Tumorigenesis and Neoplastic Progression

Mammary Carcinogenesis Is Preceded by Altered Epithelial Cell Turnover in Transforming Growth Factor- α and c-myc Transgenic Mice

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Identification of biomarkers that indicate an increased risk of breast cancer or that can be used as surrogates for evaluating treatment efficacy is paramount to successful disease prevention and intervention. An ideal biomarker would be identifiable before lesion development. To test the hypothesis that changes in cell turnover precede mammary carcinogenesis, we evaluated epithelial cell proliferation and apoptosis in mammary glands from transgenic mice engineered to develop mammary cancer due to expression in mammary epithelia of transforming growth factor α (TGF- α) or c-myc. In transgenic glands, before lesion development, epithelial cell turnover was enhanced overall compared with nontransgenic glands, indicating that aberrant cell turnover in normal epithelia may contribute to tumorigenesis. In addition, in tumor-containing glands, proliferation in normal epithelia was higher than in tumor-free transgenic glands, suggesting these cell populations influence one another. Finally, although c-myc glands displayed a uniformly high epithelial cell turnover regardless of age, cell turnover was reduced with aging in nontransgenic and TGF- α mice, indicating that some growth and death regulatory mechanisms remain intact in TGF- α epithelia. These observations support the evaluation of cell turnover as a biomarker of cancer risk and indicator of prevention/treatment efficacy in preclinical models and warrant validation in human breast cancer. (Am J Pathol 2006, 169:1821–1832; DOI: 10.2353/ajpath.2006.050675)

Development of breast cancer prevention strategies is hindered by our inability to predict who will develop the

disease and the protracted and multistep nature of the process. Women deemed at high risk for breast cancer have received preventive treatments such as selective estrogen modulators or aromatase inhibitors, and a net reduction in estrogen-positive cancers has been the result. However, resistance to estrogen modulators in initially responsive patients almost always occurs, and estrogen nonresponsive cancers make up approximately one third of breast cancers and are more difficult to treat. Clearly, additional preventive strategies are needed. However, testing the effectiveness of preventive regimens using cancer development as an endpoint takes years to decades. The identification of intermediate cellular and/or molecular biomarkers, which accurately reflect the risk of developing cancer, is critical for implementing effective breast cancer prevention strategies. Unfortunately, there is a paucity of known intermediate biomarkers.

The net gain of cells, either through excessive proliferation or failure of cell death, is a hallmark feature of cancer. Abnormal patterns of cell turnover observed in breast tissue with hormone replacement therapy may indicate a higher risk of cancer development.^{1,2} Understanding the dynamics of cell proliferation and death during carcinogenesis and in normal breast epithelia that remain healthy throughout life is critical to our understanding of the cell turnover abnormalities that accompany cancer development.

Genetically engineered mouse models of mammary cancer have been generated to mimic human disease. These mouse models demonstrate similarity in the natural history of disease progression, genomic alterations, gene and protein expression patterns, hormone-responsiveness, and metastasis to distant sites.³ In addition, the histopathology of mammary lesions in genetically engineered mice, recently reviewed by a panel of pathologists who compared tumors in mice to

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Table 1. Number of Mammary Epithelial Histotypes Identified and Evaluated for Proliferative and Apoptotic Activities

Age	Epithelial histotype (no. of mice evaluated)	Proliferation*			Apoptosis†		
		Non-TG‡ (n = 18)	TGF- α (n = 20)	c-myc (n = 17)	Non-TG (n = 18)	TGF- α (n = 24)	c-myc (17)
≤12 weeks	No. mice	5	5	4	5	5	5
	Epithelial histotype						
	Lobules	108	168	72	116	131	92
	Ducts	57	62	52	35	92	49
	Hyperplasias	0	0	19	0	0	20
>16 weeks	Tumors	0	0	0	0	0	0
	No. mice	13	15	13	13	19	12
	Epithelial histotype						
	Lobules	290	304	220	472	460	324
	Ducts	126	148	140	191	220	162
	Hyperplasias	17	39	108	20	56	117
	Tumors	0	12	15	0	13	17

*Proliferation was determined by counting BrdU-labeled epithelial cells using immunohistochemistry in histological sections (one section/mammary gland/mouse).

†Apoptosis was determined by counting pyknotic cells on histological sections stained with H&E.

‡Non-TG, nontransgenic; TGF- α , WAP-TGF- α transgenic mice; c-myc, WAP-c-myc transgenic mice.

humans, share many features with human breast cancer.⁴ Mouse models of mammary cancer also have been used in preclinical testing of preventive and therapeutic agents.⁵ Unlike xenograft models, early-to-late-stage-specific responses to chemo- or nonchemopreventives can be evaluated in genetically engineered mice. In addition, preventives can be tested for efficacy against specific oncogenes representing molecular anomalies found in clinical breast tumors, and in a physiologically relevant context. Encouraging global gene expression data revealed common molecular anomalies in tumors originating from several different transgenic mouse models of mammary cancer in which different oncogenes were overexpressed,⁶ suggesting that potential targets for prevention and therapy can be directed toward a few general molecular pathways. Regardless of the mode of intervention or the underlying molecular maladies, inhibition of proliferation is likely to be a common feature of successful treatment of breast cancer development. Data from a recent study of 21 patients support this assertion in that a high level of breast epithelial cell proliferation after neoadjuvant therapy was associated with cancer recurrence or death.⁷ Extrapolation of these observations suggests that evaluation of epithelial cell turnover activities of normal and abnormal epithelium of breast biopsies may provide another measurement of cancer risk. These data could be obtained readily from biopsies collected for the purpose of establishing the underlying character of suspicious regions found on imaging or palpation. Epithelia obtained from reduction mammoplasties could act as controls. Alternatively, a core needle biopsy of unaffected regions would provide information about site and patient-to-patient variation.

