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WAP-TAg transgenic mice and the study of dysregulated cell survival, proliferation, and mutation during breast carcinogenesis

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Understanding the process of carcinogenesis is key to developing therapies which might interrupt or reverse tumor onset and progression. Cell growth and death signals are dependent not only upon molecular mechanisms within a cell but also upon external stimuli such as hormones, cell–cell signaling, and extracellular matrix. Mouse models can be used to dissect these complex processes, to identify key signaling pathways operating at different stages of tumorigenesis, and to test the strength of specific interventions. In the WAP-TAg mouse model, carcinogenesis is initiated by expression of the Simian Virus 40 T antigen (TAg). TAg expression is triggered by hormonal stimulation, either during estrus or pregnancy. Breast adenocarcinomas (ranging from well to poorly differentiated) develop in 100% of the female mice by approximately 8–9 months of age. Three distinct stages of tumorigenesis are easily identified: an initial proliferation, hyperplasia, and adenocarcinoma. The mean time to first palpable tumor in mice which undergo at least one pregnancy is 6 months. The tumorigenic process is marked by a competition between proliferation and apoptosis and is characterized by cellular acquisition of genetic mutations and increased stromal fibrosis. Protein levels of cell cycle control genes cyclin D1, cdk2, and E2F-1 are increased in these adenocarcinomas. c-Fos protein levels are slightly increased in these cancers, while c-Jun levels do not change. Hormonal exposure alters progression. Estrogen plays a role during the early stages of oncogenesis although the growth of the resulting adenocarcinomas is estrogen-independent. Transient hormonal stimulation by glucocorticoids that temporarily increases the rate of cell proliferation results in tetraploidy, premature appearance of irreversible hyperplasia, and early tumor development. Tumor appearance also can be accelerated through over expression of the cell survival protein, Bcl-2. Bcl-2 over expression not only reduces apoptosis during the initial proliferative process but also decreases the total rate of cell proliferation. This block in cell proliferation is lost selectively as the cells transition to adenocarcinoma. The WAP-TAg model can be utilized to investigate how the basic processes of cell proliferation, apoptosis, DNA mutation, and DNA repair are modified by external and internal signals during mammary oncogenesis. *Oncogene* (2000) **19,** 1010–1019.

Keywords: breast carcinogenesis; mouse model; SV40TAg; apoptosis; proliferation

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Introduction

Breast cancer is one of the most common forms of cancer among women both in the United States and worldwide (Newcomb, 1997). Development of the disease is a multistep process proceeding from an initiated cell(s) through hyperplasia, to malignancy and depends upon the successive activation of oncogenes and inactivation of tumor suppressor genes. Hormonal factors both endogenous and exogenous influence this process. Mouse models can be used to dissect how different factors collaborate during the process of mammary carcinogenesis.

In the WAP-TAg mouse model of breast cancer, carcinogenesis is initiated by expression of Simian Virus 40 large T antigen (TAg)

Introduction of a WAP-TAg transgene into a mouse targets the expression of TAg specifically to mammary epithelial cells (Tzeng *et al*., 1993; Li *et al*., 1996a,b; Santarelli *et al*., 1996). In the transgenic line presented here, 100% of the female mice develop mammary carcinoma by the age of 6 months if they undergo at least one pregnancy. The process of tumorigenesis is marked by a competition between proliferation and apoptosis. When TAg is expressed during the first pregnancy it triggers both cell proliferation and p53-independent apoptosis (Figure 1a) (Li *et al*., 1996a; Tzeng *et al*., 1996). The rate of cell proliferation remains relatively high throughout tumorigenesis, but the incidence of apoptosis falls as the cells transition to hyperplasia and adenocarcinoma (Furth *et al*., 1999). After the first parturition, the mammary glands from transgenic mice involute normally with respect to apoptosis and tissue remodeling (Li *et al*., 1996b); however, after two to three pregnancies, the glands are not fully remodeled following involution (Figure 2). The lobule-alveolar structures in the TAg mice that resist remodeling are hyperplastic. Some but not all of the hyperplasias progress to carcinoma *in situ* and then to adenocarcinoma (Figure 1b). The adenocarcinomas which develop in the TAg mice range from well to poorly differentiated (Figure 3) (Furth *et al*., 1999).

SV40 TAg does not initiate human breast cancer, but it does dysregulate some of the same cellular pathways that are dysfunctional in human breast cancer

TAg binds to p53, the Retinoblastoma protein (pRb), and related family members (p107, p130) and functionally

inactivates them (Meitz *et al*., 1992; Dyson *et al*., 1989). Mutation of p53 is described in up to 40% of human breast cancers and inactivation of pRb and other cell cycle control proteins has also been described (Horak *et al*., 1991; Lee *et al*., 1988). As is likely for the majority of human breast tumors, exposure to the initiating oncoprotein is insufficient for full progression to breast malignancy (Hahn *et al*., 1999). Rather, time-related events which include exposure to hormonal stimulation, subsequent changes in the rate of cell proliferation or apoptosis, and acquisition of additional genetic mutations are required for development of breast cancer. In the WAP-TAg mouse model, expression levels of specific cell survival and cell cycle control proteins increase as the cells transition from the initial proliferative phase through hyperplasia to adenocarcinoma. Cyclin D1, cdk2, and E2F-1 protein levels increase significantly, whereas, less dramatic elevations in c-Fos expression are found (Figure 4b–d). Bcl-x_{long}, Bax, Bag-1 (Figure 4e) and Bad (Furth *et al*., 1999) protein expression levels are also higher in the adenocarcinomas. In contrast, expression levels of c-Jun and cyclin E protein do not change significantly at different stages of carcinogenesis. Secondary mutations appear to contribute to the process of TAg-induced carcinogenesis (Ewald *et al*., 1996). Ki-ras amplifications have been found in approximately 10% of TAg-induced mammary adenocarcinomas (M Li, M Liyanage, T Ried and PA Furth, unpublished results). It is anticipated that additional mutations (including amplifications) will be identified with further study of the TAg animals.

