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INTRODUCTION

Our ability to prevent breast cancer by rationally designed intervention requires a better understanding of the etiology of this disease. While the incidence of breast cancer continues to rise, our understanding of the causes underlying both this disease and its increasing incidence are poorly understood. Classical and molecular epidemiology has significantly improved our understanding of the etiology of several cancers such as lung, gastric, head and neck, and bladder. Most epidemiological studies of breast cancer have failed to identify major factors underlying the initiation of breast cancer even though several modifying factors that either promote or inhibit breast cancer development have been identified. A possible explanation for the inability to identify breast cancer initiating agents is suggested by the intensive epidemiological studies of breast cancer etiology in the survivors of the Hiroshima and Nagasaki A-bomb radiation exposures. These studies showed that radiation, even at low doses, causes breast cancer (1). The greatest risk of developing breast cancer was among those exposed when less than ten years of age. The risk of those exposed when 10 to 19 years old was slightly but significantly lower. Risk decreased rapidly with age at exposure thereafter; women over 40 were only minimally susceptible to radiation carcinogenesis (1).

These findings suggest that epidemiological studies that seek environmental and lifestyle factors underlying breast cancer initiation may lose much of their power by restricting their observations to cohorts of middle-aged women (the group that is usually included in most published epidemiological studies). While breast cancer frequently occurs in this middle-aged population, this group of women may have only a minimal sensitivity to the various environmental agents under study, and breast cancer initiation is likely to have occurred decades earlier under a very different environment/lifestyle than is current for this older cohort of women. In addition to studies of radiation carcinogenesis of A-bomb survivors, similar age versus risk trends have been reported in both medically irradiated patients (2), and in several epidemiological studies of cigarette smokers (3). For example, Brinton et al. report an increased relative risk to breast cancer in women who began smoking before age 17 (3). This is consistent with our *in vitro* studies with primary cultures of human breast epithelial cells in which we demonstrated the genotoxicity of cigarette smoke condensates (4).

Since the first reports of the strong dependence of age at exposure to radiation on breast cancer risk, its importance has been widely recognized; however, its underlying mechanism remains obscure. Before the current epidemiological data set became available, it was only clear that younger females were more susceptible to breast cancer initiation than older women. Accordingly, it was speculated that susceptibility was related to breast development and specifically to the increased rate of parenchymal cell division during breast maturation in the teenage girl. This hypothesis is supported by the data of Russo et al. (5) that shows that susceptibility to DMBA-induced mammary cancer in rats occurs at a period of high breast cell division as the rat becomes sexually mature. In contrast, the most recent epidemiological analysis includes cancers detected in A-bomb survivors through 1985 and shows that the age at exposure for maximum risk of breast cancer initiation by radiation is before puberty and the accompanying menarchal increase in breast cell mitosis (1). Thus the full data set suggests that some aspect of the biology of the immature human breast results in maximal sensitivity to breast cancer initiation. It should also be kept in mind that while the immature breast is most susceptible to breast cancer initiation, the maturing breast (teens to early 20's) is more susceptible to initiation than the breast of middle-aged, pre-menopausal women. This intermediate sensitivity may be rooted in the increased mitotic rate of the maturing breast parenchyma. Thus at

least two physiologic processes may underlie susceptibility to breast cancer initiation: that associated with the immature breast and that associated with the developing breast. The literature has provided little to explain the former observation mechanistically; however, we have recently generated a novel hypothesis that may explain it.

We have shown that the breast stem-cells of sexually immature rats are highly sensitive to the cytotoxic effects of ionizing radiation (6). This sensitivity decreases and then disappears as the rats begin to mature (6). These data have led to our central hypothesis that the inability of the mammary parenchyma of the immature rat to recover from radiogenic cellular damage may underlie an increased susceptibility to radiation-induced genetic lesions that lead to the genesis of breast cancer. It is further hypothesized that this sensitivity to radiation can be extended to other environmental agents. Defining and characterizing the role of the high radiosensitivity of the immature mammary gland in a rat model will provide key data for future studies to assess interspecies extrapolation to women.

