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The tumor suppressor Rb is a nuclear-phosphoprotein that controls cell proliferation, survival and differentiation and is thought to be either mutated or functionally inactivated by phosphorylation in virtually all human cancer including breast cancer. We developed transgenic mouse models to study the outcomes of upregulating the Rb pathway in the mammary gland and test whether Rb is a universal tumor suppressor. Unphosphorylatable alleles of Rb (RbΔp34 and RbΔK11, with 8 and 11 CDK sites mutated) were targeted to the mammary epithelium under control of the MMTV-LTR (proliferating and differentiating mammary epithelium) and WAP (differentiating epithelial cells) promoters. Pre-pubertal MMTV-RbΔp34 and MMTV-RbΔK11 transgenic female mice exhibited suppression of ductal growth and branching. During estrus, these transgenic females displayed enlargement of the alveolar compartment. Intriguingly, some MMTV-RbΔp34 and MMTV-RbΔK11 transgenic females developed focal hyperplasia as well as full-blown mammary adenocarcinomas. In accord with the more restricted pattern of expression, WAP-RbΔK11 transgenic mice did not exhibit the early suppression of ductal growth, seen in MMTV-RbΔK females, but about 20% developed focal hyperplasia and some developed breast tumors by one year. Though exactly the opposite of what we expect, these provocative and novel results are in accord with our emerging understanding of Rb as a major regulator of both cell proliferation and survival. To determined synergistic or suppressing effects on breast cancer, genetic crosses outlined in the Statement of Work will be carried out by breeding the RbΔK transgenic mice with MMTV- Neu and MMTV- wnt mice that are predisposed to breast cancer.

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E. Zacksenhaus Sept 26, 1999

PI - Signature Date

IV. TABLE OF CONTENTS

V. INTRODUCTION

The tumor suppressor Rb is a nuclear-phosphoprotein that controls cell proliferation, survival and differentiation and is thought to be either mutated or functionally inactivated by phosphorylation in virtually all human cancer including breast cancer. Thus, a potential therapeutic approach to breast cancer may involve targeting the G1 cyclones/cyclin dependent kinases that phosphorylate and inactivate Rb. Though we understand in great details the consequences of inactivating Rb, little is known about the effects of blocking phosphorylation of Rb on development or cancer. We developed transgenic mouse models to study the outcomes of upregulating the Rb pathway in the mammary gland and test whether Rb is a universal tumor suppressor. Intriguingly, our results indicate that expression of constitutively active Rb, suppresses ductal growth in young transgenic females but later induces outgrowth of the alveolar compartment and ultimately leads to focal hyperplasia and breast tumors. Thus, activation of the Rb pathway can induce rather than block breast cancer, presumably by inhibition of programmed cell death. Our work will not only elucidate the role of Rb during mammary gland development but will also serve as a precautionary note as to the potential risks involved in upregulating the Rb pathway for the treatment of breast cancer (and by extension - other cancers). As is the case with its main downstream target E2F1[1], both inactivation and activation of Rb may lead to cancer by dysregulating different aspects (proliferation vs. apoptosis, respectively) of cell physiology.

VI. BODY

We targeted unphosphorylatable alleles of Rb to the mammary gland of transgenic mice, in order to understand the consequences of activating the Rb pathway in vivo. Rb contains 14 conserved phosphorylation sites that dictate its interaction with cellular factors. An Rb allele with 8 CDK phosphorylation sites mutated, creating a superactive Rb[2], was placed under control of the mouse mammary tumor virus long terminal repeat (MMTV-LTR) promoter and the Whey Acid Protein

(WAP) promoter/3'UTR regulatory unit (Fig. 1). It was recently shown that while RbΔp34 can suppress growth of the osteosarcoma cell line, Saos2, only an Rb allele with three additional phosphorylation sites in the C-terminus mutated (Fig 1) can suppress growth of Rat1a cells [3]. We therefore derived a similar Rb allele, named RbΔK11, and generated MMTV- RbΔK11 constructs (Fig. 1). The MMTV-LTR often directs transgene expression to several tissues including the developing mammary epithelium[4 5], whereas WAP transgene expression is usually restricted to differentiated mammary epithelial cells in virgin females and at mid-pregnancy[6, 7]. Activity of the MMTV-RbΔK constructs was verified in vitro by transfecting cells together with an expression vector for the glucocorticoid receptor in the presence of dexamethasone[8]. Under these conditions, the transgenes were shown to localize in the nucleus (immunostaining), bind large T (co-immunoprecipitation) and suppress growth (induce a flat cell phenotype) of Saos-2 cells. Activity of the WAP RbΔp34 vector was not verified in vitro because such an assay is not available but all the junction sites in the construct were sequenced.

A total of 15 MMTV-RbΔp34 and 4 WAP-RbΔp34 transgenic founders were identified in SJLxC57BL/6J background, using Southern blot and PCR analysis (Fig. 1D). Three lines each of MMTV-LTR-RbΔp34 (#24, #8 and #30) and WAP-RbΔp34 (#40, #44, #61) were selected for further analyses. Seven MMTV-LTR-RbΔK11 lines were screened, and three (#20, #29, #7) lines were established that highly express the transgene. F1 progenies were screened for transgene expression by RT-PCR analysis of RNA isolated from the mammary gland at various developmental stages (Fig. 1E) and by whole mount staining of mammary glands to detect alterations in growth. Expression of the MMTV-RbΔK transgene suppressed the proliferation and branching of the ductal epithelium in young transgenic females. This phenotype was enhanced in homozygote MMTV-RbΔp34 and MMTV-RbΔK11 mice (Fig. 2). During pregnancy, ductal branching and alveolar proliferation proceed even in the MMTV-RbΔK transgenic females and ultimately they are able to lactate and feed their pups. Suppression of mammary duct development but not alveolar outgrowth has also been observed in MMTV-TGFβ transgenic mice[9]. In some older transgenic virgin females (>3 months), we also observed enlargement of the alveolar compartment relative to littermate mice housed in the same cage (and therefore likely in the same stage in estrus cycle) (Fig. 2). Importantly, multiple (11/~40) MMTV-RbΔp34 and WAP-RbΔp34 females at about one year of age developed focal hyperplasia (micro-tumors)(Fig. 3) and three developed full-blown mammary adenocarcinomas (Fig. 4 , and report from Dr. C. McKerlie). No breast tumor (0/>200) or focal hyperplasia (by whole mounts 0/>20) were detected in wild type littermate females in our colony. The MMTV-RbΔK11 FvB mice were generated latter, but recently a 4 month old transgenic female developed a visible solid growth in her breast after a single pregnancy. Histological examination revealed large intraductal hyperplasia with fibrosis deposition

