

AD_____

Award Number: DAMD17-03-1-0551

TITLE: A Rat Model for Human Ductal Carcinoma In Situ

PRINCIPAL INVESTIGATOR: Satyabrata Nandi, Ph.D.

CONTRACTING ORGANIZATION: University of California
Berkeley, CA 94720

REPORT DATE: September 2006

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				<i>Form Approved</i> OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE (DD-MM-YYYY) 01-09-2006		2. REPORT TYPE Final		3. DATES COVERED (From - To) 1 Sep 2003 – 31 Aug 2006	
4. TITLE AND SUBTITLE A Rat Model for Human Ductal Carcinoma In Situ				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER DAMD17-03-1-0551	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Satyabrata Nandi, Ph.D. E-Mail: nandi@berkeley.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of California Berkeley, CA 94720				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Our objective was to develop a rodent model for human DCIS and LCIS in which lesions of diverse phenotypes could be induced and characterized and their malignant potential studied in a linear manner. We tested the effect of the infusion of EGF, FGF or IGF1 on the proliferation of mammary glands in ovariectomized rats. Infusion of EGF, FGF, or IGF1 resulted in end bud formation and lobuloalveolar development within 72 hours. Intraductal proliferation resulted only in rats treated with IGF1. These results indicate that infusion of growth factors into the mammary glands induces proliferation of different kinds of structures, including end buds, lobuloalveolar structures and intraductal proliferations and that the growth factors in ovariectomized rats can support neoplastic mammary transformation. EGF or IGF-1 were infused in ovariectomized rats using Alzet pumps and treated with the chemical carcinogen N-methyl-N-nitrosourea (MNU). The cancers were varied in their estrogen and progesterone receptor expression as determined by immunocytochemistry. The objectives of the project were not achieved due to the low incidence of mammary cancers induced following treatment with growth factors and MNU making it difficult to transplant lesions of different phenotypes and characterize the malignant potential.					
15. SUBJECT TERMS rat model, ductal carcinoma in situ, lobular carcinoma in situ					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			USAMRMC
			UU	17	19b. TELEPHONE NUMBER (include area code)

Table of Contents

Introduction.....	4
Body.....	45
Materials and Methods.....	7
Results.....	11
Key Research Accomplishments.....	14
Reportable Outcomes.....	15
Conclusions.....	15
References.....	15

Introduction

Our goals were to develop methods for the induction of a large number of ductal carcinoma in situ (DCIS) and lobular carcinoma in situ (LCIS) lesions with different phenotypes, be able to detect the lesions in situ and characterize the lesions as to their cancerous potential, hormone dependence or independence, and genetic changes. DCIS and LCIS are intraductal and intralobular hyperplasias. Proliferation of these cells is a prerequisite to carcinoma. However, intraductal proliferation with the exception of the terminal end bud occurring in peripubertal rats is extremely rare and has not been found in the terminal ductal structures during development or under experimental conditions. Our laboratory has made the novel finding that treatment of rats by infusing the mammary ducts with a combination of epidermal growth factor, cortisol, and cholera toxin causes extensive intraductal proliferation in the terminal ducts with a proliferation labeling index as high as 75% (1,2). Keratinocyte growth factor (KGF) is a member of the fibroblast growth factor family (FGF 7) that is secreted by stromal cells and acts on epithelial cells. Treatment of intact rats with KGF causes massive intraductal hyperplasias (3). KGF can cause ductal growth and intraductal hyperplasias in ovariectomized mice. Concomitant treatment with estradiol and progesterone plus KGF increases intraductal hyperplasias(). The intraductal hyperplasias regress after withdrawal of the mitogenic stimulus. We thought that it would be possible to induce intraductal/intraalveolar hyperplasias by a variety of means and then treat with different chemical carcinogens to cause a large number of immortalized transformed phenotypes resembling DCIS and LCIS (4,5). These unique mitogens that cause intraductal proliferation have not been used, to our knowledge, in combination with ductal or alveolar mitogens in attempts to develop DCIS and LCIS. We believe that these treatments should result in an expanded pool of target cells for DCIS and LCIS which can then be transformed to preneoplastic and neoplastic states with well known mammary carcinogens such as N-methyl-N-nitrosourea (4), N-ethyl-nitrosourea, dimethylbenz(a)anthracene, or radiation.

Body

Ductal carcinoma in situ (DCIS) and lobular carcinoma in situ (LCIS) of the breast are proliferations of potentially premalignant or malignant breast epithelial cells within the ducts or lobules without invasion into the surrounding stroma (1-3). The detection rate has increased by over 500% in recent years due to the introduction of high-resolution mammography (1-3). Patients diagnosed with DCIS or LCIS have an increased risk for subsequent development of invasive breast cancer. Most patients diagnosed with DCIS have no symptoms and no palpable lesions. DCIS is classified into five histopathological subtypes: papillary, micropapillary, cribriform, solid, and comedo (1-3).

