Effect of c-neu/ ErbB2 Expression Levels on Estrogen Receptor α–Dependent Proliferation in Mammary Epithelial Cells: Implications for Breast Cancer Biology

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Abstract

Mammary development and tumorigenesis are profoundly influenced by signaling pathways under the control of c-erbB2/c-neu and estrogen receptor α (ERα). Signaling through ERα is essential for ductal growth during puberty. In mice overexpressing wild-type c-neu in mammary epithelial cells, Tg (c-neu), ductal growth is impaired. An impeded signaling through ERα is also observed in a subset of human mammary tumors that overexpress erbB2. However, ductal growth is also impaired in the absence of c-neu in mouse mammary epithelial cells. To resolve this apparent paradox, we examined the relationship between c-neu expression and estrogen/ERα–dependent cell proliferation in pubertal Tg (c-neu). We report that proliferation in both terminal end buds and ducts is associated with ERα-positive cells, including those that coexpress c-neu, and is abolished in the absence of circulating estradiol. Tg (c-neu) contains hyperplastic mammary ducts with high proliferative index and coexpression of both ERα and c-neu in the dividing cells. These findings suggest that c-neu promotes ERα-dependent proliferation, and that this is responsible for the presence of hyperplastic ducts. Some of the hyperplastic ducts have acinar structures, indicative of morphologic differentiation. These ducts have low proliferative index and accompanied by a vast decrease in proliferation of ERα-positive cells, including those that express c-neu. As such, c-neu has dual but opposing effects on ERα-dependent proliferation in mammary epithelial cells. Therefore, depending on the physiologic setting, ductal morphogenesis will be compromised both in the absence and overexpression of c-neu, thus explaining the paradox. (Cancer Res 2006; 66(21): 10391-8)

Introduction

The female sex steroid hormone estradiol plays an important role in the growth and differentiation of normal breast and also in the induction and/or growth of mammary carcinomas. The initial phase of mammary development accompanies puberty and results in the proliferation of epithelial cells to form a tree-like pattern of ducts commonly called ductal growth. Estradiol signaling through estrogen receptor α (ERα) is essential for ductal growth accompanying puberty (1, 2). In ovariectomized mice depleted of endogenous estrogen, implantation of pellets containing epidermal growth factor (EGF) or transforming growth factor-α (TGF-α) into regressed mammary glands can initiate ductal growth (3). It is, therefore, generally presumed that whereas estrogen is critical for triggering mammary growth, its effects are mediated by growth factors, in particular, EGF and TGF-α.

EGF and TGF-α mediate their effects through either erbB1/EGF receptor homodimers or heterodimers of erbB1 and erbB2/c-neu; erbB2 is the preferred dimerization partner for erbB1 (4, 5). ErbB2 plays a central role in proliferation and differentiation of normal mammary epithelial cells (6, 7). The absence of erbB2 results in impaired ductal growth accompanying puberty in mouse mammary glands (8, 9). However, as shown previously by our laboratory (10), ductal growth during puberty is also impeded in Tg (c-neu), transgenic mice FVB/N-Tg (MMTV-LTR-c-Neu) overexpressing inactivated erbB2 in mammary epithelial cells (11). Therefore, whereas erbB2 is essential for mediating estradiol/ERα–dependent ductal growth, overexpression of erbB2 may be incompatible with signaling through estradiol/ERα. Thus, signaling through erbB2 represents a paradox, both supporting and impeding ductal growth.

A subset of human mammary tumors that overexpress erbB2 respond poorly to treatment with antiestrogens, indicating an impeded signaling through ERα (12–16). Collectively, these observations underscore the complexities underlying the interplay between signaling through erbB2/c-neu and estradiol/ERα during mammary development and carcinogenesis. In addition, they raise the possibility that the reported incompatibility between c-neu overexpression and signaling through estradiol/ERα may be an intrinsic phenomenon and unrelated to carcinogenesis. Furthermore, immunolocalization studies have shown that in normal mammary epithelial cells, the expression of both ERα and c-neu is heterogeneous (17, 18). Together, these observations raise the possibility that the individual and combined effects of ERα and c-neu may depend on the mammary epithelial subtype and the interactions among these receptors. As yet, detailed studies using dual and triple staining have not been done. Accordingly, in our present studies using Tg (c-neu) and dual and triple immunostaining, we have examined how c-neu overexpression affects estrogen/ERα–dependent cell proliferation.

Materials and Methods

Description of mice, treatment, and tissue preparation. Tg (c-neu) mice used in these studies express the wild-type c-neu. These mice were generated by our laboratory from the parental line (11) provided by Drs. Michael Campbell and Laura Esserman. Wild-type mice (FVB/N) were from our own colony. Unless specified otherwise, all mice were 6 weeks old (pubertal mice) at the time of killing and were used either as is or after ovariectomy for 10 to 14 days. For cell proliferation studies, 5-bromo-2-deoxyuridine (BrdUrd; Sigma, St. Louis, MO) was given (160 μg/g body weight) for 2 hours before sacrifice. The mice were housed and cared for in accordance with the NIH guide to humane use of animals in research.

For histologic and immunolocalization studies, mammary tissues were collected, fixed in 4.7% formalin (same as 10% buffered formalin from Fisher Scientific, Pittsburgh, PA), dehydrated, embedded in paraffin, cut into 4- to 5-μm-thick sections, and stained with H&E.

Primary antibodies. Anti-ER-α, rabbit monoclonal antibody (SP-1, Lab Vision Corp., Fremont, CA); anti-BrdUrd, rat monoclonal antibody (Oxford Biotechnology Ltd., Oxfordshire, United Kingdom); anti-c-erbB-2/HER-2/*neu*, mouse monoclonal antibody 3B5 (Lab Vision).