(Fig. 4, and report from Dr. C. McKerlie). To complete this part of the study, we have set up a large cohort of virgin and multiparous transgenic and littermate mice to test for incidence and histopathology of breast tumors induced by unphosphorylatable Rb. In total, over 60 transgenic and wildtype littermate females were generated from the various lines and are routinely observed for evidence of breast tumors for a period of 12-16 months. At this stage females without visible tumors will be tested by whole mount staining to determine whether they developed focal hyperplasia. We will also determine whether expression of the Rb transgene suppresses apoptosis in vivo. This will be accomplished by performing TUNEL (in situ apoptosis assay[10] and references therein) on females in estrus. Our hypothesis is that activation of the Rb pathway not only suppresses growth but also blocks apoptosis and the ultimate outcome is the induction of cancer.

RT-PCR analysis revealed that expression of the MMTV-RbΔp34 transgene was highest in virgin females and reduced during pregnancy, suggesting a selection against cells expressing the transgene (Fig. 5). The observed enlargement of the alveolar compartment in some old transgenic females suggest either inhibition of cell death and/or precocious differentiation. Initial expression analysis by RT-PCR suggested that βcasein and WAP genes are frequently expressed prematurely in transgenic mice (Fig. 6). However analysis of a total of 200 females (!) revealed that the milk genes, βcasein and WAP were also expressed precociously in virgin and early pregnant females in wild type mice (data not shown). Similar precocious expression of βcasein in virgin mice was reported previously[11]. RNA in situ hybridization analysis of Rb revealed that endogenous expression of Rb concurs with expression of these milk genes during pregnancy (Fig. 7). These expression patterns are consistent with the idea that Rb may normally act as a positive regulator of differentiation and milk gene expression at mid pregnancy. Additional analysis is underway to determine whether the incidence, timing and level (by Northern blots) of expression of these milk genes and other markers is altered in the transgenic mice.

Cancer involves multiple genetic alterations that cooperate in debilitating normal control of cell growth. Cell proliferation elicited by activated oncogenes is often counteracted by apoptosis, and inhibition of cell death is a critical step in the progression of cancer. One possible mechanisms by which RbΔK induces breast tumors is by suppressing apoptosis, allowing the accumulation of oncogenic mutations. If this is true, then specific activation of oncogenes should synergize with RbΔK and increase the incidence of breast tumors in MMTV- and WAP-RbΔp34 transgenic mice.

On the other hand, it was recently shown that transgenic mice expressing E2F1 in the skin are predisposed to cancer but they are also protected from chemically-induced tumors[12]. Similarly, expression of unphosphorylatable Rb may suppress the onset of tumors induced by other oncogenes. To test these models, and in accord with the Statement of Work, we will set up a

genetic analysis to mate the MMTV-RbΔK transgenic mice with MMTV-neu, and MMTV-wnt and test for possible synergistic effects on the latency of tumors in the compound mice. The neu (erbB-2) receptor tyrosine kinase is overexpressed in 20-30% of primary breast cancer and correlates with poor prognosis(ref. 13 and references therein) MMTV-neu mice develop breast tumors after a long latency. In contrast, MMTV-wnt induce hyperplasia and tumors after a short latency[14]. For these experiments, we will use MMTV-RbΔp34#24 that initially suppresses ductal growth but later give high incidence of breast tumors. To this end, 20 compound (e.g. MMTV-RbΔp34#24 : MMTV-neu) and single transgenic mice will be set up and monitored for the appearance of breast tumors over a period of 14 months. At this stage, mice without tumors will be sacrificed and their four main glands (#3 and #4) will be analyzed by whole mounts to detect focal hyperplasia.

VII. KEY RESEARCH ACCOMPLISHMENTS

- We showed that expression of Rb is upregulated during pregnancy and peaks at lactation and involution.
- we generated transgenic mice expressing two alleles of unphosphorylatable Rb in the mammary glands under control of the MMTV and WAP promoters.
- We showed that despite the fact that early expression of unphosphorylatable Rb suppresses ductal growth, the ultimate effect of activating the Rb pathway is the induction of breast cancer.

VIII. REPORTABLE OUTCOMES

- We intend to submit a paper describing our results to a top rate journal.
- I expect the genetic crosses to generate another quality publication.
- As discussed below, several obvious and important questions are raised by this study and I will be applying to obtain funds from the US Army and other agencies to support the studies.
- I gave an oral presentation on part of the work in an NCIC organized conference on breast cancer on June, 1999.

