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13. ABSTRACT (Maximum 200 Words) The p53 and pRb tumor suppressor pathways are frequently altered in human breast cancer. Although animal models have begun to explore mechanisms for these proteins, the roles can be different depending on the cancer type. Our previous studies in a mouse brain epithelial tumor model have demonstrated the importance of pRb in tumor initiation and of p53 in tumor progression, and have established p53-dependent apoptosis as a means of tumor suppression. In this model, brain cells are induced to proliferate aberrantly by tissue-specific expression of T ¹²¹ , a small T antigen oncoprotein that inactivates pRb. This causes slow growing, but highly apoptotic tumors. Further inactivation of p53 causes a dramatic decline in cell death and rapid acceleration of tumor growth. We propose similar studies to examine the pRb and p53 roles in breast cancer. The full T antigen oncoprotein (inactivates both pRb and p53) has been shown to induce mammary tumors in transgenic mice. Here, the T ¹²¹ oncoprotein will be tissue-specifically expressed in mammary epithelium by mammary-specific promoters to test the role of pRb. Further analysis using knock out strains will address the role of p53. Such preclinical models are essential for progress in breast cancer research.				
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Final Progress Report

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Introduction

An understanding of how breast cancer develops and the design and testing of innovative therapies will require the establishment of preclinical animal models. This is because of the distinct changes in breast cells that lead to this cancer cause certain biological changes that arise within a very specific tissue environment. We are fortunate that technologies exist to introduce specific genetic changes into the experimental mouse. This allows us to determine the impact of genetic changes observed in human cancer in the context of the whole organism. The pRb tumor suppressor pathway is altered in a large fraction of human breast cancers. Yet, the impact of pRb inactivation on mammary tissue had not been tested before. During this grant period we have determined the effect on mammary tissue after inactivation pRb proteins by tissue specific expression of viral oncoprotein, T₁₂₁. We further tested the role of the p53 tumor suppressor – a gene that is also altered in about 50% of human breast cancers. These experiments have produced a valuable preclinical model of breast cancer.

Body

Original Specific aims

We have explored the function of pRb and p53 in several sites within the animal using tissue-specific promoters to express wild-type and mutant T-Ags combined with the use of knock-out mice. Here, using the same approach, we aimed to determine tumorigenesis mechanisms in mammary epithelium (ME). Our original specific aims were as follows:

- **I. To determine the impact of pRb-inactivation in mouse mammary epithelium.** The T₁₂₁ oncoprotein will be expressed in ME using the MMTV and WAP promoters. Several lines of transgenic mice (TgME-T₁₂₁) will be generated and examined for mammary-specific phenotypes. Cell cycle activity and apoptosis indexes will be determined relative to non-transgenic littermates in virgin and lactating mammary glands.
- **II. To determine the role of p53 in mammary tumor suppression subsequent to pRb inactivation.**

TgME-T₁₂₁ mice will be crossed with *p53*^{-/-} mice to generate TgME-T₁₂₁ *p53*^{+/-} and TgME-T₁₂₁ *p53*^{-/-} mice. Tumor incidence will be assessed and ME apoptosis and proliferation will be measured. If tumors ensue, tumor progression will be analyzed at the molecular, cellular and morphological levels.

Progress

Early in the funding period we developed transgenic mice using the WAP-T₁₂₁ transgene (Fig. 1). We subsequently characterized the mice for phenotypes and for expression of the transgene. These mice are summarized in a table (Fig 2A) for reference.

In the subsequent years of funding we achieved all of our aims. The results are presented briefly here and a paper describing these results in more detail is being submitted to Cancer Cell (see below).

Transgene Expression/ Western

Western blot analyses of mammary gland protein extracts demonstrated all four lines express T₁₂₁ protein of the expected size (Fig 2B, 2C) when compared to T₁₂₁ protein derived from a brain tumor of a previously characterized independent transgenic mouse line (Chen et al., 1992). T₁₂₁ expression in lines 1 and 2 was revealed only by immunoprecipitation followed by Western analysis, indicating lower levels of protein (Fig 2C). A survey of select tissues showed detectable expression was restricted to the mammary gland in lines 1-3, while expression was more promiscuous in the higher expressing line 4 (data not shown), including brain and kidney expression. Finally, as expected, T₁₂₁ expression was induced by lactation with highest levels observed 5 days postpartum (Fig 2D).