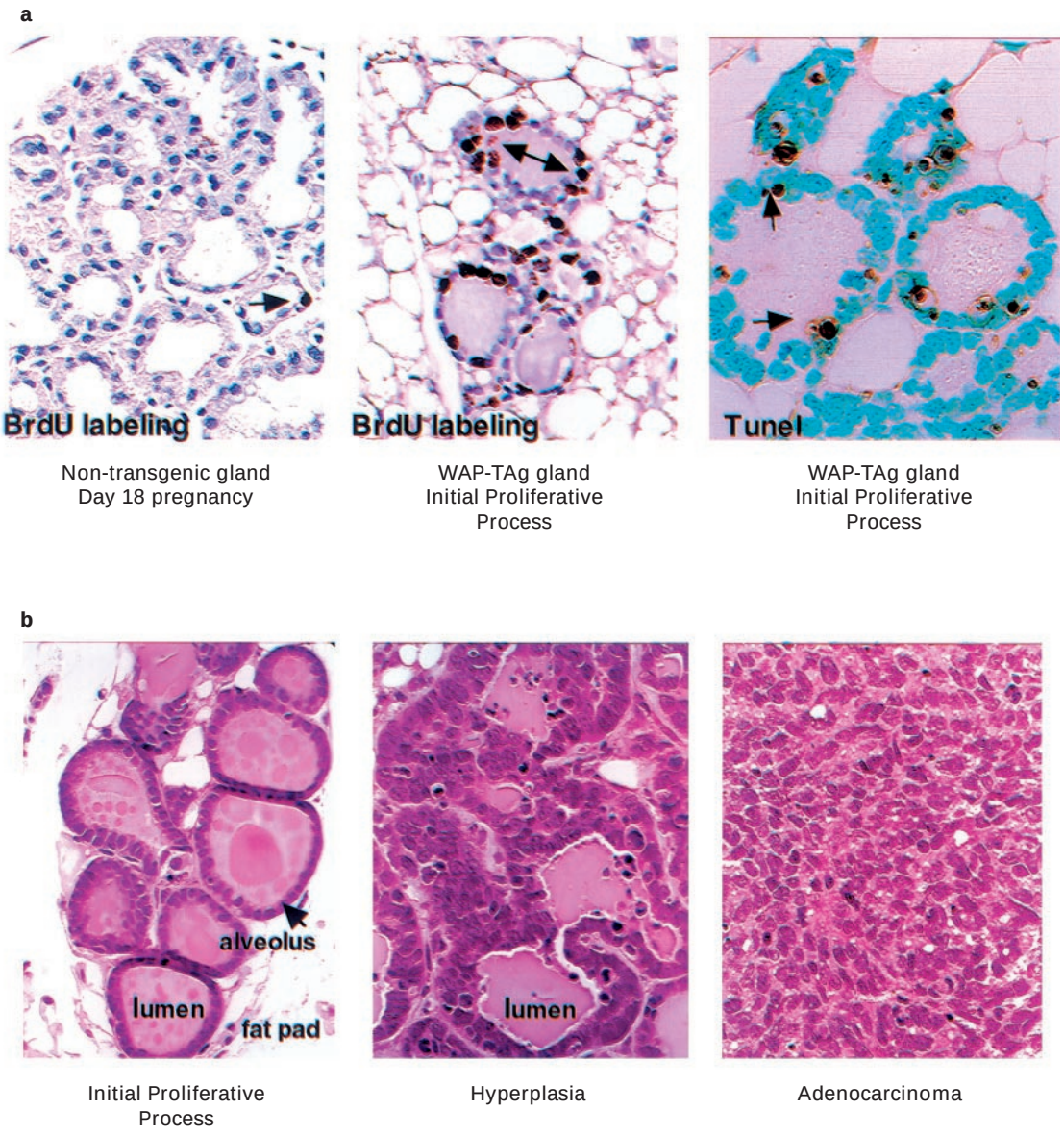

Figure 1 (**a**) Expression of TAg in mammary epithelial cells during late pregnancy induces cell proliferation and p53-independent apoptosis. Mammary epithelial cell proliferation is increased by expression of TAg. *In vivo* BrdU labeling was performed to establish the rate of proliferation of mammary epithelial cells in non-transgenic control mammary epithelial cells at day 18 of pregnancy and WAP-TAg transgenic mice at day 18 of pregnancy during the initial proliferative process. Arrows indicate representative BrdU labeled proliferating nuclei. P53-independent apoptosis is induced by expression of TAg. *In situ* staining for apoptosis demonstrates that approximately 6% of mammary epithelial cells from WAP-TAg mice during late pregnancy are undergoing apoptosis. Arrows indicate representative cells undergoing apoptosis. (**b**) Three stages of cancer progression in the WAP-TAg mouse model: the initial proliferative process at day 18 of pregnancy, hyperplasia, and adenocarcinoma. H&E stained sections/original magnification 60×

Exposure to specific hormones alters the course of tumorigenesis in the WAP-TAg model

Mammary gland development is regulated by hormones including estrogen, progesterone, prolactin, and glucocorticoid

Estrogen is essential for mammary ductal growth during the developmental stages which extend from puberty to sexual maturity and may also play a role in the pathogenesis of human breast cancer. Progesterone is required for the formation of lobule-alveolar structures during pregnancy. Intact prolactin signaling pathways are required for normal mammary gland development during pregnancy and also contribute to lactogenesis (Hennighausen *et al*., 1997). Glucocorticoid is an important lactogenic hormone required for maximal production of milk during lactation (Topper and Freeman, 1980) and may additionally contribute to cell survival. Physiological levels of lactogenic hormones, including glucocorticoid, do not block the apoptosis of mammary epithelial cells (Li *et al*., 1997), but supraphysiological levels of exogenously administered glucocorticoid can inhibit mammary epithelial cell apoptosis (Feng *et al*., 1995) and block degradation of basement membrane (Lund *et al*., 1996).

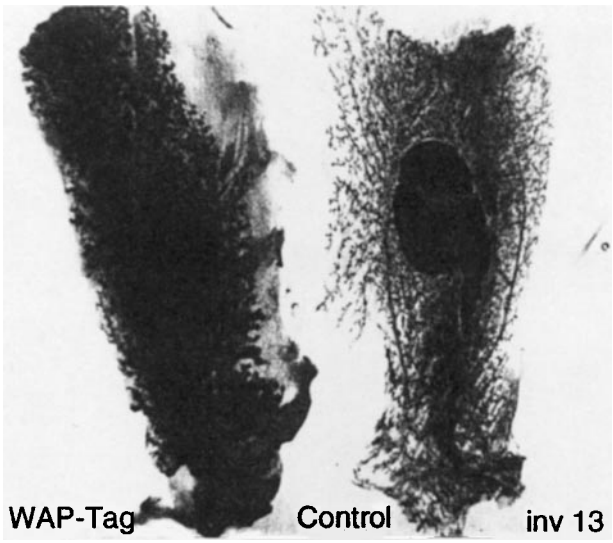

Figure 2 After serial pregnancies, mammary glands from WAP-TAg mice are not fully remodeled during involution. Note the increased tissue density in the gland from the WAP-TAg transgenic mouse at day 13 of involution following the third pregnancy as compared to the non-transgenic control gland at the same timepoint. Whole mounts of fourth (inguinal) glands

Chronic changes in estrogen exposure alter early but not the late stages of carcinogenesis in the WAP-TAg model

Changes in cyclic estrogen exposure can either accelerate or retard the appearance of first palpable mammary tumor (Santarelli *et al*., 1996). In our laboratory, ovariectomy (ovx) of TAg mice after the first pregnancy lengthened the mean time to first palpable tumor by 9 weeks (ovx: 35 ± 6 weeks (wk), $n = 5$; control: 26 ± 4 wk, $n = 21$; ($P = 0.05$)). Chronic estrogen exposure in ovariectomized mice ($n = 5$) following the first pregnancy shortened the mean time to first palpable tumor by 5 weeks as compared to placebo-treated controls ($n = 4$) ($P = 0.05$).

In contrast to the effect of estrogen on tumor development, growth of established mammary adenocarcinomas is estrogen-independent. Adenocarcinomas continue to grow at a mean rate of 0.4 cm^3/week when mice were ovariectomized at the time of first palpable tumor and an average of two new tumors appeared per mouse over the next month.

Effects of transient exposure to estrogen, prolactin, progesterone, and glucocorticoid on cell survival and oncogenesis in the WAP-TAg mouse model

Transient exposure to increased levels of glucocorticoids induces cell proliferation and the rapid develop-