Sensitivity to Radiation Cytotoxicity of Mammary Cells as a Function of Age

In order to investigate the effect of age on radiation sensitivity, we irradiated rats at various ages ranging from 1 through 12 weeks of age and assayed their mammary clonogenic (stem) cells' survival. When immature rats were irradiated with 5 Gy (500 rad) when 1, 2, 3 or 4 weeks of age, 10% of the mammary cells survived. In contrast, a 3-fold increase in survival of irradiated mammary cells was observed when mature rats at 8 or 12 weeks of age were irradiated. When complete radiation dose versus cell survival curves were generated, not only did we confirm the quantitative difference in survival of mammary cells from immature and mature rats at all doses tested, but we also found a qualitative difference in the shapes of the survival curves between pre-pubescent and post-pubescent rats. Mammary cell survival curves from irradiated 2- and 4- week-old rats were straight on a semilog plot, indicating a purely exponential cell killing. In contrast, survival curves of mammary cells from 8-week-old rats had an initial shoulder region followed by a terminal exponential portion more typical of mammalian cells. The most common explanation for an initial convex shoulder on a survival curve suggests that it reflects an ability of the cells to recover from low to moderate doses of radiation. It may be further speculated that since the major damage leading to cell death following radiation is damage to DNA, this recovery may consist of DNA damage repair. In addition to cell death, nonrepaired or misrepaired DNA damage can lead directly to mutation and thus indirectly to neoplastic transformation. It is thus speculated that the extremely high sensitivity of the immature human breast to radiation carcinogenesis may result from an increased accumulation of damaged DNA due to a diminished ability to correctly repair DNA damage.

A second period of increased sensitivity may also occur during sexual maturation and gland development. This period of intermediate sensitivity in women may be related to an increased mitotic rate during gland growth at sexual maturation (5).

The relative importance of the two periods of increased sensitivity to breast cancer susceptibility may be species dependent. In order to have the greatest chance of correlating the human and rat results, this proposal will focus on the period of highest sensitivity in the human: the immature gland. We will extend our initial observation of increased radiation sensitivity on cell killing during a homologous period in the rat and test the endpoints of DNA damage, mutagenesis

and carcinogenesis. We will also investigate possible molecular mechanisms underlying the increased radiation sensitivity of the immature mammary parenchyma.

Mechanisms Underlying Radiation Sensitivity of the Immature Mammary Gland

Knowledge of the cellular and molecular biology underlying radiation damage has been expanding rapidly over the last several years. Our group has found that epidermal growth factor (EGF) can modulate radiation sensitivity. We have shown that in two human primary epithelial cell culture systems, breast (7) and prostate (8), the removal of epidermal growth factor from a defined growth medium before and during radiation increases the radiation sensitivity of both cell types. This effect has been shown to be independent of proliferation status (7,8). Others have shown that the removal of EGF inhibits the repair of radiation DNA strand breakage (9).

The *in situ* mammary gland produces both EGF and transforming growth factor α (TGF α) which signal through the same receptor. However, it is suggested on the basis of cellular and glandular distribution that only TGF α acts within the parenchyma of the gland via an autocrine/paracrine mechanism, whereas EGF is often apically secreted and not locally active (10). In elegant studies, TGF α mRNA was not found to be present in the immature mouse mammary gland; however, it is readily detectable in the maturing gland and persists in the adult virgin gland (10).

We hypothesize that the lack of TGF α in the immature gland leads to the observed increased radiation sensitivity that could extend to mutagenic and oncogenic sensitivity in this immature tissue. We plan to test this hypothesis directly as well as to further explore alternative molecular mechanisms underlying this age-dependent increased radiation sensitivity in the mammary gland.

Establishing the cellular and molecular mechanism underlying the increased sensitivity of the immature breast to carcinogenic environmental exposures will possibly lead to better designs for breast cancer epidemiological studies and to new prevention strategies. For example, if we show that it is likely that the radiation sensitivity of the immature breast extends to chemical xenobiotics then it would suggest that epidemiological studies seeking agents that initiate breast cancer focus on young girls. Secondly, for example, if we demonstrated that this increased sensitivity of the immature breast is due to a low level of mammary gland associated TGF α then this would suggest new pharmacologic breast cancer prevention approaches using either TGF α or preferably non-peptide small molecules with TGF α activity.

PURPOSE

The overall goal of this proposal is to explore the hypothesis that the diminished ability of mammary cells from immature rats to recover from cytotoxic radiation damage may extend to an increased susceptibility to mammary carcinogenesis. If so, such a mechanism may also underlie the observation that the immature breast of pre-pubescent human females is the developmental stage most highly susceptible to breast cancer initiation.