Immunohistochemistry and double and triple immunofluorescence analyses. For BrdUrd immunohistochemistry, a standard procedure, as described previously (19), was used. The antigen-antibody complexes were detected using Vectastain Avidin-Biotin Complex kits (Vector Laboratories, Burlingame, CA) For immunofluorescence analyses, staining was done on paraffin sections, prepared identically for immunohistochemistry. Slides were deparaffinized in Histo-Clear (National Diagnostics, Atlanta, GA) and rehydrated in a series of graded alcohol concentrations. Antigen retrieval was done by microwaving the slides in antigen unmasking solution (Vector Laboratories) for 21 minutes. Tissue sections were incubated for 1 hour at room temperature with Super Block Blocking Buffer (Pierce, Rockford, IL).

Incubations with all primary antibodies were done overnight at 4°C, whereas incubations with secondary antibodies (Alexa 488 anti-rabbit or Alexa 568 anti-rat, Molecular Probes, Eugene, OR) were done at for 1 hour at room temperature. Incubation with each primary antibody was followed by treatment with its corresponding secondary antibody. Blocking buffer was applied before incubation with the next primary antibody. The slides were counterstained with 4′,6-diamidino-2-phenylindole (Sigma-Aldrich, St. Louis, MO) and mounted with Vectashield (Vector Laboratories).

Image acquisition and processing. Immunofluorescence images were obtained using a ×40, 0.75 numerical aperture Zeiss Neofluor objective on a Zeiss Axiovert equipped with epifluorescence. A multiband-pass dichoric mirror, barrier filter, and differential wavelength filter wheel combination was used to selectively excite fluorochromes in sequence. Images were captured using a scientific-grade 12-bit charged coupled device (KAF-1400, 1317 × 1035, 6.8 μm^2 pixels) on Xillix digital camera (Vancouver, Canada). Relative intensity of images were maintained when constructing figures by using Scilimage (TNO Institute of Applied Physics, Delt, The Netherlands) to scale the 12-bit data to a common 8-bit scale using the data set minimum and maximum. False color was assigned accordingly. Internal
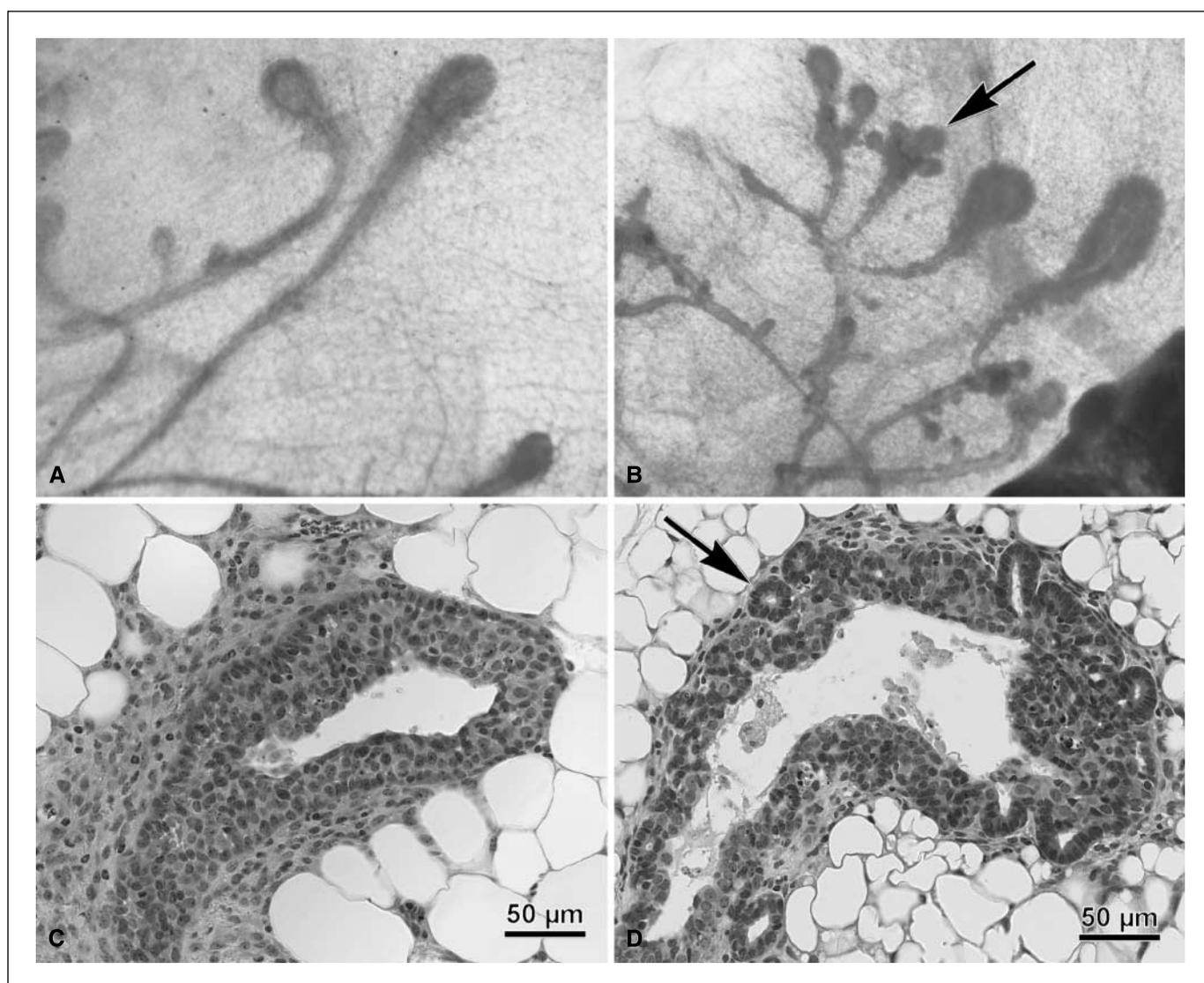

