Journal of Cellular Biochemistry 17G:66–72 (1993)

The Introduction of Activated Oncogenes to Mammary Cells *In Vivo* Using Retroviral Vectors: A New Model for the Chemoprevention of Premalignant and Malignant Lesions of the Breast

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Abstract Breast cancer is an important disease site for chemopreventive intervention. The current *in vivo* rodent models for breast cancer do not adequately approximate the human disease. Thus, we developed a new series of models based on the direct introduction of activated oncogenes into *in situ* mammary ductal cells in the rat. So far we have introduced both activated *ras* and *neu* into the mammary parenchyma of the rat. Both oncogenes cause the development of mammary carcinomas without any additional exogenous intervention. The activated *neu* gene is, however, 200 times more penetrant than the activated *ras* gene. The carcinomas induced by both genes are more aggressive than those induced by chemical carcinogens. They are more often transplantable, locally invasive, and metastatic. Tumors arising from the introduction of the *neu* oncogene have a greater histopathological resemblance to human breast cancers than those associated with *ras* insertion. For example, within a week post-introduction of *neu*, lesions resembling ductal carcinomas *in situ* are observed in the rat mammary gland. These do not occur following *ras* introduction or exposure to chemical carcinogens. These models are helping to better define the cellular and molecular events associated with the multistage progression of breast cancer. They are also being used to develop models to evaluate chemopreventive approaches for breast cancer. For example, in the area of cancer prevention we have shown that hormonal intervention is more effective in preventing *neu*-initiated breast cancers than *ras*-initiated breast cancers. We have also shown that the chemopreventive monoterpene, *d*-limonene, can prevent *ras*-induced mammary tumors in this model. We therefore feel that these models will be useful in identifying and characterizing agents for chemoprevention in breast cancer, as well as in defining and evaluating intermediate biomarkers for chemoprevention trials.

Key words: Breast cancer, hormonal therapy, mammary, monoterpene, *neu*, *ras*

Breast cancer is one of the most common malignancies in American women. Unfortunately, there has been a steady, slightly rising incidence of this disease over the last half century, with little change in therapeutic success. It is clear that this disease requires the development of new, organ-specific, targeted therapeutic strategies. One such strategy is disease prevention. While endocrine prevention strategies are currently undergoing clinical evaluation, these strategies are not likely to prevent all types of this malignancy, and may also be associated with both acute and chronic toxicities. Thus, alternative or supplemental prevention strategies need to be developed and evaluated. Many potential chemopreventive agents have been proposed; however, many still require the completion of preclinical efficacy evaluation, hopefully to be followed by human trials.

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Rodent models are needed for both preclinical agent evaluation and to develop and evaluate biomarkers for human Phase II testing. Such rodent models are required due to the lack of good *in vitro* human breast transformation models, and the inability of current *in vitro* models to evaluate most histopathological biomarkers. Current *in vivo* models for breast cancer prevention studies are quite limited and, in many aspects, inadequate. Mouse models are often confounded by viral etiologies not seen in women. In addition, unlike human mammary carcinomas, those of the mouse are rarely hormonally dependent. Rat models are superior to mouse models for chemoprevention; rat mammary carcinoma lacks a viral etiology and is often, but not always, hormonally responsive.

The rat mammary models most often used in chemoprevention studies are those in which tumors are induced by chemical carcinogens such as *N*-nitroso-*N*-methyl urea (NMU) and dimethylbenz(*a*)anthracene (DMBA). The appropriateness of these models to screen breast-specific chemopreventive agents and trial biomarkers may be less than ideal. Human breast cancer has not, for the most part, been associated with environmental chemical initiation. Furthermore, chemically induced mammary cancers that arise in the rat are different than human cancer in their histopathology and stage of progression, as well as their molecular profile. For example, chemically induced tumors rarely if ever metastasize, and do not routinely invade the surrounding tissue. The characteristic molecular lesion of rodent breast tumors is activation of the *ras* oncogene. This genetic alteration is rarely seen in human breast tumors. In contrast, human breast cancer is often associated with amplification of the *neu* oncogene.