IX. CONCLUSIONS

Studies on Rb-deficient mice revealed that several cell types undergo ectopic DNA synthesis and apoptosis and display incomplete differentiation[10] (Fig. 8, below). In addition Rb+/- heterozygote mice develop pituitary tumors and other malignancies[15]. Since the Rb pathway is so often disrupted in human cancer, activation of the pathway by expression of unphosphorylatable Rb or by soluble CDKIs is an attractive approach for reversing malignancy by gene therapy. Although

we understand in great detail the consequences of inactivating Rb, little is known about the outcome of activating this tumor suppressor in vivo.

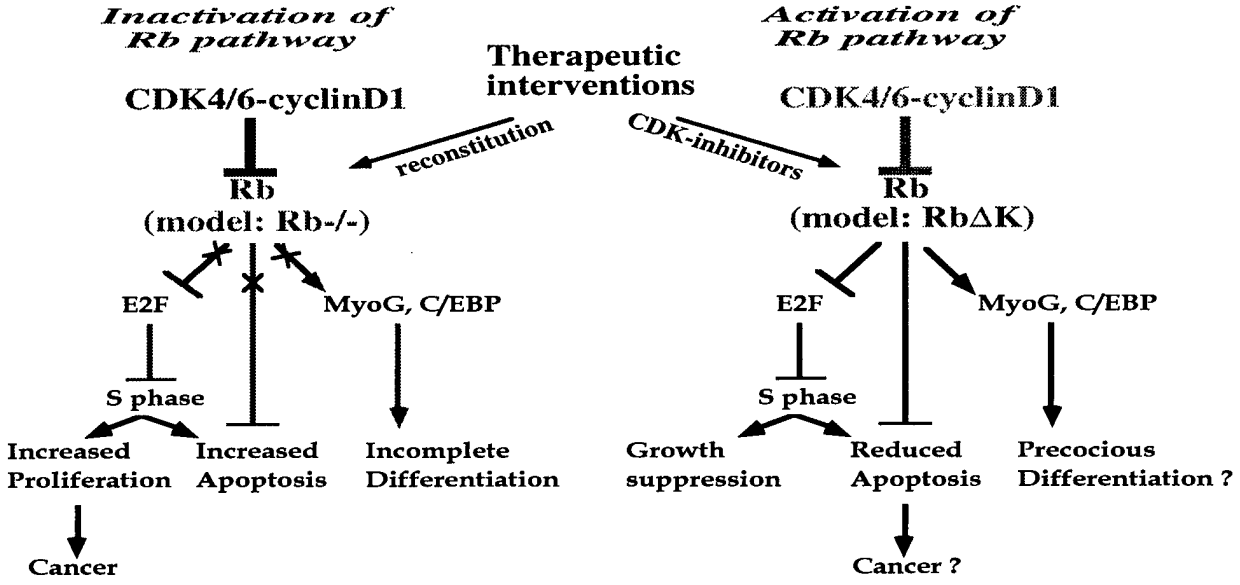

Fig. 8. *A model for activation of the Rb pathway.*

Unexpectedly, our analysis of transgenic mice expressing unphosphorylatable Rb alleles in the mammary gland reveals that MMTV-RbΔK transgenic mice display initial growth suppression but later develop breast tumors. In addition, WAP-RbΔK transgenic mice also develop breast tumors. The effect of upregulating the Rb pathway may vary depending on the tissue and developmental stage. The most obvious effect of RbΔK in most tissues may be inhibition of cell proliferation. In tissues such as mammary epithelium, where cells undergo continuos proliferation and apoptosis the ultimate effect of activating the Rb pathway may be inhibition of apoptosis and cancer. In addition, the expression level of RbΔK may be critical. If most of the Rb pool is kept underphosphorylated - cell proliferation may cease. Only if a fraction of the Rb pool is underphosphorylated, such as in the case of MMTV/WAP-RbΔK transgenic mice or following therapeutical treatment with CDK inhibitors, that a cell may be protected from apoptosis but may still proliferate. These issues may be resolved by expressing physiological levels of RbΔK, using a conditional Cre/lox based knock-in approach that we have initiated.

Another important issue is that unphosphorylatable Rb suppresses both proliferation and apoptosis and ultimately cause cancer. In as much as cancer often involves the inactivation of both Rb and p53, the protection of cells from tumorigenicity may require the activation of both Rb and p53 (or another pro-apoptotic factor). If we identify the mechanisms by which Rb suppresses apoptosis in the mammary gland and find a way to block the apoptosis by RbΔK the ultimate effect may be inhibition of breast cancer. These current and future studies may not only enhance our

understanding of this tumor suppressor gene pathway but may also provide critical information for future development of Rb-pathway/cell cycle -based therapy for breast cancer.