Figure 1. Structure of terminal end buds in mammary glands of pubertal wild-type and Tg (c-neu) mice. *A* and *C*, whole mount (magnification, ×50) and H&E-stained section (magnification, ×400) of normal TEB of wild-type mouse, respectively. Note that the growing tip of each duct contains a single TEB. *B* and *D*, whole mount and H&E-stained section of abnormal TEB of Tg (c-neu), respectively. Note the presence of multiple TEBs at the tips of some ducts and the large size of some TEBs. The presence of acini in the abnormal TEB (*D*) is indicated by the arrows.

Table 1. Analyses for cell proliferation in mammary glands of pubertal wild-type and Tg (c-neu)

Genotype	Structures	Endocrine status	BrdUrd-positive cells (% total cells)		
			I	II	III
Wild type	Normal TEB	Intact	NF	5.4 ± 2.31	23.9 ± 3.03
		Ovx	NF	NF	NF
	Normal ducts	Intact	0.2 ± 0.15	2.8 ± 0.59	NF
		Ovx	0.1 ± 0.06	1.7 ± 0.31	NF
c-neu	Normal TEB	Intact	NF	NF	NF
	Abnormal TEB	Intact	NF	7.3±1.19*	21.3 ± 2.32
		Ovx	NF	1.4 ± 0.35	NF
	Normal Ducts	Intact	0.3 ± 0.16	2.7 ± 0.47	NF
		Ovx	0.04 ± 0.03	2.2 ± 0.46	NF
	Hyperplastic Ducts	Intact	0.2 ± 0.10	3.5 ± 0.42	30 ± 4.02
		Ovx	0.1 ± 0.04	4.1 ± 0.61	NF

NOTE: Mammary glands from intact and ovariectomized wild-type mice and Tg (c-neu) were analyzed for BrdUrd immunoreactivity as described in text. Based on the number of BrdUrd-positive cells, each structure was roughly classified into three groups: BrdUrd labeling index <1 (group I), 1 to 10 (group II), and >10 (group III).

Abbreviation: NF, not found.

*Abnormal TEB of ovariectomized Tg (c-neu) is significantly lower compared with its counterpart in intact mice ($P < 0.05$).

standardization was achieved by comparing only images stained with the same antibodies in the same experiment, captured with identical variables, and scaled and displayed identically. Image analyses were achieved using the R3D2 software (20).

Quantitation. The percentage of immunopositive cells in different structures/genotypes was obtained by counting a minimum of 500 cells per gland. For each experimental group, a minimum of three mice was analyzed, and mammary glands for each mouse were analyzed in triplicate. The data are presented as percentages ± SE. The differences between the various experimental groups were analyzed by Student's *t* test and were considered significant when $P < 0.05$ was obtained.

Results

Mammary glands of pubertal Tg (c-neu) contain terminal end buds with altered structures and hyperplastic ducts with high BrdUrd labeling index. In postnatal mice, the initial phase of mammary epithelial growth is triggered at puberty (21) and requires signaling through estradiol/ERα (1, 2). Therefore, to determine the effect of c-neu overexpression on estrogen/ERα–dependent proliferation, we examined the mammary glands of pubertal Tg (c-neu). In pubertal mice, the sites of highest mitotic rates reside in unique structures called terminal end buds (TEB) that initiate ductal elongation (21). The number of TEBs in mammary glands of pubertal Tg (c-neu) was equivalent to those in wild-type mice but consisted of two types; some had structures resembling the wild-type TEBs, whereas the majority (~80%) were abnormal. The abnormal TEBs had larger diameters with asymmetrical outlines and denser cores and contained acinar structures (Fig. 1, compare *C* and *D*). The terminal ends were also abnormal with multiple branch points and side buds that emanated from either the neck or the tip (Fig. 1, compare *A* and *B*).

An examination of the proliferative status of abnormal TEBs in Tg (c-neu), using BrdUrd labeling as an index, revealed a high percentage of BrdUrd-positive cells, comparable with normal TEBs of wild-type mice (Table 1). Upon ovariectomy (hence, depletion of estrogens), mammary glands of wild-type mice lacked TEBs and abnormal TEBs were reduced in Tg (c-neu) to ~25% of that found in intact mice; these TEBs had a very low BrdUrd labeling index (Table 1).

Mammary ducts are derived from proliferating cells in TEBs (21); this is accomplished as TEBs move forward by invading the fat pad and mature ducts appear behind the TEBs. The mature ducts in wild-type mice are known to be relatively quiescent, with low BrdUrd labeling index (Table 1). Mammary glands of pubertal Tg (c-neu) contained both normal and hyperplastic ducts; ~50% of all ducts were hyperplastic. Similar to wild-type mice, normal ducts of Tg (c-neu) had relatively few BrdUrd-positive cells (Table 1). The number of BrdUrd-positive cells varied considerably among hyperplastic ducts (Table 1) and could be roughly classified into three groups: BrdUrd labeling index <1 (group I), 1 to 10 (group II), and >10 (group III). Upon ovariectomy, group III hyperplastic ducts, with high BrdUrd labeling index, were virtually eliminated; in intact mice, ~26% of all ducts had BrdUrd labeling index >10, whereas in ovariectomized mice, it was <4%. Thus, BrdUrd labeling indices in hyperplastic ducts of ovariectomized Tg (c-neu) were mostly in the range of 1 to 5 (Table 1). Ovariectomy also reduced the number of BrdUrd-positive cells in normal ducts of both Tg (c-neu) and wild-type mice (Table 1).

