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The purpose of this project is to test whether deregulated cyclin E in the mammary epithelia predisposes to chromosomal instability and hence mammary tumorigenesis. To test this hypothesis based on *in vitro* work we have crossed transgenic mice expressing either wild-type human cyclin E or a hyperstable mutant ($T_{380}A$) with mice heterozygous at either the p53 or Rb loci. If genomic instability is induced by deregulated expression in the mammary epithelia, we anticipate an increased penetrance and decreased latency of tumorigenesis. In the first 12 months we have successfully established the 9 genotypes of mice necessary for this project and have established protocols for histopathological and karyotype analysis. Pathological findings observed to date are all in keeping with published genotype-specific tumor types with the exception of those in the newly established strains, the significance of which will not be established until greater numbers have been studied. Although this project is in its early stages, all practical objectives to achieve its completion have been met. I have an excellent working relationship with two experienced pathologists (both veterinary and medical) to maximise interpretation of the results.

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INTRODUCTION

The purpose of this project is to test whether deregulated cyclin E in the mammary epithelia predisposes to chromosomal instability and hence tumorigenesis. To test this hypothesis based on in vitro work (Spruck *et al.*, 1999) we crossed transgenic mice expressing either wild-type human cyclin E or a hyperstable mutant (T380A) with mice heterozygous at either the p53 or Rb loci. If genomic instability is induced by deregulated expression in the mammary epithelia, we anticipate an increased penetrance and decreased latency of tumorigenesis. Neither heterozygosity of p53 or Rb predisposes to mammary tumorigenesis in mice (Harvey *et al.*, 1995; Jacks *et al.*, 1994) except in the BALB/c background (Kuperwasser *et al.*,). In the transgenic mice 10% develop mammary tumors in the wildtype cyclin E strain (CycE) (Bortner & Rosenberg, 1997) and 25% develop tumors in the hyperstable transgene strain ($T_{380}A$; unpublished). In both cases tumor appearance occurs no earlier than 11 months of age.

BODY

In the first year of this project it was intended to meet several technical objectives (in boldface):

Technical objective 1.	Generation of founder mice
Task 1. Months 1-2.	**Cross breed transgenic and heterozygous strains.**
Task 2. Months 2-6.	**Genotype offspring and identify mice appropriate as founders.**
Technical objective 2.	Characterization of tumor kinetics and types
Task 3. Months 5-36.	**Weekly examination by palpitation and recording approximate tumor size.**
Task 4. Months 8-36.	**Terminal necropsy, tumor collection and histopathology.**
Technical objective 3.	Genomic instability
Task 5. Months 1-6.	**Establishment of primary cell culture and chromosome painting techniques.**
Task 6. Months 12-36	Karyotype analysis of cells derived from 'normal' and tumor mammary specimens from all genotypes.

All technical objectives set for the first 12 months have been achieved.

Generation of founder mice

Founder mice were procured in a timely fashion from a variety of sources; Rb heterozygotes from Dr. Jean Wang University of California at San Diego (6 male; $Rb^{+/-}$), p53 heterozygotes from Taconic Farms (1 male and 1 female $p53^{+/-}$) both the Cyclin E (4 breeding pairs) and hyperstable cyclin E transgenics (4 breeding pairs) from Dr. Donna Bortner (Glaxo-Wellcome).

No problems were encountered in genotyping or establishing colonies for the Rb, p53 or CycE strains. However, establishing a colony for the $T_{380}A$ strain was more problematic as 2 females died within 2 months (with no progeny) and 1 breeding pair failed to produce progeny. The remaining pair successfully produced 3 litters before the female developed a mammary tumor at 13 months of age. Once colonies were established cross breeding was initiated to generate 4 doubly engineered strains; $p53^{+/-}$ CycE, $Rb^{+/-}$ CycE, $p53^{+/-}$ $T_{380}A$ and $Rb^{+/-}T_{380}A$ as outlined in the original proposal. Again no problems were encountered except that the founder CycE mice all appeared to be heterozygous at the transgenic locus which necessitated the development of an assay to determine transgene zygosity.

Transgene zygosity and localization

PCR genotyping was rapidly established for all engineered loci. However, while the locus of p53 and Rb mutation was well characterized the locus of the (random) transgenic insertions were not and PCR genotyping yielded only a yes or no answer and did not address the issue of a) zygosity at each locus and b) the copy number either within the genome or at each locus of insertion.