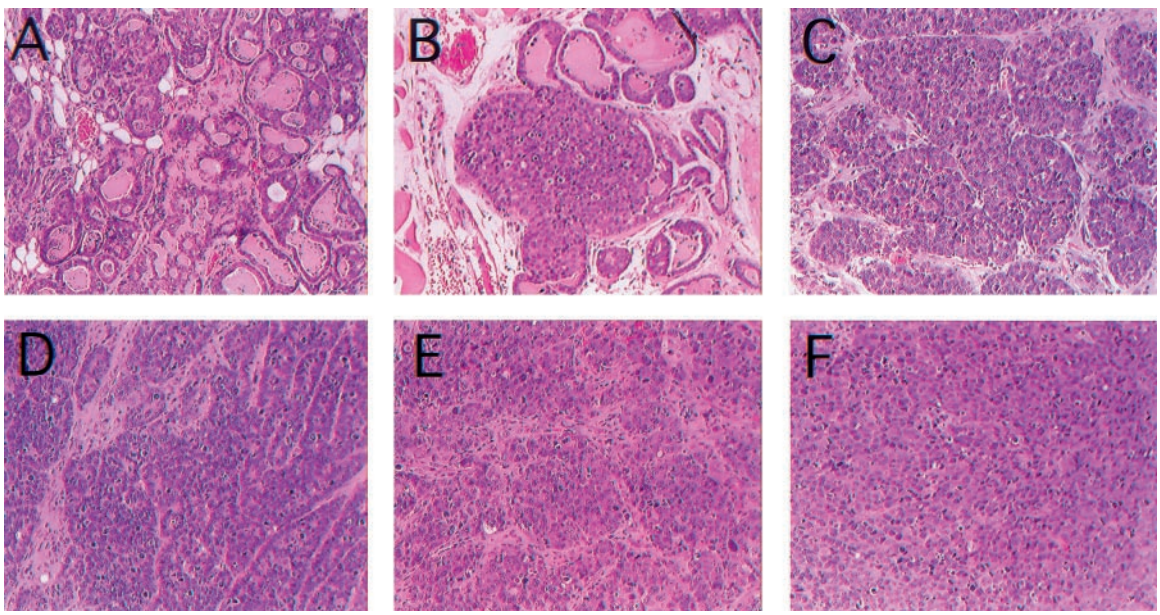

Figure 3 Tumor histology in the WAP-TAg mice. The adenocarcinomas that develop in the WAP-TAg mice demonstrate a range of histological types. Alveolar-type structures are retained in the more differentiated tissue (**a,b**), but are lost in the poorly differentiated adenocarcinomas (**f**). Fibrosis is prominent in some of these adenocarcinomas (**d,e**)

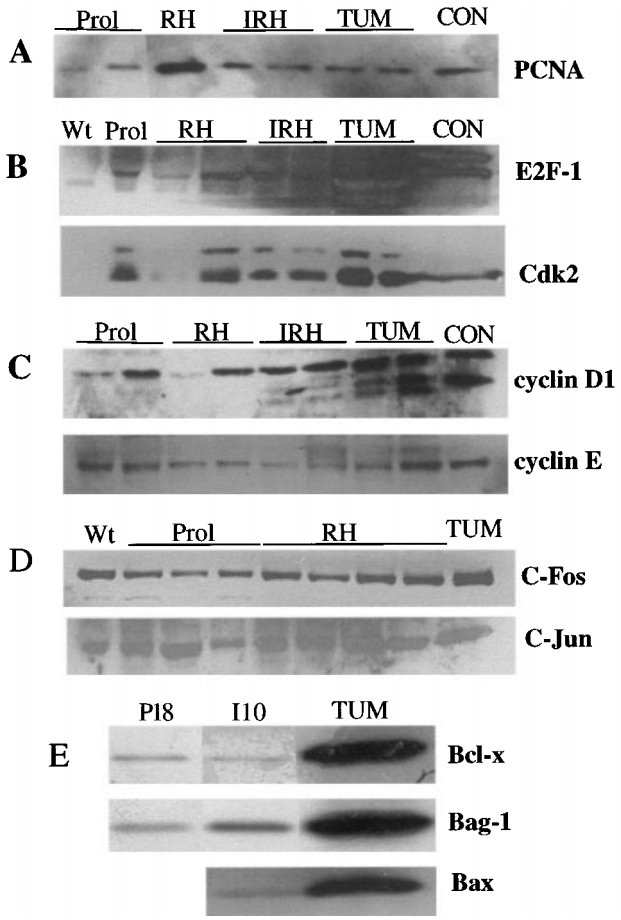

Figure 4 Protein expression levels of PCNA, E2F-1, cdk-2, cyclin D1, cyclin E, c-Fos, c-Jun, Bcl-x, Bag-1 and Bax during the course of tumorigenesis. Proteins were extracted from mammary gland tissue of wild-type, non-transgenic control and WAP-TAg mice at different stages of tumor development and analysed by Western blot. Prol = Initial proliferative process in WAP-TAg mice at day 18 of the first pregnancy. RH = Reversible hyperplasia following glucocorticoid exposure. (See text for description of model). IRH = Irreversible hyperplasia. TUM = Poorly differentiated adenocarcinoma (see Figure 3f). CON = Cell lysate of EGF-stimulated human carcinoma cell line A-431 used as control. Wt = Wild-type, non-transgenic control gland. P18 = Day 18 of first pregnancy during initial proliferative process. I10 = Day 10 of involution following the first pregnancy

ment of hyperplasia in the WAP-TAg mice (Figure 5a). In contrast, transient exposure to increased levels of estrogen, prolactin, or progesterone at the time of involution does not alter cell proliferation (Figure 5b–d). The effect of glucocorticoid is dose-dependent; in the WAP-TAg mice, the maintenance of lactogenic hormone stimulation through suckling does not produce hyperplasia whereas exogenous glucocorticoid does (Figure 5f). Similarly, in normal, non-transgenic control mice, exposure to lactogenic hormones does not block apoptosis (Li *et al*., 1997), while high levels of glucocorticoids can block both apoptosis and tissue remodeling (Feng *et al*., 1995; Lund *et al*., 1996).

In WAP-TAg mice apoptosis of mammary epithelial cells and involution proceed normally following the first parturition with the gland fully remodeling (Figure 6a; Li *et al*., 1996b). However, mice exposed to glucocorticoid at the time of the first involution develop a degree of hyperplasia (Figure 6b) comparable to that seen after three pregnancies in untreated WAP-TAg mice (Figure 6c). Cell cycle analyses of the glucocorticoid-treated hyperplastic tissue demonstrate increased S phase entry and tetraploidy (Figure 6d–e; Table 1). There is no significant change in the percentage of apoptotic cells in the glucocorticoid-treated WAP-TAg mice as compared to control WAP-TAg mice that are suckled to maintain lactogenic hormone stimulation (gluc-exp: 5.9% vs control: 5.1%). The glucocorticoid-treated mice exhibit accelerated tumor development and demonstrate a significant increase in the number of tumors per mouse using a log-rank test ($P = 0.0006$) (Figure 7).

Glucocorticoid-exposed WAP-TAg mice as a model for reversible and irreversible hyperplasia

The glucocorticoid-treated WAP-TAg mice can be exploited as a model for reversible and irreversible mammary hyperplasia. Following transient glucocorticoid exposure, the hyperplastic cells within the mammary tissue begin to undergo apoptosis with partial hyperplastic tissue regression (Figure 8a–c). In the 30 days following transient glucocorticoid exposure, 95% of the multi-layered hyperplastic alveoli regress to a single cell layered alveoli; however, 5% of the tissue does not fully regress and foci of abnormal tissue that resemble carcinoma *in situ* persist (Figure 8d). These persistent foci are likely to be responsible for the accelerated tumor development and increased tumor multiplicity found in the glucocorticoid-treated TAg mice (Figure 7).

Development of more effective approaches to the treatment of pre-malignant lesions is an important area of focus for breast cancer biologists. This model of reversible and irreversible hyperplasia progressing to cancer can be utilized to explore how different therapeutic approaches may shift the balance from progression to regression. The model can be used to test agents for their ability to influence mammary hyperplasia regression and prolong or prevent tumor formation. The role of specific signaling pathways can also be examined. For example, levels of Stat5 phosphorylation are abnormally high in the glucocorticoid-exposed mammary tissue (M Li, S Ren and PA Furth, unpublished results). Further investigation, including breeding experiments in progress with mice carrying functional deletions of Stat5a and 5b, will reveal if abnormal activation of Stat5 is a contributing factor to more rapid disease progression. The same approach can be applied to investigate other regulatory candidates. Finally, this model can be used with gene array and other technologies to identify known or novel genetic pathways that might contribute to disease reversal or progression.