The comedo subtype is associated with high nuclear grade, aneuploidy, high proliferation rate, HER2/neu gene amplification or overexpression, and a clinically more aggressive behavior. The histopathological classification is made complex because the different subtypes may contain elements of other subtypes and high nuclear grade can be found in any of the subtypes and there is no single unified classification. DCIS and LCIS do not fully express the malignant phenotype, as at presentation they lack the ability to invade and metastasize. It is of major importance to identify and understand why some in situ lesions progress to a malignant state and others do not (1,2).

A thorough understanding of the factors that influence the progression to malignancy of DCIS and LCIS is needed. However, the necessary experimental studies to sequentially follow the progression of a lesion with its accompanying phenotypic and genotypic changes cannot be performed in humans. Additionally, surgical samples of human DCIS are difficult to obtain for xenograft studies in nude mice.

Significant similarities can be found between breast cancers in humans and rats. Human and rat cancers are thought to originate in the terminal ductal structures (4,5), cancers can be hormone dependent or independent (6), and a full term pregnancy provides protection from breast cancer in humans and carcinogen induced breast cancer in rats (7). Additionally, the rat experimental system has been used as model for the development of tamoxifen treatments currently used in humans (8).

A human xenograft model for DCIS is available for studies. It is based on a premalignant variant of the MCF 10 human breast cell line (9). Upon transplantation into nude mice it produces a mass that has the morphology of a comedo DCIS and after several weeks it becomes invasive. This model has potential for studying the progression of DCIS to malignancy but it only represents a single lesion.

Transgenic mice have been produced that are models for DCIS. The WAP promoter linked to SV40 large T antigen (10) and a component of rat prostate steroid binding protein linked to SV40 large T antigen (11) have been used to target the mammary epithelial cells. The mice develop ductal atypia, DCIS, and in time infiltrating ductal carcinoma. All of the carcinomas have been found to be hormone independent. The hormone dependence of the DCIS and carcinomas was not described.

Intraductal proliferations, DCIS, LCIS, and carcinomas can be induced in rats by carcinogen treatment (5,12). It is hypothesized that there is a stepwise progression of these lesions through these morphological stages but it has been difficult to directly study these progressions. Genes involved in cell cycle control including cyclins, cyclin-dependent kinases and cyclin-dependent kinase inhibitors are altered in the immortalized ducts and early alveolar hyperplasias in the mouse (13). There is an increase in MAP kinase associated with cancer progression in ovariectomized rats (14). It has been demonstrated in C3(1)/SV40 large T antigen (TAg) transgenic mice that *bax* is a critical suppressor of mammary cancer progression at the stage of preneoplastic mammary lesion development through the upregulation of apoptosis, but this protective effect is lost during the transition from preneoplasia to invasive carcinoma(15). Transplantation studies of lesions have provided evidence that carcinomas can arise from lobular or ductal lesions however; there is disagreement as to which lesions are the main preneoplastic lesions in the rat (5,16-18). The difficulty in studying these lesions is that there are few lesions after carcinogen treatment, the lesions can be in any one of twelve mammary glands, the lesions are extremely small and difficult to find in situ, and there has been an inability to expand the lesions in vivo to determine their neoplastic potential and hormone dependency.

We hypothesized that lesions with different phenotypes could be induced by varying the mammogenic mitogen around the time of initiation with a carcinogen. We have previously demonstrated in vitro and in vivo in mice and rats that the mitogenic environment around the time of carcinogen exposure determines the phenotype and genotype of the resultant mammary cancers (19-21).

Our goals were to develop methods for the induction of a large number of DCIS and LCIS lesions with different phenotypes, be able to detect the lesions in situ and characterize

the lesions as to their cancerous potential and hormone dependence or independence. We believed that these goals were attainable in view of our previous studies (21,22) and studies from other laboratories (23). DCIS and LCIS are intraductal and intralobular hyperplasias. Proliferation of these cells is a prerequisite to carcinoma. However, intraductal proliferation with the exception of the terminal end bud occurring in peripubertal rats is extremely rare and has not been found in the terminal ductal structures during development or under experimental conditions. We have made the novel finding that treatment of rats by infusing the mammary ducts with a combination of epidermal growth factor (EGF), cortisol, and cholera toxin (CT) causes extensive intraductal proliferation in the terminal ducts with a proliferation labeling index as high as 75% (22). Keratinocyte growth factor (KGF) is a member of the fibroblast growth factor family (FGF 7) that is secreted by stromal cells and acts on epithelial cells. Treatment of intact rats with KGF causes massive intraductal hyperplasias (24). KGF can cause ductal growth and intraductal hyperplasias in ovariectomized mice. Concomitant treatment with estradiol and progesterone plus KGF increases intraductal hyperplasias (25). The intraductal hyperplasias regress after withdrawal of the mitogenic stimulus. We thought that it would be possible to induce intraductal/intralobular hyperplasias by a variety of means and then treat with different chemical carcinogens to cause a large number of immortalized transformed phenotypes resembling DCIS and LCIS. These unique mitogens that cause intraductal proliferation have not been used, to our knowledge, in combination with ductal or alveolar mitogens in attempts to develop DCIS and LCIS. We believed that these treatments would result in an expanded pool of target cells for DCIS and LCIS which could then be transformed to preneoplastic and neoplastic states with well known mammary carcinogens such as N-methyl-N-nitrosourea or N-ethyl-nitrosourea.