An alternative to chemically induced rodent mammary cancers for modeling human cancer is to construct transgenic rodents in which activated oncogene expression is targeted to the mammary gland using mammary-specific promoters, such as mouse mammary tumor virus (MMTV) and whey acidic protein (WAP) promoters..To date, most of these models have been confined to mice; however, rat models are currently being developed. The use of transgenic models has several theoretical and practical limitations. Practically, there is limited ability to produce certain valuable mice, such as those expressing high levels of activated *neu*, due to its systemic and local toxicities. Theoretically, the screening of chemoprevention agents is confounded by the use of exogenous hormones or altered physiological states such as pregnancy, which trigger the mammary-specific promoters that express the genes of interest. Finally, the evaluation of biomarkers in a transgenic model is complicated by the fact that all cells of the transgenic animal contain a specific genetic change with downstream physiological effects, unlike human tumors which develop surrounded by physiologically and genetically normal tissue.

Due to these limitations in the currently available rodent breast cancer models, we sought to develop alternate approaches to model the etiology of breast cancer in a rodent system. Such a model could be useful to study the etiology of the human disease and to develop new strategies for breast cancer prevention and therapy.

THE MODEL

We have devised methods to incorporate selected genes *in situ* into mammary parenchymal cells. This approach is similar to methodology used in gene therapy. The gene of interest is subcloned into the retroviral vector JR [1], which is a derivative of LNL6 [2]. The gene is under control of the MMLV long terminal repeat (LTR); in principle, however, it could be controlled by any desired internal promoter. This vector is then introduced into a packaging cell line as previously described [1]. We have found that amphotropically packaged vectors are more efficient than ecotropically packaged vectors to infect rat mammary epithelial cells. Replication-defective retroviral vectors are collected from the culture media of these packaging cells and concentrated to 30- to 100-fold by centrifugation through a sucrose shelf. This vector is then mixed with polybrene plus a vital tracking dye, and infused into the central mammary ducts of recipient rats. Following infusion, the viral particles enter the ductal mammary cells which abut the glands' lumen. Following reverse transcription, virally encoded DNA is incorporated into the mammary genome. This incorporation requires mammary cell division in close temporal proximity to transfection. This process is maximized by increasing circulating levels of prolac-

tin, the mammary hormonal mitogen, during the period surrounding transfection.

In our initial studies, these procedures were evaluated using the reporter gene β-galactosidase. We demonstrated that up to ~1% of the cells could be functionally transfected. Surprisingly, transfected cells did not significantly lose the ability to express β-galactosidase over a three-month follow-up interval [1].

ONCOGENE TRANSFER

Our initial use of this model involved the introduction of activated forms of *ras* and *neu* oncogenes into rat mammary parenchyma. When v-Ha-*ras* with codon 12 and 59 mutations was used to infect less than 0.1% of the mammary epithelial cells, carcinomas developed without additional exogenous modifications. Each infected individual gland (12 glands/rat) had approximately a 50% chance of developing a carcinoma. Thus, not every infected cell progressed to cancer, suggesting that additional endogenous cellular events were required in addition to an activated *ras* incogene to convert infected cells to cancer. Interestingly, *ras*-infected cells were susceptible to hormonal promotion as observed in chemically or radiation-initiated mammary glands. Mammary carcinomas developing after *ras*-vector infection morphologically resembled chemically induced carcinomas. However, *ras*-induced carcinomas were more readily transplantable into syngenic recipient rats, and more often locally invaded the surrounding muscle. Unlike chemically induced tumors, *ras*-induced carcinomas were only occasionally associated with distant metastasis [1].