SPECIFIC AIMS

In order to achieve our overall goals, we will address the following specific questions using a rat model:

1. Does the sensitivity to cell killing by ionizing radiation in immature glands extend to various classes of xenobiotic chemical carcinogens including those acting via bulky adducts (DMBA) and alkylating small adducts (NMU)?
2. Does the irradiation of cells from immature mammary glands (in contrast to mature glands) result in a higher sensitivity to the induction of specific locus mutations? Is the spectrum of mutations different in cells from immature and mature glands?
3. Is the immature gland more sensitive to the scopol carcinogenic effect of radiation?
4. Does irradiation of the immature gland (in contrast to the mature gland) result in a) more extensive DNA damage, b) more poorly repaired damage, or c) a greater induction of apoptotic cell death?
5. Is the lack of TGF α production by cells of the immature mammary gland related to the increased sensitivity of radiation-induced cell killing?
6. How is the spectrum of gene expression in the immature and mature mammary glands different with regard to genes which could directly or indirectly confer altered cellular recovery capacity following cytotoxic and genotoxic damage?

BODY - Final Report

Aim 1: Does the sensitivity to cell killing by ionizing radiation in immature glands extend to various classes of xenobiotic chemical carcinogens including those acting via bulky adducts (DMBA) and alkylating small adducts (NMU)?

The purpose of the aim was to investigate the *in vivo* cytotoxicity of 3 versus 8 week old F344 mammary gland following exposure to either NMU or DMBA using a mammary cell transplantation assay. This aim has been completed.

Materials and Methods

Animals

Virus-free F344 female and male rats were obtained from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). The breeding of the rats to create 3 week old F344 female donor rats was performed at our facility. All other F344 female rats were obtained directly from Harlan Sprague-Dawley, Inc. All rats were provided with Teklad Lab Blox chow (Harlan Teklad, Madison, WI) and acidified water ad libitum. The rats were housed under a 12 hour light and 12 hour dark cycle.

Carcinogen Treatment

At either 3 or 8 weeks of age, F344 female rats (n=2-5 rats) were treated with either N-nitroso-N-methylurea (NMU) or 7,12-dimethylbenz[a]anthracene (DMBA). The NMU was given as a single tail-vein injection at a dose of 80 mg/kg body weight. The NMU was dissolved in 10mg/ml acidified saline, pH 5.0 with acetic acid. The DMBA was administered as a single gastric intubation at a dose of 80 mg/kg body weight. The DMBA was prepared in 20 mg/ml sesame oil, heated in boiling water until dissolved and cooled to room temperature prior to administration.

Mammary Cell Transplantation Assay

Twenty-four hours after carcinogen treatment, the inguinal mammary fat pads of the treated, donor rats were excised following removal of all lymph nodes, and placed into a dish containing DMEM medium (Gibco, Grand Island, NY). The tissue was scissor minced and transferred into a flask containing collagenase (Worthington; type III, 200 mg/ml). After a two hour digestion at 37°C, DNase (Sigma; 7.5 units/ml) was added to each flask and the digestion continued for an additional 15 minutes. Following centrifugation, the cell pellet was washed and further digested with 0.05% trypsin and 0.02% EDTA for 10 minutes at 37°C. The cells were again pelleted, washed and resuspended for filtration. The cell suspension was filtered through 53µ nylon mesh and the mammary cells in the filtrate were collected, washed, resuspended and counted using a hemocytometer. The cell suspension was serially diluted 1:1 in media. To each of the final dilutions, a equal volume of 50% rat brain homogenate was added. A volume of 0.06 mls of each of the cell dilutions was injected into three sites in the interscapular white fat pad of 8-10 week old recipient rats. These recipient rats had received grafts 2 weeks before transplantation of the pituitary tumor MtT-F4, which induces the transplanted mammary cells to grow and differentiate. At 3 weeks after the transplantation of the mammary cells, the interscapular fat pads were removed, fixed, stained with alum carmine and examined using a dissecting microscope for the development of alveolar units.