Our experimental objectives were to develop methods for the induction of intraductal and intralobular proliferation using combinations of mammogenic hormones, growth factors, and CT. Methods were attempted to better visualize hyperplastic areas in situ by using systemic trypan blue injection or intraductal infusion of the dye. The best methods were selected for induction of intraductal proliferation and intralobular proliferation, the rats were treated with N-methyl-N-nitrosourea (MNU) in attempts to immortalize and transform the proliferations to a preneoplastic state. MNU was the carcinogen of choice because it is a highly efficient mammary carcinogen in the rat, has low toxicity, and has a very short half-life at physiological pH so that their effect occurs within a defined short period of time (26,27). Selected lesions were to be transplanted to the gland free mammary fat pads of syngeneic female hosts to determine their neoplastic potential and whether they continue to proliferate or not following ovariectomy of the host. Microarray was to be done to identify differentially expressed genes in the preneoplastic lesions with differing neoplastic potential.

Our specific aims were:

Specific Aim 1. Development of methods for the induction of DCIS and LCIS in inbred Lewis rats (months 1-12).

- a) intralobular hyperplasia (3 rats/treatment).
- b) Quantitate proliferation by immunocytochemistry and confirm intraductal or intralobular hyperplasia by histology.
- c) Transform treatment induced hyperplasia by administration of MNU (5 rats/group)

- d) Transplant transformed lesions to gland-free fat pads of host rats and determine the morphology of the outgrowths.

Specific Aim 2. Characterization of lesions as to their neoplastic potential and hormone dependency (months 12-36)

- a) Select lesions for expansion by serial transplantation based on their ability to proliferate and their neoplastic potential (5 rats/group).
- b) Characterize resultant outgrowths and cancers as their ovarian hormone dependence.

Specific Aim 3. Identification of biomarkers to distinguish between DCIS/LCIS with or without neoplastic potential (months 18-36).

- a) Identify differentially expressed genes in DCIS/LCIS with different hormonal responsiveness and different neoplastic potential by microarray technology.
- b) Confirm microarray results by immunocytochemistry or real time polymerase chain reaction.

Materials and Methods.

Animals. Sprague Dawley rats were purchased from Charles River Laboratories (Wilmington, MA) and kept in our vivarium for 1 week before initiation of the experiment. Rats were kept in a temperature controlled room with a 12 h light and 12 h dark schedule and fed Teklad 8640 (Teklad, Madison, WI) and water ad libitum.

Growth Factors and Hormones. Recombinant growth factors KGF, FGF α , IGF-1 and HGF were obtained from US Biologicals (Swampscott, MA) and EGF, bovine growth hormone (bGH) and prolactin (Prl) were obtained from Sigma (St. Louis, MO). Cholera toxin and corticosterone were obtained from Sigma (St. Louis, MO).

N-methyl N-nitrosourea (MNU) Injection. N-methyl N-nitrosourea (MNU) was obtained from Sigma (St. Louis, MO) and dissolved to a concentration of 10 mg/ml in 0.9 % sodium chloride adjusted to pH 5.0 which had been warmed to 50-60 °C. At 7 weeks of age a single i.p.injection of 50 mg/kg MNU was given to each female Sprague Dawley rat.

Hormone Treatment. Capsules were prepared as previously described (13). Control capsules were prepared with 30 mg of microgranular cellulose (Sigma, St. Louis, MO). Thirty milligrams of progesterone (P) (Sigma, St. Louis, MO) capsules were packed without cellulose, and 17 β -estradiol E₂ (Sigma, St. Louis, MO) capsules were also mixed with cellulose to produce capsules that totaled 30 milligrams (13, 14). All capsules were readied for implantation by incubation at 37 °C overnight in Dulbeccos's Modified Eagle's Medium (Invitrogen, Grand Island, NY) (13).