Establishing the colonies rapidly demonstrated that all of the founder mice of the CycE strain were infact heterozygous at a single locus of insertion (see figure 1). Crossbreeding and outbreeding of the $T_{380}A$ strain also demonstrated that this was a single locus of insertion. Cross breeding to the $p53^{+/-}$ and $Rb^{+/-}$ strains further demonstrated that the locus of transgene insertion for both the CycE and $T_{380}A$ strains were inherited independently of the p53 or Rb loci and of gender thus demonstrating that these loci were not located on chromosomes 11 or 14 (Hsieh *et al.*, 1989; Rotter *et al.*, 1984) nor sex-linked.

Determination of transgene zygosity was somewhat simplified by the fact that there are only single insertion loci for each transgene. Zygosity at this locus was determined by a combination (to increase confidence) of genetics where available (i.e. if a male founder gave rise to progeny that were all positive for the transgene when outcrossed) and a semiquantitative PCR assay using Cks1 primers as an internal multiplex control (in which the ratio of transgene/Cks1 control is twice that of a heterozygote). In the manner described above and in the original proposal we have established colonies for all 9 genotypes required for the study.

Characterization of tumor kinetics and types

During the course of the breeding program it became apparent that a careful study of the animals involved was necessary to ensure that all pertinent information is obtained. The breeding is maintained in a facility to which access is restricted. Therefore a protocol was developed in which females were bred to have two or three litters in the controlled facility and then shipped to a facility to which I have daily access. A regimen has now been established so that all animals are routinely examined twice a week (Mondays and Fridays) and any that warrant closer watch are inspected daily. This has ensured that a) fewer animals are lost without the opportunity of an informative necropsy and b) animal discomfort/distress when tumors develop is kept to a minimum. These routine inspections have also provided an invaluable insight into the clinical symptoms associated with the pathology development.

At this early stage of the project few mice have developed tumors and only a small number have reached the end-point age at which terminal necropsy is performed (12 months for strains carrying $Rb^{+/-}$ genotype and 18 months for all other strains). However, during the course of this work I have developed an excellent working relationship with Dr Kent Osborn, DVM, Ph.D. (Assistant director of the animal facility). Routinely I perform necropsy and histological evaluation and consult Dr Osborn on cases that warrant further examination or differential diagnosis. Dr Osborn is an extremely keen veterinary pathologist hosting a weekly comparative pathology rounds (which I attend regularly and at which several of my cases have been presented for discussion). I have developed a full necropsy and histology protocol (including optimal fixation for morphology and immunohistochemistry) and have developed the skills necessary for histological diagnosis for all but the most challenging cases.

Pathology is a new and fascinating discipline for me. Over the course of these studies I have become aware for a need to carefully consider all aspects of mouse physiology in interpreting necropsy and histopathological findings. No cases better illustrate this than some of the Rb heterozygotes in which pituitary neoplasia are a common finding. In some of the $Rb^{+/-}$ mice diffuse hyperplasia of the mammary glands with accompanying galactorrhea was noted. Prolactin secretion likely plays a role in the etiology of diffuse mammary hyperplasia and therefore a regulatory role in β-lactoglobulin expression, and hence the cyclin E transgenes (see next section).

Pathology summary

A summary of pathological findings to date are presented below.

Genotype	Findings	Incidence*	Age
Wild type	Hydronephrosis (unilateral; subclinical)	1/2 (50%)	18 months
$Rb^{+/-}$	Pituitary adenoma	11/11 (100%)	9-12 months
	Medullary thyroid carcinoma	10/11 (90%)	9-12 months
$p53^{+/-}$	Generalized lymphoma	1/3 (33%)	7 months
	Osteosarcoma (rib cage)	1/3 (33%)	13 months
	Pituitary adenoma/adenocarcinoma	1/3 (33%)	15 months
CycE	None		
$T_{380}A$	Mammary adenocarcinoma	1/3 (33%)	11 months
$p53^{+/-}$ CycE	Osteosarcoma (vertebra)	1/1 (100%)	6 months
$Rb^{+/-}$ CycE	None		
$p53^{+/-}$ $T_{380}A$	Thymic Lymphoma	2/2 (100%)	7-10 months
$Rb^{+/-}$ $T_{380}A$	Mammary adenosquamous carcinoma	1/1 (100%)	4 months

*It is important to note that most of these findings are made in mice that have died prematurely and that other mice of a similar age are still alive. Therefore incidence does not reflect the incidence in the population as a whole but only those necropsied to date.

