A Short-term Rat Mammary Carcinogenesis Model for the Prevention of Hormonally Responsive and Nonresponsive *In situ* Carcinomas

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Abstract Preclinical models that accurately reproduce specific aspects of human disease etiology are invaluable for the initial development and evaluation of chemopreventive agents. We developed a novel, short-term prevention model, which is particularly useful for assessing the efficacy of a compound to prevent hormonally responsive and nonresponsive *in situ* carcinomas. In this model, carcinogenesis is induced by a high titer of neu-containing, replication-defective retrovirus. The multiplicity and size of the resulting *in situ* carcinomas are scored in whole-mounted, aluminum carmine–stained mammary glands at 15 days postinfusion. These *in situ* carcinomas represent a distinct biological time point in the development of neu-induced mammary cancer in the rat. They are characterized by high rates of proliferation (40.0%; $P < 0.0001$) and apoptosis (2.8%; $P < 0.005$) compared with mammary carcinomas. The majority of *in situ* carcinomas regress spontaneously after 20 days postinfusion. The *in situ* carcinomas at 15 days postinfusion exhibit hormonal responsiveness. The effects of the chemoprevention agents tamoxifen, celecoxib, and targretin on hormonally responsive and nonresponsive *in situ* carcinomas recapitulate those observed on mammary carcinomas at 12 and 18 weeks postinfusion for intact and ovariectomized rats, respectively. Neu-induced *in situ* carcinomas in the rat represent etiologically relevant intermediate time points of mammary carcinogenesis. Our prevention model represents a cost-efficient *in vivo* system to determine whether the preventive effects of a compound extend to hormonally nonresponsive mammary lesions, for which new chemoprevention approaches are needed.

Rodent models of mammary carcinogenesis have been shown to recapitulate histopathologic morphology and molecular characteristics of various malignancies of the human breast (1, 2). They are vital for the development and preclinical evaluation of chemopreventive agents. Both rat and mouse mammary cancer models have advanced the field of chemoprevention by showing the efficacy of several pharmacologic and natural compounds to prevent mammary cancer *in vivo*.

Hormonal responsiveness, the ability to prevent mammary tumors by physiologic or chemical hormone ablation, is a hallmark of mammary cancer. The NSABP Breast Cancer Prevention Trial (P-1) showed that 49% of invasive breast cancers were prevented by the selective estrogen response modulator tamoxifen (3). This corresponds to a 69% prevention rate for estrogen receptor–positive breast tumors, whereas no prevention benefit was observed on estrogen receptor–negative tumors. Despite investigative chemoprevention efforts to target hormonally nonresponsive breast tumors, selective estrogen response modulators remain the clinical standard of care for the prevention of breast cancer. Consequently, the population of breast tumors not prevented by selective estrogen response modulators remains a major cause of mortality from this disease. More efforts need to be undertaken to develop chemoprevention agents specifically targeting hormonally nonresponsive breast cancer.

The neu-induced retroviral rat mammary carcinogenesis model exists in two distinct hormonal configurations. In intact rats, ~50% of mammary carcinomas are hormonally responsive and, thus, can be prevented by treatments with the selective estrogen response modulator tamoxifen. In ovariectomized rats, mammary carcinomas are uniformly hormonally nonresponsive and tamoxifen treatment is inefficacious for the prevention of these tumors. The model recapitulates the hormonal responsiveness evident in human breast cancers and has been used to investigate the efficacy of multiple chemoprevention agents to prevent the development of hormonally nonresponsive mammary carcinomas (4, 5). However, it might be cost-prohibitive to investigate the efficacy of a large number of potential compounds in this long-term prevention model with mammary carcinomas as end points.

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We have therefore developed a short-term prevention model in which hormonally responsive and nonresponsive *in situ* carcinomas are scored as end points.

The prevention of precancerous lesions should be a primary effort in the field of chemoprevention. The majority of invasive breast tumors arise from *in situ* carcinomas (6) and the treatment of ductal carcinomas *in situ* in women is considered a viable strategy for the prevention of invasive breast carcinomas (7). Because our current understanding of the molecular changes that govern the progression from ductal carcinomas *in situ* to invasive breast cancer is inadequate, the clinical care for ductal carcinomas *in situ* varies greatly, ranging from mastectomy to excision and radiation to excision alone with considerable variation in recurrence rates (8). Preclinical models of early end points in mammary carcinogenesis are therefore urgently needed to elucidate the mechanisms by which the progression from *in situ* carcinoma to mammary carcinoma occurs.

A variety of preclinical models for premalignant mammary cancers in the laboratory rat have been developed over the past 40 years. The most widely used are chemical carcinogen–induced models using the polycyclic hydrocarbon 7,12 dimethylbenz(*a*)anthracene or the direct-acting *N*-methyl-*N*-nitrosourea (9). Difficulties in adaptation of these models as chemoprevention models arise from the fact that chemical induction of mammary carcinogenesis in the rat results in a wide spectrum of premalignant lesions that coexist at the same time (9). This report shows that retrovirally induced mammary lesions arise faster and result in functionally uniform preventable end points.

The neu-induced retroviral *in situ* carcinoma rat model is a short-term prevention model in which the multiplicity and size of such lesions are scored in whole-mounted, carmine-stained mammary glands. We characterized neu-induced *in situ* carcinomas as uniform, distinct, and transient time points of mammary carcinogenesis in the rat, as well as their potential to develop into mammary carcinomas. The chemopreventive effects of the selective estrogen response modulator tamoxifen, the cyclooxygenase-2 inhibitor celecoxib, and the rexinoid targretin on hormonally responsive and nonresponsive *in situ* carcinomas are discussed.