Tumors and Tumor Palpation. Rats were palpated weekly and length and width of tumors were measured with calipers. When tumors reached 1 cm or larger they were removed and fixed in phosphate buffered formalin for 18-24 h and then transferred to 70% ethanol before embedding in paraffin and sectioning. All tumors were then sectioned at 5 μ m and stained with

hematoxylin and eosin and the histological type of mammary cancer determined. Tumors types were cribiform, papillary and comedo. Rats were terminated when their third mammary tumor reached 1 cm as required by our University of California Animal Care and Use Committee. All University of California ACUC guidelines were followed. The experiment was terminated at 39 weeks following MNU injection. At the termination of the experiment all rats were necropsied and their mammary glands removed, fixed in phosphate buffered formalin (10%), defatted in acetone for 2 days and stained with iron hematoxylin. Also, at this time all previously palpated tumors were removed, fixed in phosphate buffered formalin (10%) for 18-24h, transferred to 70% ethanol, embedded in paraffin and 5 µm sections obtained for H&E and ER/ PR staining.

Experiment #1: Effect of intraductal infusion of hormones and growth factors on proliferation of the mammary gland in intact Sprague Dawley rats. Fifty seven female Sprague Dawley rats were obtained from Charles River (Wilmington, MA) and at 6 weeks the #3 and #4 mammary glands were injected with 15 µl of growth factors or hormones dissolved in PBS (19 groups of 3 rats each). One rat from each group was sacrificed at 24 hours and 2 rats at 72 hour and the #3 and #4 glands were taken and fixed in phosphate buffered formalin (10%), defatted in acetone for 2 days and stained with iron hematoxylin or alum carmine. For time line see Figure 1.

Figure 1. Effect of intraductal infusion of hormones and growth factors on mammary proliferation in intact Sprague Dawley rats

ID injection	Terminate	Terminate
↑	↑	↑
45 Days	24 hr	72 hr

Experiment #2: Effect of intraductal infusion of EGF, IGF-1, and FGF on proliferation of the mammary gland in ovariectomized Sprague Dawley rats. Nine female Sprague Dawley rats obtained from Charles River (Wilmington, MA) were ovariectomized at 45 days of age. The rats were divided into three groups of 3 rats each and one group was injected intraductally with 15 µl of 1 µg of recombinant epidermal growth factor (EGF) dissolved in PBS into the left #3 and #4 mammary glands and on the right side with 5 µg of recombinant EGF. The other two groups of rats were injected intraductally either with recombinant fibroblast growth factor acidic (FGFa) or recombinant insulin like growth factor one (IGF-1) similarly with 1 µg into the left #3 and #4 mammary glands and 5 µg on the right #3 and #4 mammary glands. One rat from each group was sacrificed at 24 hours and 2 rats at 72 hours and the #3 and #4 glands were taken and fixed in phosphate buffered formalin (10%), defatted in acetone for 2 days and stained with iron hematoxylin or alum carmine. For time line see Figure 2.

Figure 2. Effect of intraductal infusion of EGF, IGF-1 and FGF on mammary proliferation in ovariectomized Sparague Dawley rats

OVX	ID Injection	Terminate	Terminate
↑ 45 Days	↑ 60 Days	↑ 24 hr	↑ 72 hr

Experiment #3: Effect of growth factor exposure with pumps followed by MNU injection on mammary carcinogenesis. From Charles River 131 female Sprague Dawley rats were obtained at 21 days of age. At 25 days of age 2 were terminated, 109 were ovariectomized and 20 were left intact. To check for complete ovariectomy rats were smeared for a week before initiation of Alzet pump and hormonal pellet implantation. At 46 days of age, 2 intact and 2 ovariectomized rats were terminated in order to compare mammary glands prior to growth factor treatment. Alzet pumps from Alza Corporation which expressed 0.5 µl/hr were used to deliver 10 µ of growth factor a day for 1 week were implanted subcutaneously into rats (Figure 3). Five days after pump implants at 51 days of age MNU (50 mg/kg) was injected i.p. and the pumps were removed 2 days later when the rats were 53 days of age. Also at 53 days of age, 2 intact and 2 ovariectomized rats were terminated in order to compare the initial effects of MNU injection. The rats were palpated and weighed weekly until the termination of the experiment 9 months after MNU injection. All mammary glands were taken and fixed in phosphate buffered formalin (10%), defatted in acetone for 2 days and stained with iron hematoxylin. For details of experimental protocol see Figure 3.

Administration of growth factors with pumps were an attempt to induce hormone dependent and hormone independent mammary tumors using different growth factors.

Different Experiment Groups. Sprague Dawley rats (groups II-VII) were ovariectomized at 25 days of age. Pumps were implanted at 46 days of age into intact and ovariectomized rats. Five days after pump implants at 51 days of age, rats were injected i.p. with MNU (50 mg/kg) and the pumps removed 2 days later when the rats were 53 days of age. Upon removal of the pumps at 53 days of age, silastic capsules containing various steroids were implanted and were exchanged every 60 days until the termination of the experiment 9 months after MNU injection. On days 25, 46 and 53 of age rats from each different group were also terminated and mammary glands removed to determine the initial conditions of the mammary gland.

Figure 3.

Termination Schedule of All Treatment Groups. Rats in the 3rd and 4th terminations had been injected with MNU.