Incidental deaths have resulted from complication during delivery of pups and euthanasia required because of dermatitis. Exclusion criteria include females that have not littered twice. Diagnosis of carcinoma is based upon unequivocal local invasion, involvement of local or distant lymph nodes or evidence of metastases.

To date 3 instances of mammary neoplasia have been recorded; 2 in the $T_{380}A$ strain (one in a nulliparous female and therefore excluded) and 1 in the $Rb^{+/-}T_{380}A$ strain. In all three cases the kinetics of tumor development has been rapid with growth from undetectable to a mass of approximately 1 cm within a week. While it is encouraging for the hypothesis that a single $Rb^{+/-}T_{380}A$ female has developed a mammary neoplasm very much earlier than the control $T_{380}A$ strain (and of a different tumor type) it is important to note that there are older animals alive at present and that no conclusions should be drawn until all the mice for each genotype have been accounted for.

It is also important to report that in 2 of the female $Rb^{+/-}$ mice necropsied, diffuse mammary hyperplasia with associated galatorrhea was noted. This is indicative of hyperprolactinemia and is an important finding since the cyclin E transgenes are under the control of the β-lactoglobulin promoter which is prolactin responsive. Histopathological analysis and other reports (Park *et al.*, 1999) support a cellular origin of these tumor from the pars intermedia and not from the pars distilis in which the mammotrophic cells are found. Hyperprolactinemia is a common finding in neoplasia of the pars distilis. However, these tumors have consistently tested negative for prolactin expression by immunohistochemistry in contrast to the pituitary tumor from a p53 heterozygote. It is concluded that the most likely cause of this diffuse mammary hyperplasia is a destruction of the bridge between the hypothalamus (secreting prolactin inhibitory factor) and the pituitary causing elevation of circulating prolactin. It is obviously important to consider pituitary hyperplasia/neoplasia and concurrent diffuse mammary hyperplasia when considering mammary tumorigenesis in the $Rb^{+/-}$CycE and $Rb^{+/-}T_{380}A$ strains. The finding of diffuse mammary hyperplasia will likely constitute an exclusion criterion in these mice. This condition has only been noted in mice with relatively large pituitary tumors and was accompanied by mild hydrocephalus and clinical symptoms of general poor health and morbidity.

Pituitary adenoma of the pars distilis is a relatively common finding in mice from a C57/BL6 x C3H mixed background (Maronpot, 1999) but has not been reported in a $p53^{+/-}$ strain. It is, therefore, unlikely that this will be a common lesion complicating this study but its incidence will be considered in cases concurrent with mammary neoplasia and will also constitute an exclusion criterion..

It is a possibility that leaky expression of the β-lactoglobulin promoter in other tissues may contribute to acceleration of $p53^{+/-}$ and $Rb^{+/-}$ genotype-specific tumors in the doubly engineered mice. This may be relevant in the light of two instances of thymic lymphoma in $p53^{+/-}T_{380}A$ mice at relatively early ages. However, there are a number of other animals that have exceeded this age and the relevance of this finding will only become apparent as more mice are studied.

Some female mice have been used at midlactation (2 weeks post partum) for the purposes of histological evaluation and analysis of transgene expression by western blotting and immunohistochemistry (figure 2) and developing primary cultures.

Genomic instability

To determine genome instability and to characterize tumor cell karyotypes we are developing a culture protocol for normal and tumor mammary cells and will analyze these cells by karyotype chromosome painting. To date, normal mammary epithelial culture has been achieved with limited success. Proliferation of normal primary cells was easily maintained in the presence of serum or pituitary extract. However we needed to use defined medium to avoid expression of the transgenes. Using a defined serum-free medium with supraphysiological insulin levels (Imagawa *et al.*, 1982) we have achieved limited growth before senescence obviates further expansion of the culture. The limitations of this have been overcome by seeding directly onto coverslips in small culture plates or limited use of adult male horse serum. Even with this methodology there is considerable variation in growth dependent upon seeding density. While normal primary cell epithelia are well characterized and the normal karyotype is well established those of mammary tumor line are less so and will be dependent upon the karyotypic lesions specific for each tumor. Despite the paucity of mammary tumor tissue to date we have managed to propagate cells from both tumors for a limited time *in vitro.* Cells from the first tumor samples were slow to proliferate and were eventually lost to infection but not before establishing that insulin levels were key to growth. The second tumor line was propagated successfully for two passages before loss due to over dilution. We believe that these cells required close contact which was lost when passaged at a ratio of 1:3.