Materials and Methods

Neu-induced retroviral *in situ* carcinoma rat model

All animal experiments were done at our facility under protocols approved by the University of Wisconsin Medical School Animal Care and Use Committee. Virgin Wistar-Furth female rats were obtained from Harlan Sprague-Dawley at 6 wk of age. All rats were group housed in suspended wire cages and maintained at a 12 h light/12 h dark cycle, receiving Teklad lab meal (#8604) and acidified water *ad libitum*. After 1 to 2 wk of acclimation, at ~50 to 60 d of age, all rats underwent retroviral infusion with the pJRneu vector, which induces mammary carcinogenesis by expressing the activated Her-2/neu oncogene. The construction and generation of the pJRneu retroviral vector have been previously described (10, 11). Details on retroviral gene transfer into the mammary epithelium of the laboratory rat

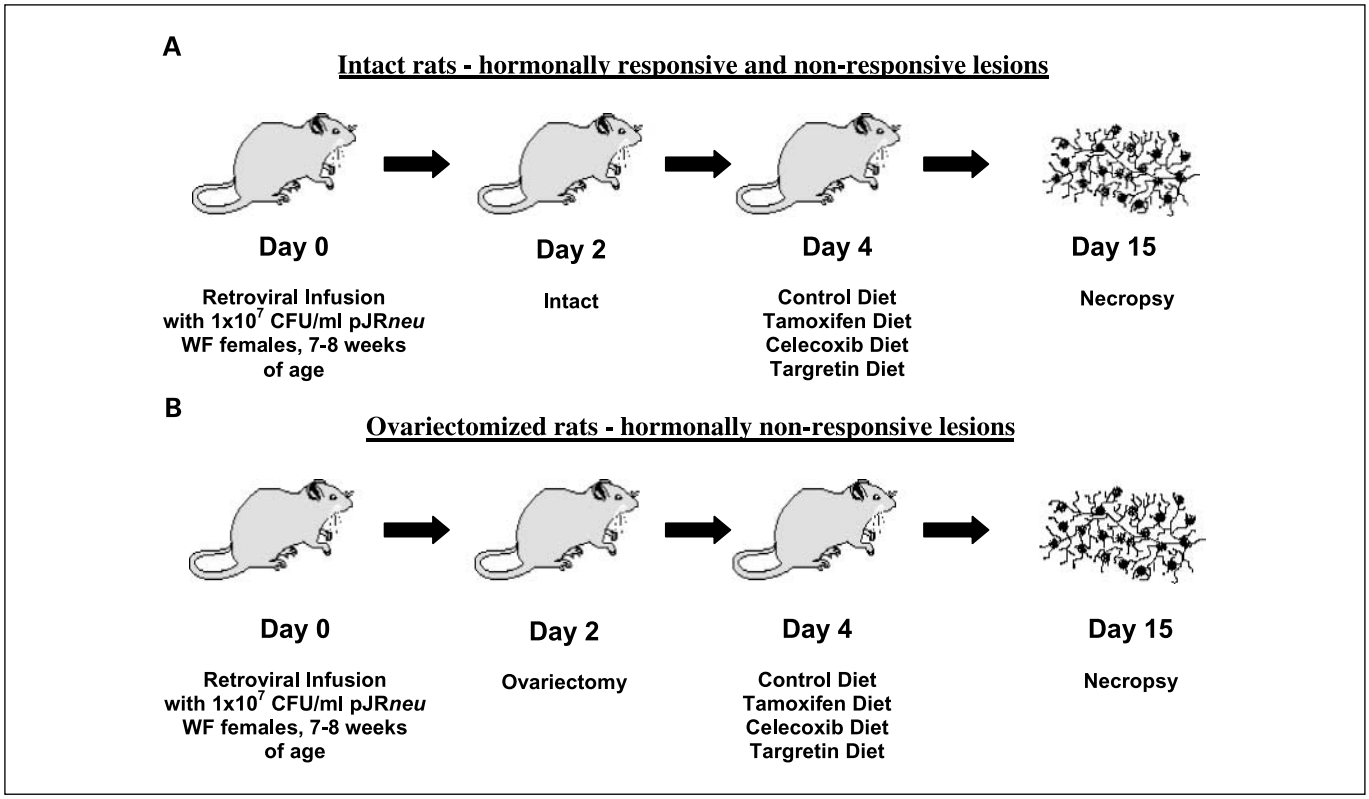

Fig. 1. The neu-induced retroviral *in situ* carcinoma rat model. The neu-induced retroviral *in situ* carcinoma rat model exists in two distinct hormonal configurations. The *in situ* carcinomas of intact animals (*A*) exhibit a mixed hormonal response, whereas those of the ovariectomized configuration (*B*) are hormonally nonresponsive.