<u>Groups</u>	<u>1st Term</u> <u>25 days</u>	<u>2nd Term</u> <u>46 days</u>	<u>3rd Term</u> <u>53 days</u>	<u>4th Term</u> <u>11 months</u>	
1. Intact (no pump)	2(20)	2(18)	2(16)	16	= 22
2. OVEX (no pump)	0(19)	2(17)	2(15)	15	= 19
(a) empty pellets			(a) 8 (b) 7	(a) 8 (b) 7	
(b) 30 mg P					
3. OVEX + EGF (pump)	0(18)	0(18)	2(16)	16	= 18
(a) 20 µg E			(a) 8 (b) 8	(a) 8 (b) 8	
(b) 20 µg E + 30 mg P					
4. OVEX + IGF ₁ (pump)	0(18)	0(18)	2(16)	16	= 18
(a) 20 µg E			(a) 8 (b) 8	(a) 8 (b) 8	
(b) 20 µg E + 30 mg P					
5. OVEX + OV Tr (no pump)	0(18)	0(18)	0(18)	18	= 18
(a) 20 µg E			(a) 9 (b) 9	(a) 9 (b) 9	
(b) 20 µg E + 30 mg P					
6. OVEX + EGF (pump)	0(18)	0(18)	2(16)	16	= 18
(a) 20 µg E			(a) 8 (b) 8	(a) 8 (b) 8	
(b) 20 µg E + 30 mg P					
7. OVEX + IGF ₁ (pump)	0(18)	0(18)	2(16)	16	= 18
(a) 20 µg E			(a) 8 (b) 8	(a) 8 (b) 8	
(b) 20 µg E + 30 mg P					

Results

Experiment #1: Effect of Intraductal Infusion of Hormones and Growth Factors on Proliferation of the Mammary Gland. Seven week old Sprague Dawley rats (nineteen groups of 3 rats each) were injected intraductally through the nipple into the #3 and #4 mammary glands with 15 μ l of material dissolved in PBS. One rat per group was sacrificed at 24 h and the 2 remaining rats per groups at 72 h. At termination their mammary glands were removed, fixed in phosphate buffered formalin overnight, defatted with acetone and stained either with alum carmine or iron hematoxylin. The glands were then microscopically examined for the presence of end buds (EB), lobuloalveolar ductal structures (LAD) and intraductal proliferations (IDP) and photographed, and areas which showed IDPs were histologically confirmed by cutting out, embedding and staining with H&E. All 19 groups were subjectively scored for presence of EB, LAD, and IDP. See Table 1.

Table 1.

Group	Treatment (per 15 μ l)	24 HR			72 HR		
		EB	LAD	IDP	EB	LAD	IDP
1	PBS	-	-	-	+	+	-
2	CT (1 ng)	+	++	-	+	++	-
3	CT (10 ng)	+	+++	-	+	+++	-
4	CT (100 ng)	+	+++	-	+	+++	-
5	EGF (100 ng)	+	++	+	+	++	-
6	EGF (1 μ g)	+	++	+	+	++	+
7	EGF (5 μ g)	++	+	+	++	++	+
8	FGFa	+	++	+	+	+++	+
9	FGFa	+	+++	+	+	+++	+
10	Heregulin (100 ng)	+	++	+	+	++	+
11	Heregulin (1 μ g)	+	++	+	+	++	+
12	IGF-1 (1 μ g)	+	++	-	+	++	-
13	IGF-1 (5 μ g)	+	++	-	+	++	-
14	KGF (100 ng)	+	+	-	+	++	+
15	KGF (400 ng)	+	+	-	+	++	+
16	E (10 ng)	+	+	-	+	+	-
17	E + P (10 ng + 5 μ g)	+	+	-	+	+	-
18	bGH (5 μ g)	+	++	-	+	+	-
19	PRL (5 μ g)	+	++	-	+	+	-

Only the infusion of the growth factors EGF, FGF, heregulin, and KGF induced intraductal proliferations in intact rats. The infusion of the hormones, E, P, bGH, and Prl resulted in the stimulation of the proliferation of end buds, and lobuloalveolar-ductal structures.

Experiment #2: Effect of Intraductal Infusion of EGF, IGF-1 and FGF on Proliferation of the Mammary Gland. Nine female Sprague Dawley rats obtained from Charles River (Wilmington, MA) were ovariectomized at 45 days of age. The rats were divided into three groups of 3 rats each and one group was injected intraductally with 15 μ l of 1 μ g of epidermal growth factor (EGF) dissolved in PBS into the left #3 and #4 mammary glands and on the right side with 5 μ g of recombinant EGF. The other two groups of rats were injected intraductally either with recombinant fibroblast growth factor acidic (FGFa) or recombinant insulin like growth factor one (IGF-1) similarly with 1 μ g into the left #3 and #4 mammary glands and 5 μ g on the right #3 and #4 mammary glands. One rat from each group was sacrificed at 24 hours and 2 rats at 72 hour and the #3 and #4 glands were taken and fixed in phosphate buffered formalin (10%), defatted in acetone for 2 days and stained with iron hematoxylin. The glands were examined for the presence of EB, LAD, and IDP and subjectively scored. See Table 2.