BrdUrd- and ERα-positive cells colocalize in TEBs and ducts of wild-type mice and Tg (c-neu). The foregoing studies established that cell proliferation in both TEBs and ducts of Tg (c-neu) was dependent on ovarian steroids. Next, to examine whether this proliferation was associated with ERα-positive cells, we did dual immunolocalization studies. These studies revealed colocalization of immunoreactive BrdUrd and ERα both in abnormal TEBs of Tg (c-neu; Fig. 2A, a) and in normal TEBs of pubertal wild-type mice (Fig. 2A, b). BrdUrd-positive cells in normal ducts of both Tg (c-neu) and wild-type mice were also positive for ERα (Fig. 2B, a and b). Similarly in hyperplastic ducts of Tg (c-neu), with both low and high BrdUrd labeling index, BrdUrd-positive cells expressed ERα (Fig. 2B, c and d).

Dual immunolocalization studies also revealed that the percentages of ERα-positive cells were not significantly different between
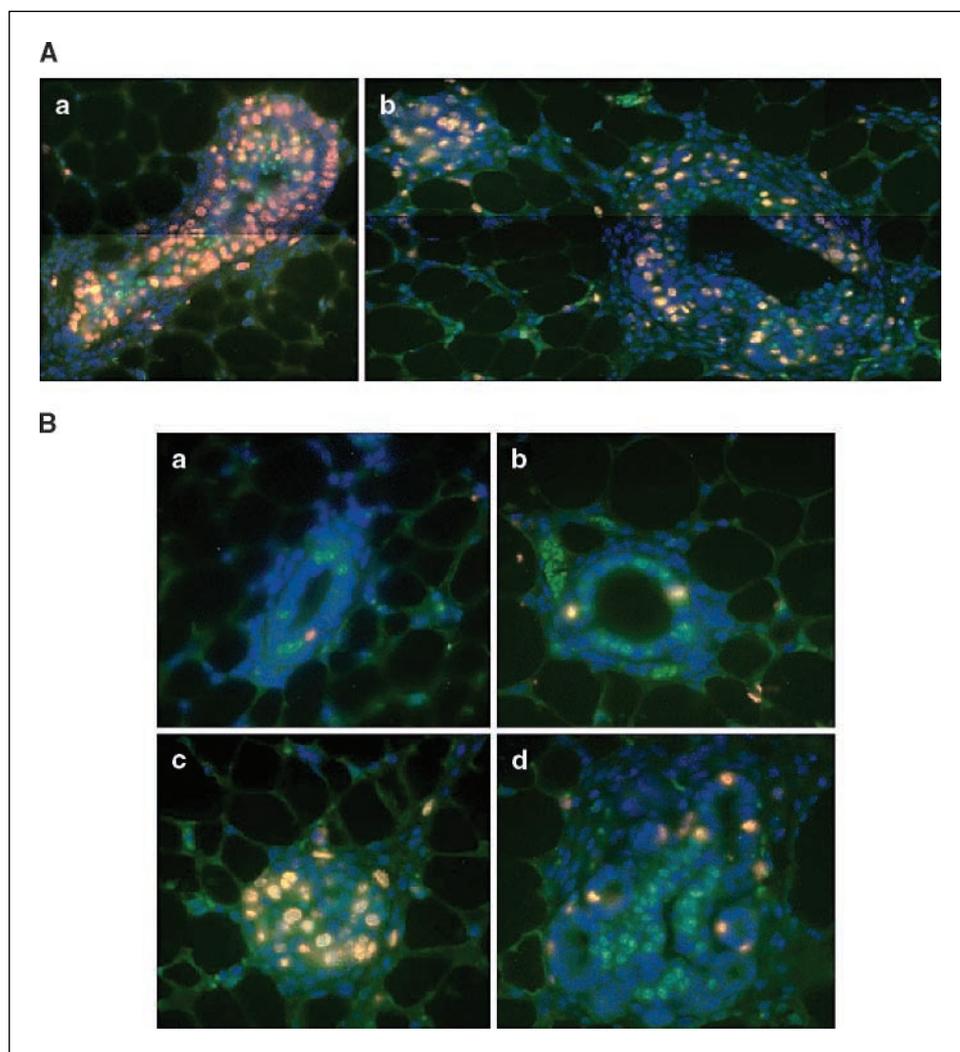
Figure 2. Analyses for ERα and proliferating ERα-positive cells in pubertal wild-type mice and Tg (c-neu). Cells positive for ERα and/or BrdUrd were identified using dual immunolocalization, as described in text. Digital images show cells positive for 4′,6-diamidino-2-phenylindole (*blue*), positive for BrdUrd (*red*), positive for ERα (*green*), and positive for both ERα and BrdUrd (*yellow/orange*). *A, a* and *b,* normal TEB of wild-type mouse and abnormal TEB of Tg (c-neu), respectively. Magnification, ×400. *B, a* and *b,* normal ducts of wild-type mouse and Tg (c-neu), respectively; *c* and *d,* hyperplastic ducts of Tg (c-neu).

(*a*) TEBs of Tg (c-neu) and wild-type mice, (*b*) normal ducts of Tg (c-neu) and wild type mice, and (*c*) hyperplastic ducts with varying rates of proliferation (Fig. 3*A*). Thus, differences in the BrdUrd labeling index among the various structures in both the genotypes did not seem to be due to differences in the percentages of total ERα-positive cells. On the other hand, in all morphologic structures, the BrdUrd labeling index was related to the percentages of proliferating ERα-positive cells (i.e., cells positive for both BrdUrd and ERα; Fig. 3*B*).