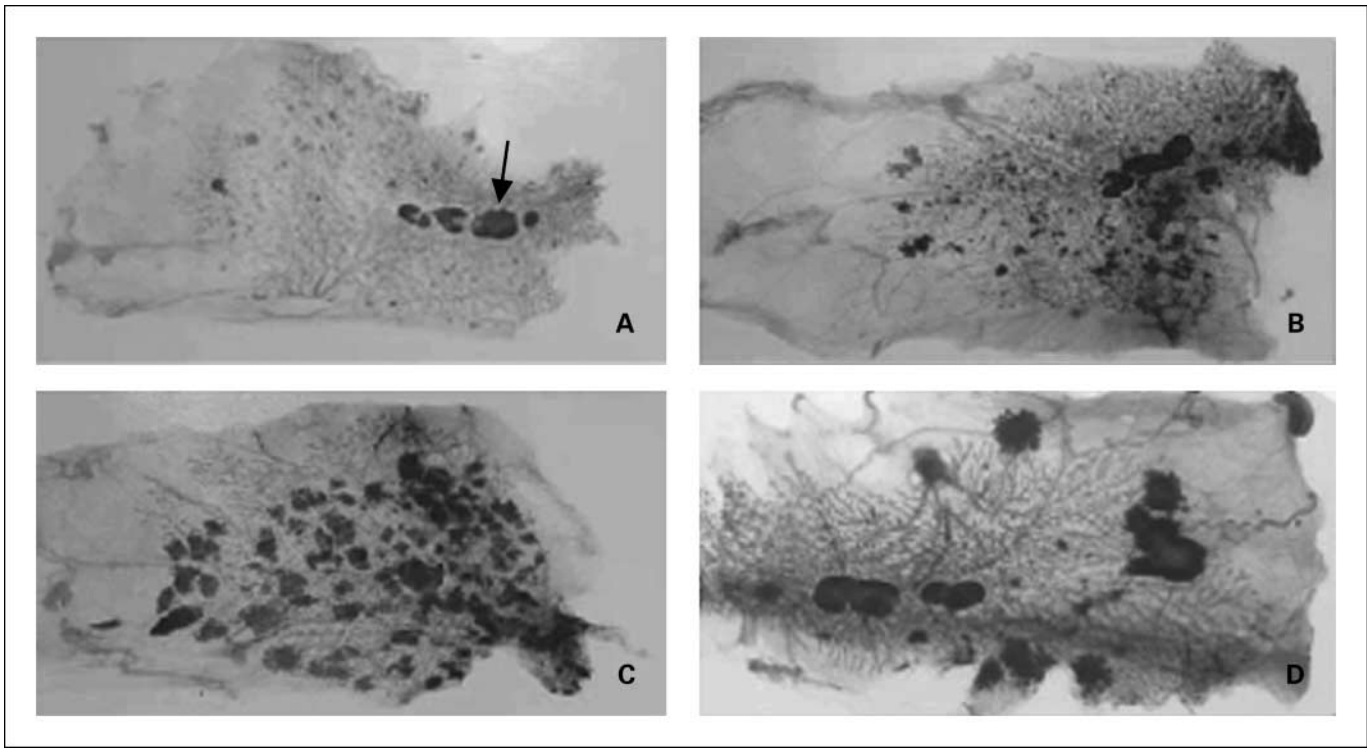

Fig. 2. Time course following the development and progression of neu-induced mammary lesions. Neu-induced mammary lesions in intact Wistar-Furth rats are visualized in whole-mounted, carmine-stained abdominal mammary glands at 10 d (*A*), 13 d (*B*), 16 d (*C*), and 27 d (*D*) postinfusion. Lymph nodes are marked in *A* (*arrow*).

(12) and the application of this technology for chemoprevention in the neu-induced retroviral rat carcinogenesis model (4, 5) have also been published. A 15-μL suspension of replication-defective amphotropic retrovirus containing the activated neu oncogene was infused into the central ducts of the abdominal (fourth) mammary glands. All rats were infused with a viral titer of 1×10^7 colony-forming units (CFU)/mL. At 2 d postinfusion, a portion of the rats underwent a bilateral ovariectomy. At 4 d postinfusion, the rats were randomly assigned to the treatment groups and the administration of the experimental diets was begun. Tamoxifen was administered at a dose of 2 mg/kg diet, celecoxib at 1,200 mg/kg diet, and targretin at 250 mg/kg diet. The study was terminated 15 d postinfusion. The abdominal mammary glands were excised and whole-mounted onto microscope slides. They were fixed in buffered formalin, defatted via acetone treatment, rehydrated through ethanol gradients, stained with aluminum carmine, dehydrated through ethanol gradients, and cleared in xylenes. The stained slides were then transferred to mineral oil and photographed. The carmine stain visualized the ductal tree within the mammary gland as well as major anatomic landmarks such as the central duct and lymph nodes within the gland. The *in situ* carcinomas induced by the retrovirus appeared as nodules on the ductal structure within the mammary gland. Both the number of mammary lesions and the size of the lesions were scored using Image J (NIH), an open source application for data analysis (13).

Chemopreventive agents

Tamoxifen was purchased from Sigma. Celecoxib (LKT Laboratories, Inc.) and targretin (Onyx Scientific) were obtained through the Division of Cancer Prevention Repository. All experimental diets were dry mixed in Teklad 4% fat rodent meal (Harlan Teklad), which was also used as control diet. All diets were prepared fresh weekly and stored at −20°C. Rats were provided fresh diet twice weekly.