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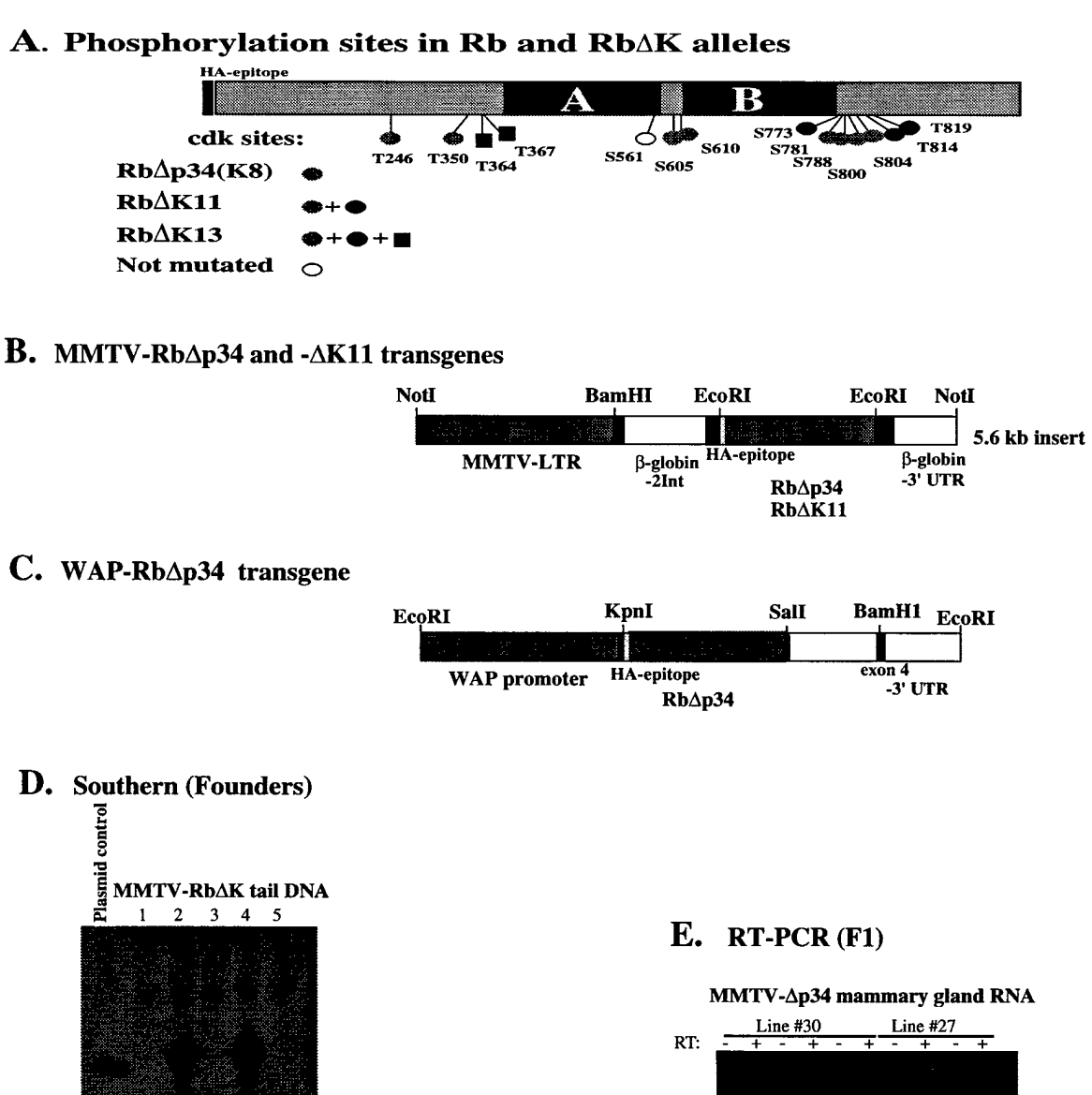

Fig. 1. Generation of transgenic mice expressing unphosphorylatable Rb alleles in the mammary gland

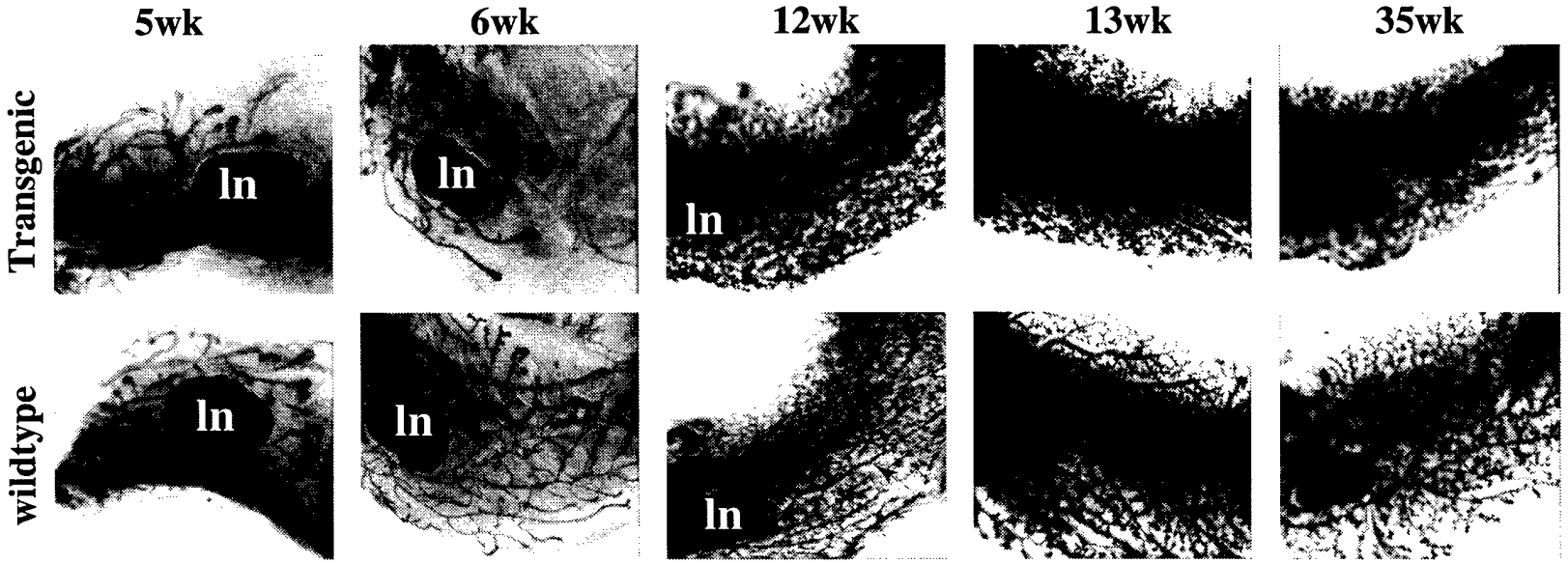

Fig. 2. RbΔK transgenes induce early suppression of ductal growth and late enlargement of the alveolar compartment at estrus. MMTV-RbΔK11 is shown at 6 weeks; the other panels represent MMTV-RbΔp34. All wildtypes represent littermates caged together with transgenic mice. Note that ducts are masked by alveolar outgrowth in old transgenic mammary glands and can only be seen in wildtype mice in post-pubertal animals. Whole mounts - iron hematoxilin.