In the interim, karyotypic analysis and development of the chromosome painting techniques were established in a well defined mouse embryonic fibroblast cell line C3H/10T 1/2. These cells are very amenable to mitotic arrest using nocodazole and colcemid. In these tetraploid cells we were able to identify 4 chromosomes for each of chromosome 11 and 14 (to which the Rb and p53 loci localize) with a small translocation of chromosome 11 (see figure 4). The quality of mitotic spreads available for mammary tumor lines will depend upon the ease with which they are arrested in mitosis.

PCR analysis from mammary tumor explants indicated that in both the $T_{380}A$ mammary tumor and $RbT_{380}A$ mammary tumor the Rb locus was lost. A residual band was apparent in both tumors for p53 although it is not clear whether this represented contaminating normal connective tissue, a mutation in the p53 locus with loss of the other copy or a combination of the two. We are currently developing an assay to PCR from paraffin embedded archival material by micro dissection which will eliminate contamination by normal tissue cells. Furthermore this assay may reveal clonal differences in multilobular tumors.

KEY RESEARCH ACCOMPLISHMENTS

Established in the first 12 months;

- All mouse strain colonies established
- Necropsy and histological protocol established (and expanding knowledge of pathology)
- Short-term primary cell culture developed.
- Karyotypic analysis (chromosome 11 and 14 minimally) established.

REPORTABLE OUTCOMES

None to date

CONCLUSIONS

The initial objectives of this project have been met with few technical problems. An appreciation of the potential caveats to pathological interpretation have been identified and a meeting between myself, and two pathologists, Dr. Kent Osborn (The Scripps Research institute) and Dr. John Lee (Sheffield, U.K.) has been arranged for 17th -20th June 2001 to ensure that all possible data requirements and parameters are covered. It is anticipated that during the next few months more mammary tumors will allow cell lines to be established and tumor karyotype analysis to begin.

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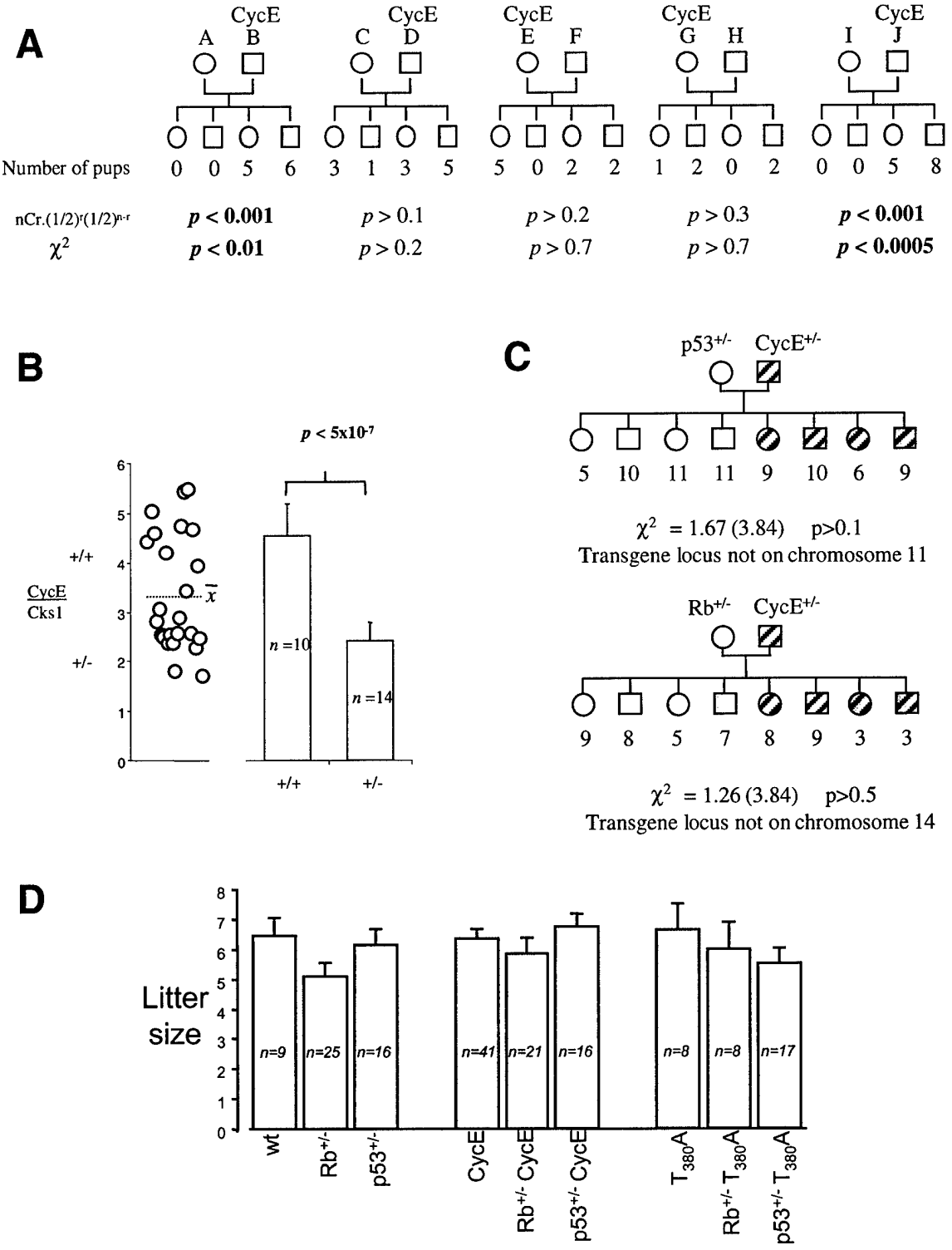