Our objective in this study was to test the hypothesis that increased cell turnover in normal mammary epithelium is a common feature preceding and accompanying cancer development. To accomplish this we evaluated proliferative and apoptotic activities in lobules, ducts, hyperplasias, and tumors in transgenic mice overex-

pressing transforming growth factor (TGF)- α or c-myc in mammary epithelia before and during tumorigenesis. We also characterized normal proliferation and apoptosis in wild-type non-TG mice that remained mammary cancer-free. We report that changes in cell turnover preceded the development of mammary hyperplasias and tumors and that cell turnover events were qualitatively similar for both mouse models.

Materials and Methods

Mice

WAP-TGF- α line 3573-2 transgenic mice, designated TgN(WAPTgfa)215Bri and WAP-c-myc line 3507-1, TgN(WAPMyc)212Bri, have been described.⁸ These mice were generated in the (C57BL/6xSJL)F2 background and were backcrossed into the FVB/N (Taconic, Germantown, NY) strain at least 15 generations before use in this study. In this report, wild-type non-TG FVB/N mice are designated as non-TG, WAP-TGF- α mice are designated as TGF- α , and WAP-c-myc mice are designated c-myc mice. All mice were housed separately from males and in Association of and Accreditation of Laboratory Animal Care, International-accredited facilities and handled in accordance with the Guide for the Care and Use of Laboratory Animals. All studies were approved by the institutional animal care and use committee.

Sampling Design, Age, and Degree of Lobularization

The numbers of mice and epithelial histotypes assessed for proliferation and apoptosis is presented in Table 1. Eighteen non-TG, 20 TGF- α , and 17 c-myc virgin mice were evaluated for proliferation. For apoptotic assessments, the same mammary glands from non-TG mice were evaluated. For TGF- α , mice the same mammary glands were evaluated, and mammary glands from four additional animals also were as-

sessed. For *c-myc* mice, an additional mouse ≤ 12 weeks was evaluated and mammary glands from a single mouse >16 weeks were not available for evaluation. When nulliparous female mice of the FVB/N strain reach 12 weeks of age, arborization of the ductal epithelial tree is complete, and ducts extend to the edges of the mammary fat pad. Therefore, data analysis was carried out on mice segregated into two physiologically relevant age groups: mice ≤ 12 weeks with immature mammary glands and mice >16 weeks with fully mature mammary glands. The age distributions for mice ≤ 12 weeks and >16 weeks are as follows. For mice ≤ 12 weeks, ages ranged from 51 to 81 days with mean $\pm$ SE of 65 ± 5 , 67 ± 6 , and 65 ± 1 for non-TG, TGF- α , and *c-myc* mice, respectively. For mice >16 weeks, ages ranged from 132 to 286 days with mean $\pm$ SE of 194 ± 11 , 176 ± 9 , and 194 ± 11 days for non-TG, TGF- α , and *c-myc* mice, respectively. There were no statistical differences between groups (data not shown). A single cross-section of one to two mammary glands was evaluated for each mouse. The total number of epithelial histotypes evaluated for cellular proliferation and apoptosis also are presented in Table 1. The morphology of epithelial histotypes (ie, lobules, ducts, hyperplasias, and tumors) is shown in Figure 1. In addition, animals were categorized by the degree of lobular development. A modified version of the staging system developed by Fata and colleagues¹¹ was used in which lobular development was graded from 0 to 3 with increasing lobularization. In this study lobular development with an average of <1.2 alveolar lumens/lobule was graded as stage 0 whereas versus ≥ 1.2 alveolar lumen/lobules in which animals were graded as $\geq$ stage 1.

Histology and Immunohistochemistry

Female virgin mice were injected with 200 mg/kg body weight BrdU (no. B5002; Sigma, St. Louis, MO), a nucleotide analog incorporated into DNA of proliferating cells, and sacrificed 1 to 2 hours later. The fourth mammary gland from each mouse was collected. Tissues were fixed in 10% neutral buffered formalin at room temperature for 18 to 24 hours, dehydrated in ethanols, embedded in paraffin, sectioned ($6 \mu\text{m}$), and mounted on glass slides. Mice were not staged for estrus, but the extent of lobular development was determined by counting the number of alveolar lumens/lobule. This method was a direct modification of a system developed by Fata and colleagues¹¹ who found that a higher degree of lobular development was positively correlated with circulating progesterone levels.

BrdU Labeling for the Identification of Proliferating Cells

Methods used for immunohistochemistry were described previously.⁹ Briefly, slides were deparaffinized in Citri-Solv (Fisher Scientific, Pittsburgh, PA), hydrated in graded alcohols, and exposed to 0.5% H_2O_2 in methanol to block

endogenous peroxidase activity. Slides then were boiled in a solution of 0.1 mol/L Tris for antigen retrieval and incubated overnight with rat monoclonal anti-BrdU (MAS-250; Accurate-Scientific, Westbury, NY) at a dilution of 1:40 in 0.5% powdered milk dissolved in phosphate-buffered saline (PBS). The following day slides were rinsed with PBS, treated with rat-specific link antibody (BioGenex, San Ramon, CA) for 30 minutes, rinsed, incubated with the enzyme label peroxidase streptavidin (BioGenex) for 30 minutes, and treated with 3,3'-diaminobenzidine (no. D-4293; Sigma) for up to 10 minutes. Slides were counterstained in Nuclear Fast Red (Polyscientific, Bay Shore, NY) for 1 minute, dehydrated, and mounted under a glass coverslip. Cells labeled with BrdU displayed brown nuclear stain. Positive control tissues included duodenum or lymph nodes from mice used in this study. Negative controls were mammary gland or duodenum incubated with a nonspecific antibody.

Identification of Apoptotic Cells

Slides were deparaffinized in Citri-Solv (Fisher Scientific), hydrated in graded alcohols, and stained with hematoxylin and eosin (H&E). Apoptotic cells were identified at $\times 400$ magnification by the presence of pyknotic nuclei and retracted cytoplasm. For some mammary glands, terminal dUTP nick-end labeling (TUNEL) staining was carried out as described previously¹⁰ using the *in situ* cell death detection kit, POD (no. 1 684 817; Roche, Penzberg, Germany). Apoptotic cells were identified by addition of fluorescein-labeled nucleotides to DNA ends by terminal deoxynucleotidyl transferase. Anti-fluorescein-specific antibody Fab fragments conjugated to peroxidase were applied to the tissue, followed by 3,3'-diaminobenzidine. Slides were counterstained with nuclear fast red and mounted under glass coverslips.