Table 2.

Group	Treatment (per 15 μ l)	24 HR			72 HR		
		EB	LAD	IDP	EB	LAD	IDP
1	1 μ g EGF	+	+	-		+	-
	5 μ g EGF	+	+	-	+	+	-
2	1 μ g FGFa	+	+	-		+	-
	5 μ g FGFa	+	+	-	+	+	-
3	1 μ g IGF-1	-	++	+	+	+	-
	5 μ g IGF-1	-	++	+	+	+	-

Summary of results

Infusion of IGF-1 but not EGF or FGF resulted in the induction of intraductal proliferations in ovariectomized rats.

Experiment #3: Effect of Growth Factor Exposure with Pumps followed by MNU injection on Mammary Carcinogenesis. Summary of incidence of rats with mammary tumors over total rats in the group (% of rats with mammary tumors in parentheses). See Tables 3. For ER and PR results of mammary tumors see Tables 4 and 5.

Table 3. Incidence of mammary cancer in ovariectomized in MNU treated Sprague Dawley rats

Treatment	Incidence	Total No. Tumors
Intact	16/16 (100%)	47
OVX, Control	3/29 (10.3%)	3
OVX, EGF	4/30 (13.3%)	9
OVX, IGF-1	6/28 (21.4%)	9

Approximately six months after carcinogen treatment 100 % of the intact control rats developed mammary cancers. There were few mammary cancers in the growth factor treated groups either treated or not treated with estradiol and progesterone for promotion. The mammary cancer incidence was 10.3% in the ovariectomized control rats, 13.3% in the EGF treated group and 21.4% in the IGF-1 treated group (Table 3). Immunocytochemical analysis of the mammary cancers from the intact controls determined that ER and PR were highly expressed in the cancer cells, The breakdown of the combined groups are described in Table 4, and the breakdown of all the various subgroups is shown in Table 5.

Previously, we have found that infusion of growth factors directly into the mammary nipple results in rapid proliferation of the mammary epithelium in ovariectomized rats and when treated with carcinogen up to 75% of the animals develop mammary cancers.

Summary of ER and PR of Mammary Tumors. Total ER or PR incidence is expressed as over total tumors in group (% of mammary tumors positive or negative for ER and PR in parentheses). See Table 4.

Table 4. Immunocytochemistry of Er and Pr of intact and ovariectomized Sprague Dawley rats (combined groups).

Treatment	Er+ Pr+	Er+ Pr-	Er- Pr+	Er- Pr-
Intact	38/47 (80.9%)	3/47 (6.4%)	4/47 (8.5%)	2/47 (4.3%)
OVX, Control	2/3 (66.7%)	0/3 (0%)	0/3 (0%)	1/3 (33.3%)
OVX, EGF	4/9 (44.4%)	2/9 (22.2%)	1/9 (11.1%)	2/9 (22.2%)
OVX, IGF-1	5/9 (55.6%)	1/9 (11.1%)	0/9 (0%)	3/9 (33.3%)

The incidence of mammary tumors positive for both Er and Pr was high in the intact controls. The incidence of mammary tumors positive for both Er and Pr was lower in the ovariectomized rats treated with EGF or IGF-1.

Comparison ER negative and PR negative mammary tumors from intact and ovariectomized rats over total tumors (% of mammary tumors ER and PR negative in parentheses). See Table 5.

Table 5. Immunocytochemistry of Er and Pr of intact and ovariectomized Sprague Dawley rats.

Group	Treatment	Er+ Pr+	Er+ Pr-	Er- Pr+	Er- Pr-
I	Intact	38/47 (80.9%)	3/47 (6.4%)	4/47 (8.5%)	2/47 (4.3%)
II A	OVX control capsule	0/1	0/1	0/1	1/1
II B	OVX 30mg P	0	0	0	0
III A	OVX 20ug E	0	0	0	0
III B	OVX 20ug E+30mg P	2/2	0/2	0/2	0/2
IV A	EGF control capsule	0/1	1/1	0/1	0/1
IV B	EGF 30mg P	0	0	0	0
V A	EGF 20ug E	0/1	0/1	1/1	0/1
V B	EGF 20ug+30mg P	4/7	2/7	0/7	1/7
VI A	IGF-1 control capsule	0/1	1/1	0/1	0/1
VI B	IGF-1 30mg P	0/3	0/3	0/3	3/3
VII A	IGF-1 20ug E	4/5	1/5	0/5	1/5
VII B	IGF-1 20ug+30mgP	1/1	0/1	0/1	0/1

The incidence of mammary tumors positive for both Er and Pr was high in the intact rats. It was difficult to determine the effect of growth factors on the Er or Pr incidence because of the low number of mammary tumors induced.