Impact of T₁₂₁ Expression

Histopathological analysis of mammary gland from multiparous line 2 founder and a multiparous line 3 F1 shows the impact of Rb perturbation is several fold. Compared to an age- and parity-matched control tissue it's evident from H&E stained sections that the normal architecture and morphology of the lactating mammary tissue is disturbed (Fig. 3 A, E, I). In contrast to normal tissue where acini consist of a single layer of secretory epithelia with milk-filled lumen, transgenic animals have lower density of atypical acini, consistent with atrophy. Observed acini atypia ranged from pleomorphic nuclei (Fig 3E, arrows) to multifocal low-grade MIN lesions (Cardiff et al., 2000) in the terminal ductal lobular units (TDLUs)

T₁₂₁ -positive mammary epithelial cells are associated with tumorigenic effects (Fig. 3B,F,J). The line 2 founder animal is mosaic for T₁₂₁ protein expression with distinct regions of expressing and non-expressing cells (Fig. 3F), whereas T₁₂₁ expression in the line 3 F1 animal is in secretory epithelium distributed throughout the entire gland (Fig. 3J). Increased levels of proliferation, indicated by PCNA staining levels, is also observed in transgenic animals (Fig. 3 C,G,K), concomitant with increased levels of apoptosis assayed by TUNEL staining (Fig 3 D,H,L). Quantification of apoptosis reveals a correlation between protein expression levels (Fig. 2B) and the percentage of apoptotic cells (Fig. 4A). Consistent with a model for cell-autonomous functioning of T₁₂₁, the pattern of abnormalities of morphology, proliferation and apoptosis, in the mosaic

animal mimic the regionalized T₁₂₁ expression pattern, and conversely, where T₁₂₁ protein is absent the tissue appears normal.

Role of p53 in Apoptosis

To investigate the impact of germ-line loss of p53 on apoptosis levels induced by T₁₂₁ we mated transgenic line 3 animals to mice harboring *p53* null alleles to generate transgenic and non-transgenic females of three classes with respect to their *p53* genotype (+/+, +/-, -/-). Transgene expression was induced by pregnancy, and mammary glands were examined during late pregnancy. As expected, non-transgenic mice showed no appreciable apoptosis in mammary glands regardless of *p53* status (Fig. 4B). However, in transgenic animals, decreased levels of p53 activity were correlated with lower levels of apoptosis. The mean percentage of apoptotic cells in transgenic animals wild type for *p53* was 21%, in *p53* heterozygous animals the percentage decreased to 9%, and in *p53* null animals, the mean percentage was further reduced to 5% (Fig. 4B). Thus, as in another epithelial tissue (the choroids plexus), p53 likely suppressed tumor growth via its apoptotic functions.

Role of p53 in Proliferation

In a transgenic model of mammary tumors initiated by activated Ras, inactivation of p53 did not result in a reduction of apoptosis. Rather, loss of p53 was associated with increased proliferation of the mammary epithelium. To determine whether p53 inactivation also impacted proliferation in TgT₁₂₁ mammary tissue, glands from pregnant mice were assessed for the expression of nuclear PCNA using immunohistochemistry. Unlike the tumors initiated by activated Ras, p53 heterozygosity or inactivation had no significant impact on the level of cell proliferation (Fig. 5). This experiment indicates that the same tumor suppressor can have distinct mechanisms of action depending on the nature of the initiating lesion.

Rb Inactivation Predisposes to Tumorigenesis

Following transgene induction, all female mice of the expressing lines showed mammary gland abnormalities (Fig 2A). While the two lower expressing lines, lines 1 and 2, were able to nurse pups and appeared grossly normal, both had hyperplastic TDLUs associated with increased levels of proliferation and apoptosis. However, females from neither low-expressing line developed adenocarcinomas (data not shown). All females from higher expressing lines, lines 3 and 4, failed to nurse pups due to lactation defects and also ultimately developed mammary tumors (see below). Because expression in line 4 was also detected in non-mammary tissues, we focused our efforts on characterization of line 3. The median time following transgene induction until palpable tumors appeared was 10 months, and within 18 months, all mice succumbed to mammary tumors (Fig. 6A, line 4 data not shown).