In contrast, when retroviral vectors containing the mutated *neu* oncogene were used to infect *in situ* mammary epithelial cells, carcinomas developed in higher frequencies with shorter latencies when compared to *ras*-infected cells (Fig. 1). In addition, *neu*-infected cells were over 200 times more likely to progress to carcinomas [3,4]. Inter-

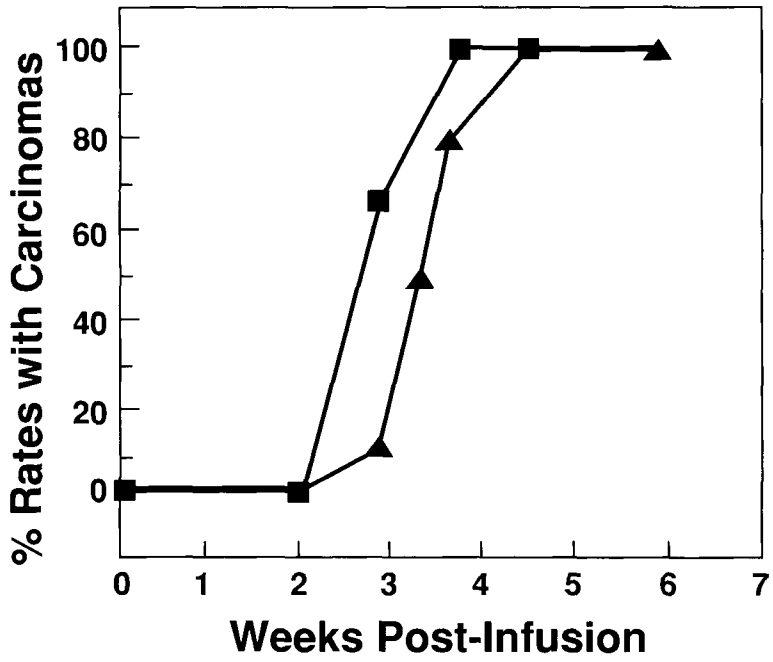

Fig. 1. Kinetics of tumor induction following *neu* oncogene transfer. WF (▲) and Cop (■) rats were pretreated with perphenazine, and 15 μl of $1x10^7$ CFU/ml JT-*neu* retrovirus suspension were infused into each gland. Tumor development was monitored by palpation. (From ref. 3, with permission of the publisher).