Proliferation and apoptosis

All animals were infused in accordance with the procedure for neu-induced retroviral *in situ* carcinomas with a retroviral titer of 1×10^7 CFU/mL. Rats were group-housed and received control diet (Teklad #8604) and acidified water *ad libitum*. Twenty-five female Wistar-Furth rats were randomized into five time-point groups, which were sacrificed 15, 18, 21, 24, and 27 d postinfusion. At necropsy, the abdominal mammary glands were excised, fixed in buffered formalin, and paraffin embedded. Consecutive slices were stained with H&E for histologic evaluation or used for proliferation and apoptosis assays.

The proliferation index of *in situ* carcinomas was evaluated by Ki-67 staining (14, 15). Immunohistochemical procedures were done according to standard protocols using the primary antibody VP-K452 (Vector Labs) to detect the Ki-67 epitope. Primary antibody binding was visualized by horseradish peroxidase–conjugated secondary antibody, the VECTASTAIN ABC Elite System (Vector Labs), and diaminobenzidine as the chromogen.

The apoptotic index of mammary carcinomas was evaluated by terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling (16) using the TdT-FragEL DNA Fragmentation Detection Kit QIA 33 (Calbiochem). Slides were processed according to the manufacturer's recommendations.

Ki-67 and terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling staining were evaluated by light microscopy. For each time point, 20 *in situ* carcinomas were randomly chosen for proliferation and apoptosis analysis. Approximately 500 cells in several random fields were evaluated for proliferating or apoptotic cells for each lesion.

Statistical analysis

The statistical analysis of the proliferation and apoptosis analysis, as well as the *in situ* carcinoma multiplicity and size comparisons, was done by Wilcoxon rank-sum (Mann-Whitney) test.

Results

The neu-induced retroviral *in situ* carcinoma rat model

The neu-induced retroviral *in situ* carcinoma rat model (Fig. 1) reproducibly induces *in situ* carcinomas using a viral titer of 1×10^7 CFU/mL. This high retroviral titer minimizes the number of animals needed and ensures reliable statistical power. The number of *in situ* carcinomas induced in each mammary gland can be regulated by adjusting the retroviral titer, which controls the multiplicity of infection.

Figure 1 outlines the method for inducing hormonally responsive and nonresponsive *in situ* carcinomas in our short-term model. Following retroviral infusion, the rats remain untreated for 2 days after infusion to allow for reverse transcription and stable integration of the retrovirus into the ductal epithelium. After this time period, the ovariectomized group undergoes bilateral ovariectomies while the intact group retains normal hormone functions. Ovariectomy in neu-induced rats is associated with reductions of >75% in estrogen receptor and nearly 90% in progesterone receptor levels in the resulting mammary carcinomas (17). Both hormonally responsive and nonresponsive *in situ* carcinomas arise in intact rats, whereas the *in situ* carcinomas in ovariectomized animals are uniformly hormonally nonresponsive.

In a preliminary time course series experiment, early lesions on the ductal structure were visible starting day 10 postinfusion (Fig. 2A). The number and size of these lesions increased until approximately day 13 postinfusion (Fig. 2B). After day 13, there was no appreciable increase in number of lesions but the lesion size increased steadily through day 16 (Fig. 2C) and day 19 (data not shown) postinfusion. At day 27 postinfusion, when tumors are generally palpable in the living rat, the majority of lesions had spontaneously regressed (Fig. 2D). Day 15 postinfusion was decided on as the end point in our prevention model as the *in situ* carcinomas were sufficiently large to be easily counted without the confounding effects of overlapping lesions.