Expression patterns of c-neu and its relationships to cells positive for ERα and/or BrdUrd in TEBs and ducts of wild-type mice and Tg (c-neu). Next, using dual immunolocalization, we examined the relationships between c-neu and/or ERα expression and cell proliferation. In normal TEB of wild-type mouse, c-neu expression was below detectable levels, whereas its expression was higher in abnormal TEBs of Tg (c-neu; Fig. 4*A*, compare *a* and *b*). Similar to TEBs, c-neu expression was below the level of detection in ducts of wild-type mice, whereas its expression was robust in both normal and hyperplastic ducts of Tg (c-neu; Fig. 4*B*, compare *b-d* with *a*). In addition, as found with adult mice (17), expression of c-neu was heterogeneous among the various structures of pubertal Tg (c-neu). Dual immunolocalization studies also revealed that both the TEBs and ducts of Tg (c-neu) contained cells positive for

both c-neu and BrdUrd (Fig. 4*A, b*; Fig. 4*B, b-d*; Fig. 5). As shown in Fig. 5, there was a positive relationship between BrdUrd labeling index and proliferating c-neu−positive cells; as such, the percentages of cells positive for both c-neu and BrdUrd were higher in ducts with higher proliferation (group II and III ducts), compared with either group I or normal ducts. On the other hand, there was an inverse relationship between the percentage of c-neu-positive cells and overall BrdUrd labeling index (Fig. 5).

Both normal and hyperplastic ducts of Tg (c-neu) contained dual staining c-neu/ERα–positive cells (Fig. 4*C, b-d*). However, in all morphologic structures, the majority (∼75%) of c-neu-positive cells were ERα negative.

Proliferating cells in mammary ducts of pubertal c-neu mice consist of distinct subpopulations. The mammary ducts of pubertal Tg (c-neu) have both ERα/BrdUrd– and c-neu/BrdUrd– positive cells, suggesting that both c-neu- and ERα-positive cells undergo proliferation. However, due to the heterogeneous distribution of ERα-, c-neu-, and BrdUrd-positive cells, the direct contribution of c-neu on ERα-dependent proliferation at the level of individual cells was not clear. Therefore, triple immunolocalization techniques were used to simultaneously detect cells positive for immunoreactive c-neu, ERα, and BrdUrd. Group I ducts, with BrdUrd labeling index < 1, were inconsistent because

very few BrdUrd-positive cells were present against a large background of ERα- and c-neu-positive cells, and hence, the data are not shown. The distribution of cells positive for c-neu, ERα, and c-neu/ERα in group II ducts (BrdUrd labeling index, 1-10) and group III (BrdUrd labeling index >10) is shown in Fig. 6A. As may be seen, the percentages of ERα-positive cells (with and without coexpression of c-neu) in hyperplastic ducts were in good agreement with results from dual immunolocalization experiments (compare Fig. 3A with Fig. 6A). Similarly, the percentages of c-neu-positive cells (with or without coexpression of ERα) was in the range seen in dual immunolocalization studies (compare Fig. 5 with Fig. 6A).

Triple immunostaining experiments also confirmed that both ERα- and c-neu-positive cells undergo proliferation. Most importantly, these experiments revealed the presence of triple-labeled BrdUrd/c-neu/ERα–positive cells (Fig. 6B). Figure 6B also shows that the percentage of triple-labeled cells is higher than double-labeled c-neu/BrdUrd–positive cells. Triple immunostaining also revealed a small population of BrdUrd-positive cells that were negative for both ERα and c-neu. Thus, four populations of proliferating cells were identified in ducts of pubertal Tg (c-neu) mice: ERα/c-neu/BrdUrd, ERα/BrdUrd, c-neu/BrdUrd and BrdUrd only.

Discussion

The present studies were undertaken to identify the reasons for the paradoxical effects of c-neu on ductal morphogenesis in pubertal mice (i.e., the impairment of ductal morphogenesis both in its absence and overexpression in mammary epithelial cells). At present, the general consensus is that signaling through ERα is essential for mammary epithelial cell proliferation. However, some controversy exists regarding the ability of ERα-positive cells to proliferate (22, 23). Accordingly, to determine the effect of c-neu overexpression on estrogen/ERα–dependent proliferation during puberty, the proliferative status of ERα-positive cells in mammary glands of pubertal Tg (c-neu) was examined using BrdUrd labeling. The ERα-positive cells labeled for BrdUrd, in TEBs (Fig. 2A and Fig. 3B) and ducts (Fig. 2B and Fig. 3B) of both wild-type mice and Tg (c-neu). Proliferation in both TEBs and ducts of Tg (c-neu) and wild-type mice was mostly abolished after ovariectomy (Table 1). Together, these observations show that proliferation in TEBs and ducts of pubertal mice is ovarian dependent and associated with ERα-positive cells. In all cases, the number of proliferating ERα-positive cells correlated with the BrdUrd labeling index (Fig. 3B) and not with the number of total ERα-positive cells. This evidence suggests that when the rate of proliferation in mammary glands is low, the number of proliferating ERα-positive cells will also be low and, hence, may escape detection. Therefore, the inability to detect proliferating ERα-positive cells in mammary glands does not necessarily indicate that ERα-positive cells cannot undergo proliferation, as suggested previously (22, 24).