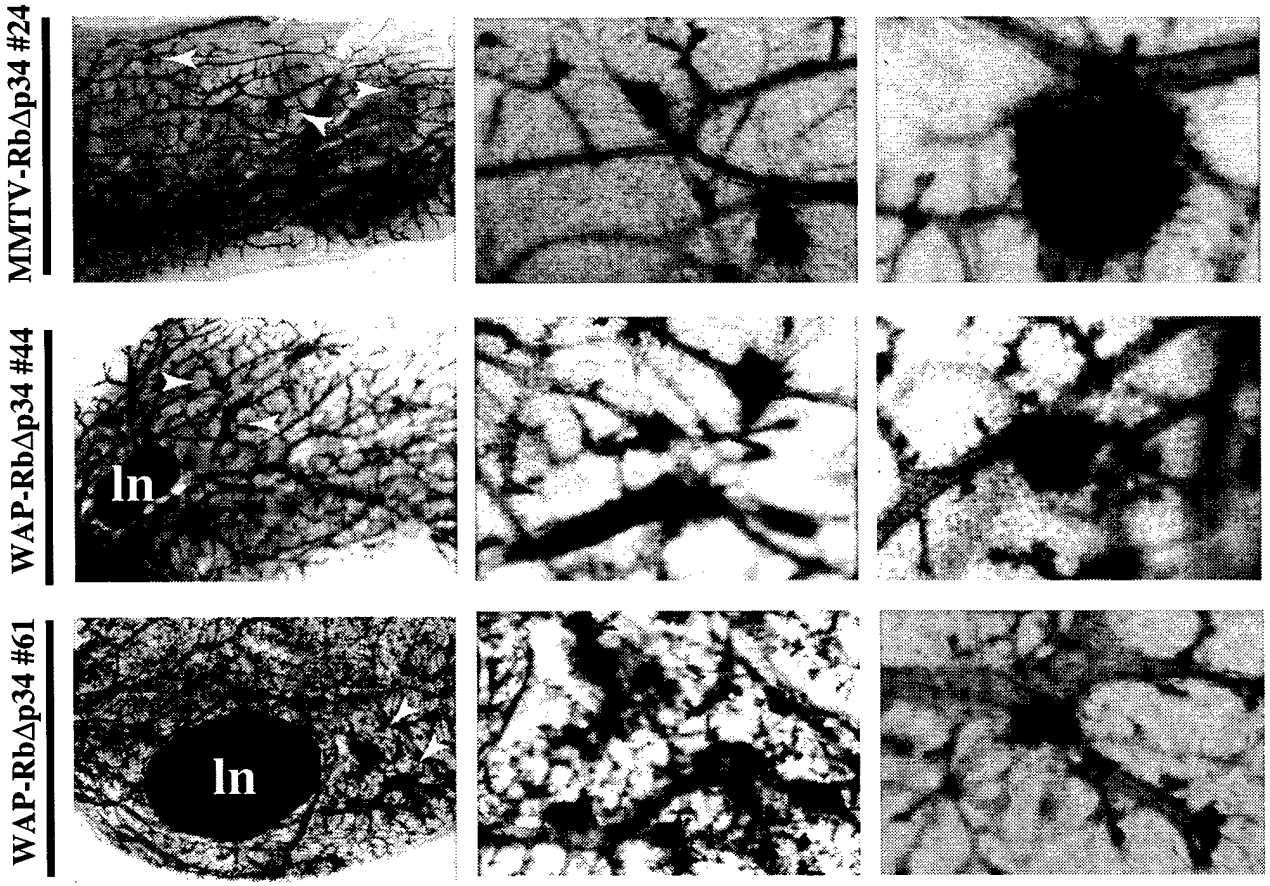

Fig. 3. Hyperplastic lesions in MMTV-RbΔp34 and WAP-RbΔp34 mice. ln - lymphnode.