WF = Wistar-Furth
Cop = Copenhagen
CFU = colony-forming units

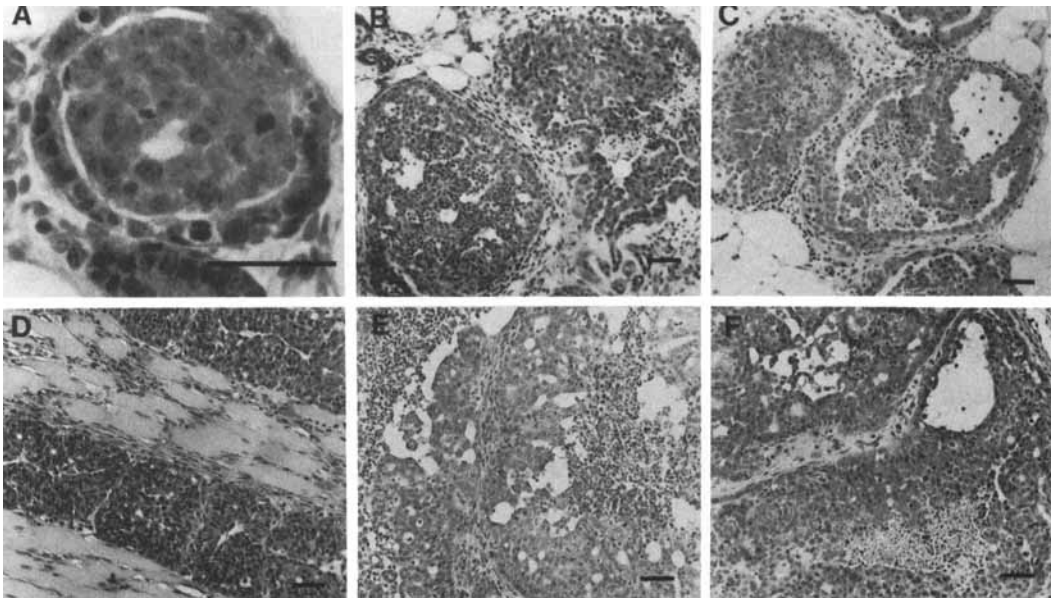

Fig. 2. Histopathological examination of the *neu*-induced mammary carcinomas. (A) Microcarcinoma in a WF rat 6 days after infection. Note the normal mammary ducts below the microcarcinoma. The clear space is the fat tissue. (B) Mammary carcinomas with cribriform comedo type morphology resected from a WF mammary gland 14 days after infection. Note the central necrosis in the tumor on the right. (C) A similar cluster of mammary carcinomas from a Cop rat mammary gland 14 days after infection. Central necrosis is also apparent in these tumors. (D) Muscle invasion by a WF carcinoma 16 weeks after infection. (E) WF mammary carcinomas 3 weeks after infection. Note the extensive necrosis on the right and left, characteristic of cribriform comedo morphology. Similar carcinomas were frequently found in Cop rats (F). Bars, 50 μm. (From ref. 3, with permission of the publisher.)

WF = Wistar-Furth
Cop = Copenhagen

estingly, the histopathological appearance and morphological progression of these cancers resembled human breast cancer much more than *ras*-associated or carcinogen-initiated mammary cancers. Six days post-*neu* infection, multiple micro-carcinomas (Fig. 2, Panel A) were found throughout the mammary gland. Within 14 days post-infection, these lesions progressed to *in situ* carcinomas with a cribriform-comedo morphology (Fig. 2, Panels B, C) resembling the histopathology of the equivalent human carcinoma at this progression stage. Over time, large aggressive cancers developed that were quite invasive (Fig. 2, Panel D). This similarity to the progression of human breast cancer suggests that the *neu* gene transfer model would be useful to evaluate early biomarkers such as micro-carcinoma, carcinoma *in situ*, and *neu* activation. In addition, this model should provide an improvement in the preclinical evaluation of breast prevention agents and strategies. Thus far, we have evaluated the efficacy of these models using two diverse classes of breast prevention agents as detailed below.

PREVENTION STUDIES

Hormonal

The most highly documented approach to the prevention of breast cancer in women is hormonal manipulation which reduces circulating estrogen levels, modifies estrogen metabolism, or blocks hormone cellular activities. Examples of these manipulations include ovariectomy to reduce circulating estrogen levels or the use of antiestrogenic drugs such as tamoxifen to block

the cellular effects of estrogen. Similar approaches are used to treat breast cancer, but are usually maximally effective only in a subset of cancers that have functional estrogen receptors.

We used our retrovirus gene transfer mammary carcinogenesis model to ask whether ovariectomy could prevent rat mammary carcinomas induced by either the mutated *ras* or *neu* oncogene. We also asked if the results of a prevention study could be generalized to a therapeutic setting and *vice-versa*.

Isoeffective titers of the *ras*- and *neu*-encoding retroviral replication-defective virus were infused into the central mammary duct of Wistar-Furth (WF) rats. Two days post-infection, half of the rats receiving each virus were ovariectomized. All rats were then followed for mammary tumors by weekly palpation.

The results of this experiment highlighted a major difference in the response of activated *ras*- and *neu*-induced tumors to early ovariectomy. Ovariectomy did not significantly affect the latency (Fig. 3) or number of carcinomas developing per rat (Fig. 4) following *ras* infection. In contrast, ovariectomy both significantly extended latency (Fig. 3) and reduced the average number of developing carcinomas (Fig. 4) following *neu* infection [4].

Interestingly, this dramatic difference between the prevention effects of ovariectomy for *ras*- and *neu*-induced tumors did not extrapolate to a therapeutic setting. If ovariectomy was done on rats bearing established (>25 mm^2 cross-sectional area) *ras*- or *neu*-induced mammary carcinomas, a similar regression response was observed. Specifically, 55% of *ras*-induced tumors regressed following ovariectomy, while 63% of *neu*-induced tumors regressed following this procedure [4].