Expression of Bcl-2 family members and tumorigenesis in the WAP-TAg mouse model

The Bcl-2 gene family, survival of mammary epithelial cells, and breast oncogenesis

Bcl-2 and related family members play an important role in survival of normal mammary epithelial cells

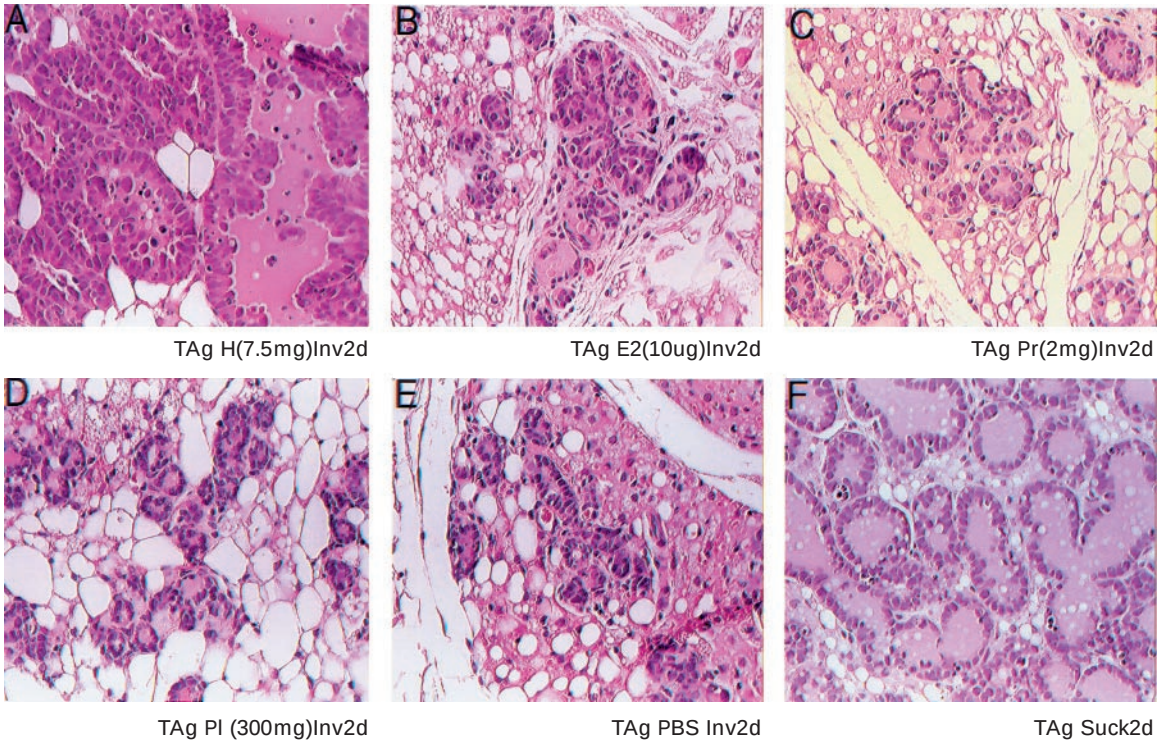

Figure 5 Transient exposure to exogenous glucocorticoids results in the rapid development of hyperplasia in the WAP-TAg transgenic mice. (**a**) Hyperplasia develops within 4 days of initial exposure to hydrocortisone (H). (**b–d**) No hyperplasias develop after exposure to estrogen (E2), progesterone (Pr), or prolactin (P1). (**e**) No hyperplasias are found in phosphate buffered saline (PBS)-injected control tissues. (**f**) Maintenance of high levels of endogenous glucocorticoids by stimulation of lactogenic hormones through suckling results in expansion of alveolar structures but not hyperplasia. Initial injections made at day 18 of first pregnancy. Tissue was taken 4 days later at day 2 of involution (Inv2d) or day 2 of suckling (Suck2d). H&E stained sections/original magnification 40×

and imbalanced expression of Bcl-2 family survival and death factors can alter the response to treatment in human breast cancer (Schorr *et al*., 1999a,b). Changes in expression patterns of cell survival and cell death factors is one factor that may contribute to development and progression of human breast cancer. The WAP-TAg mouse model can be utilized to explore how specific genetic and cellular factors impact on apoptosis and cell proliferation at different stages of the disease. To date we have tested the roles of Bcl-2 over expression, loss of Bax expression, and the combination of Bcl-2 over expression with loss of Bax in this model.

Over expression of Bcl-2 inhibits mitosis at early stages of TAg-induced carcinogenesis but anti-mitotic activity is lost as the cells transition to carcinoma. In contrast, the anti-apoptotic action of Bcl-2 is maintained throughout tumorigenesis

Simultaneous over expression of Bcl-2 with TAg in mammary epithelial cells inhibits TAg-induced mitotic activity during the initial proliferative process. Significantly, this activity is lost in mammary adenocarcinomas (Furth *et al*., 1999). In contrast, the anti-apoptotic action of Bcl-2 transgene persists throughout tumorigenesis. The mechanism responsible for the selective loss of the Bcl-2-directed anti-mitotic activity remains under investigation. One hypothesis is that increased expression levels of specific cell cycle control proteins (Figure 4a) may titrate out or otherwise negate the Bcl-2 inhibitory activity. Interestingly, anti-proliferative activity also has been observed in adenocarcinomas triggered by dimethylbenz(a)-anthracene-treated (DMBA) treatment of WAP-Bcl-2 transgenic mice, but this effect is maintained throughout tumorigenesis (Murphy *et al*., 1999). The different behavior of Bcl-2 in these models may be explained by the type of signaling pathways disrupted. In the WAP-TAg model, disruption of p53 function, alteration of the activity of pRb, p107, and p130, and interruption of transforming growth factor-β (TGF-β) function have all been found. While the database is incomplete, activity of at least some of these pathways may be intact in DMBA-induced cancers (Furth *et al*., 1999). In the end, as one might predict, simultaneous over expression of Bcl-2 with TAg accelerates the appearance of palpable tumors; in contrast, the appearance of tumors is delayed in the DMBA model.

Interactions between loss of Bax function and gain of Bcl-2 function in the WAP-TAg model

WAP-TAg mice with different levels of Bax expression were tested for the time to first palpable tumor. Neither partial nor complete loss of Bax function by itself accelerated tumorigenesis in the WAP-TAg mice (Figure 9a). In contrast, a trend towards delayed tumor development was seen in the WAP-TAg/Bax−/− mice. While this requires further investigation, it is possible that this is due to systemic effects secondary to loss of Bax function. Bax−/− mice are reported to exhibit mammary ductal hypoplasia (Shibata *et al*., 1999) which could be consistent with