Tumor Phenotypes

Terminal stage neoplasms appear to resemble poorly to moderately differentiated invasive ductal adenocarcinoma in humans. Morphologically, we designate these mammary adenocarcinomas as mixed solid (Fig. 7A) and glandular (Fig. 7B) carcinoma with necrosis and fibrosis, and comedo (Fig. 7C) (Cardiff et al., 2000). General characteristics observed among these tumors include irregular glands and cords of epithelial cells with large pleomorphic nuclei and a delicate chromatin pattern with inverted nuclear:cytoplasmic ratio. Tumors are commonly infiltrating a dense, highly cellular fibrous connective tissue. These tumors are accompanied with strong peripheral host responses including granulocytes, histiocytes, and angiogenesis. Low and high grade multifocal MIN lesions (Cardiff et al., 2000) originating in the TDLU are suggestive of a progressive model of tumorigenesis; however, further study of these hyperplasias is required to confirm this hypothesis.

Mammary Tumor onset and growth are accelerated by p53 absence

To investigate the impact of germ-line loss of p53 on tumor onset and growth kinetics, animals harboring a single p53 null allele were monitored for mammary tumors. As expected, a subset of $p53^{+/-}$ mice succumbed to non-mammary tumors (either thymic lymphomas or sarcomas) consistent with published reports (Jacks et al., 1994). Mammary tumors, however, were detected significantly earlier in $TgT_{121}p53^{+/-}$ compared with animals harboring 2 wild type p53 alleles (Fig. 6A, $p < 0.0001$). Furthermore, once palpable, $TgT_{121}p53^{+/-}$ tumors grew significantly faster than the p53 wild type counterparts (Fig. 6B). These studies indicate that p53 heterozygosity increases the rate of tumor growth and/or progression.

Selective Pressure for p53 Loss

Since apoptosis was significantly reduced in $TgT_{121}p53^{+/-}$ mammary tissue, it was possible that p53 heterozygosity was sufficient for tumor acceleration. To assess the selective pressure for p53 inactivation, real-time PCR analysis was employed to determine whether the wild-type p53 allele was lost in $TgT_{121}p53^{+/-}$ tumors. Eight of ten tumors indeed showed loss of the single wild type p53 allele, indicating that the apoptosis reduction observed in $TgT_{121}p53^{+/-}$ mammary epithelium was not sufficient for tumor progression. This indicates that significant selective pressure, likely due to the remaining apoptosis, favored cells that had inactivated p53. In addition, loss of p53 may contribute to tumor progression by additional mechanisms.

Tissue	Genotype	^a 2 ^{-AverageΔΔCt}	No. of wild type p53 alleles
muscle	p53+/+	1.0	2
muscle	p53+/+	0.51	1.75
muscle	p53+/+	0.39	1.5
muscle	p53+/+	0.32	1.25
muscle	p53+/+	0.31	1
tumor-1(line7)	TgWAPT121, p53+/+	0.73	2
tumor-2	TgWAPT121, p53+/+	0.90	2
tumor-3	TgWAPT121, p53+/+	0.91	2
spleen	TgWAPT121, p53+/-	0.22	1
tumor-4	TgWAPT121, p53+/-	0.34	1
tumor-5	TgWAPT121, p53+/-	0.25	1
tumor-6	TgWAPT121, p53+/-	0.12	0
tumor-7	TgWAPT121, p53+/-	0.08	0
tumor-8	TgWAPT121, p53+/-	0.13	0
tumor-9	TgWAPT121, p53+/-	0.10	0
tumor-10	TgWAPT121, p53+/-	0.07	0
tumor-11	TgWAPT121, p53+/-	0.04	0
tumor-12	TgWAPT121, p53+/-	0.16	0
tumor-13	TgWAPT121, p53+/-	0.15	0

Key Research Accomplishments

1. We have established a model of mammary cancer that is based on genetic aberrations frequently observed in human breast cancer.
2. We have shown that inactivation of the Rb pathway elicits similar responses in multiple cell types.
3. We have demonstrated that apoptosis induced by Rb inactivation is largely dependent on p53, and that the level of apoptosis is dose-dependent with respect to p53.
4. We have shown that heterozygosity at the p53 locus accelerates the development of mammary tumors thus providing a system by which to study tumor progression.

Reportable Outcomes

Much of the above list of accomplishments is included in a manuscript in preparation for publication.

Conclusions

Stated in research accomplishments above.

References

Wu, H. Simin, K., Cardiff, R. and T. Van Dyke (2002) "Specific inactivation of the Rb pathway in mammary epithelium predisposes to adenocarcinoma that is accelerated by p53 heterozygosity" in preparation.

Appendices

Figures 1-7 attached.