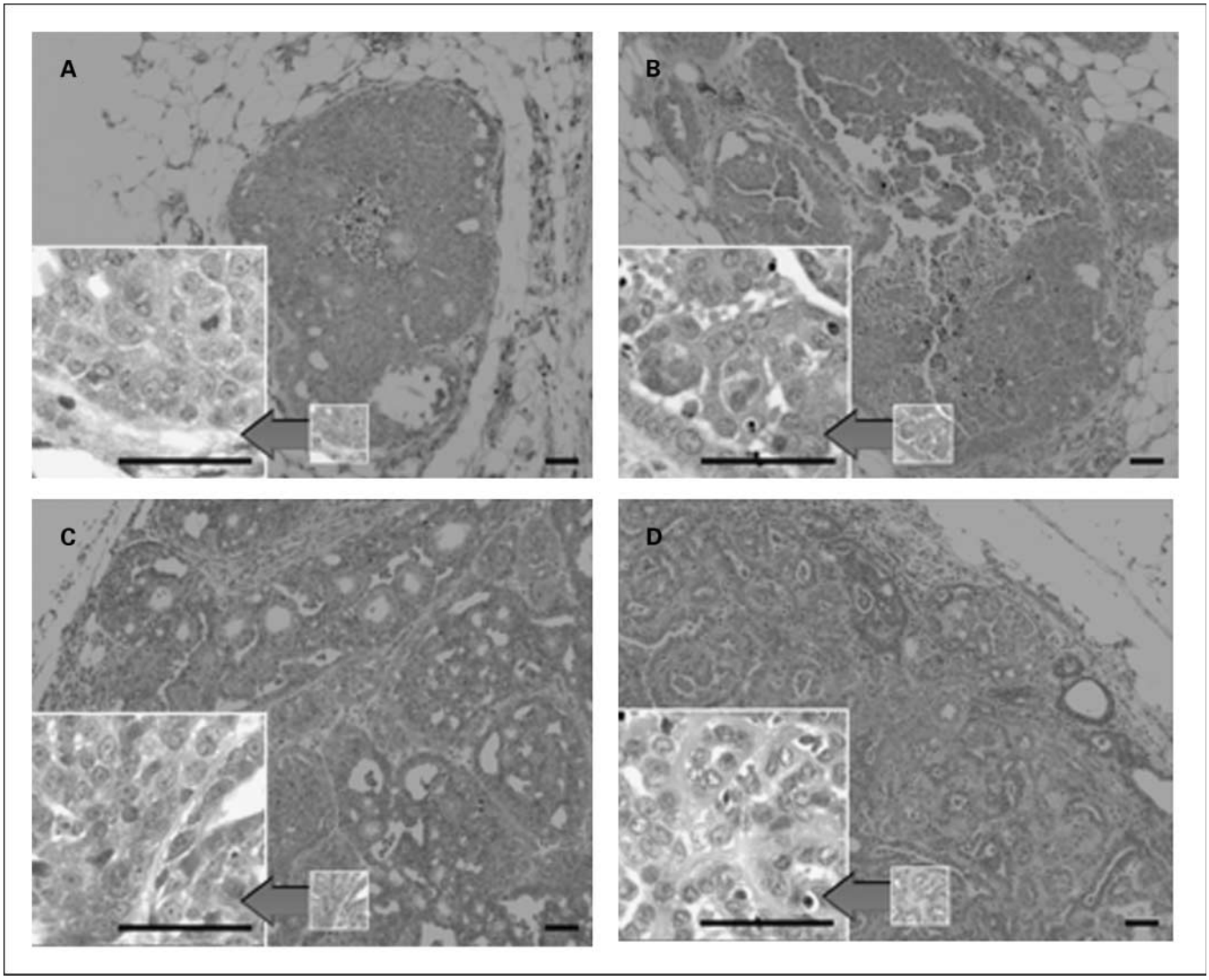

Fig. 3. Histopathologic morphology of neu-induced mammary lesion. Histopathologic morphology of *in situ* carcinomas at 15 d postinfusion (*A* and *B*), stable tumors at 27 d postinfusion (*C*), and mammary carcinomas at 12 wk postinfusion (*D*). Reference line, 50 μm.

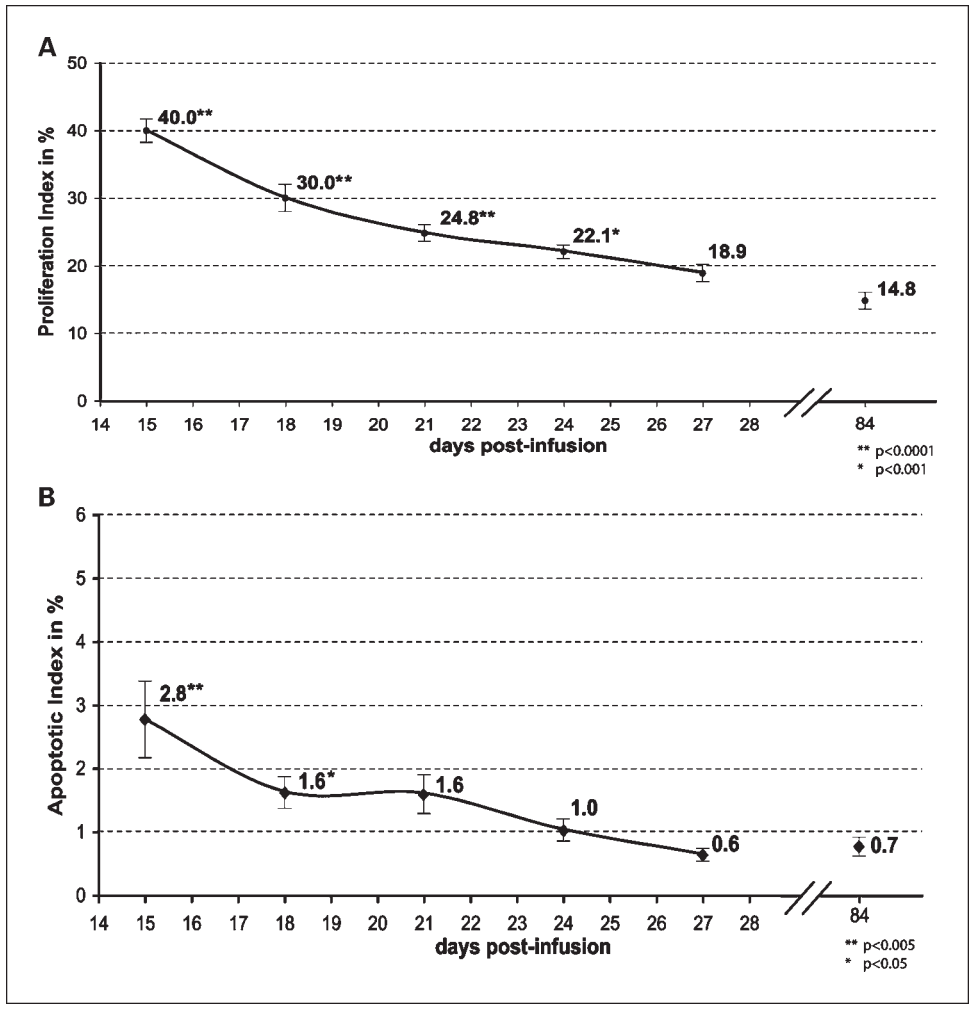

Fig. 4. Proliferation and apoptotic indices during the progression from *in situ* carcinomas into mammary carcinomas. Proliferation indices (*A*) and apoptotic indices (*B*) decrease rapidly during the progression from *in situ* carcinomas at 15 d postinfusion into early mammary carcinomas at 27 d postinfusion, approaching the rates of mammary carcinomas at 84 d postinfusion. Averages are labeled; *bars,* SE. Asterisks, significant differences from the 84-d value. The day 84 postinfusion values have been published previously (4).