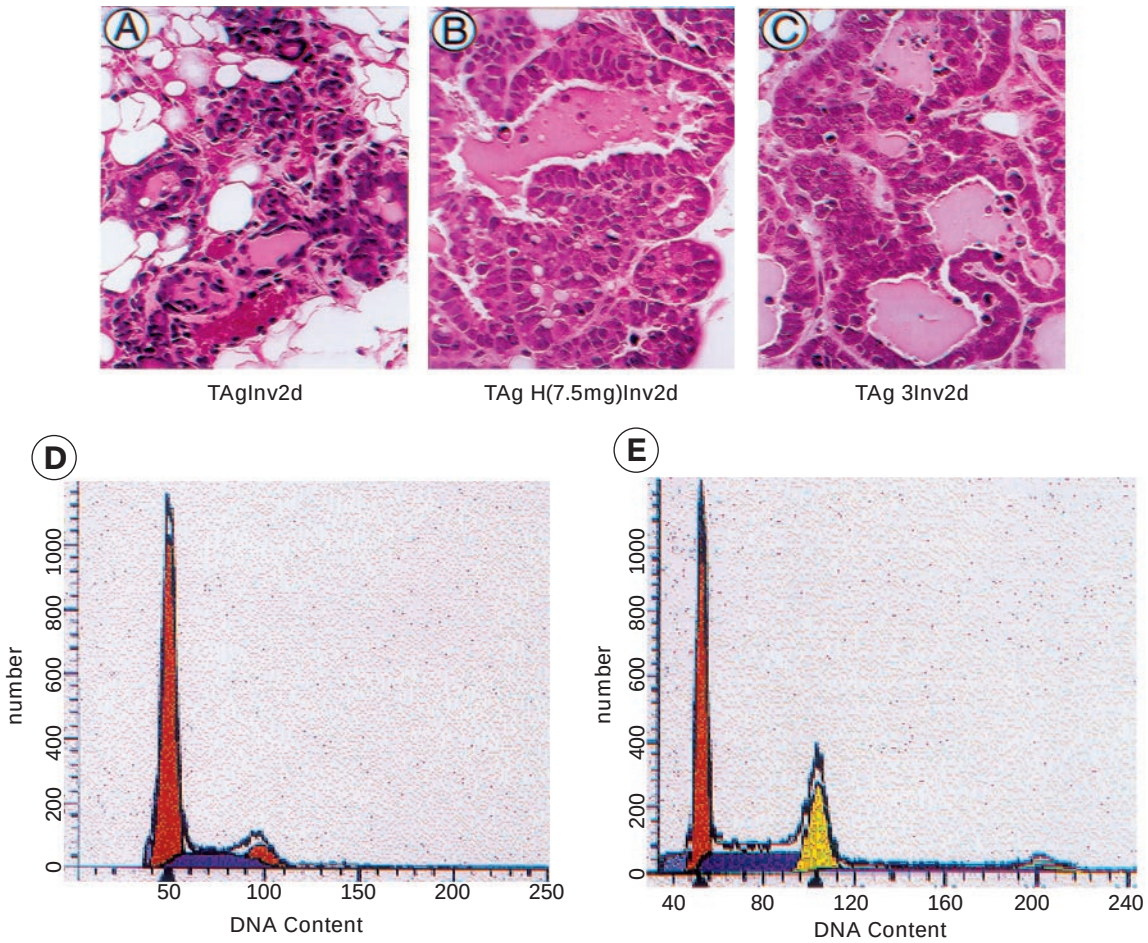

Figure 6 The hyperplasia found in glucocorticoid-exposed WAP-TAg transgenic mice is comparable to that found after three pregnancies in untreated TAg mice. (**a**) No hyperplasia is found after the first pregnancy at day 2 of involution (Inv2d) in untreated TAg mice. (**b**) Extensive hyperplasia is found in glucocorticoid-treated mice after the first pregnancy at day 2 of involution. (**c**) Hyperplasia is found in untreated TAg mice at day 2 of involution after the third pregnancy. (**d**) Representative DNA histogram of cells collected from a WAP-TAg transgenic mouse after 2 days of suckling following the first pregnancy (see Figure 5f). These epithelial cells are diploid and the mean percentage of cells in S phase is approximately 22% (Table 1e) Representative DNA histogram of cells collected from a glucocorticoid-treated WAP-TAg transgenic mouse at day 2 of involution. A significant percentage of the cells are tetraploid and the mean percentage of cells in S phase is 38%. (Table 1). TAg: WAP-TAg transgenic mice. H: hydrocortisone. 3Inv2d: day 2 of involution following the third pregnancy. (**a-c**): H&E stained sections/original magnification 40×

Table 1 Glucorticoid administration increased S phase fraction and induced tetraploidy

		Diploid			*Tetraploid*			
		G0/G1	*G2/M*	*S*	*G0/G1*	*G2/M*	*S*	*Total (%)*
Ctrl[a]	Mean	66.9	10.8	22.3	0	0	0	0
	s.e.m.	2.9	0.3	2.7	0	0	0	0
Glu-exp[b]	Mean	61.0	1.5	37.5	47.2	5.2	29.4	27.0
	s.e.m.	3.4	1.5	2.2	12.0	1.81	7.5	7.0
P=		0.28	0.02	0.006	0.01	0.02	0.01	0.01

[a]WAP-TAg mice ($n=3$); [b]Glucocorticoid exposed WAP-TAg mice ($n=6$). Values compared using paired *t*-tests. *P*-values are listed

delayed tumor development in the WAP-TAg/Bax −/− model. In the C3(1)/SV40-TAg mouse model, partial loss of Bax expression resulted in a higher incidence of tumors at 19–21 weeks of age despite no change in tumor incidence before or after this timepoint. The WAP-TAg model did not demonstrate a similar phenomenon.

The absence of a significant effect on tumorigenesis with either partial or complete loss of Bax expression in the WAP-TAg model could be due to the development of resistance to Bax-induced apoptosis during mammary tumorigenesis. Rates of apoptosis fall from nearly 6% during the initial proliferative phase to approximately 1.5% in the mammary adenocarcinomas of WAP-TAg mice (Furth *et al*., 1999). During this transition levels of Bax, Bcl-x_{long} (Figure 4e) and Bad protein rise (Furth *et al*., 1999). It is possible that the high levels of Bax do not effectively induce apoptosis in tumors due to its interaction with Bcl-x_{long}, which is also highly expressed in these tumors. If that is the case, loss of Bax function by itself might not have a marked effect. Additional experiments will be required to measure the effectiveness of Bax alone in inducing apoptosis at the different stages of WAP-TAg-induced

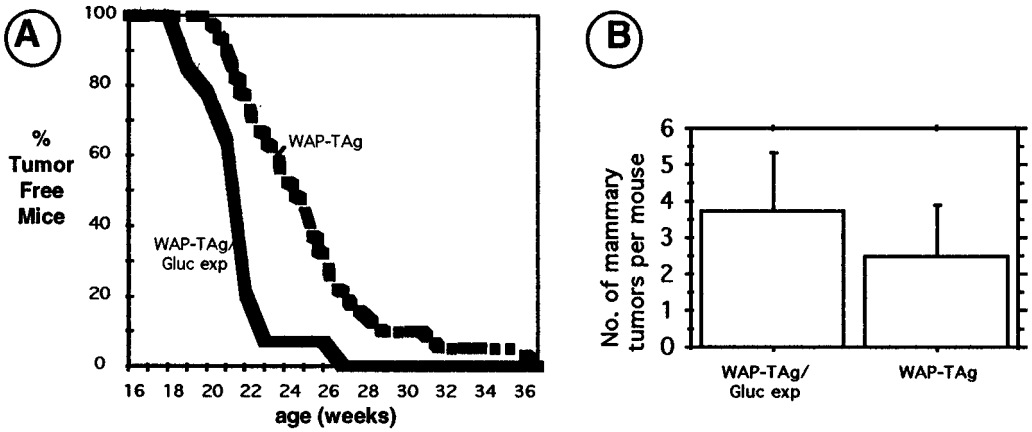

Figure 7 The rapid development of hyperplasia in the glucocorticoid-treated WAP-TAg mice results in both accelerated tumor development and an increase in the total number of tumors per mouse. (**a**) Percentage of tumor-free mice graphed as a function of age (in weeks). Glucocorticoid-treated (Gluc-exp) WAP-TAg mice: ($n=14$). Control, unexposed WAP-TAg mice: ($n=20$). The difference in the tumor incidence curves was highly significant ($P=0.0006$). (**b**) Mean number of tumors in the glucocorticoid-treated WAP-TAg mice was significantly higher than that found in the non-exposed WAP-TAg mice ($P<0.05$ by paired t-test). Tumor multiplicity measured at the time the largest tumor was 1 cm in diameter. Gluc-exp WAP-TAg mice: ($n=18$). Control WAP-TAg mice: ($n=19$). Standard deviation indicated