In a pilot study, we compared apoptotic activity in normal and hyperplastic epithelia using two methods: a histological method evaluating the number of pyknotic cells on H&E-stained tissue sections (Figure 1F) with an immunohistochemical method of identifying cells with degraded DNA nucleotides using a TUNEL assay. In mammary glands from three non-TG, three TGF- α , and three *c-myc* mice, the percentage of cells identified as apoptotic were determined by these two methods in adjacent histosections and were not different for lobules or ducts ($P \leq 0.05$). As a result, the most direct approach of evaluating histosections for pyknotic cells was used.

Proliferative and Apoptotic Activities

Epithelial histotypes including lobules, ducts, hyperplasias, and cystic and solid tumors were evaluated for BrdU labeling (LI) and apoptotic labeling (AI) indices. As previously described,⁹ lobules were defined as containing one to four adjacent alveolar lumens in cross-section, each surrounded by a single layer of luminal epithelial cells. Ducts could be found in longitudinal and cross-section. Ducts in cross-section con-

Table 2. Focal Nature of Proliferation as Measured by the Percentage of Epithelial Histotypes with at Least One BrdU-Labeled Cell

Age	Epithelial histotype	Non-TG	TGF- α	c-myc
		Mean $\pm$ SE (no. of mice)*		
≤ 12 weeks	Lobules	32 $\pm$ 17 ^{a,x} (4/5)	50 $\pm$ 19 ^{a,x} (5/5)	89 $\pm$ 4 ^{b,x} (4/4)
	Ducts	44 $\pm$ 19 ^{a,x} (4/5)	58 $\pm$ 21 ^{a,x} (5/5)	94 $\pm$ 4 ^{b,x} (4/4)
	Hyperplasias			100 $\pm$ 0 (3/4)
>16 weeks	Lobules	7 $\pm$ 3 ^{a,x} (7/13)	47 $\pm$ 9 ^{b,x} (12/15)	89 $\pm$ 5 ^{b,x} (12/13)
	Ducts	30 $\pm$ 8 ^{a,x} (10/13) [†]	77 $\pm$ 6 ^{b,y} (14/15)	89 $\pm$ 4 ^{b,x} (13/13)
	Hyperplasias	43.2 (2/13)	84 $\pm$ 11 ^y (9/15)	100 $\pm$ 0 ^x (13/13)
	Tumors		100 $\pm$ 0 ^y (6/15)	100 $\pm$ 0 ^x (6/13)

Individuals with epithelia in which an entire histotype (lobules, ducts, or hyperplasias) did not exhibit BrdU-labeled cells were excluded from these calculations.

*Number of animals that exhibited proliferating cells in the respective epithelial histotype/all animals examined.

[†]Significantly reduced compared with lobules from non-TG mice <12 weeks. Statistical significance at $P < 0.05$. See Statistical Analysis for further details.

^{ab}Statistical differences between non-TG and each transgenic depicted by different letters within rows (Student's *t*-tests).

^{xy}Statistical differences between histotypes depicted by different letters within columns (ANOVA). Independent statistical analysis was performed for each age category. Comparisons between age groups but within genotype were carried out using Student's *t*-test.

tained secretions within a single lumen, which generally had a larger diameter than an alveolar lumen of a lobule. Terminal endbuds in animals ≤ 12 weeks of age were grouped with lobules. Structures containing five or more adjacent lumens were classified as lobular hyperplasias. These structures, which resemble lobules at mid-pregnancy, also have been called hyperplastic alveolar nodules.⁸ Cystic tumors were lined with a single layer of simple and/or complex papillary epithelium and were present in TGF- α mice but were not found in non-TG or c-myc mice. Solid tumors contained tightly packed epithelium with few or no cystic spaces. To minimize selection bias, for each slide representing a single individual, at least 10 normal-appearing ducts and 10 normal-appearing lobules were examined, providing a minimum of 500 cells per histotype per slide with the following exceptions: in one TGF- α mouse >16 weeks, only nine lobules were evaluated, and in a single c-myc mouse, there were zero lobules to evaluate because the gland was filled with hyperplasias. Fewer than 10 ducts ($n = 5, 7, 8, 9, 9$) were present in slides representing five TGF- α glands. For hyperplasias, a minimum of 10 hyperplasias and at least 1000 cells were evaluated. For cystic and solid tumors, all epithelial cells visible in cross-section were counted. When slides contained multiple tumors, each was counted separately. BrdU staining of normal ducts, lobules, and hyperplasias is shown in Figure 1. The number of epithelial histotypes identified and evaluated for proliferative and apoptotic activities is depicted in Table 1.

Proliferation

To describe the focal nature of proliferative activity in mammary glands of non-TG and transgenic mice, the percentage of epithelial histotypes that contained any BrdU-labeled cells for each individual was determined and averaged over all mice for each mouse group (non-TG, TGF- α , c-myc). These values are depicted in Table 2. Next, within each BrdU-containing epithelial unit, we also determined the percentage of BrdU-labeled cells and report these as BrdU labeling indices. A BrdU labeling index (LI) was calculated by dividing the number of BrdU-labeled cells by the total number of cells counted for each epithelial histotype, ie, lobules, ducts, hyperplasias, and tumors. The mean LI for each histotype within an individual then was averaged over all individuals within a mouse group, and these data are represented in Table 3.