Histopathologic evaluation of neu-induced *in situ* carcinomas

The neu-induced *in situ* carcinomas of our short-term prevention model display varying degrees of nuclear atypia at 15 days postinfusion (Fig. 3A and B). They exhibit solid, acinar, and papillary growth patterns and are frequently characterized by areas of central necrosis. Stable tumors at 27 days postinfusion (Fig. 3C) are larger, with an overall more pronounced degree of architectural derangement and cellular atypia but in general exhibit similar morphologic features. Mammary carcinomas at 12 weeks postinfusion (Fig. 3D) exhibit a range of morphologic features including papillary and acinar patterns. The general morphology of neu-induced *in situ* carcinomas and mammary carcinomas does not seem to be affected by ovariectomy or chemoprevention regimen.

Proliferation and apoptotic analysis

Neu-induced *in situ* carcinomas at day 15 postinfusion represent a distinct intermediate time point in the mammary carcinogenesis process, characterized by high levels of both proliferation and apoptosis. Proliferation rates of *in situ* carcinomas averaged 40.0% at 15 days postinfusion (Fig. 4A). In this time course analysis, the mean proliferation rate decreased steadily after 15 days until, by day 27 postinfusion, it approached levels similar to those of mammary carcinomas at 12 weeks postinfusion. The mean apoptotic index of *in situ* carcinomas at day 15 postinfusion was 2.8% (Fig. 4B). Following the day 15 postinfusion time point, apoptotic levels sharply decreased over time. By day 27 postinfusion, the apoptotic index had normalized to that of mammary carcinomas at 12 weeks postinfusion.

Progression rates of neu-induced *in situ* carcinomas

We compared the multiplicity of *in situ* carcinomas at day 15 postinfusion to the multiplicity of mammary carcinomas at 84 days postinfusion in intact rats and at 126 days postinfusion for ovariectomized rats. The retroviral titers were identical for each corresponding short-term and long-term model. In intact animals, the proportion of mammary carcinomas present at day 84 postinfusion compared with *in situ* carcinomas present at day 15 postinfusion is ~5% (Table 1). In ovariectomized rats, the ratio of mammary carcinoma at 126 days postinfusion to *in situ* carcinomas present at day 15 postinfusion is ~1% (Table 1).

Chemopreventive effects of tamoxifen, celecoxib, and targretin on neu-induced *in situ* carcinomas

The chemopreventive effects of tamoxifen were limited to hormonally responsive *in situ* carcinomas arising in intact rats. Dietary tamoxifen reduced the multiplicity of *in situ*

Table 1. Multiplicity of intermediate *in situ* carcinomas and mammary carcinomas induced by identical retroviral titers

Model	INT/OVX	End point	Viral titer (CFU)	Duration (d)	No. rats	Multiplicity
Short-term	INT	*In situ* carcinomas	7.5×10^4 CFU	15	12	8.7 lesions/gland
Long-term	INT	Carcinomas	7.5×10^4 CFU	84	15	0.43 carcinomas/gland
Short-term	OVX	*In situ* carcinomas	5×10^5 CFU	15	12	22.7 lesions/gland
Long-term	OVX	Carcinomas	5×10^5 CFU	126	25	0.24 carcinomas/gland

Abbreviations: INT, intact rat model; OVX, ovariectomized rat model.

carcinomas in intact rats by 38% (Fig. 5A; $P = 0.0007$) and lesion size was reduced by 57% (Fig. 5C; $P < 0.0001$). No changes in multiplicity (Fig. 5B) or size (Fig. 5D) of *in situ* carcinomas were observed in the ovariectomized, tamoxifen-treated animals.

Dietary treatment with the cyclooxygenase-2 inhibitor celecoxib resulted in a 32% (Fig. 5A; $P = 0.004$) reduction of *in situ* carcinoma multiplicity in intact rats. Whereas a marginal reduction of *in situ* carcinoma size might be discerned in intact rats following celecoxib treatment (Fig. 5C), it failed to reach levels of statistical significance. Celecoxib treatment did not result in modulation of multiplicity (Fig. 5B) or size (Fig. 5D) of *in situ* carcinomas in ovariectomized rats.