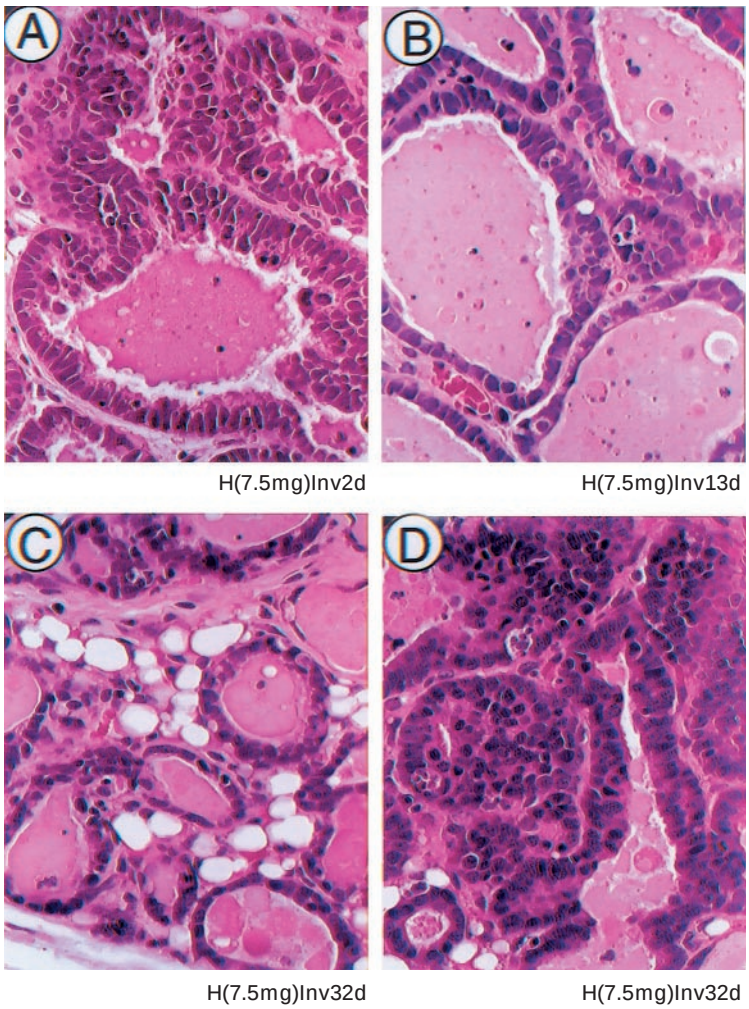

Figure 8 Both reversible and irreversible hyperplasia are induced in the glucocorticoid-treated WAP-TAg mice. (**a**) Extensive hyperplasia is found in glucocorticoid-treated WAP-Tag mice at day 2 of involution. (**b**) Regression of hyperplasia at day 13 of involution. (**c**) Further regression of hyperplasia at day 32 of involution (typical of 95% of the exposed tissue). (d) Foci of irreversible hyperplasia and possible carcinoma *in situ* found at day 32 of involution. H = Mammary tissue from glucocorticoid (hydrocortisone) treated WAP-TAg mice. Inv13 days = 13 days involution. Inv32days = 32 days involution

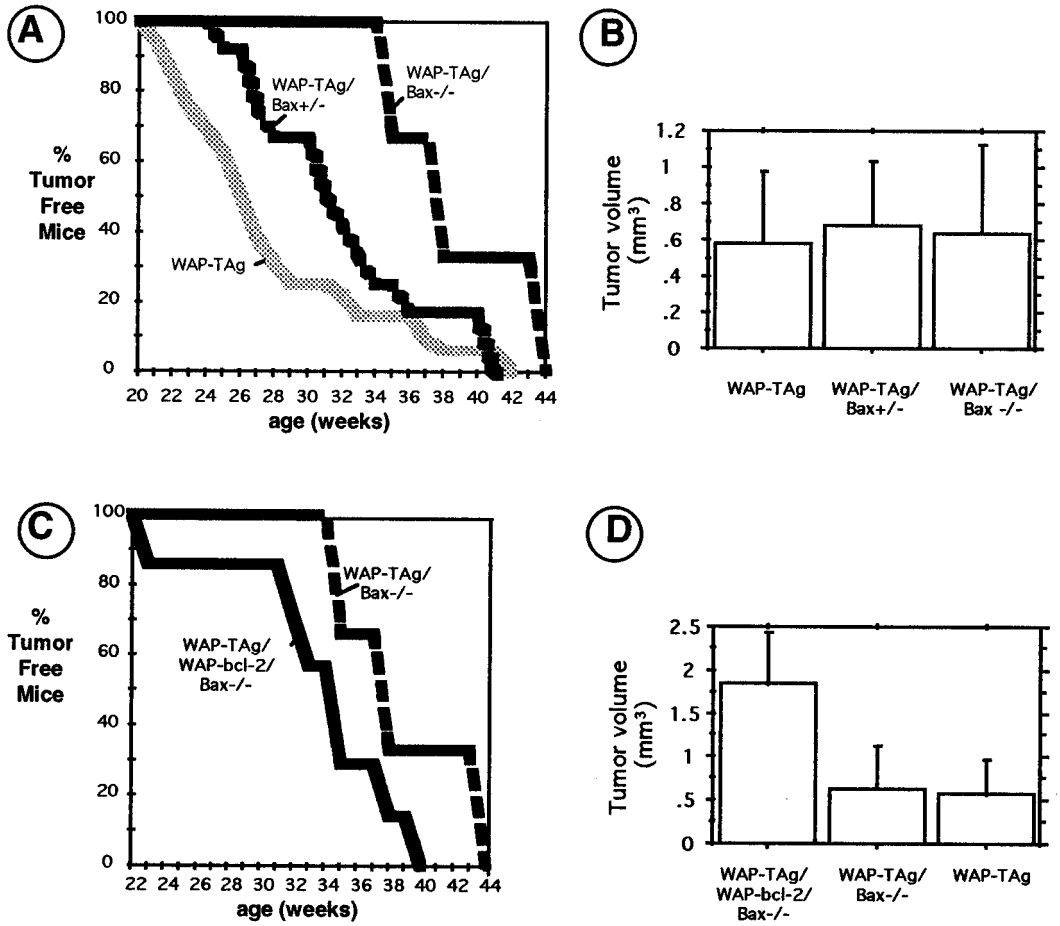

Figure 9 Effect of partial and complete loss of Bax function by itself and in combination with gain of Bcl-2 function on tumor development in WAP-TAg transgenic mice. (**a**) Percentage of tumor free mice graphed as a function of age (in weeks). WAP-TAg transgenic mice: ($n=23$). WAP-TAG/Bax+/− transgenic mice: ($n=12$). WAP-TAg/Bax−/− transgenic mice: ($n=3$). The tumor incidence curves were not significantly different. (**b**) Comparison of mean tumor volume in WAP-TAg, WAP-TAg/Bax+/−, and WAP-TAg/Bax−/− transgenic mice. Standard deviation indicated. There was no significant difference in mean tumor volume between the three genotypes. (**c**) Percentage of tumor free mice graphed as a function of age (in weeks). WAP-TAg/WAP-bcl-2/Bax−/− transgenic mice: ($n=6$). WAP-TAg/Bax−/− transgenic mice: ($n=3$). The tumor incidence curves were not significantly different. (**d**) Comparison of mean tumor volume in WAP-TAg/WAP-bcl-2/Bax+/−, and WAP-TAg/Bax−/− transgenic mice. Standard deviation indicated. Tumor volume in the WAP-TAg/WAP-bcl-2/Bax−/− transgenic mice was significantly greater than that in WAP-TAg transgenic mice ($P<0.008$) and WAP-TAg/Bax+/− transgenic mice ($P<0.005$)

tumorigenesis and to determine the mechanism of any resistance to Bax identified.