The percentages of transplantation sites with an alveolar unit(s) identified was calculated for each cell dilution. This data was fit to the transplantation model of Porter et al. to give an alveolar dose 50% of AD₅₀ (11). An AD₅₀ is defined as the number of cells per injection required to produce at least one alveolar growth in 50% of the graft sites. This number can then be used to determine the frequency of clonogenic stem-like cells or the clonogenic fraction in the cell suspension (12,13). Clonogen survival following carcinogen exposure is estimated by dividing the value of the AD₅₀ of the control, non-treated group by the AD₅₀ of the treated group.

Results

Aim 1 - Table I. Cytotoxic effects of chemical carcinogens on rat mammary cells.

F344 donor mammary gland	Control		NMU		DMBA	
	AD ₅₀	Clonogen Survival	AD ₅₀	Clonogen Survival	AD ₅₀	Clonogen Survival
3 week - a	549	x	2046	27%	nd	nd
3 week - b	306	x	1034	30%	489	63%
3 week - c	344	x	1081	32%	407	85%
8 week - a	322	x	529	61%	516	62%
8 week - b	372	x	555	67%	516	72%

The final results obtained from the cell transplantation assays may be seen in the table above. The clonogen survival frequency of 8 weeks of age F344 mammary glands exposed to either 80 mg/kg NMU or DMBA for 24 hours averages 70%. This same frequency of clonogen survival is observed in 3 week old F344 mammary glands exposed to DMBA; however, NMU treatment of 3 week old F344 mammary gland shows an increase in cytotoxic effect, resulting in a clonogen survival frequency of ~30%. We conclude that immature (3 week old) mammary cells are more sensitive to cell killing following NMU exposure ($p < 0.0001$) than following DMBA exposure ($p = 0.2192$). Mature (8 week old) mammary cell appear slightly, but not significantly sensitive to cell killing following either NMU or DMBA exposure ($p = 0.3678, 0.1881$ respectively). NMU, a direct acting carcinogen, and DMBA, an indirect acting carcinogen, exert their cytotoxic effects by different molecular mechanisms. The results from these studies suggest 8 week old mammary gland exposed to either NMU or DMBA and 3 week old mammary gland exposed to DMBA are equally sensitive to induced cytotoxicity. In contrast, it is possible that the mammary gland of the 3 week old rats accumulate damage to a greater extent or are deficient in repairing the damage caused by the NMU as compared to the 8 week old rats treated with NMU. In addition, it appears that the 3-fold increase sensitivity to cell killing in immature mammary cells when exposed to NMU is similar to the 3-fold increased sensitivity in immature mammary cells exposed to ionizing radiation.

Aim 2: Does the irradiation of cells from immature mammary glands (in contrast to mature glands) result in a higher sensitivity to the induction of specific locus mutations? Is the spectrum of mutations different in cells from immature and mature glands?

The purpose of this aim is to determine whether the spectrum of ionizing radiation-induced mutations produced in the immature and mature rat mammary gland differs, possibly correlating with the differential survival of rat mammary epithelial cells.

Materials and Methods

Big Blue Transgenic Rat Mutagenesis Assay - Immature Big Blue transgenic rats (3 weeks of age upon exposure) will be bred in house under an agreement with Stratagene. Mature Big Blue transgenic rats (8 weeks of age upon exposure) will be purchased and housed under the same conditions as the immature rats for at least one week prior to dosing. Using a balanced block design, the rats will be dosed with 0, 20, or 50 mg/kg NMU by tail vein injection. There will be three rats per age per group per expression period in order to ensure isolation of sufficient quantities of RMECs. Three groups will be used at each age and dose (except the single pilot 20 mg/kg group) to provide triplicate samples. After the expression period of 1, 3, or 5 weeks of housing under standard conditions, the rats will be sacrificed. Primary RMECs will be isolated as described above with the following modifications: 1) There will be no trypsinization step. 2) Organoids caught on the filter will be used. 3) After the fast stick, the cells will be pelleted and flash frozen. Genomic DNA will be isolated by a procedure provided by Johan deBoer. Briefly, samples will be dounced and nuclear pellets will be collected, treated with a lysis solution, and dialyzed against TE buffer. Genomic DNA will be stored for the short term at 4° C prior to use.

Genomic DNA will be packaged and plated according to Stratagene's instructions (14,15). Briefly, 8µL of genomic DNA will be added to a red tube of Transpack packaging extract, followed by incubation at 30° C for 90 minutes. To that will be added 12 µL from a blue tube of Transpack packaging extract and the contents will be incubated an additional 90 minutes at 30° C. Sterile SM buffer will be added, chloroform will be added, a pretiter will be performed, the packaged DNA will be plated on large plates and incubated overnight. Blue mutant plaques will be scored and verified by replating; mutant frequencies (# blue plaques/# total plaques) will be determined. Alternative packaging and scoring methods are available.