The retinoid X receptor–selective retinoid targretin was efficacious for the prevention of both hormonally responsive and nonresponsive *in situ* carcinomas. Dietary targretin decreased

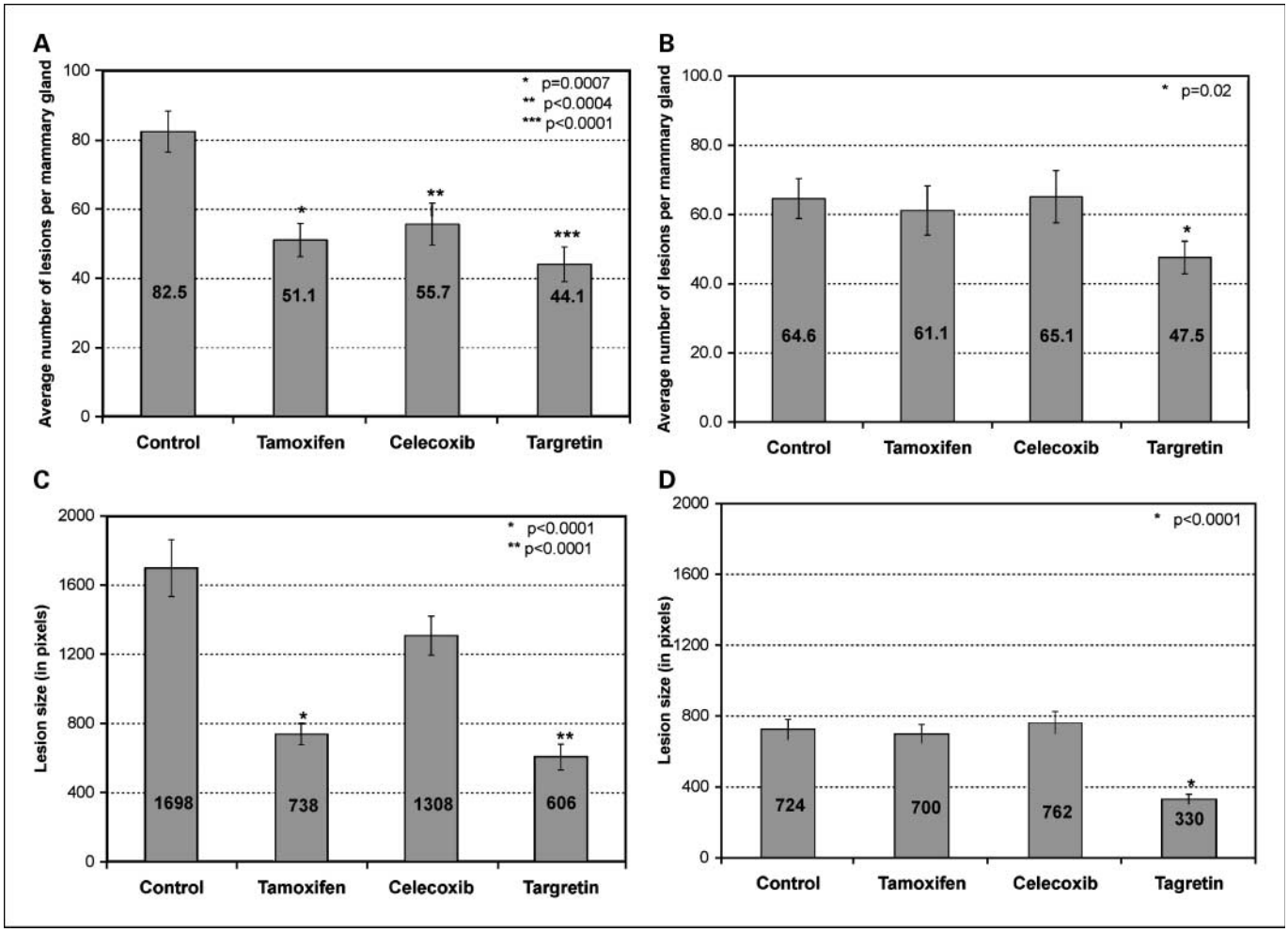

Fig. 5. Chemoprevention of *in situ* carcinomas using tamoxifen, celecoxib, and targretin. The multiplicity of *in situ* carcinomas within the abdominal mammary glands for each treatment is shown in intact (*A*) and ovariectomized (*B*) rats. The average size of *in situ* carcinomas, as measured by pixels within lesion area, is shown for each treatment in intact (*C*) and ovariectomized (*D*) rats. Group means are indicated; *bars,* SE.

the multiplicity of *in situ* carcinomas by 47% (Fig. 5A; $P < 0.0001$) and 26% (Fig. 5B; $P = 0.02$) in intact and ovariectomized rats, respectively. Targretin treatment also reduced lesion size by 64% (Fig. 5C; $P < 0.0001$) in intact rats and by 54% (Fig. 5D; $P < 0.0001$) in ovariectomized animals.

Discussion

The neu-induced *in situ* carcinoma model presents a reliable and convenient method to induce distinct, transient intermediate end points of mammary carcinogenesis in the rat. Aside from sharing histopathologic features with their human ductal carcinomas *in situ* counterparts and their ability to spontaneously regress or progress into mammary carcinomas, neu-induced *in situ* carcinomas have molecular characteristics relevant for the study of human breast cancer development. For instance, cyclooxygenase-2 expression is up-regulated in neu-induced *in situ* carcinomas (5) compared with mammary carcinomas in our long-term model and only low baseline levels in normal mammary gland tissue. This recapitulates a feature of human Her-2/neu–positive ductal carcinomas *in situ*, in which cyclooxygenase-2 overexpression is found more frequently than in invasive breast carcinomas (18), whereas it is virtually absent from normal breast parenchyma (19). In addition, we provided evidence that hormonal responsiveness, a highly relevant characteristic of human breast cancer etiology, can be accurately assessed in this short-term chemoprevention model.