Figure 1 Transgene Construction

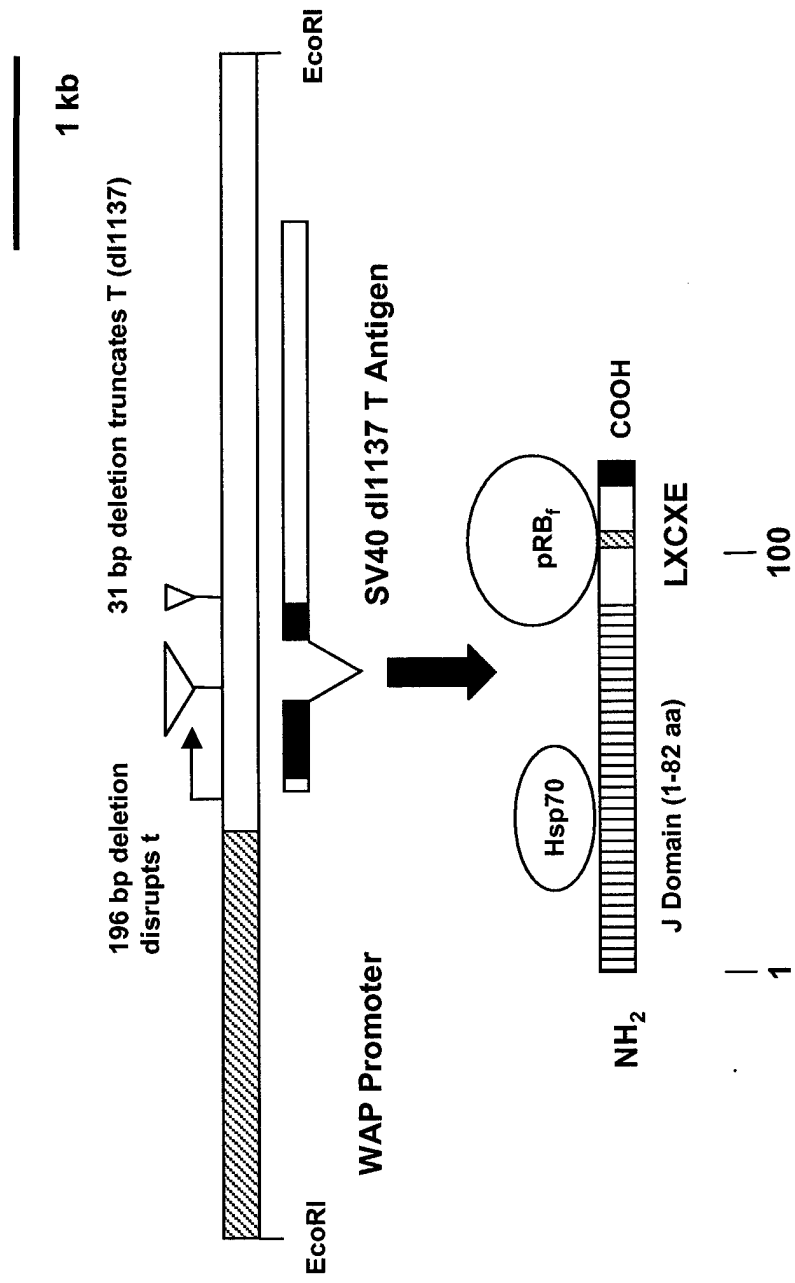


Figure 2

A.

Transgenic Line	Expression		Gross Phenotype	Mammary Gland Abnormalities
	Tissue	Protein		
1	lactating MG	+	normal	Ap, Pr, Hyp
2 (Mosaic)	lactating MG	+	normal	Ap, Pr, Hyp
3	lactating MG	++++	FTN	At, Ap, Pr, MIN, Adeno-Ca ^a
4	lactating MG, Brain, Kidney	++++	FTN, Death ^b	At, Ap, Pr, MIN, Adeno-Ca ^a