Figure 1. Genetic and transgene analysis in establishing the mouse genotypes. **A**. Out crossing the Cyclin E mice to identify mice homozygous for the transgene locus. Male mice B and J were homozygous while male D and females E and G are heterozygous. **B.** A typical semiquantitative multiplex PCR assay to determine transgene zygosity in CycE mice. This was used in conjunction with genetic analysis to identify homozygous mice to establish the new strains. **C.** Out crossing known heterozygotes established that the transgene locus is on neither chromosome 11 or 14 nor on either of the sex-chromosomes. Each of the four progeny genotypes are represented by different shading.. Analysis of the $Rb^{+/-}$ crosses yielded a significantly lower representation of $Rb^{+/-}$ progeny. However, this was peculiar to these crosses as this was not the case when greater numbers were considered (see D). **D.** No significant variation of litter size within the nine genotypes established. Essentially the same results have been established for the $T_{380}A$ strains.

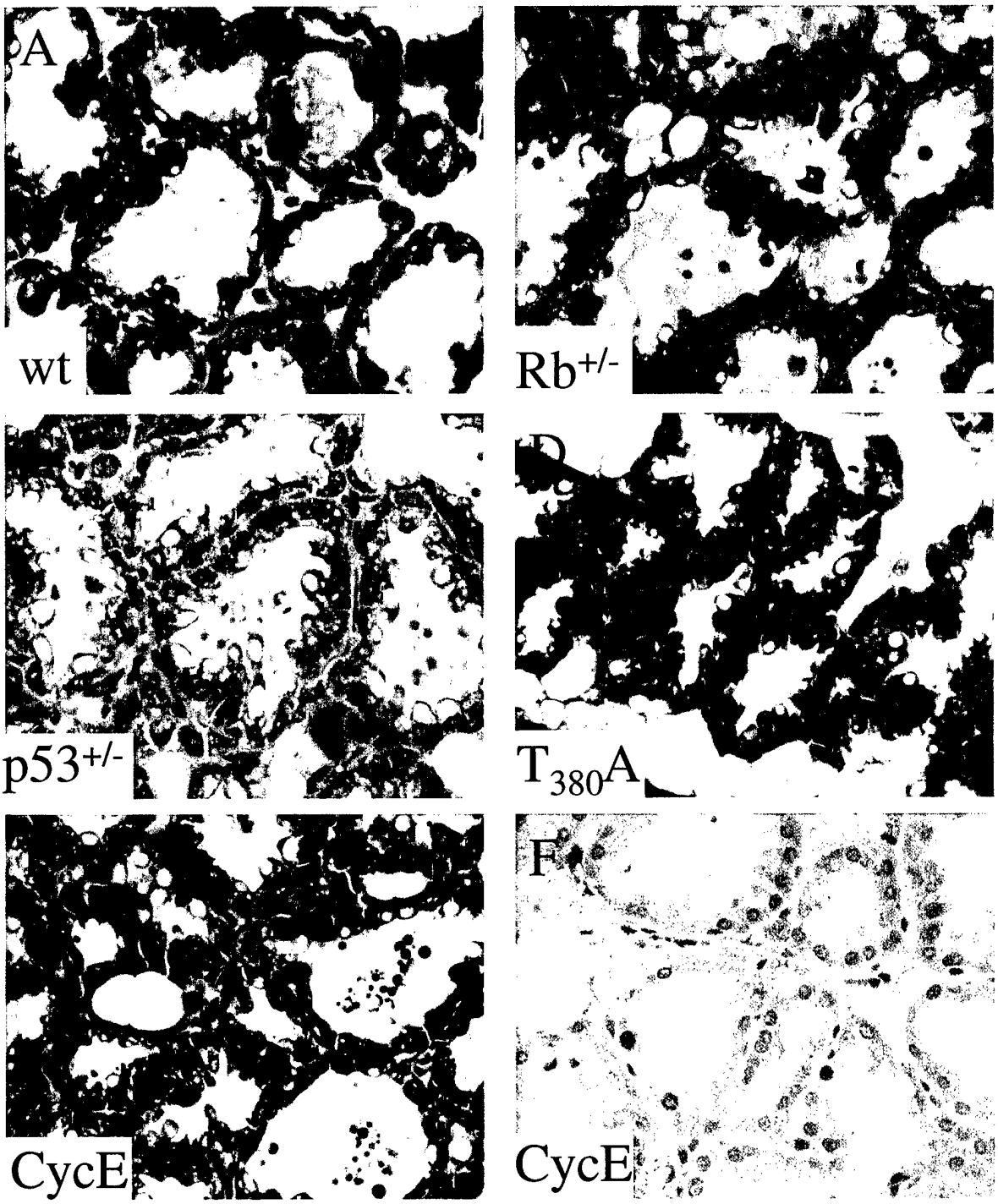

Figure 2. Representative photomicrographs of mid-lactation mammary glands from the five founding strains of mice. **A-E** . H&E staining 400x. No overt differences in general morphology are noted except a tendency for fewer lactating lobules in $T_{380}A$ mice which also display a poorer survival rate with first litters. Interpretation of cellular morphology and possible preneoplastic lesions is awaiting collaboration with experienced pathologists in June 2001. There were no overt papillary structures in either of the transgenic strains such as those reported by Bortner and Rosenberg (1997) despite positive immunostaining in the epithelia for cyclin E. **F.** Immunohistochemistry on midlactation mammary gland from a cyclin E mouse. Mammary glands were fixed in Bouin's fixative and incubated with a mouse monoclonal antibody (HE12) raised against human cyclin E and with which there is no crossreactivity with mouse cyclin E. Several nuclei display intense staining (AEC; red). Generally most nuclei display varying degrees of staining but in weaker staining nuclei the signal is masked by haematoxylin counterstaining.