The chemopreventive effects of the selective estrogen response modulator tamoxifen are well established in the literature. We observed a 38% reduction in multiplicity of *in situ* carcinomas in our short-term model, which is consistent with reductions of 33% to 49% in mammary carcinoma multiplicity in our long-term prevention model published previously (5). Tamoxifen was not efficacious for the prevention of hormonally nonresponsive *in situ* carcinomas in ovariectomized rats, which is also reflected in our long-term prevention studies (4, 5). The significant reduction in size of hormonally responsive *in situ* carcinomas following short-term treatment with tamoxifen is also consistent with observations in long-term models of neu-induced (4, 5) and chemically induced (20) mammary carcinomas.

The significant reduction in multiplicity of *in situ* carcinomas associated with celecoxib treatment in intact rats is in agreement with data from our long-term prevention model (5) and those of other carcinogen-induced rat models (21, 22) in which tumors tend to be uniformly hormonally responsive. The lack of effect of celecoxib on *in situ* carcinoma size in intact rats is also consistent with our observations in neu-induced mammary carcinomas where it is accompanied by failure to modulate proliferation or apoptotic rates (5). The effects of celecoxib on tumor size in carcinogen-induced rat models seem to be less consistent. Whereas one study using the 7,12 dimethylbenz(*a*)anthracene rat model reported no effects on tumor volume (22), another found an 81% reduction of tumor volume following celecoxib treatment at the same dose of 1,500 ppm (21). Consistent with our previous findings that efficacy of celecoxib is limited to hormonally responsive mammary carcinomas (5), we observed no effect of celecoxib treatment on lesion multiplicity or size in ovariectomized rats. Whereas the literature cites celecoxib-modulated reductions in tumor multiplicity, but not in size, in an estrogen receptor–negative mouse model of mammary carcinogenesis (23), these findings must be understood in context, as tamoxifen treatment also causes significantly decreases in tumor multiplicity in this preclinical model (24). Importantly, a recent randomized, placebo-controlled prevention trial with celecoxib in women at high risk for breast cancer showed no modulation of the proliferation maker Ki-67 in breast epithelial cells after celecoxib treatment (25). This result is consistent with our findings that proliferation rates are not affected by celecoxib treatment (5) and might explain why the sizes of celecoxib-treated mammary carcinomas and *in situ* carcinomas seem to be unmodulated in our experiments, despite reductions in multiplicity of these end points.

The significant efficacy of the rexinoid targretin to prevent both hormonally responsive and nonresponsive *in situ* carcinomas in our short-term model mirrors the effects of targretin in our neu-induced mammary carcinoma prevention models (4), albeit with slightly lower magnitudes. This is consistent with the literature, which reports that both hormonally responsive mammary carcinomas in carcinogen-induced rat models (26, 27) and estrogen receptor–negative mammary tumors in transgenic mice (28, 29) are preventable by targretin treatment. The targretin-mediated reductions of lesion size in intact rats were mirrored in the results of our long-term prevention model (4). Whereas targretin treatment showed no significant effect on tumor volume in ovariectomized rats in our long-term model, targretin caused strong reductions in proliferation rates and significant increases in apoptotic rates in hormonally nonresponsive mammary carcinomas (4). We postulated that the 84% reduction in tumor multiplicity resulted in too few carcinomas included in the tumor size analysis to unveil a significant effect of targretin treatment on the size of mammary carcinomas in ovariectomized rats. In our short-term model, targretin treatment resulted in a 54% reduction in the size of hormonally nonresponsive *in situ* carcinomas in ovariectomized rats. These data are suggestive of a role for rexinoids in the prevention of hormonally nonresponsive breast cancer.

Our report suggests that the progression from *in situ* carcinomas at 15 days postinfusion into stable, palpable mammary carcinomas with proliferation and apoptotic rates equivalent to those of mammary carcinomas in our long-term models takes a mere 12 days. This short and reproducible duration, along with the ability to adjust the multiplicity of arising lesions to a desired level, could make the model adaptable for the study of molecular mechanisms underlying the progression from *in situ* carcinoma to carcinoma stage. With recent advances in microimaging technology (30), it might be possible to identify progressing and regressing lesions at various stages of progression and characterize them molecularly.

It should be stated that the high rate of *in situ* carcinoma regression is most likely related to the fact that these lesions are hyperproliferative and eventually outgrow their blood supply within the mammary gland. No regression was observed in the 3 days preceding or following the day 15 postinfusion end point. Our data contain no unequivocal evidence ruling out the possibility that a compound could differentially affect progressing and regressing lesions. However, the fact that the unique prevention patterns of the three compounds with regard to their effects on multiplicity and size

across hormonal configuration mirror one another for the two distinct end point suggests the absence of such an artifact and is consistent with the notion that the mechanisms for the prevention of *in situ* carcinomas and mammary carcinomas are functionally related.

Based on the evidence presented, the use of intermediate *in situ* carcinomas as preventable end points in this short-term model is a valid initial method for assessing the efficacy of a compound for preventing hormonally responsive and nonresponsive mammary cancer. As potential chemopreventive agents for breast cancer are being developed, especially by high-throughput methodologies in *in vitro* models (31), cost-effective *in vivo* prevention models are vital for their initial preclinical efficacy assessment. Its short duration, the use of fewer animals and high statistical power should make the neu-induced retroviral *in situ* carcinoma model an excellent choice for this undertaking. In addition, the amount of chemopreventive agent required for efficacy testing is significantly less in our short-term model compared with other preclinical models, an important economic consideration, especially for testing precious quantities of novel agents in drug development.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Cancer Prevention Research

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