A possible interaction between gain of Bcl-2 function and loss of Bax function was found (Figures 9c,d). Tumors tended to appear at a younger age in the WAP-TAg/WAP-bcl-2/*Bax*−/− mice as compared to the WAP-TAg/*Bax*−/− mice and the size of these tumors was significantly increased over the average size found in the WAP-TAg mice ($P<0.008$). Gain of Bcl-2 can synergize with loss of Bax during mammary gland involution (Schorr *et al*., 1999a).

Summary

The WAP-TAg mouse model can be used to explore how changes in cell proliferation and apoptosis impact on tumorigenesis. One strength of this model is that it can be used to study events during the different stages of tumorigenesis, not only its final outcome. The relative contributions of specific gene products or signaling pathways to cell proliferation, apoptosis, and mutation can be assessed in a stage-specific approach. Underlying mechanisms and the development of early interventions that may be used to halt or retard cancer progression are important focus areas for this model.

Materials and methods

Mouse models, hormone administration, mammary gland biopsies, ovariectomy, and preparation of mammary gland whole mounts

Mouse models: Transgenic mice carrying a WAP-TAg hybrid gene and their non-transgenic littermates were used for the β-estradiol, prolactin, progesterone, glucocorticoid, and ovariectomy experiments. Non-transgenic and transgenic NMRI mice carrying a WAP-TAg transgene were identified using a polymerase chain reaction (PCR) based approach (Li *et al*., 1996a). Transgenic NMRI/C57bl/6/SV129 mice carrying a WAP-TAg transgene and either haploid or complete functional deletion of the Bax gene were identified using another PCR approach (Schorr *et al*., 1999a).

Hormone administration Glucocorticoid: Groups of five transgenic and five non-transgenic mice were exposed to exogenous glucocorticoids, on day 18 and the day of parturition of the first pregnancy, by subcutaneous injection

of 7.5 mg hydrocortisone (Merck, West Point, PA, USA). Ten μg β-estradiol (Sigma Chemical Co, St Louis, MO, USA) and 2 mg progesterone (Sigma) were administered at the same two timepoints as used for glucocorticoid. Two hundred mg prolactin from sheep pituitary (Sigma) was injected twice per day for 4 days at days 18, 19, and 20 of pregnancy and the day following parturition. β-estradiol-slow-release pellets (0.025 mg/pellet, 3 week release) and placebo pellets (Innovative Research of America, Sarasota, FL, USA) were embedded under the back skin lying between the shoulder blades after first parturition for the experiments utilizing pellets. Four days after initial exposure to glucocorticoid and 2 days after parturition, mammary gland biopsies were performed to remove the left side fourth (inguinal) mammary glands as described previously (Li *et al*., 1996a). Mice were not allowed to suckle their young; therefore, these glands were collected at day 2 of involution. Control mammary glands were removed from mice that had undergone either 2 days of mammary gland involution or had been suckled for 2 days. After the first biopsy, a second biopsy was performed 13 days later to remove the right side fourth (inguinal) gland and the mice were euthanized 32 days after exposure to glucocorticoids with the remaining mammary glands collected at autopsy. For the acute estrogen, progesterone, and prolactin experiments, mice were euthanized at day 2 of the involution and the mammary glands collected at autopsy. All glands collected were physically divided for histology and protein analyses. Specimens for histology were fixed in 10% formalin while samples for protein analysis were frozen in liquid nitrogen and stored in -70°C for Western blot analysis.

Ovariectomy Mice were anesthetized with 0.7 ml of 0.175% avertin (Li *et al*., 1996a) and positioned ventral side down on a dissection board. The fur was shaved off the skin overlying the ovaries and the area was wiped with 70% ethanol. The ovarian fat pads were localized and a small lateral incision was made through the skin and peritoneum above the ovarian fat pad. The fat pad was then grasped with a blunt forceps and taken out of the mouse by careful excision using small scissors. The uterine horn was subsequently replaced into the abdomen and the incision was closed using wound clips. The mouse was wrapped with a disposable laboratory paper towel, placed back in the cage, and observed during recovery.

Whole mount preparation Mammary gland specimens were spread on glass slides and fixed in Carnoy's solution (100% ethanol : chloroform : glacial acetic acid at 6 : 3 : 1) for at least 1 h at room temperature. Following fixation, the glands were washed with 70% ethanol for 15 min and water for 5 min. Then the glands were stained in carmine alum solution (1 g carmine (Sigma); 2.5 g aluminum potassium sulfate (Sigma) in 500 ml water) at 4°C overnight. The tissue was then dehydrated and mounted on glass using routine methods (Li *et al*., 1996). Experimental protocols were approved by the Institutional Animal Care and Use Committees.

Histological examination and in situ *detection of programmed cell death*

Mammary gland specimens were fixed in 10% neutral formalin solution and embedded in paraffin using routine methods (Li *et al*., 1996). Five-micrometer tissue sections were used for hematoxylin and eosin staining or for *in situ* detection of apoptosis using Apoptag (Oncor, Gaithersburg, MD, USA). Histologic specimens were examined under the microscope for the presence or absence of hyperplasia and carcinoma *in situ*. Reversible and irreversible hyperplasia was characterized by single to multilayered epithelial cells with nuclear atypia organized around a lumen. Irreversible hyperplasia that did not regress during post-pregnancy involution of the gland was surrounded by increased connective tissue. Apoptotic frequency was determined by counting the number of apoptotic cells within a population of 1000 cells as described previously (Li *et al*., 1996a).

Immunoprecipitations and Western blot analyses

For Western blot analysis, protein was extracted as described previously (Li *et al*., 1996a). Forty μg of protein were fractionated on 14% Tris-glycine gels (Novex, San Diego, CA, USA) for PCNA, c-Fos, and c-Jun analyses. Eighty μg of protein were fractionated on 14% Tris-glycine gels (Novex) for cyclin D1, cyclin E, E2F-1, and cdk-2 analyses. Twenty μg of protein were fractionated on 14% Tris-glycine gels (Novex) for Bcl-x_{long}, Bax, and Bag-1. Proteins were transferred onto poly vinlidene difluoride (DVDF) membrane using a NOVEX Western blot transfer apparatus. After transfer and blocking with buffer (5% nonfat milk/20 mM Tris base, pH 7.6/137 mM NACl) for 1 h at room temperature, the membranes were incubated with either anti-cyclin D1 (#06 – 137, 1 : 1000 dilution, Upstate Biotechnology, Lake Placid, NY, USA), anti-cyclin E (#06 – 459, 1 : 1000 dilution, Upstate Biotechnology), anti-cdk-2 (#sc-163, 1 : 500 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-E2F1 (#sc-193, 1 : 500 dilution, Santa Cruz Biotechnology), anti-PCNA (#sc-56, 1 : 500 dilution, Santa Cruz Biotechnology), anti-c-Fos (#sc-52, 1 : 2000 dilution, Santa Cruz Biotechnology), anti-c-Jun (#sc-1694, 1 : 1000 dilution, Santa Cruz Biotechnology), anti-Bcl-x (#sc-1041, 1 : 200, Santa Cruz Biotechnology), anti-Bag-1 (sc#-939, 1 : 200, Santa Cruz Biotechnology) or anti-Bax (#sc-493, 1 : 200, Santa Cruz Biotechnology) at 4°C overnight. Membranes were exposed to the appropriate peroxidase-conjugated secondary antibody (goat anti-mouse IgG, #sc-2005, 1 : 5000 dilution or goat anti-rabbit IgG, #sc-2004, 1 : 5000 dilution, Santa Cruz Biotechnology) for 1 h at room temperature. Proteins were then visualized using the ECL detection system (Amersham, Arlington Heights, IL, USA).