In data collected from mice >16 weeks, we compared percentages of epithelia with any BrdU-labeled cells and LI of those with label to a more traditional method of evaluating BrdU-labeled cells, which entails simply counting the number of labeled cells and dividing by all cells counted for an epithelial histotype. Using the traditional method, epithelial units devoid of any BrdU-labeled cells are incorporated in the LI. We compared BrdU labeling using this traditional method with our measurements described above. The overall measure of results was similar (data not shown). We therefore chose our more informative method, which breaks down the measure of proliferation from a single value into two values: the percentage of epithelial histotypes that contained any

Figure 1. Photomicrographs of epithelial histotypes evaluated for proliferation using immunohistochemical localization of BrdU and for apoptosis by identifying pyknotic cells in non-TG (A–C), TGF- α (G–K), and c-myc (D–F, L–P) mice. BrdU-labeled cells are stained brown, and slides were counterstained with nuclear fast red (A–D, G–I, L–N). Lobules and ducts in non-TG (A), TGF- α (G), and c-myc (L) mice have similar morphology although BrdU labeling is greater in transgenic mice. C: Terminal end buds contained numerous BrdU-labeled cells. Hyperplasias in non-TG mice (B) are significantly smaller than in TGF- α (H, I) and c-myc (M, N) mice and have fewer BrdU-labeled cells. Tumors in TGF- α mice were complex with cystic spaces and papillary growth (J, K) compared with solid tumors of c-myc (O, P), which have higher levels of cell turnover. Duodenal sections containing extensive labeling of BrdU acted as positive controls (D) whereas negative controls were adjacent sections subjected to the same immunohistochemical protocol but without exposure to anti-BrdU antibody (E). Pyknotic cells in H&E-stained slides are depicted by arrows (F). Original magnifications: $\times 40$ (C, J, O); $\times 100$ (A, G–H, L–M); $\times 200$ (B, D, P); $\times 400$ (E–F, I, K, N). Scale bars = 50 μ m.

Table 3. Percentage of BrdU-Labeled Cells (LI) Per Epithelial Histotype

Age	Epithelial histotype	Non-TG	TGF- α	c-myc
		Mean $\pm$ SE (no. of mice)*		
≤ 12 weeks	Lobules	5.6 $\pm$ 1.9 ^{a,x} (4/5)	11.0 $\pm$ 3.9 ^{a,x} (4/5)	16.2 $\pm$ 1.2 ^{b,x} (4/4)
	Ducts	4.9 $\pm$ 3.5 ^{a,x} (4/5)	9.2 $\pm$ 5.4 ^{a,x} (5/5)	6.3 $\pm$ 0.9 ^{a,y} (4/4)
	Hyperplasias			18.0 $\pm$ 2.2 ^x (3/4)
>16 weeks	Lobules	5.9 $\pm$ 1.5 ^{a,x} (7/13)	7.6 $\pm$ 1.1 ^{a,x} (12/15)	12.9 $\pm$ 1.5 ^{b,x} (12/13)
	Ducts	1.4 $\pm$ 0.1 ^{a,x} (10/13)	4.7 $\pm$ 0.9 ^{b,x} (14/15)	6.6 $\pm$ 0.8 ^{b,y} (13/13)
	Hyperplasias	1.6 (2/13)	5.3 $\pm$ 1.3 ^x (9/15)	18.1 $\pm$ 1.8 ^{x,z} (13/13)
	Tumors		7.4 $\pm$ 0.6 ^x (6/15)	24.9 $\pm$ 3.0 ^z (6/13)

Only epithelial units with at least one BrdU-labeled cell were included in these calculations.

*Number of animals that exhibited BrdU-labeled cells/all animals examined.

^{ab}Statistical differences between non-TG and each transgenic depicted by different letters within rows (Student's *t*-tests).

^{xyz}Statistical differences between histotypes depicted by different letters within columns (ANOVA). Independent statistical analysis was performed for each age category. Comparisons between age groups but within genotype were carried out using Student's *t*-test. Statistical significance at *P* < 0.05. See Statistical Analysis for further details.

proliferating (BrdU-labeled) cells, and, within those proliferating epithelial units, the fraction of BrdU-labeled cells.

Finally, we measured two features of mammary epithelial apoptotic activity. First, we calculated for each epithelial histotype (lobules, ducts, hyperplasias, and tumors) the percent that displayed at least one pyknotic cell. This allowed an evaluation of the focal nature of apoptotic activity in the gland of non-TG or transgenic mice and is reported separately for each epithelial histotype in Table 4. Second, for those epithelial units with at least one pyknotic cell, we calculated apoptotic indices. An apoptotic index (AI) was calculated by dividing the number of pyknotic cells by the total number of cells counted for each epithelial histotype. The mean AI for each histotype within an individual then was averaged over all individuals within a mouse group. This provided a measure of the percentage of pyknotic cells for each epithelial histotype and is reported as AI in Table 5.

Statistical Analyses

Two-tailed Student's *t*-test were used to determine significant differences between the percentage of each epithelial histotype (lobules, ducts, hyperplasias, and tumors), which contained BrdU-labeled cells or pyknotic cells, as well as LI or AI for each mouse group (non-TG, TGF- α , and c-myc).

Analysis of variance was used to compare proliferative and apoptotic activities between epithelial histotypes within a mouse group. Separate analyses were performed for each age group (≤ 12 weeks and >16 weeks). Comparisons between age groups but within genotype were carried out using Student's *t*-test. Spearman correlation coefficients were determined for LI and AI in lobules, ducts, hyperplasias, and tumors from non-TG, TGF- α , and c-myc animals. Within each mouse group, Student's *t*-tests were used to determine whether cell turnover activities were altered with the extent of lobular development (<1.2 versus ≥ 1.2 number of lumens/lobule), and linear regression was used to depict the relationship. Linear regression also was used to describe the relationship between age and measurements of cell turnover activities within each mouse group. Data comparisons with *P* values ≤ 0.05 were considered significant. All statistical analyses were carried out using Prism Version 4.0 (GraphPad, Inc., San Diego, CA).