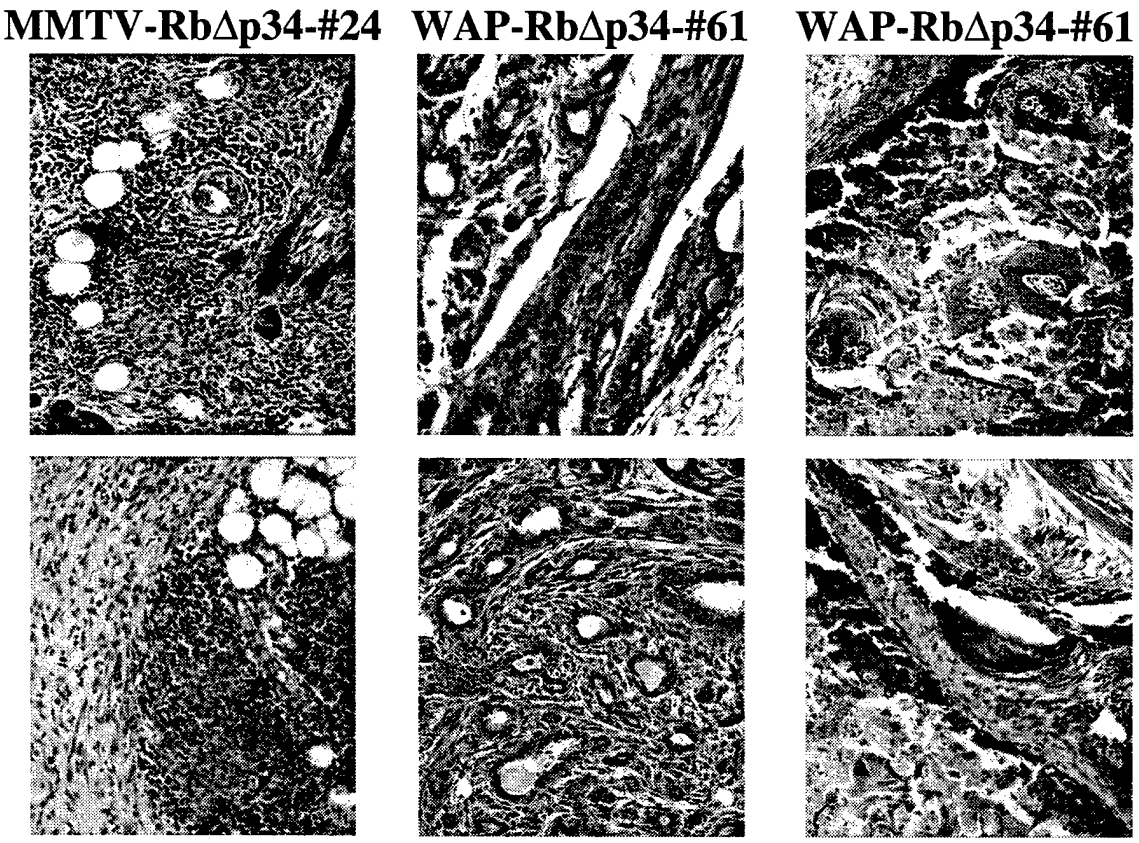

Fig. 4. Mammary adenocarcinomas in MMTV-RbΔp34 and WAP-RbΔp34 transgenic mice (original x200)

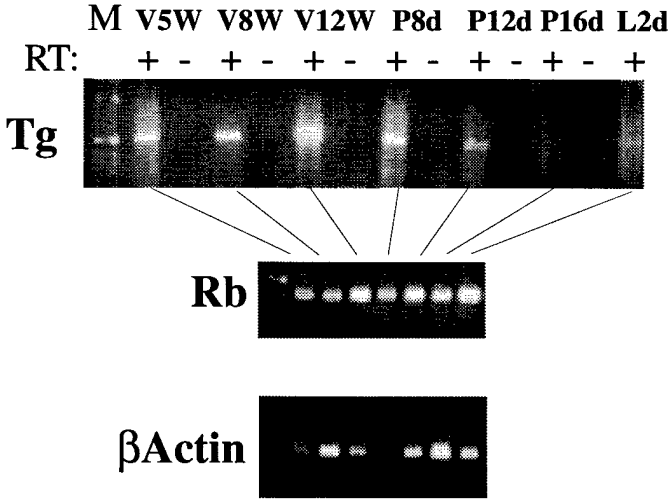

Fig. 5 Expression of the MMTV-RbΔp34#24 transgene in virgin (V) females and during pregnancy (P) and lactation (L).