Mutant phage stock will be used as a template in the polymerase chain reaction (PCR) using Stratagene's commercially available forward and reverse primers according to manufacturer's instructions in the Big Blue Cyclist DNA Sequencing kit. Sequencing gels will be run by the McArdle core facility. Jackpot mutations (the exact same mutation at the same site in the same group) will be excluded from numerical analysis to control for clonal expansion. Mutation frequencies and spectra will be compared between the immature and mature RMECs.

Results

At the beginning of this update period, it had been demonstrated that although the Big Blue transgenic mutagenesis assay could be performed in the laboratory (by the ability to detect limited mutants in the spleens of irradiated Big Blue mice), the system was not practical for the detection of ionizing radiation-induced mutations in rat mammary epithelial cells. The system is not well suited for the detection of large insertions or deletions, which are thought to be the critical lesions induced by ionizing radiation. However, the system is well suited for the detection of point mutations, which are induced by alkylating agents, including N-nitroso-N-methylurea, which also causes age-differential cytolethality in rat mammary epithelial cells. It was determined that work during this update period would focus on optimizing the Big Blue transgenic mutagenesis system to examine age-differential effects on mutant frequency, mutation frequency, and mutation spectra in immature and mature rat mammary epithelial cells.

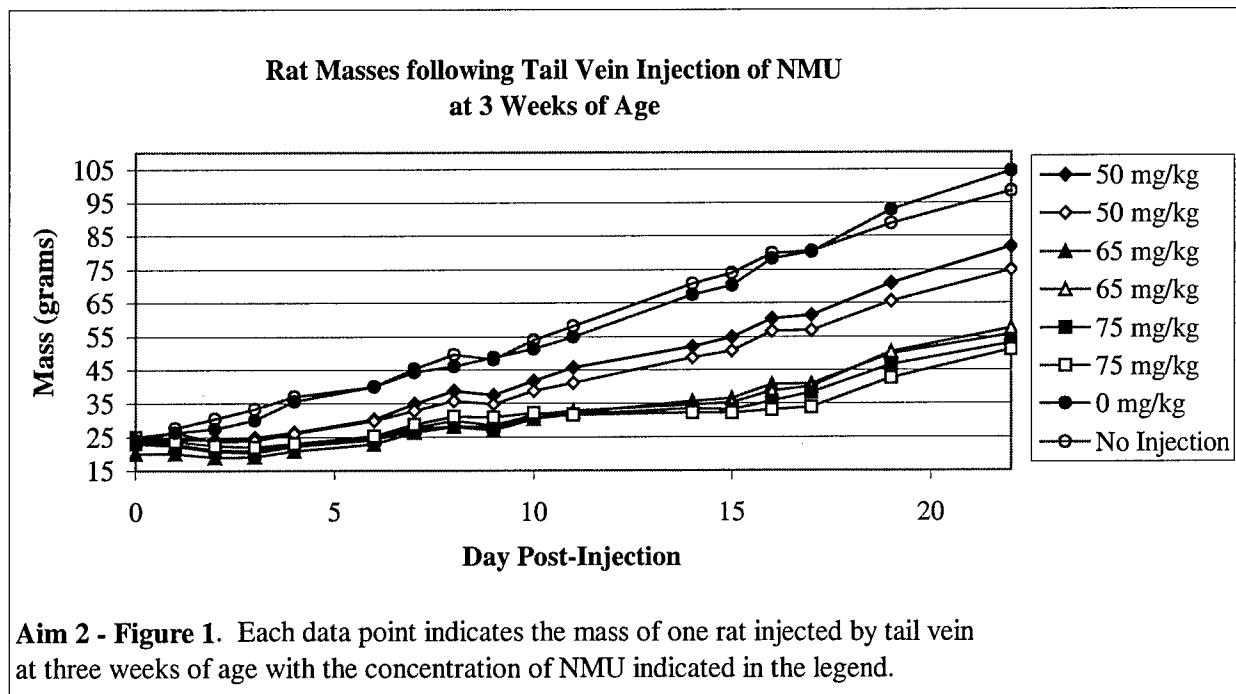
Because the doses of NMU used in the survival studies produced some gross toxicity after 24 hours and the rats used in Stratagene's Big Blue transgenic mutagenesis system would have to survive with minimal toxicity for an extended period, the first studies conducted period were to determine the optimal dose for use in the mutagenesis assay. The highest dose used was 75 mg/kg because of the known toxicity at 80 mg/kg (16). The lowest dose chosen was the lowest dose demonstrated in the literature to produce tumors, 50 mg/kg. Additionally, gross parameters of animal mass and organ mass as a percentage of body mass at time of necropsy were analyzed.