Cell cycle analysis

DNA flow cytometry was performed on paraffin embedded tissue samples. Three 50 microns sections were cut from each sample for DNA analysis (Demeril *et al*., 1996). Nuclear suspensions were made using a modification of Hedley's method (Hedley *et al*., 1983). Tissue sections were initially deparaffinized in xylene for two 50 min intervals at 25°C. Rehydration is accomplished by two treatments in decreasing concentrations of ethanol (100, 95, 70 and 50%) ending with deionized water. A 50 min incubation step at to 25°C is required for each ethanol concentration. To make a single cell suspension, the sample is treated with a 0.5% pepsin solution (Sigma) at 37°C for 30 min. The final nuclear suspension is stained with propidium iodide using the Vindelov technique (Vindelov *et al*., 1983). DNA flow analysis is performed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). Approximately 20 000 events are collected per sample, and the parameters used for gating are FL2-W versus VL2-A. A tissue was considered diploid if only one G0/G1 peak was identified. Tetraploid tissue exhibited a large dominant peak in the 4N region of the DNA histogram. If G0/G1 peaks were identified between the 2N and 4N region of the DNA histogram, the tissue was designated as having a DNA aneuploid stem line. DNA cell cycle analysis was performed using Modfit LT software (Verity Software, Topsham, ME, USA). Two 4 microns sections are requested (before and after DNA analysis) as histologic adequacy checks.

Labeling index using BrdU

BrdU labeling studies were performed on two non-transgenic control and two WAP-TAg transgenic mice at day 18 of pregnancy. BrdU (Amersham) was injected intraperitioneally (1 ml/100 g body weight). Mice were euthanized 2 h later and both fourth (inguinal) mammary glands were collected for analysis. The mammary tissue was fixed overnight in 10% neutral buffered formalin at 4°C. Tissues were embedded in paraffin, sectioned at 5 microns, and mounted on glass slides. BrdU-labeled mammary epithelial cells were detected using a BrdU staining kit (Zymed Laboratories, Inc., San Francisco, CA, USA) with all procedures performed at room temperature. Tissue sections were deparaffinized in xylene, then rehydrated in a graded series of alcohol, and quenched with 3% H_2O_2 in absolute methanol for 10 min at room temperature. Sections were then washed with phosphate buffered saline (PBS) and the tissue was digested with proteinase-K (20 μg/ml PBS) for 15 min. The sections were washed in distilled H_2O and then denatured in 4N HCl for 20 min. After washing with PBS and incubating with blocking solution (BrdU Staining Kit, Zymed) for 10 min, the tissue sections were incubated with the monoclonal anti-BrdU for 40 min. Slides were then washed in PBS followed by incubation with streptavidin-peroxidase for 10 min. Sections were washed again in PBS followed by incubation with the chromogen, diaminobenzidine (DAB), for approximately 3 min. After washing with distilled H_2O, tissues were counterstained with hematoxylin and viewed by light microscopy.

Statistical analyses

Cell cycle analyses Data were analysed using a one-way analysis of variance. The root transformation of raw data was used for analysis of the percentage distribution of cells in the cell cycle. $P<0.05$ was considered significant. Results are presented as arithmetic means $\pm$ s.e.m. of non-transformed data.

Time to first palpable tumor, tumor volume, and number of tumors Means and standard deviations were calculated and paired *t*-tests performed using Stat View 5.4 (Abacus Concepts, Inc., Berkeley, CA, USA). $P\leqslant 0.05$ was considered significant.

Survival curves of glucocorticoid exposed and control mice Tumor incidence curves were compared using the log-rank test utilizing Prism, version 2.0 (GraphPad Software, Inc., San Diego, CA, USA). $P<0.05$ was considered significant.

Abbreviations

WAP, whey acidic protein; TAg, Simian virus 40 large T antigen; WAP-TAg, a transgenic mouse model of breast cancer progression in which TAg expression is targeted to mammary epithelial cells using the WAP promoter; pRb, Retinoblastoma protein; H&E, hematoxylin and eosin; PCNA, proliferating cell nuclear antigen; PBS, phosphate buffered saline; DAB, diaminobenzidine; WT, wild-type nontransgenic control mice; ovx; ovariectomy; wk, weeks; PCR, polymerase chain reaction; DMBA, dimethylbenz(a)anthracene; TGF-β, transforming growth factor-β.

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References

Demeril D, Laucirica R, Fishman A, Owens RG, Grey MM, Kaplan AL and Ramzy I. (1996). *Cancer, 77,* 1494–1500.

Dyson N, Buchkovich K, Whyte P and Harlow E. (1989). *Cell,* **58,** 249–255.

Ewald D, Li M, Efrat S, Auer G, Wall RJ, Furth PA and Hennighausen L. (1996). *Science,* **273,** 1384–1386.

Feng Z, Marti A, Jehn B, Altermatt HJ, Chicaiza G and Jaggi R. (1995). *J. Cell Biol.,* **131,** 1095–1103.

Furth PA, Bar-Peled U, Li M, Lewis A, Laucirica R, Jager R, Weiher H and Russell RG. (1999). *Oncogene,* **18,** 6589–6596.

Hahn WC, Counter CM, Lundberg AS, Beijersbergen RL, Brooks MW and Weinberg RA. (1999). *Nature,* **400,** 464–468.

Hedley DW, Friedlander ML, Taylor IW, Rugg CA and Musgrove EA. (1983). *J. Histochem. Cytochem.,* **31,** 1333–1335.

Hennighausen L, Robinson GW, Wagner KU and Liu W. (1997). *J. Biol. Chem.,* **272,** 7567–7569.

Horak E, Smith K, Bromley L, LeJeune S, Greenall M, Lane D and Harris AL. (1991). *Oncogene,* **6,** 2277–2284.

Lee EYH, To H, Shew JY, Bookstein R, Scully P and Lee WH. (1988). *Science,* **241,** 218–221.

Li M, Hu J, Heermeier K, Hennighausen L and Furth PA. (1996a). *Cell Growth Differ.,* **7,** 3–11.

Li M, Hu J, Heermeier K, Hennighausen L and Furth PA. (1996b). *Cell Growth Differ.,* **7,** 13–20.

Li M, Liu X, Robinson G, Bar-Peled U, Wagner KU, Young WS, Hennighausen L and Furth PA. (1997). *Proc. Natl. Acad. Sci. USA,* **94,** 3425–3430.

Lund R, Romer J, Thomasset N, Solberg H, Pyke C, Bissell MJ, Dano K and Werb Z. (1996). *Development,* **122,** 181–193.

Meitz JA, Unger T, Huibregtse JM and Howley PM. (1992). *EMBO J.,* **11,** 5013–5020.

Murphy KL, Kittrell FS, Gay JP, Jäger R, Medina D and Rosen JM. (1999). *Oncogene,* **18,** 6597–6604.

Newcomb PA. (1997). *J. Mammary Gland Biol. Neoplasia,* **2,** 311–318.

Santarelli R, Tzeng YJ, Zimmermann C, Guhl E and Graessmann A. (1996). *Oncogene,* **12,** 495–505.

Schorr K, Li M, Bar-Peled U, Lewis A, Heredia A, Lewis B, Knudson CM, Korsmeyer S, Jager R, Weiher H and Furth PA. (1999a). *Cancer Res.,* **59,** 2541–2545.

Schorr K, Li M, Krajewski S, Reed JC and Furth PA. (1996b). *J. Mammary Gland Biol. Neoplasia,* **4,** 153–164.

Shibata M-A, Liu M-L, Knudson CM, Shibata E, Yoshidomo K, Bandey T, Korsmeyer SJ and Green JE. (1999). *EMBO J.,* **18,** 2692–2701.

Topper YJ and Freeman CS. (1980). *Physiol. Rev.,* **60,** 1049–1060.

Tzeng Y-J, Guhl E, Graessmann M and Graessmann A. (1993). *Oncogene,* **8,** 1965–1971.

Tzeng YJ, Gottlob K, Santarelli R and Graessmann A. (1996). *FEBS Lett.,* **380,** 215–218.

Vindelov LL, Christensen IJ and Nissen NI. (1983). *Cytometry,* **3,** 323–327.