Together these data suggest that endocrine therapy would be useful for both the prevention and treatment of breast cancer with activated *neu*. These data also suggest that endocrine therapy may be more effective in preventing than treating breast cancer. Sixty percent of established cancers responded to late ovariectomy, while over 85% of developing breast carcinomas with *neu* activation were prevented by early ovariectomy.

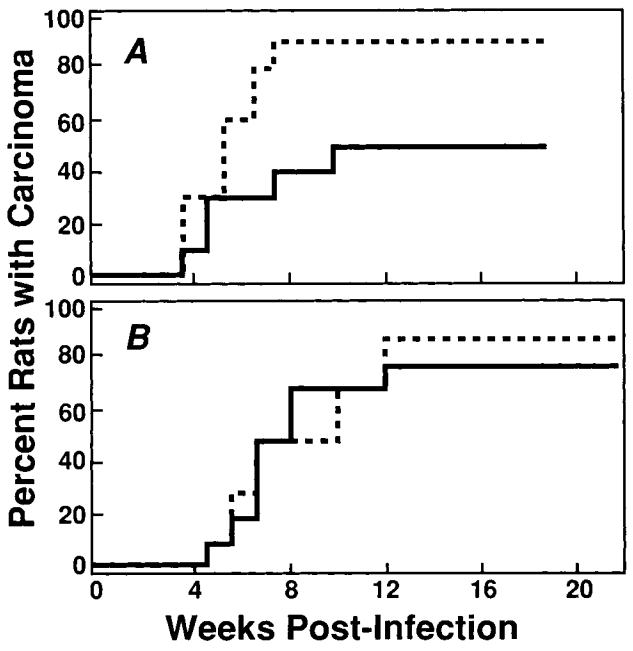

Fig. 3. Effects of early ovariectomy on the latency of mammary carcinoma development following *neu* (A) and *ras* (B) oncogene transfer into rat mammary epithelial cells *in situ*. About 15 µl of retrovirus vector suspension at $1x10^5$ CFU/ml (*neu*) or $2x10^7$ CFU/ml (*ras*) were infused into each of the 12 glands in every rat by central duct cannulation as described in the text. Two days after infusion, one group of rats (——, n = 10) was ovariectomized, while another group was left intact (- - -, n = 10). (From ref. 4, with permission of the publisher.)