In addition to ERα-positive proliferating cells, hyperplastic ducts of pubertal Tg (c-neu) also contained ERα-negative proliferating cells (Fig. 6B). However, the ERα-negative population was significantly smaller compared with ERα-positive proliferating cells. It is well established that the turnover rate of ERα is related to its biological activity (25, 26). Therefore, we speculate that ERα-negative proliferating cells represent the population in which ERα had been degraded as a result of their responsiveness to ERα-mediated signaling. A similar scenario has also been proposed to explain the presence of proliferating ERα-negative cells in mammary glands of ovariectomized adult mice treated with estradiol (23).

The hallmark of Tg (c-neu) is overexpression of erbB2 in mammary epithelial cells. As such, if c-neu expression inhibited ERα-dependent proliferation, c-neu positive cells would be expected to be segregated from ERα/BrdUrd–positive cells The hyperplastic mammary ducts of pubertal Tg (c-neu) have BrdUrd-labeled cells that express both ERα and c-neu. Furthermore, in these ducts, the percentage of triple labeled cells (ERα[+]/c-neu[+]/BrdUrd+) were twice that of dual-labeled c-neu/BrdUrd positive cells (Fig. 6B). These observations indicate that c-neu expression is compatible with ERα-dependent proliferation and suggest that c-neu and ERα may act in synergy to promote cell proliferation in mammary ducts. Normal ducts in pubertal Tg (c-neu) also over express c-neu (Fig. 4B, b) and have high percentages of ERα-positive cells (Fig. 3A). However, these ducts have low BrdUrd labeling index (Table 1). This suggests that c-neu promotion of ERα-dependent proliferation is not simply dictated by the magnitude of c-neu and ERα expression but tied to the ability of cells to proliferate in response to estradiol/ERα. Therefore, we propose that c-neu plays a permissive role in ERα-dependent proliferation. A permissive role for c-neu is also supported by the observations that the impairment in ductal elongation is only delayed but not permanent in the absence of c-neu (9) but is permanent in the absence of ERα (1).
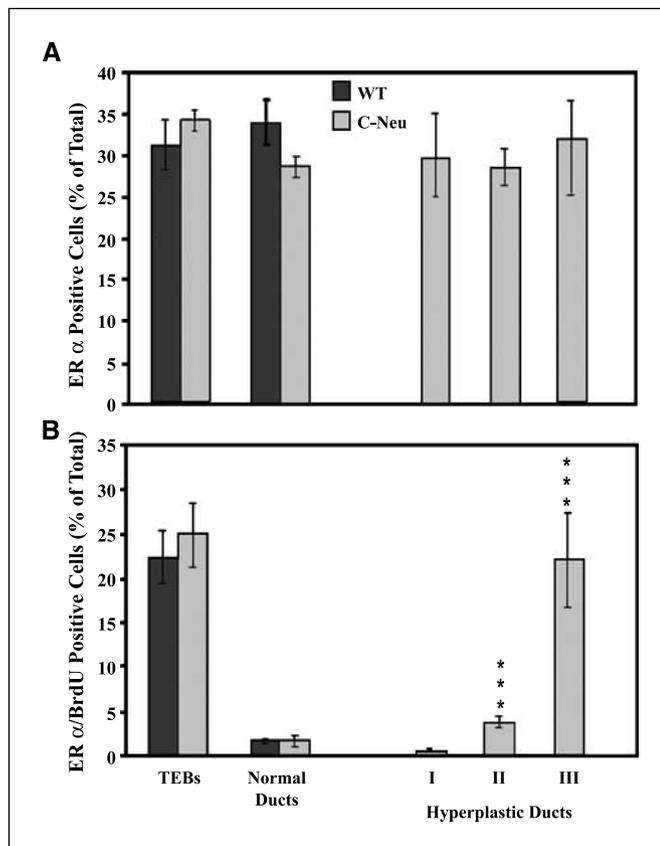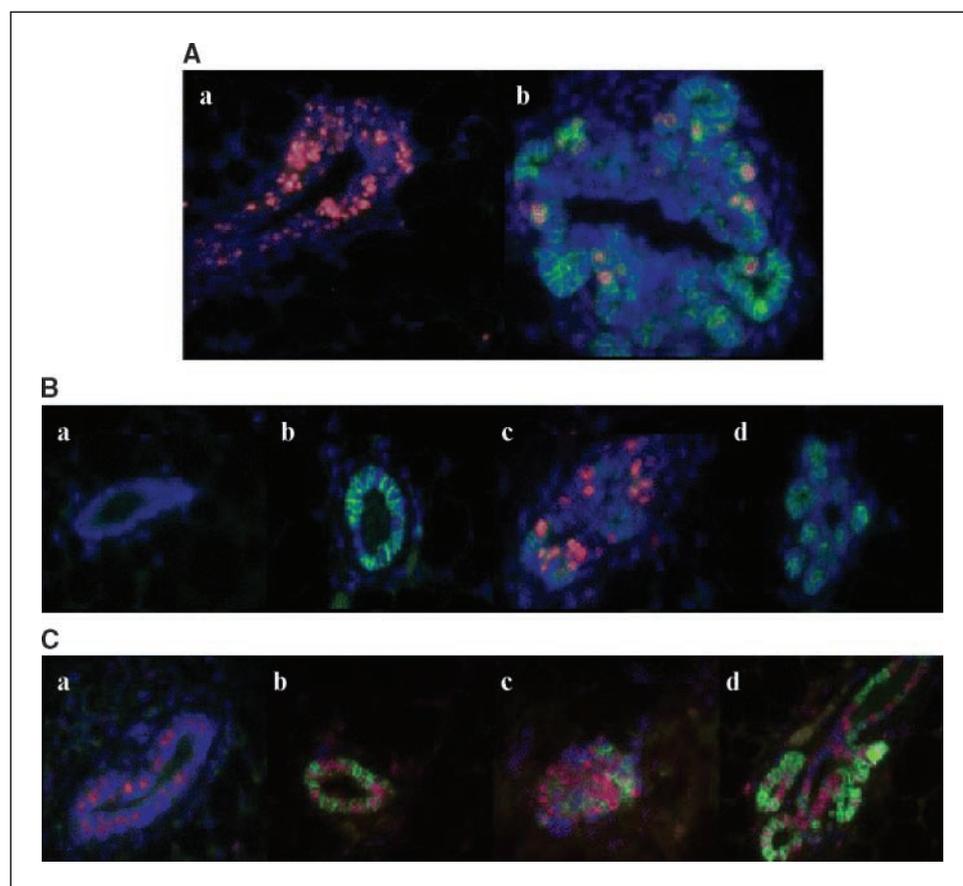

