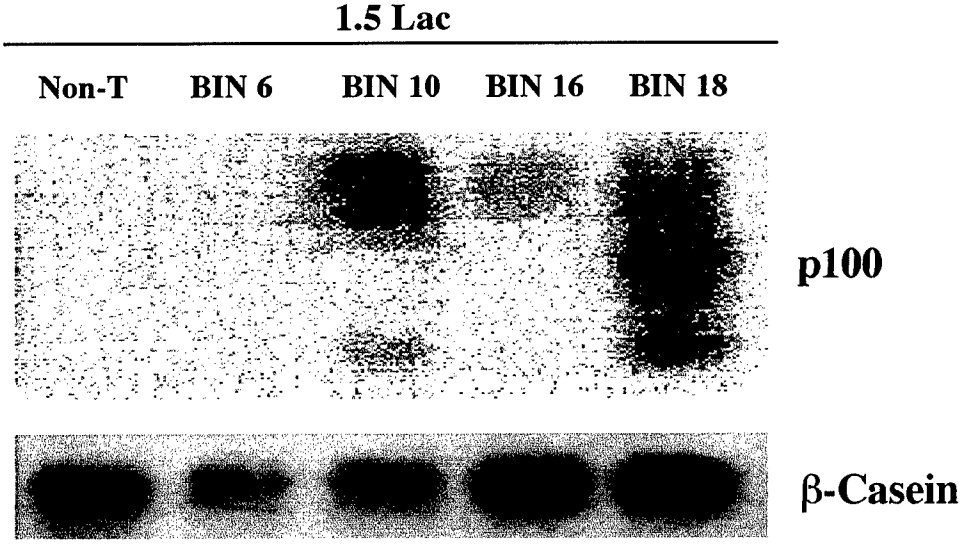
2) Generation of four lines of mice carrying a mammary specific p100 transgene.

3) Basic characterization of lines.

4) Accumulation of major repository of tissue, RNA, protein, whole mount and paraffin section samples.

Figure 1 : Northern Analysis of Transgene / β-Casein Expression

Figure a



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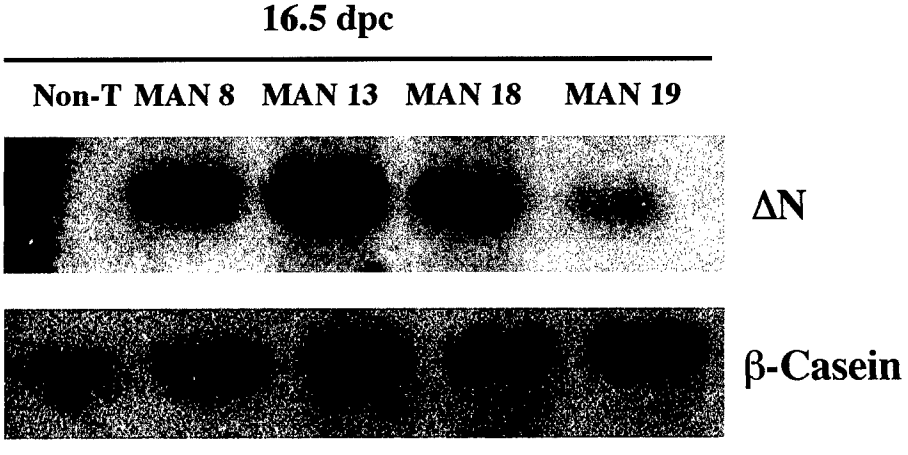
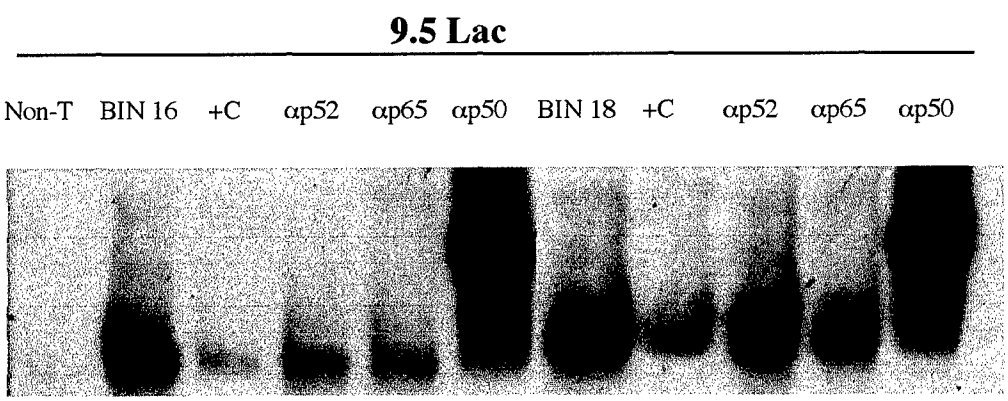