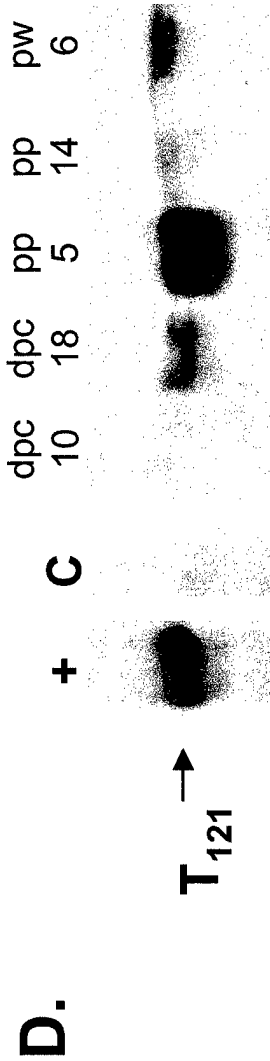
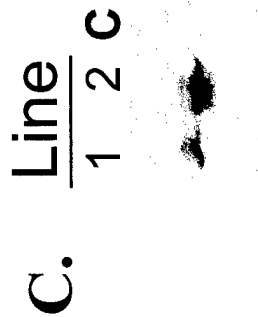
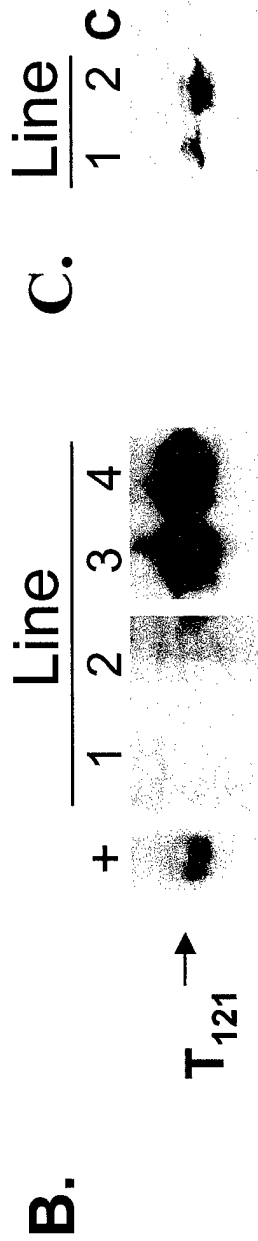