Results

Proliferation Patterns in Non-TG Mice

In non-TG mice ≤ 12 weeks, BrdU-labeled cells were found in one third of lobules and one half of ducts (Tables 2 and 3). In mice >16 weeks, the percentage

Table 4. Focal Nature of Apoptosis as Measured by the Percentage of Epithelial Histotypes with at Least One Pyknotic Nucleus

Age	Epithelial histotype	Non-TG	TGF- α	c-myc
		Mean $\pm$ SE (no. of mice)*		
≤ 12 weeks	Lobules	45 $\pm$ 5 ^{a,x} (5/5)	14 $\pm$ 2 ^{b,x} (5/5)	48 $\pm$ 11 ^{a,x} (5/5)
	Ducts	82 $\pm$ 8 ^{a,y} (5/5)	38 $\pm$ 5 ^{b,x} (5/5)	63 $\pm$ 6 ^{b,x} (5/5)
	Hyperplasias			100 $\pm$ 0 (1/5)
>16 weeks	Lobules	13 $\pm$ 2 ^{a,x} (13/13) [†]	39 $\pm$ 4 ^{b,x} (19/19) [†]	38 $\pm$ 5 ^{b,x} (12/12)
	Ducts	41 $\pm$ 4 ^{a,y} (13/13) [‡]	67 $\pm$ 6 ^{b,y} (19/19) [‡]	60 $\pm$ 6 ^{b,y} (12/12)
	Hyperplasias	75 ^y (2/13)	96 $\pm$ 4 ^y (12/19)	91 $\pm$ 3 ^y (12/12)
	Tumors		89 $\pm$ 5 ^y (6/19)	100 $\pm$ 0 ^y (5/12)

Only epithelial units with pyknotic nuclei were included in the calculations.

*Number of animals that exhibited pyknotic cells in the respective epithelial histotype/all animals examined.

^{†‡}Significantly reduced compared with lobules and ducts, respectively, from non-TG mice <12 weeks. Statistical significance at *P* < 0.05. See Statistical Analysis for further details.

^{ab}Statistical differences between non-TG and each transgenic depicted by different letters within rows (Student's *t*-tests).

^{xyz}Statistical differences between histotypes depicted by different letters within columns (ANOVA). Independent statistical analysis was performed for each age category. Comparisons between age groups but within genotype were carried out using Student's *t*-test.

Table 5. Percentage of Pyknotic Cells (AI) Per Epithelial Histotype

Age	Epithelial histotype	Non-TG	TGF- α	c-myc
		Mean $\pm$ SE (no. of mice)*		
≤ 12 weeks	Lobules	5.0 $\pm$ 0.3 ^{a,x} (5/5)	5.6 $\pm$ 0.5 ^{a,x} (5/5)	5.0 $\pm$ 0.6 ^{a,x} (5/5)
	Ducts	2.2 $\pm$ 0.3 ^{a,y} (5/5)	3.0 $\pm$ 0.2 ^{b,y} (5/5)	3.9 $\pm$ 0.5 ^{b,x} (5/5)
	Hyperplasias			3.9 $\pm$ 0.2 ^x (3/5)
> 16 weeks	Lobules	5.8 $\pm$ 0.4 ^{a,x} (13/13)	5.5 $\pm$ 0.4 ^{a,x} (19/19)	6.5 $\pm$ 0.4 ^{b,x} (12/12) [†]
	Ducts	3.0 $\pm$ 0.2 ^{a,y} (13/13) [†]	3.3 $\pm$ 0.3 ^{a,x} (19/19)	4.8 $\pm$ 0.5 ^{b,x} (12/12)
	Hyperplasias	4.6 ^{xy} (2/13)	3.4 $\pm$ 0.5 ^x (12/19)	5.0 $\pm$ 0.6 ^x (12/12)
	Tumors		3.8 $\pm$ 0.4 ^x (6/19)	13.1 $\pm$ 1.6 ^y (5/12)

Only epithelial units with at least one pyknotic nucleus were included in the calculations.

*Number of animals that exhibited pyknotic cells/all animals examined.

[†]Significantly different than the same histotype observed in the same mouse group <12 weeks. Comparisons between age groups but within genotype carried out using Student's *t*-test. Statistical significance at $P < 0.05$. See Statistical Analysis under Materials and Methods for details.

^{ab}Statistical differences between non-TG and each transgenic depicted by different letters within rows (Student's *t*-tests).

^{xy}Statistical differences between histotypes depicted by different letters within columns (ANOVA). Independent statistical analysis was performed for each age category. Comparisons between age groups but within genotype were carried out using Student's *t*-test.

of lobules with BrdU-labeled cells was reduced to 7% (Table 2). This reduction likely reflected the loss of terminal endbuds in FVB/N mice at ~ 12 weeks of age once ductal arborization of the mammary fat pad is complete. LI was similar in glands from both age groups (Table 3). According to our definition of hyperplasia, ie, lobules containing ≥ 5 ductules, mammary glands from 2 of 13 non-TG mice were found to contain a total of 17 hyperplasias. Half of the hyperplasias contained BrdU-labeled cells and those with BrdU-labeled cells had a LI of 1.6 (Tables 2 and 3, respectively). We were also interested in the effects of lobularization and aging on cell turnover dynamics. In non-TG animals, proliferative activity was not altered with degree of lobular development.

TGF- α Mice >16 Weeks Display Enhanced Proliferation

Differences in proliferation were not found in TGF- α mice compared with non-TG mice ≤ 12 weeks. However, in mice >16 weeks, the overall percentage of proliferation in lobules of glands from TGF- α mice was sevenfold higher than in non-TG glands (Table 2). Instead of a reduction in proliferation typically observed in non-TG mice >16 weeks com-

pared with mice ≤ 12 weeks, proliferation remained high in glands from TGF- α mice >16 weeks: lobular LI was approximately twofold higher than in age-matched non-TG mice and ductal LI was threefold higher (Table 3). In addition, in contrast to non-TG mice, which did not display differences in proliferative levels with lobular development, there was a significant increase in ductal proliferation in TGF- α glands with greater lobular development ($P = 0.024$, Figure 2A), suggesting a growth factor effect *in vivo*. Furthermore, correlations between proliferative activities and degree of lobular development were also identified in TGF- α glands ($r^2 = 0.31$, $P = 0.02$ and $r^2 = 0.38$, $P = 0.009$, for lobules and ducts, respectively). Hyperplasias in TGF- α glands were different from hyperplasias found in non-TG glands in that the majority of hyperplasias in TGF- α mice 1) displayed a twofold greater level of BrdU-labeled cells than in non-TG glands and 2) had significantly more cells (see details in the Lobules and Hyperplasias are Larger in Transgenic Mice and Develop Earlier section). All TGF- α tumors displayed BrdU-labeled cells (Table 2). There was an elevated LI for ductal epithelium in TGF- α versus non-TG glands. In addition, LI was similar in hyperplasias and tumors found in TGF- α glands. Finally, normal lobules residing in tumor-bearing mammary glands displayed significantly increased proliferative levels (70% with BrdU-labeled cells) compared