Figure 2 : NF-κB Binding Activity in Transgenics

Figure a



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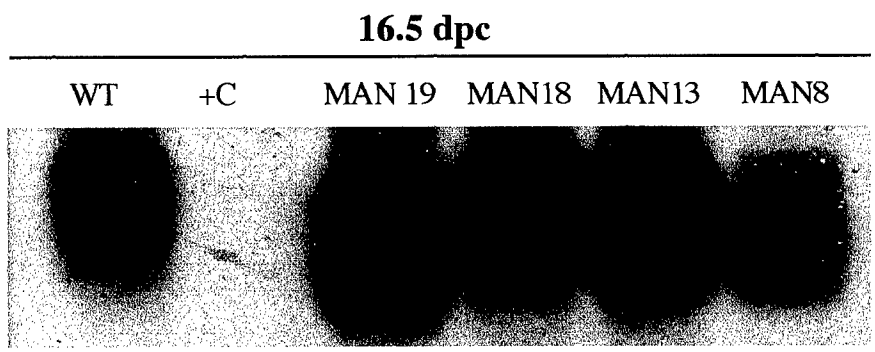
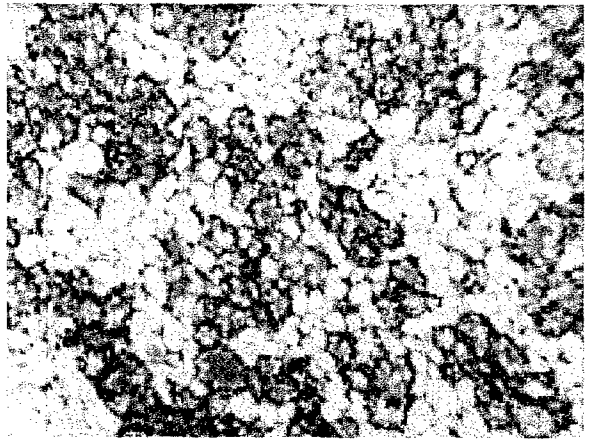
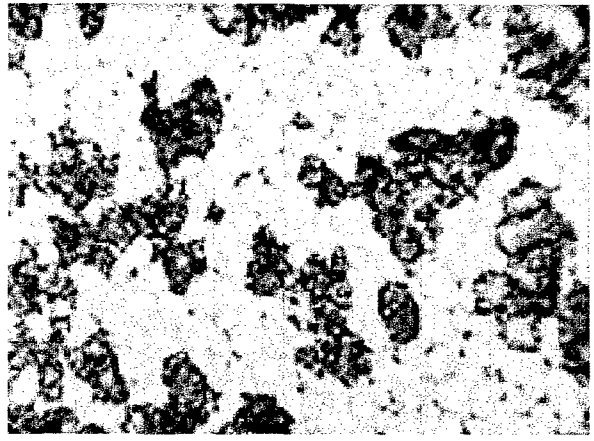


Figure 3 : BrdU incorporation at 16.5dpc/BIN transgenics

Intestine/ control



Non-Transgenic A



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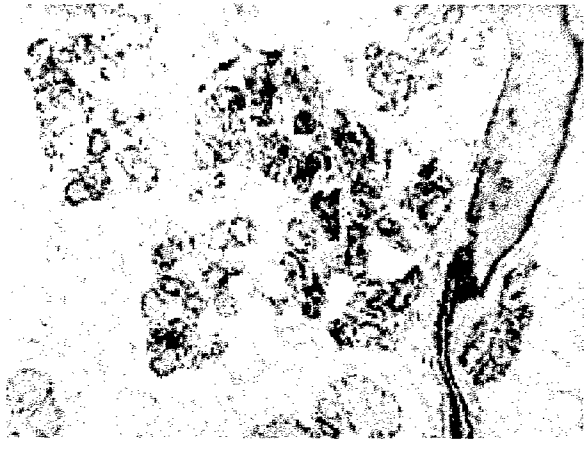

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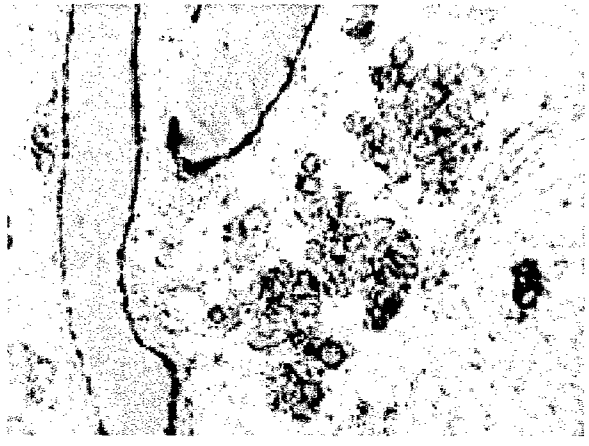