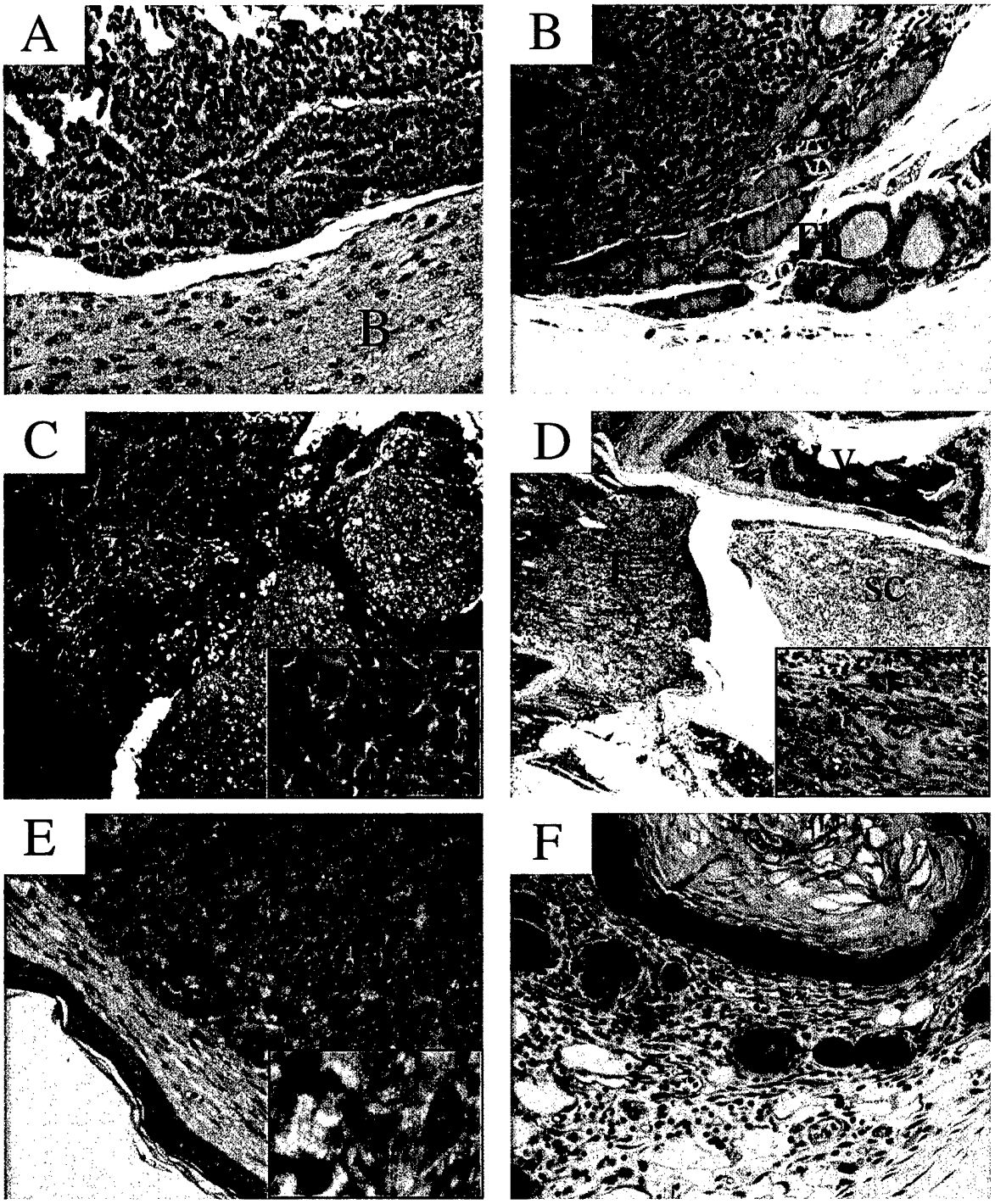

Figure 3. Representative photomicrographs of tumors. **A**. Pituitary adenoma in an $Rb^{+/-}$ mouse (H&E 200x) showing mitotic figures (arrowheads) in the tumor (T) with compression but no invasion of the brain (B). **B**. Medullary thyroid carcinoma in an $Rb^{+/-}$ mouse (H&E 200x) showing uninvolved thyroid follicles (Th) and numerous mitotic figures (arrowheads) in the tumor (T). **C**. Pituitary adenoma/adenocarcinoma in a $p53^{+/-}$ mouse (H&E 200x) showing considerable pleiomorphism and karyomegaly within the tumor (T) and possible involvement of the trigeminal nerve (tg). Numerous mitotic figures (arrowheads) are present with karyomegaly and several overtly aberrant mitoses (insert 400x). **D**. Osteosarcoma at the level of T7 veterbra in a $p53^{+/-}$ CycE mouse (H&E 20x) showing complete occlusion of the vertebral canal by the tumor (T) severing the spinal cord (sc) and presenting with clinical paraplegia. T8 vertebra (v). Insert shows the presence of mitotic figures (arrowheads) and classic osteoid deposition. **E**. Mammary adenocarcinoma in a $T_{380}A$ mouse (H&E 200x) showing numerous mitotic figures (arrowheads) some of which display bridging chromosomes (inset 600x). **F**. Mammary adenosquamous carcinoma in a $Rb^{+/-}T_{380}A$ mouse (H&E 200x) showing characteristic squamous metaplasia, keratin deposition and numerous mitotic figures (arrowheads) of the mammary epithelia with normal fibrous connective tissue.