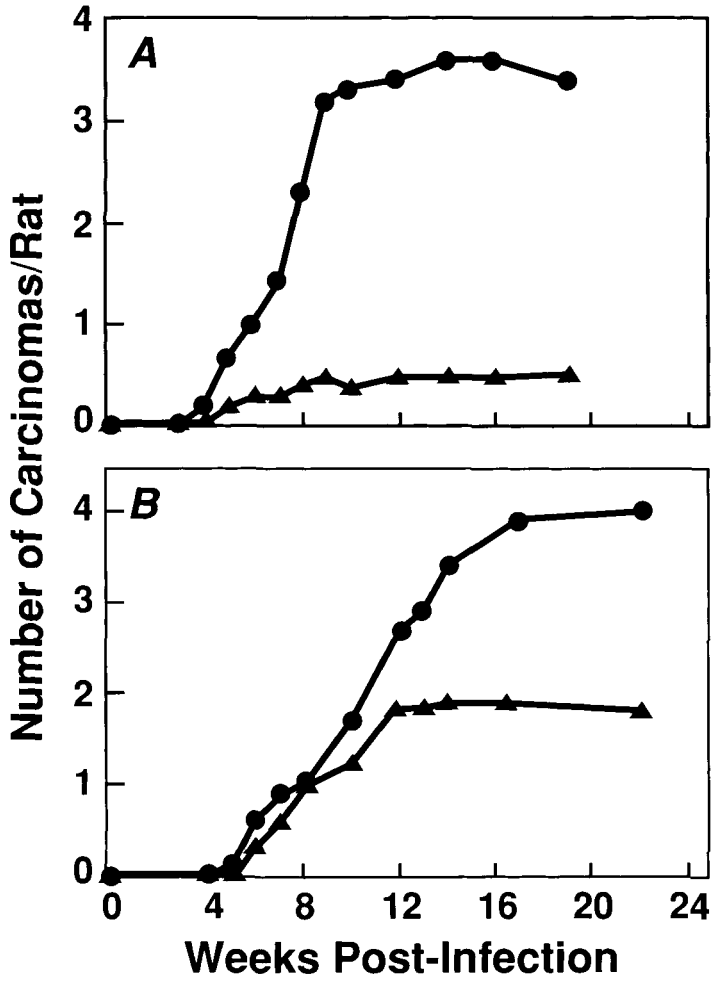

Fig. 4. Modulation of tumor incidence by early ovariectomy following *neu* (A) and *ras* (B) oncogene transfer into rat mammary epithelial cells *in situ*. The experimental procedures are given in Figure 3 and the text. Ovariectomized rats (▲, n = 10) and intact rats (●, n = 10) were compared. (From ref. 4, with permission of the publisher.)

Chemoprevention With Monoterpenes

Monocyclic monoterpenes, such as the orange oil component *d*-limonene, have shown broad applications in preventing chemically induced rat mammary carcinomas [5]. Limonene prevents DMBA-initiated mammary cancer when fed during either the initiation or the promotion/progression period [6]. Limonene also prevents mammary cancers initiated by the direct-acting carcinogen NMU when given during the promotion/progression period [7]. Interestingly, limonene's preventive activity extends to fully progressed cancer [8]. When rats with growing mammary carcinomas of 1 g or larger are fed limonene, the majority of cancers fully regress [9].

Limonene prevents mammary carcinogenesis at the initiation stage in the DMBA model by reducing the formation of DMBA-DNA adducts [10] via the induction of hepatic Phase II detoxification enzymes [11].

The terpenes' promotion/progression and therapeutic efficacy may result, at least in part, from their multiple cellular activities. These include the upregulation of genes such as the insulin-like growth factor II (IGF-II)/mannose 6-phosphate receptor, which facilitates the activation of latent transforming growth factor-β (TGF-β) [12]. In addition, terpenes and their metabo-

lites act as competitive inhibitors of the enzymes that prenylate proteins. Limonene and its *in vivo* metabolites can selectively block isoprenylation of small G proteins such as *ras* p21 [13]. This latter activity led us to test limonene's ability to prevent the development of rat mammary carcinomas following the *in situ* retroviral transfer of activated *ras* in mammary parenchyma.

Mammary carcinomas were induced in rats by intraductal infection with v-Ha-*ras*-containing retrovirus. Half of the rats were fed limonene-containing diets from infection through the end of the experiment. Rats were necropsied 18 weeks post-infection. The control rats developed an average of 7.6 carcinomas per rat, while those on limonene-enriched diets developed only 1.2 carcinomas per rat. No toxicity or weight loss was seen at necropsy in the limonene-fed rats. Thus, limonene was highly effective in preventing *ras*-initiated mammary carcinomas (unpublished observations).

CONCLUSIONS

We have developed a novel alternative rodent model to study the etiology of breast cancer, and for use in breast cancer prevention studies. In this model, activated oncogenes such as *ras* or *neu* can be directly transfected into mammary parenchyma *in situ* using "gene-therapy" retroviral vectors. Tumors which arise following infection with vectors containing either *ras* or *neu* are more aggressive than chemically induced mammary carcinomas. Those induced by *neu* histopathologically resemble human breast cancer at the various stages of progression. These models are ideal to evaluate early biomarkers that could be used in Phase II prevention trials, as well as new potential breast cancer preventive agents and strategies.

ACKNOWLEDGEMENT

This work is supported by PHS, NIH Grant CA38128.

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