Figure 2. The degree of lobular development affected proliferation (A) and apoptosis (B). In non-TG glands, the extent of lobular development did not significantly alter proliferation (A), but higher levels of pyknosis were found in glands with more extensive lobular development (B, $P = 0.017$ for alveoli). Proliferation was also higher in ducts ($P = 0.008$) when lobular development was more extensive (data not shown). In TGF- α glands, both proliferative and apoptotic levels were elevated when glands displayed greater lobular development. In c-myc mice all glands displayed a more extensive lobular development, and there was not a correlation between lumen number and cell turnover activities.

Figure 3. Compared with tumor-free glands, the percentage of lobules and ducts containing BrdU-labeled cells increased significantly in tumor-bearing TGF- α mice ($P = 0.019$) and *c-myc* ($P = 0.032$), respectively. Note the relatively low percentage of BrdU-containing alveoli compared with ducts in TGF- α mammary glands whereas in *c-myc* mice ~80 to 90% of both epithelial histotypes contain BrdU-labeled cells. Data were analyzed using Student's *t*-tests comparing values for each mouse group and histotype in mammary glands with tumors compared with tumor-free glands.

with levels of normal lobules in tumor-free TGF- α glands (29%, Figure 3).

c-myc Mice of All Ages Have High Proliferative Activity

Unlike the relatively low percentage of BrdU-labeled cells found in non-TG mice, nearly 100% of lobules, ducts, hyperplasias, and tumors contained BrdU-labeled cells in *c-myc* mice (Table 2). Differences in proliferative levels were observed already in ≤ 12 -week-old *c-myc* mice in that alveolar LI was approximately threefold elevated compared with age-matched non-TG mice (16.2 versus 5.6%, respectively; Table 3). Increased proliferation in lobules compared with ducts also was found in *c-myc* mice > 16 weeks, suggesting *c-myc* effects were greatest in lobules. Unlike TGF- α glands, epithelial proliferation in *c-myc* glands displayed progressive increases in LI from ducts to tumors so that LI in ducts $<$ lobules $<$ hyperplasias $<$ tumors (6.6, 12.9, 18.1, and 24.9%, respectively; Table 3). In addition, the percentage of normal ducts containing BrdU-labeled cells was elevated significantly in *c-myc* glands with tumors (97%) compared with tumor-free glands (82%, Figure 3). Finally, not only did *c-myc* glands display, on average, larger lobules than non-TG and TGF- α glands (see the Lobules and Hyperplasias are Larger in Transgenic Mice and Develop Earlier section), but the extent of proliferation was near 90% regardless of lobular size (Table 2; Figure 2).

Proliferation of Normal Epithelium in Aging Mice

We also were interested in the effects of aging on proliferation. In non-TG mice, proliferation was decreased with aging in mice between 18 and 40 weeks, as manifested by a reduction in the percentage of BrdU-labeled cells (Figure 4). In TGF- α or *c-myc* mice > 16 weeks, there were no changes in proliferative levels with aging.

Figure 4. The effect of aging on epithelial proliferation in mice > 16 weeks (range, 132 to 286 days; 18 to 41 weeks). Linear regression analysis revealed a reduction in the percentage of ducts with BrdU-labeled cells with age in 13 non-TG mice ($r^2 = 0.31$, slope = -0.40). Proliferation was not altered with aging in age-matched transgenic mice.

Apoptotic Activity in Non-TG Mice

In non-TG mice ≤ 12 weeks, levels of pyknosis were one third to one half greater than in mice > 16 weeks (Tables 4 and 5). Although the extent of lobular development was not associated with altered proliferative activities in non-TG mice, higher levels of pyknotic lobular and ductal cells were observed in mice with mammary glands with more extensive lobular development ($P = 0.017$ for alveoli, Figure 2B; $P = 0.008$ for ducts, data not plotted).

Transgenic Mice Displayed Altered Apoptotic Activity in Both Age Groups

TGF- α mice ≤ 12 weeks displayed a reduced level of apoptosis compared with age-matched non-TG mice. This reduction was manifested in the percentage of alveoli and ducts with pyknotic cells: 14 versus 45% for lobules and 38 versus 82% in ducts for TGF- α versus non-TG glands, respectively (Table 4). This relationship shifted when non-TG mice reached > 16 weeks of age, as the apoptotic percentage dropped to half that of younger non-TG mice ≤ 12 weeks. In TGF- α mice > 16 weeks, apoptotic levels increased to levels 1.5- to 3-fold higher than age-matched non-TG mice (Table 4). Nearly all TGF- α hyperplasias and tumors contained pyknotic cells although AI levels were the same as those found in normal epithelia of both non-TG and TGF- α glands (Table 5).