Figure 3. Prevalence of ERα- and/or BrdUrd-positive cells in different morphologic structures of pubertal wild-type mice and Tg (c-neu). The data were derived from dual immunolocalization experiments as described in Fig. 2. Hyperplastic ducts: I, II, and III correspond to ducts with BrdUrd labeling index <1, 1 to 10, and >10 respectively. *A*, ERα-positive cells. *B*, cells positive for both ERα and BrdUrd. ***, ERα/BrdUrd–positive cells are significantly higher in hyperplastic ducts II and III compared with either ducts I or normal ducts (*P* < 0.001).

Figure 4. Analyses for c-neu expression in proliferating and ERα-positive cells in mammary glands of pubertal wild type and Tg (c-neu). *A* and *B,* digital images of cells positive for c-neu and/or BrdUrd. *A, green,* c-neu; *red,* BrdUrd; *blue,* 4′,6-diamidino-2-phenylindole (nuclei). *B, a* and *b,* TEB from wild-type mouse and Tg (c-neu), respectively. *C, a* and *b,* normal ducts of wild-type mouse and Tg (c-neu), respectively; *c* and *d,* hyperplastic ducts of Tg (c-neu). Digital images show cells positive for c-neu and ERα: *green,* c-neu; *red,* ERα; *blue,* 4′,6-diamidino-2-phenylindole.

A distinguishing feature of the mammary phenotype of pubertal Tg (c-neu) is the presence of numerous short side buds. The side buds do not extend but exhibit acinar differentiation. In mammary glands of pubertal Tg (c-neu), ∼ 50% of all ducts were hyperplastic, and more than half of the hyperplastic ducts were associated with acinar structures. In wild-type mice acinar development, indicative of morphologic differentiation, usually occurs in adult females especially during pregnancy a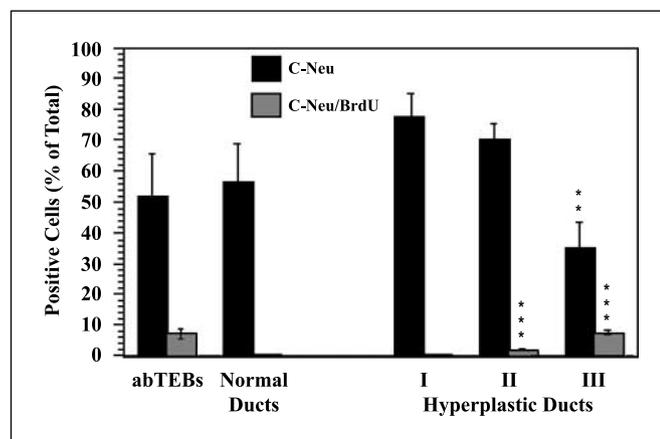nd bears an inverse relationship to estrogenic sensitivity and cell proliferation (2). Indeed, in Tg (c-neu), hyperplastic ducts with more acini had fewer BrdUrd-positive cells (Fig. 2*B*, compare *c* and *d*; Fig. 4*B*, compare *c* and *d*). The number of proliferating c-neu/ERα–positive cells (with or without coexpression of c-neu) was also reduced in group II ducts, with lower BrdUrd labeling indices (Fig. 6*B*). An inverse relationship between c-neu-positive cells and overall BrdUrd labeling index was also noted (Fig. 5). Therefore, we propose that in pubertal Tg (c-neu), c-neu has dual but opposing effects; that is, it promotes ERα-dependent proliferation (accounting for the presence of hyperplastic ducts) and also causes premature acinar differentiation with a concomitant decrease in ERα-dependent proliferation. If this were so, ductal morphogenesis, which is facilitated by ERα-dependent proliferation, will be compromised both in the absence of c-neu (8, 9) and when c-neu is overexpressed (10).