Key Research Accomplishments

- Demonstrated that a single infusion of growth factors or mammogenic hormones infused through the nipple of the mammary gland results in rapid proliferation of the mammary epithelium within 72 hours in intact rats (Table 1).
- Demonstrated that a single infusion of EGF, FGF, or IGF-1 into ovariectomized rats results in the rapid proliferation of ducts and lobules (Table 2).
- Infusion of IGF-1 but not EGF or FGF into ovariectomized rats results in the induction of intraductal proliferations (Table 2).
- Demonstrated that the growth factor IGF-1 is capable of supporting mammary neoplastic transformation in ovariectomized rats (Table 3).
- We found that ovariectomized rats treated with growth factors at the time of carcinogen administration produce a higher percent hormone independent mammary cancers but the overall incidence of mammary cancer induced was low (Table 4).

Reportable Outcomes

None

Conclusions

Taken together, these results indicate that 1) proliferation of the mammary epithelium can be induced quickly by intraductal infusion of mammogenic hormones and growth factors. 2) The pattern of proliferation varies with the hormone or growth factor used. Interestingly, intraductal proliferations could only be induced by the infusion of growth factors into the ducts but not by infusion of hormones into ducts. Intraductal proliferations are only seen in the normal mammary gland in end buds. Intraductal proliferations are observed following the administration of carcinogen and are thought to be the primary carcinogenic lesion. 3) The induced proliferations can be neoplastically transformed with a chemical carcinogen. These findings suggest that it should be feasible to induce proliferation of the mammary epithelium with different agents and neoplastically transform them resulting in lesions with different morphologies and different phenotypes and genotypes. However, it might be more productive to develop means for a more efficient neoplastic transformation by determining the optimal time of proliferation following treatment with growth factors and once that is established to transplant the mammary epithelial cells within a short time after treatment with carcinogen and select outgrowths of the treated cells that actively proliferate and give rise to lesions of differing phenotypes

References

1. Silverstein, M.J. Ductal Carcinoma In Situ of the Breast. (2000) *Annu. Rev. Med.* 51: 17-32.
2. Libby, A.L., Zellars, R.C., O'Connell, P., and Allred, D. C. (1999) Biological characteristics of premalignant breast disease. In *Molecular Pathology of Early Cancer*. Srivastava, S. ed. IOS Press. 79-95.
3. Allred, D.C. and Mohsin, S.K. (2000) Biological features of premalignant disease in the human breast. *J. Mam. Gland Biol. And Neoplasia*, 5: 351-364.
4. Cardiff, R.D. and Wellings, S.R. The comparative pathology of human and mouse mammary glands. (1999) *J. Mam. Gland Biol. Neoplasia* 4: 105-122.
5. Russo, J., Gusterson, B.A., Rogers, A.E., Russo, I.H., Wellings, S.R., and van Zweiten, M.J. (1990) Comparative study of human and rat mammary tumorigenesis. *Lab invest.* 62:244-278
6. Welsch, C.W. (1985) Host factors affecting the growth of carcinogen-induced rat mammary carcinomas: A review and tribute to Charles Brenton Huggins. *Cancer Res.* 45: 3415-3443.
7. Russo, I.H., and Russo, J. (1998) Role of hormones in mammary cancer and initiation. *J. Mam. Gland Biol. And Neoplasia* 3: 49-62.
8. Ip, C. (1996) Mammary tumorigenesis and chemoprevention studies in carcinogen treated rats. *J. Mam. Gland Biol. Neoplasia* 1: 37-48.