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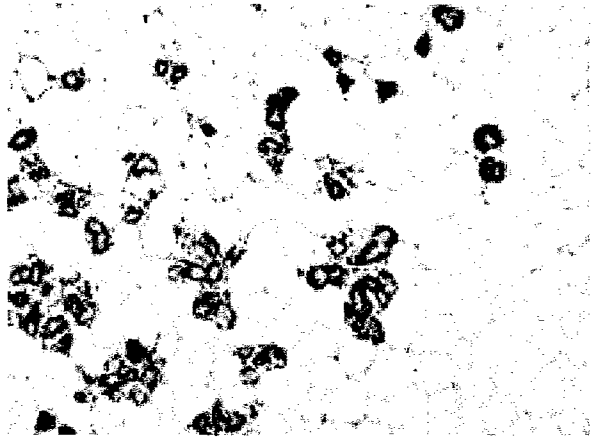
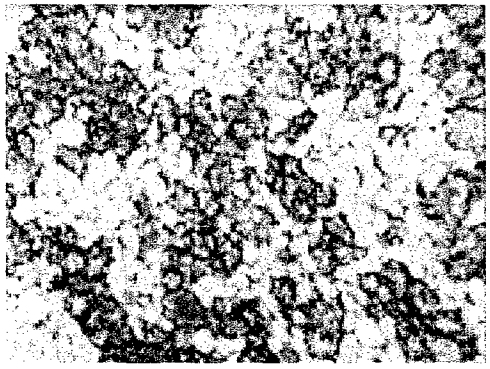


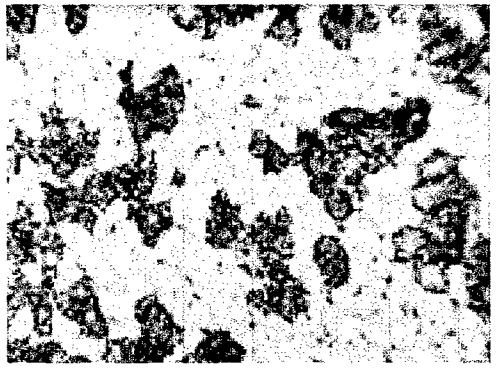
BIN 18

Figure 4 : BrdU incorporation 16.5dpc/MAN transgenics

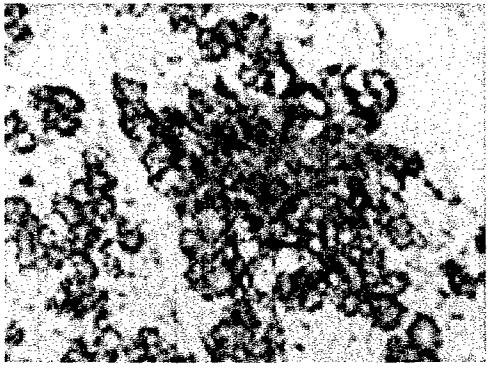
Intestine/ control



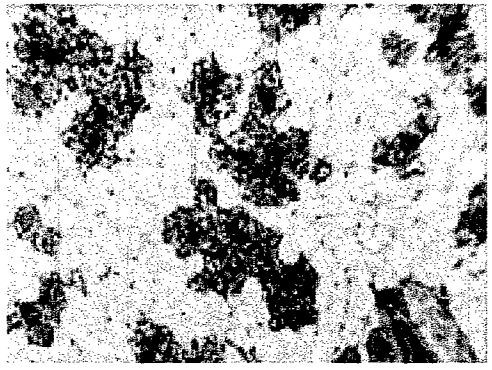
Non-Transgenic A



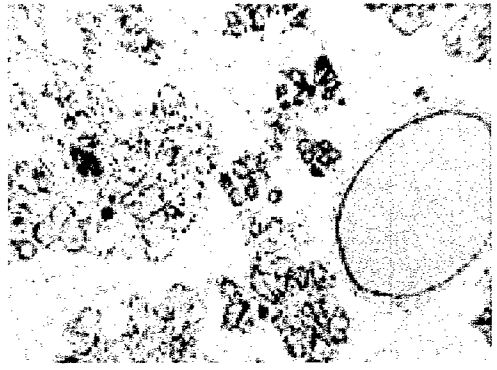
Non-Transgenic B



MAN 8



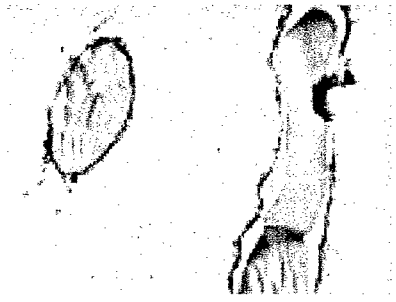
MAN 13



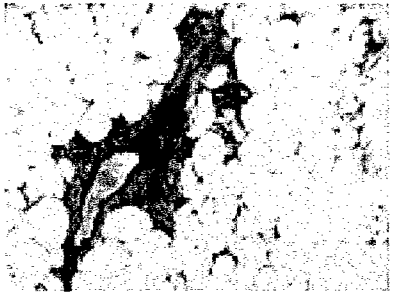
MAN 19

Figure 5 : H&E of old mammary

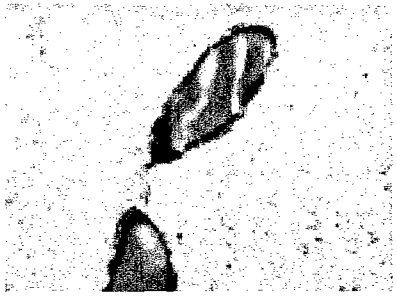
Non-Transgenic
vir 10 months



BIN 10
vir 12 months



BIN 16
vir 10 months



BIN 18
vir 11 months