Figure 3

T₁₂₁ Expression Induces High Proliferation and Apoptosis

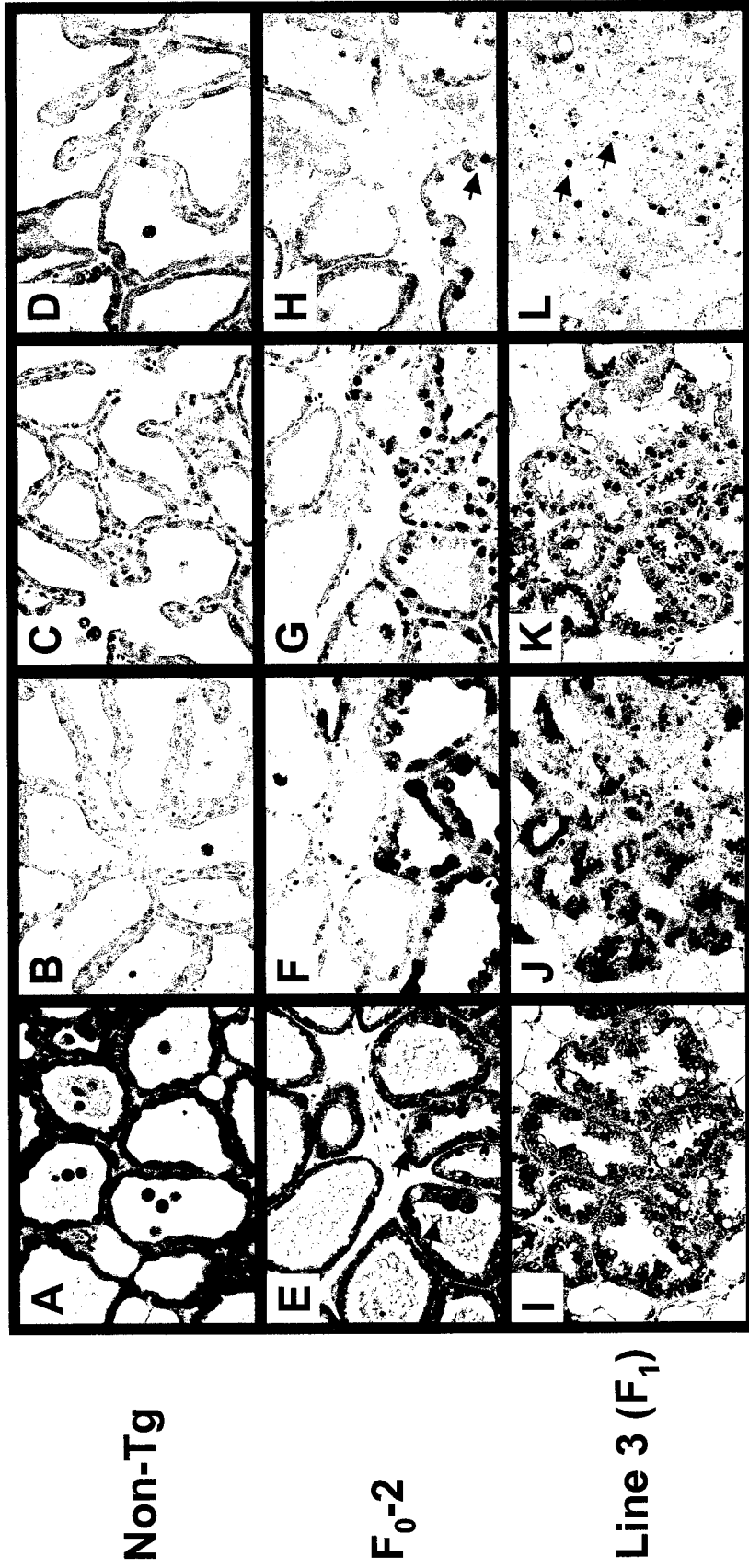


Figure 5 Proliferation

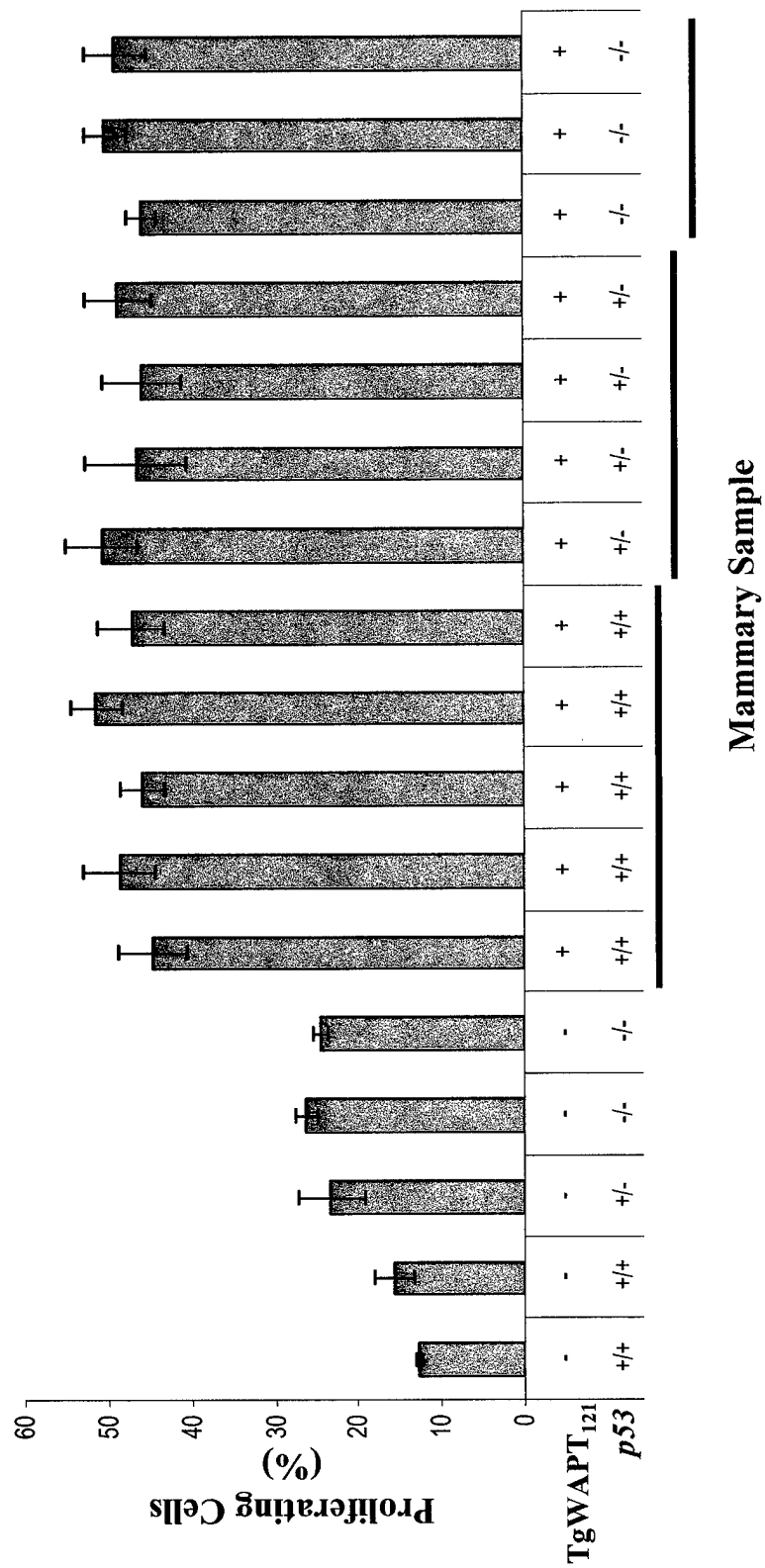