9. Miller, F.R., Santner, S.J., Tait, L., and Dawson, P.J.(2000) MCF10DCIS.com xenograft model of human comedo ductal carcinoma in situ. *J Natl Cancer Inst.* 92:1185-6.
10. Schulze-Garg, C., Lohler, J., Gocht, A., and Deppert, W. (2000) A transgenic mouse model for the ductal carcinoma in situ (DCIS) of the mammary gland. *Oncogene* 19: 1028-1037.
11. Green, J. E., Shibata, M.-A., Yoshidome, K., Liu, M., Jorcyk, C., Anver, M.R., Wigginton, J., Wiltout, R., Shibata, E., Kaczmarczyk, S., Wang, W., Liu, Z., Calvo, A., and Couldrey, C. (2000) The C3(1)/SV40 T-antigen transgenic mouse model of mammary cancer : ductal epithelial targeting with multistage progression to carcinoma. *Oncogene* 19: 1020-1027.
12. Thompson, H.J., McGinley, J.N., Wolfe, P., Singh, M., Steele, V.E., and Kelloff, G. (1998) Temporal sequence of mammary intraductal proliferations, ductal carcinomas in situ and adenocarcinomas induced by 1-methyl-1-nitrosourea in rats. *Carcinogenesis*. 19:2181-5
13. Said TK, Moraes RC, Singh U, Kittrell FS, Medina D.(2001)Cyclin-dependent kinase (cdk) inhibitors/cdk4/cdk2 complexes in early stages of mouse mammary preneoplasia. *Cell Growth Differ* 12:285-95.
14. Gudmundur Thordarson, Adrian V. Lee, Meghan McCarty, Katharine Van Horn, Oriana Chu, Yu- Chien Chou, Jason Yang, Raphael C. Guzman, Satyabrata Nandi, and Frank Talamantes (2001) Growth and characterization of *N*-methyl-*N*-nitrosourea-induced mammary tumors in intact and ovariectomized rats. *Carcinogenesis* 22: 2039-2047.
15. Shibata MA, Liu ML, Knudson MC, Shibata E, Yoshidome K, Bandey T, Korsmeyer SJ, Green JE. (1999) Haploid loss of *bax* leads to accelerated mammary tumor development in C3(1)/SV40-TAg transgenic mice: reduction in protective apoptotic response at the preneoplastic stage. *EMBO J* 18:2692-701
16. Beuving, L.J., Faullkin, L.J., DeOme, K.B., and Bergs, V.V., Hyperplastic lesions in the mammary glands of Sprague Dawley rats after 7,12 dimethylbenz(a)anthracene treatment. (1967) *J Natl.Cancer Inst.* 39: 423-429.
17. Haslam, S.Z., and Bern, H.A. (1977) Histopathogenesis of 7,12 dimethylbenz(a)anthracene-induced rat mammary tumors. *Proc. Natl. Acad. Sci. USA* 74: 4020-4024.
18. Hwang, S.I., Guzman, R.C., Swanson, S.M., and Nandi, S. (1996) Hormone dependent and independent mammary tumor development from *N*-methyl-*N*-nitrosourea treated rat mammary epithelial xenografts in the nude mouse: multiple pathways and *H-ras* activation. *Cancer Lett.* 196 101:123-34.
19. Nandi, S. Guzman, R.C., and Yang, J. (1995) Hormones and mammary carcinogenesis in mice rats, and humans: A unifying hypothesis. *Proc.Natl. Acad. Sci. USA* 92: 3650-3657.
20. Chou, Y.-C., Guzman, R.C., Swanson, S.M., Yang, J., Lui, H.M., Wu, V., and Nandi, S. Induction of mammary carcinomas by *N*-methyl-*N*-nitrosourea in ovariectomized rats treated with epidermal growth factor. (1999) *Carcinogenesis* 20: 677-684.
21. Guzman, R.C., Imagawa, W., Yang, J., Miyamoto, S., and Nandi, S. (2000) Chemical –carcinogen-induced transformation of primary cultures of mouse mammary epithelial cells grown inside collagen gels. In: *Methods in Mammary Gland Biology and Cancer Research*, Ip, M.M. and Asch, B.B. eds. Kluwer Academic/Plenum Publishers, New York.125-137.

22. Popnikolov, N., Yang, J., Guzman, R.C., Swanson, S.M., Thordarson, G., Collins, G., Talamantes, F., and Nandi, S. (1995) In vivo growth stimulation of collagen gel embedded normal human and mouse primary mammary epithelial cells. *J. Cell. Physiol.* 165: 51-60.
23. Thompson, H.J. and Singh, M. (2000) Rat models for premalignant breast disease. *J. Mam. Gland Biol. And Neoplasia*, 5: 409-420.
24. Ulich, T.R., Yi, E.S., Cardiff, R., Yin, S., Bikhazi, N., Biltz, R., Morris, C.F., and Pierce, G.F. (1994) Keratinocyte growth factor is a growth factor for mammary epithelium in vivo. *Am. J. Path.* 144: 862-868.
25. Yi, E., Bedoya, A., Lee, H., Kim, S., Housley, R., Aukerman, S., Tarpley, J., Starnes, C., Yin, S., Pierce, G., and Ulich, T. (1994) Keratinocyte growth factor causes cystic dilation of the mammary glands of mice. *Am. J. Path.* 145: 1015-1022.
26. Swanson, S.M., Guzman, R.C., Tsukamoto, T., Huang, T.T., Dougherty, C.D., and Nandi, S. (1996) N-Ethyl-N-nitrosourea induces mammary cancers in the pituitary-isografted mouse which are histologically and genotypically distinct from those induced by N-methyl-N-nitrosourea. *Cancer Lett.* 102:159-65.
27. Thompson, H.J. and Adlakha, H. (1991) Dose responsive induction of mammary gland carcinomas by the intraperitoneal injection of 1-methyl-1-nitrosourea. *Cancer Res.* 51: 3411-3415.

Personnel

Raphael C. Guzman, Research Biologist
Edward Blank, Staff Research Associate III
Anne Wong, Staff Research Associate I