Apoptotic Activity in Aging Mice

The percentage of lobules with pyknotic cells was reduced with age in non-TG mice between 18 to 40 weeks (Figure 5A). Glands from TGF- α mice of similar age mimicked the pattern displayed in non-TG glands, in that apoptotic levels also decreased with age (Figure 5B). However, tumors that developed in older TGF- α mice displayed a greater level of pyknosis than those found in younger mice (Figure 5B). However, apoptosis in *c-myc*

Figure 5. The effect of aging on apoptosis in mice >16 weeks (range, 132 to 286 days; 18 to 41 weeks). **A:** In 13 non-TG mice, the percentage of lobules with pyknotic cells decreased significantly with age ($r^2 = 0.28$, slope = -0.073). **B:** In 19 TGF- α mice, the percentage of ducts and lobules with pyknotic cells also was reduced with age ($r^2 = 0.31$, slope = -0.28 ; and $r^2 = 0.22$, slope = -0.30 , respectively). The percentage of tumors with pyknotic cells increased with the age of the animal ($r^2 = 0.76$, slope = 0.37 ; $n = 5$). There were no changes in apoptotic activities observed with age in *c-myc* mice.

mammary epithelia, unlike that of non-TG and TGF- α mice, was not altered with adult aging.

Lobules and Hyperplasias Are Larger in Transgenic Mice and Develop Earlier

We also wished to determine whether the average cell number for lobules and hyperplasias was different in non-TG and transgenic mice. A lobule consisted of a grouping of alveoli made up of secretory epithelial cells with the potential to produce milk products during lactation. An average lobule in non-TG mice consisted of a total of 23.1 ± 1.1 (mean $\pm$ SE) cells together comprising, on average, 1.2 alveolar lumens. A typical lobule in TGF- α mice was larger, consisting of 27.8 ± 2.1 cells arranged in 1.4 alveolar lumens, whereas lobules in *c-myc* mammary glands typically consisted of a total of 28.0 ± 1.9 cells arranged in 2.0 alveolar lumens. The average cell number/lobule was significantly higher in *c-myc* glands compared with non-TG glands ($P = 0.03$). Hyperplasia incidence, latency, size (number of lobules/hyperplasia), and cell turnover dynamics also differed significantly in non-TG compared with transgenic mice (Tables 2 through 5). First, the incidence of hyperplasias was lower in non-TG mice and latency longer. Only 2 of 18 non-TG mice displayed a total of 17 hyperplasias. Second, hyperplasias were found exclusively in mice >16 weeks and not in mice ≤ 12 weeks unlike *c-myc* mice. Third, hyperplasias were significantly smaller in size in non-TG glands, which contained 5 to 13 ductules (6.7 ± 0.5 , mean $\pm$ SE). In contrast, 9 of 20 TGF- α mice displayed hyperplasias and all were >16 weeks. Hyperplasias were more numerous ($n = 39$) and larger (range, 5 to 31 alveolar lumens; 11 ± 1 , mean $\pm$ SE; $P = 0.006$) than those found in non-TG mice. In *c-myc* mice, hyperplasias were identified in three of four mice <12 weeks and in all 13 *c-myc* mice >16 weeks of age and were significantly larger, containing between 5 to 74 ductules (15 ± 1.3 , mean $\pm$ SE; $P = 0.006$ compared with non-TG). Finally, the cell turnover characteristics

were markedly lower in hyperplasias found in non-TG versus transgenic glands. BrdU-labeled cells and pyknotic cells could be identified in nearly all hyperplasias in transgenic mice but were found in 43 and 75%, respectively, in non-TG mice (Tables 2 and 4). LI also was higher for hyperplasias in transgenic glands compared with non-TG glands (Table 3), whereas AI was elevated significantly only for hyperplasias in *c-myc* mice (Table 5).

LI and AI Are Correlated Only in Hyperplasias of c-myc Mice

Proliferative and apoptotic activities in lobules, ducts, and hyperplasias in adjacent sections in a subset of five non-TG, five TGF- α and five *c-myc* mice were evaluated for LI and AI. Only in hyperplasias in *c-myc* mice was a positive correlation found ($r = 0.57$).

Discussion

We have demonstrated that elevated proliferative and apoptotic levels are present before mammary lesion development in both WAP-TGF- α and WAP-*c-myc* transgenic mouse models compared with wild-type mice despite the distinctly different molecular mechanisms underlying tumorigenesis in these models. We also have demonstrated a pattern of cell turnover in mammary glands of wild-type mice that shifts with lobular development and with adult aging.

In this study, there was a significant reduction in cell turnover activity in glands of non-TG mice >16 weeks compared with non-TG mice ≤ 12 weeks. This shift coincided with completion of ductal arborization and the loss of terminal endbuds, which in non-TG and transgenic mice of this study was completed by 12 weeks of age. We identified high levels of proliferation and apoptosis in ductal regions distant from terminal endbuds (Tables 2 to 4), which may represent regions where lobules develop and recede coincident with the

Figure 6. Schematic representation of the overall patterns of proliferation (A) and apoptosis (B) in normal epithelium in mammary glands of adult non-TG, WAP-TGF- α , and WAP-c-myc mice with fully developed mammary glands between 18 and 41 weeks of age (132 to 286 days). Lobular and ductal data were derived from counting cells, which were BrdU labeled (A) or pyknotic (B). Overall lobular and ductal proliferation and apoptosis were higher in transgenic versus non-TG mice. In TGF- α glands, cell turnover pattern reflected the pattern of proliferation and apoptosis of non-TG glands; however, the overall levels were higher. Proliferative activities in c-myc glands were 3- to 10-fold greater than non-TG mice and similarly high in both lobules and ducts (A). Proliferation in c-myc gland was greater than in TGF- α glands (A). Patterns of apoptosis were similar in transgenic and non-TG mice in that ductal apoptotic levels were greater than lobular apoptotic levels.

estrous cycle. Similar to a previous report in C57BL/6 mice, we also found that apoptotic activity was enhanced in non-TG FVB/N mice with more extensive lobular development (Figure 5).¹¹ Finally, we believe this is the first report of a gradual decrease in both proliferative and apoptotic activity in mammary glands of aging wild-type mice (Figures 3 and 4). It is important to emphasize that this finding was observed in a population of healthy mature mice ranging from 18 to 40 weeks of age. Although we did not test fertility for each mouse in this study, in our colony, breeder females often reproduce well past 40 weeks of age. A reduction in cell proliferation may be an intrinsic cellular and tissue mechanism that protects against the expansion of cells that may be cancer prone. Then again, a decrease in apoptosis suggests that the mammary epithelium may be less capable of eliminating aberrant cells with age. Importantly, the fact that both components of cell turnover are altered with age may be important for understanding means in which one can prevent and treat cancer.