At present, we can only speculate on the mechanisms linking overexpression of c-neu and premature acinar differentiation and arrest in ERα-dependent proliferation. As shown in Fig. 4*B* and *C*, in ducts with acinar structures, c-neu-positive cells were more prevalent in the acini. Increased expression of c-erbB2 (by transfection) in MCF-7 human mammary tumor cells inhibits estrogen-dependent cell growth and induces a differentiated phenotype (27). Overexpression of oncogenic erbB2 in human mammary tumor cells results in growth arrest due to premature senescence (28). In fact, there is an emerging consensus that overexpression of growth modulators, including oncogenes, can lead to premature growth arrest as a protection against tumor development (28–32). Therefore, it is possible that premature differentiation/growth arrest in mammary ducts of pubertal Tg (c-neu) is a response to protect
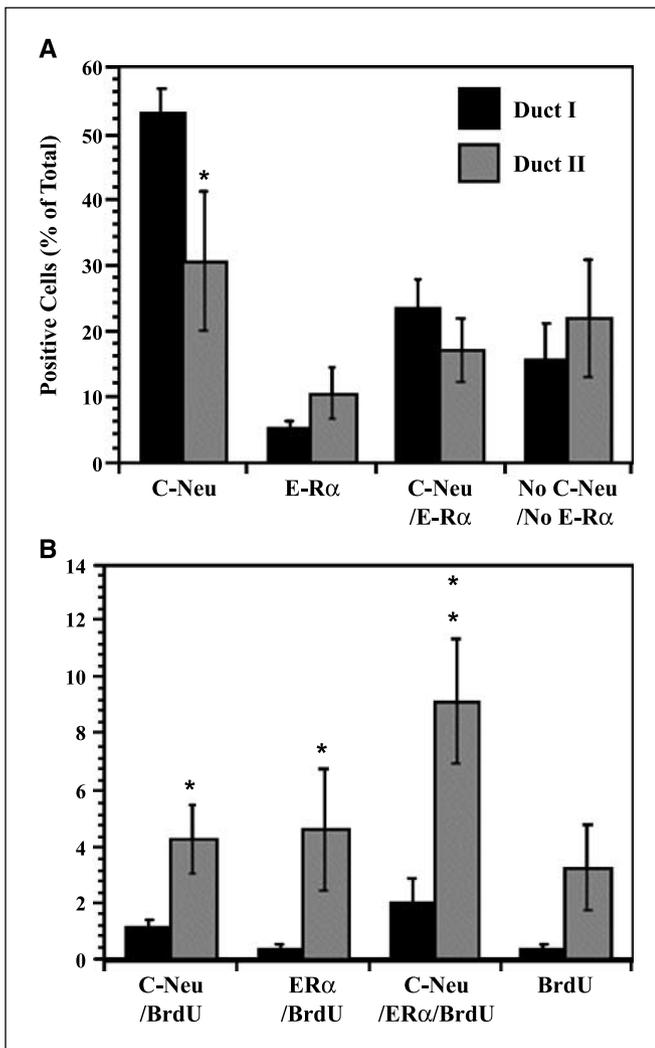

Figure 5. Prevalence of c-neu- and/or BrdUrd-positive cells in TEBs and ducts of pubertal Tg (c-neu). The data were derived from dual immunolocalization experiments as described in Fig. 4. Hyperplastic ducts: I, II, and III correspond to ducts with BrdUrd labeling index <1, 1 to 10, and >10, respectively. ***, c-neu/BrdUrd–positive cells are significantly higher in hyperplastic ducts II and III compared with either ducts I or normal ducts (*P* < 0.001).

Figure 6. Identification of subpopulations of proliferating cells in hyperplastic ducts of pubertal Tg (c-neu). The data were obtained from triple immunolocalization experiments as described in text. Ducts II and III correspond to ducts with BrdUrd labeling index 1 to 10 and >10, respectively. *A,* distribution of c-neu- and/or ERα-positive cells. *, c-neu-positive cells are significantly lower in ducts II compared with ducts I ($P < 0.05$). *B,* distribution of proliferating ERα- and/or c-neu-positive cells. Compared with ducts II, ducts III contain significantly more (*) c-neu/BrdUrd ($P < 0.05$) and (**) c-neu/ERα/BrdUrd–positive cells ($P < 0.01$). Ducts II contain significantly less (*) c-neu/BrdUrd–positive cells compared with c-neu/ERα/BrdUrd–positive cells ($P < 0.05$).

against tumor development that can result from hyperproliferation, associated with overexpression of c-neu. If this were so, considering that hyperproliferation is predominantly associated with ERα-positive cells (Fig. 6), the decrease in proliferation accompanying premature differentiation would also be expected to occur in these cells; indeed, this is the case (Fig. 6). As such, these observations suggest that the ultimate result of the synergy between c-neu- and ERα-dependent proliferation is to delay tumor development by causing premature differentiation/growth arrest. Yet, starting at ~8 months of age, mammary tumors begin to appear in Tg (c-neu; ref. 11). The proliferation accompanying tumor onset is most likely not due to a reversal of growth arrest in ERα-positive cells because tumors arising in Tg (c-neu) are ovarian independent for growth and contain very few ERα-positive cells,[3] suggesting that tumors may originate from ERα-negative cells. Indeed, mammary ducts of pubertal Tg (c-neu) contain a subpopulation of proliferating c-neu-positive/ERα-negative cells (i.e., cells positive for only c-neu and BrdUrd; Fig. 6), and these cells constitute the majority of proliferating cells in tumors.[3] At present, we do not know whether proliferating c-neu/ERα–negative cells represent an independent population or they arise from proliferating c-neu/ERα–positive cells through asymmetric cell division. Regardless, our present observations suggest that, within the context of tumor development, the fates of ERα-positive and ERα-negative cells may be different in mammary epithelial cells overexpressing c-neu. Similar to tumors arising in Tg (c-neu), human mammary tumors overexpressing erbB2 exhibit a loss of ERα-positive cells and are resistant to endocrine therapy (12–16). At present, the precise mechanisms responsible for this phenomenon remain elusive. To this end, our present observations provide a relevant framework for identifying the cellular origin of tumors arising from mammary epithelial cells overexpressing c-neu and the mechanisms responsible for their failure to respond to antiestrogen therapy.

Acknowledgments

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[3] Our unpublished observations.

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Effect of c-neu/ ErbB2 Expression Levels on Estrogen Receptor α–Dependent Proliferation in Mammary Epithelial Cells: Implications for Breast Cancer Biology

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