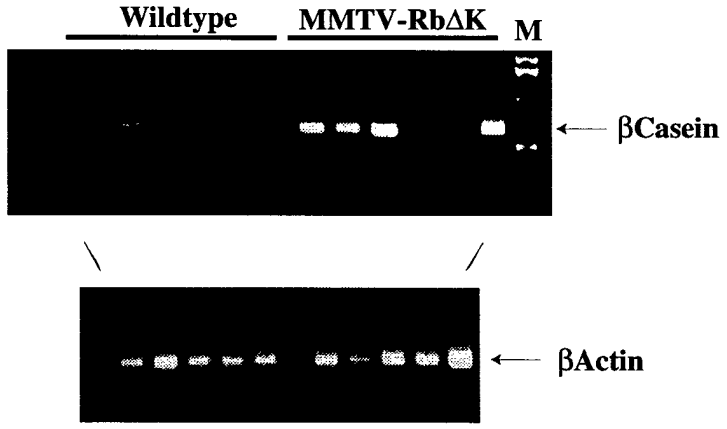

Fig. 6. Expression of βCasein in nulliparous transgenic females

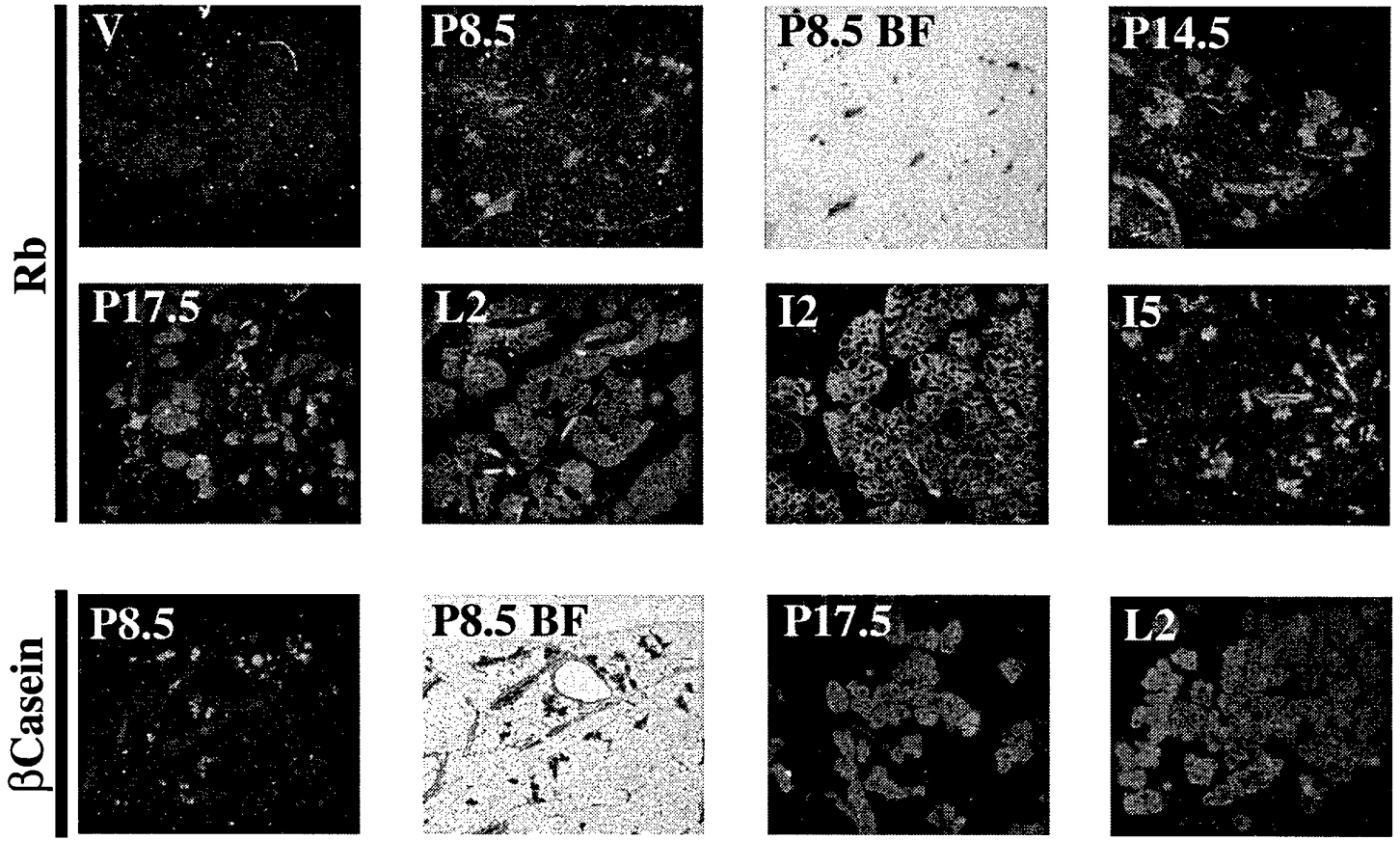

Fig. 7. RNA ISH analysis of Rb and βCasein in the mammary gland of virgin (V), pregnant (P), lactating (L) and involuting (I) mice. BF - bright field

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06 August 1999

Necropsy Report Number: SHSC 99024

Principal Investigator:	Dr. Eldad Zachsenhaus	Fund:	external
Department:	UHN	Ext:	

Species:	Murine	Sex:	female
Strain:	SJL/C57BL/6J *Tg*	Colour:	?
Birth date/Age:	various	Weight:	not given
I.D. No.:	82Tg WAP-OP34 one year-old virgin female sacrificed 02/07/99		
	5Tg MMTV-OP34 one year-old parous female sacrificed 02/07/99		
	61Tg WAP-OP34 female sacrificed 03/06/99		

Type of animal housing: __?_Conventional ____SPF
__Barrier ___Containment

Problems (include diagnosis if established):

1. These mice are from a transgenic colony with an increasing incidence of tumors
2.

Relevant History (include date(s) of onset of problems):

No tumors/lesions have been found in wild-type animals from this colony to date.

Summary of recent procedures/therapy (medical/surgical):

none reported

Specimen submitted: Prepared slides

Time of death: ?

Euthanized: ?

Method/route: n/a

Histological Examination:

82Tg WAP-OP34 one year-old virgin female sacrificed 02/07/99

Tumor - The histological sections are thick and of poor quality, making definitive morphology of cell type difficult. There are large areas of relatively normal mammary tissue on the slide. The most striking feature of each section on the slide is an expansive but well circumscribed tumor mass (Figure 1) that is characterized primarily by a mixed growth pattern. The cells lining acinar structures, tubules and stromal papillary projections have basally oriented nuclei and eosinophilic apical cytoplasm (Figure 2). Stromal cells are not a prominent feature.

Figure 1.

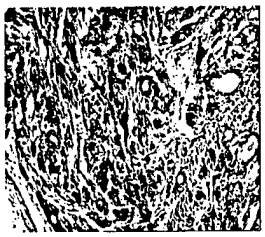

Figure 2.

Diagnosis - Well-differentiated tubulo-acinar mammary adenocarcinoma

Comment - Adenocarcinomas in mice are commonly derived from alveolar cells and are typically preceded by hyperplastic alveolar nodules. Viral (MMTV) factors, inherited susceptibility, and hormonal stimuli determine onset, incidence, and types of 'spontaneous' mammary tumors in the mouse. Incidence rates of mammary tumor sin mice harboring viruses are high (70-100%) by age 1 year. However, mouse strains and stocks without infectious viruses have intermediate of low incidence of mammary gland tumors. C57BL/6 mice in particular are reported in several large studies of both aged virgin and breeder females to have a very low to zero incidence rate of mammary tumors. Consequently, a transgenic colony with mutant-animal specific incidence of mammary tumors at 10-15% should be considered significant and warrants further characterization.