Although increases in cell turnover were a common feature of normal epithelia in both TGF- α and c-myc mammary glands, there were distinct differences in the pattern of cell turnover between these two transgenic models. First, in mice ≤ 12 weeks, lobular proliferation in TGF- α and non-TG glands was similar, whereas levels in c-myc glands were approximately twofold to threefold higher. Proliferation subsided in non-TG mice > 16 weeks, but remained high in transgenic mice. Second, in glands from TGF- α mice > 16 weeks, proliferative activity was greater in ducts than lobules, whereas glands of c-myc mice displayed equivalent levels of proliferation even though in both models, transgene expression was directed by the WAP promoter. Elevated lobular proliferation was correlated with development of lobular hyperplasias in glands from both transgenic mouse lineages (Tables 2 and 3, Figure 6).

Principal differences in epithelial cell turnover between non-TG and TGF- α mice were observed already in mice ≤ 12 weeks, manifested as a twofold to three-

fold reduction in apoptosis in normal epithelium of TGF- α glands. A reduction in epithelial apoptosis was seen previously in other models of TGF- α transgenic mice^{12,13} and in bitransgenic c-myc-TGF- α ¹⁴ and prolactin-TGF- α mice¹⁵ compared with the single transgenic c-myc- or prolactin-overexpressing mice. Reduced apoptosis coupled with augmented cell proliferation can promote an environment in which defective cells normally destined for programmed cell death remain alive. If these cells also have a growth advantage, as may be the case with cells expressing growth factors, the result can be hyperplasia and cancer development. However, apoptosis in normal mammary epithelia from TGF- α mice was suppressed only in mice ≤ 12 weeks and actually increased to levels higher than non-TG mice in animals > 16 weeks. In addition, unlike non-TG glands, epithelia in TGF- α glands displayed an increase in proliferation coinciding with increased stage of lobularization, suggesting a growth effect not seen in non-TG glands (Figure 2). The fact that nearly all TGF- α mice eventually develop hyperplasias and tumors indicates that enhanced cellular proliferation overwhelms the enhanced apoptotic responses, resulting in a net gain of cells.

It is uncertain in transgenic mice whether lobular hyperplasias are the precursor lesion of end-stage tumors. In TGF- α mice, heterogeneous end-stage lesions include features reminiscent of fibrocystic changes in women,¹⁶ such as adenomas, fibroadenomas, and adenocarcinomas, making it difficult to ascribe a simple tumor precursor histotype. However, transplanted TGF- α hyperplasias recapitulate many end-stage lesion and tumor histotypes (T.A.R.-H. and E.P.S., unpublished data), suggesting that cells from hyperplastic lobules have the potential to be tumor precursors in this model. In c-myc mice, intermediate lesions other than lobular hyperplasias with various degrees of dysplasia (Figure 1M) were not found. In non-TG mice, hyperplasias were intrinsically different from transgenic hyperplasias using several criteria, and they likely represent a variant of normal lobular development. First, incidence was low (2 of 13 animals

>16 weeks of age). Second, non-TG hyperplasias contained approximately fivefold fewer alveolar lumens than those found in transgenic mice (Figure 1, B, H, and M). Third, proliferative and apoptotic levels in hyperplasias from non-TG mice were significantly lower than in transgenic mice (Tables 2 to 5). These data indicate that lobular hyperplasias display variable cell turnover characteristics and those that precede or accompany tumor development are distinguishable from large lobules/small hyperplasias that appear to have no potential for cancer development. Hyperplasias that are larger and highly proliferative may be a cellular feature (biomarker) worthy of future evaluation in preclinical testing of preventive regimens.

TGF- α and *c-myc* are molecules that frequently are activated in breast cancer in women. TGF- α is overexpressed in 30 to 70% of human breast tumors examined^{17,18} and, depending on the study, *c-myc* amplification, expression, or rearrangement occurs in the majority of breast cancers.¹⁹ In this study, *c-myc* mice displayed a uniformly high epithelial cell turnover (Tables 2 to 4) before and coincident with lesion development. High levels of cell turnover were maintained regardless of epithelial type, size of lobules, degree of lobularization, and age, indicating that in *c-myc*-expressing cells the signal was dominant, overriding normal cell growth and death signals. Finally, a stepwise increase in cell turnover from normal epithelia to hyperplasias to tumors suggests a progressive increase in this measure during tumor progression. The overall high level of cell turnover in *c-myc* in normal and abnormal epithelia may be explained by the autocrine nature of the downstream effects of this transcription factor. The impact of TGF- α is more diverse, despite the fact that both transgenes were directed by the whey acidic protein (WAP) promoter.²⁰ This diversity may be explained by the autocrine, paracrine, and endocrine nature of TGF- α signaling. Numerous factors impact downstream TGF- α signaling,^{21–23} which are accompanied by more highly variable proliferative and apoptotic responses compared with *c-myc* expressing cells (Tables 2 to 5). It also may explain the diverse set of end-stage TGF- α lesions.

We have found that enhanced cell turnover in lobules and ducts precede morphological evidence of tumorigenesis in two distinct transgenic mouse models of breast cancer. The identification and validation of early markers that denote an increased risk of breast cancer development and that can be used to predict the success or failure of a preventive therapeutic regimen is a major goal of breast cancer prevention research. The degree of cell turnover in lesions is already a biomarker used in human breast cancer. In addition, alterations in proliferation have been measured from core needle biopsies of breast tissue taken before and after cancer treatment.²⁴ Our data from transgenic mouse models indicate that cell turnover activities in normal-appearing mammary epithelium before the onset of lesions should be evaluated as a potential biomarker of breast cancer risk in women and of the

efficacy of cancer prevention regimens in preclinical animal models.

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