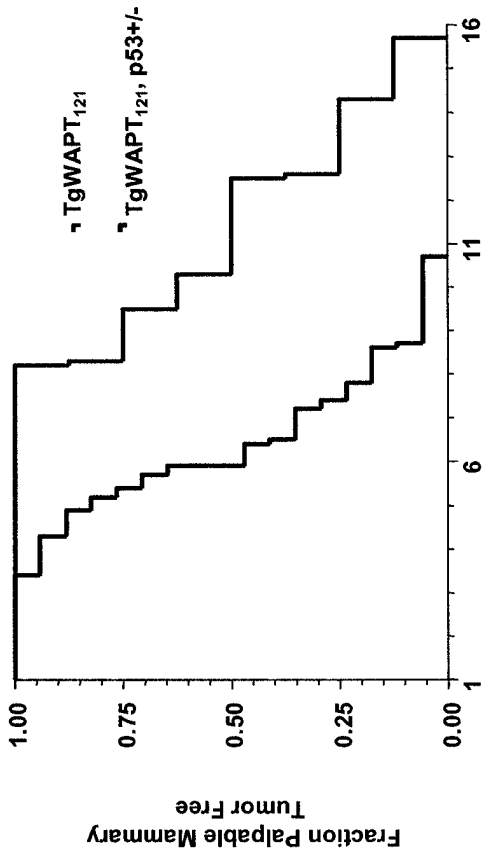
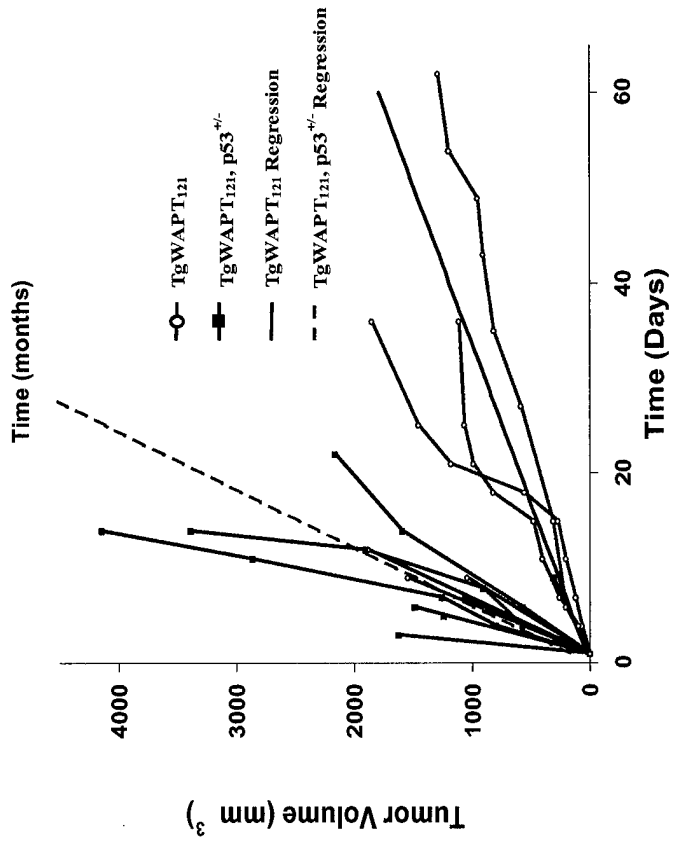


Figure 6 Tumor Growth Rates



A.



B.

Figure 7 Tumor Morphologies