53Tg MMTV-OP34 one year-old parous female sacrificed 02/07/99

Tumor - The histological sections are thick and of poor quality. Staining contrast is underdeveloped. However, the development and mild fibroplasia of the large areas of relatively normal mammary tissue on the slide is within normal limits for an aged parous female (Figure 3). There is a large poorly delineated tumor mass that is characterized primarily by a solid growth patter but with multiple cystic areas (Figure 4). Confluent strands of round cells (Figure 5) characterize the cells, which dominate the multiple nodules of tumor. There are residual acinar structures concentrated at the periphery of the tumor, but also as isolated islands within the central mass of the tumor.

Diagnosis - Solid mammary adenocarcinoma with cysts

Comment - See comments on murine mammary adenocarcinomas above.

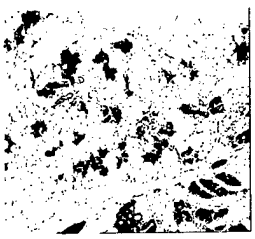

Figure 3.

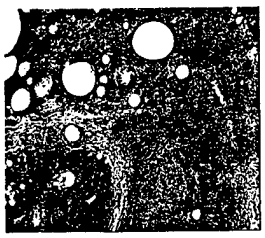

Figure 4.

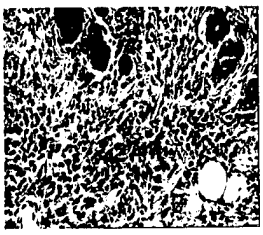

Figure 5.

Histological Examination (cont.d):

61Tg WAP-OP34 female sacrificed 03/06/99

Tumor - The histological sections are thick and of poor quality. Sections of the tumor mass in particular are torn and folded. Adjacent to a small area of relatively normal mammary tissue (Figure 6) are several massively dilated and neoplastic tubules separated by prominent stromal tissue. The most striking feature of this tumor is the central and characteristic radiating keratinization (Figure 7) and peripheral growth of basaloid cells (Figure 8).

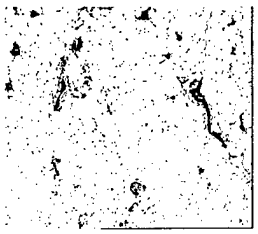

Figure 6.

Figure 7.

Figure 8.

Diagnosis - Well-differentiated papillary mammary adenocarcinoma

Comment - Mammary tumors in mice of ductal origin in particular are rare and few studies are available to provide incidence data. These neoplasms generally arise spontaneously in susceptible strains or can be induced by chemical carcinogens.

Histological Examination:

MMTVΔKII #29 5M MGT? Slide 7

Mammary gl - Multiple histological sections of a mammary tissue show a histologically normal lymph node within mammary gland. The morphology of the surrounding mammary gland is interpreted to be normal (Figure 1) including occasional examples of alveoli with pigment, an incidental finding (Figure 2). The single lymph node captured in cross section within the mammary fat pad shows typical murine lymph node architecture characterized by a parenchymal mass of lymphoid tissue encapsulated by a fibrous capsule. The underlying marginal sinus overlies the outer cortex which is made up of poorly delineated follicles and the inner cortex (paracortex) which is composed of a constitutively normal population of more loosely organized but well-differentiated lymphocytes (Figure 3). The medulla is composed of normal sinuses, containing macrophages and lymphocytes, and cords containing scattered lymphocytes.

MMTVΔKII #29 5M MGT? Slide 10

Mammary gl - Multiple histological sections of mammary tissue show a histologically normal lymph node. The majority of the surrounding mammary tissue is relatively normal, but there are mutlifocal examples of intraductular hyperplasia (Figure 4). The proliferation is characterized by cystic and early papillary hyperplasia.

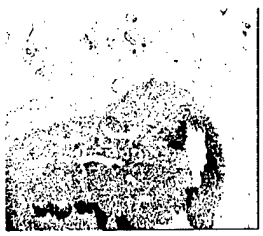

Figure 1.

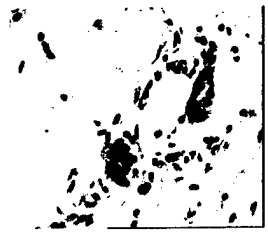

Figure 2.

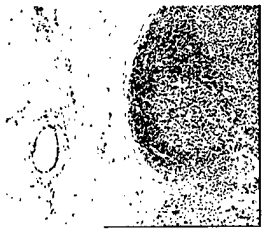

Figure 3.

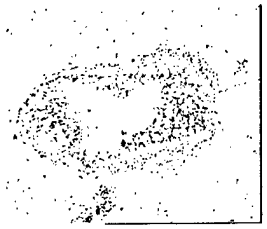

Figure 4.

MMTVΔKII #29 5M MGT? Slide 17

Mammary gl - Multiple histological sections of a mammary tissue show a histologically normal lymph node. The surrounding mammary tissue contains examples of normal glandular morphology, intraductal hyperplasia, and moderate to marked fibrosis. In one foci, there is significant deposition of fibrous stroma at the expense of the adipose tissue (Figure 5).

Diagnosis - Intraductal mammary hyperplasia and mammary gland fibrosis
Histologically normal lymph node

Comment - Intraductal hyperplasia in the mouse is generally considered uncommon but may be observed in preexisting mammary ducts or in ducts derived from tubular or lobular hyperplasia. It is frequently associated with experimental tumor induction or can occur at the periphery of ductal carcinoma. There was no carcinoma in any of the tissue sections submitted for examination. Fibrosis typically becomes conspicuous in glands of parous mice beginning at about 18 months of age. The incidence and degree of fibrosis is highly variable, but fibrosis is typically more extensive in parous than in nulliparous older mice.

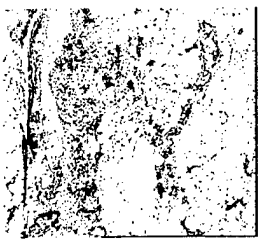

Figure 5.

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