Aim 2 - Figure 1 displays the effect on body mass of NMU administered to Fischer 344 rats at three weeks of age by tail vein injection. Injection did not have a significant effect as shown by the lack of difference between the saline injected control (0 mg/kg in Aim 2 - Figure 1) and non-injected control (0 in Aim 2 - Figure 1). All doses resulted in an initial decrease in body mass relative to control rats. This decrease was more prolonged in the 65 mg/kg and 75 mg/kg rats than in the 50 mg/kg rats. By the end of the study all rats were gaining weight at a similar rate. No prolonged gross toxicity was observed.

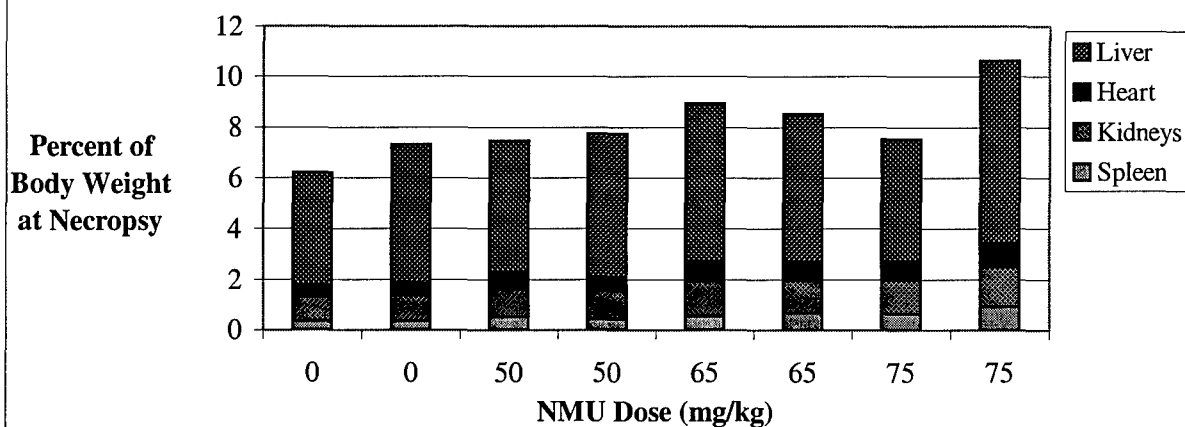
Aim 2 - Figure 2 displays the effect on specific organs of NMU administered to Fischer 344 rats at three weeks of age by tail vein injection. Organ masses were normalized to body mass at time of necropsy. NMU-induced effects were seen as enlargement of the spleen, kidneys, and heart as percentages of body mass. These effects were less pronounced in the 50 mg/kg rats than in those treated with higher doses. Because 50 mg/kg NMU produced the most minimal effects on specific organs and whole body mass, that dose was used in a pilot Big Blue transgenic mutagenesis experiment.

The first experiment performed was to determine the optimal expression period, the time between treatment and sacrifice of the animal for isolation of mammary epithelial cell genomic DNA that would produce the greatest mutant frequency at each age (Aim 2 - Figure 3). The same expression period would be used for both ages. The results confirm that mutants can be detected after treatment of rats with NMU by tail vein injection and that there is an age-dependent difference in mutant frequency in rat mammary epithelial cells. The expression period appeared to be more crucial in the mature mammary epithelial cells as there were greater differences in mutant frequency with expression period in the mature rats. Thus, the Big Blue transgenic mutagenesis system has been

established for the detection of NMU-induced mutations in rat mammary epithelial cells. Our data indicate that immature rat mammary epithelial cells are more susceptible to the mutagenic effects of NMU than mature rat mammary epithelial cells.

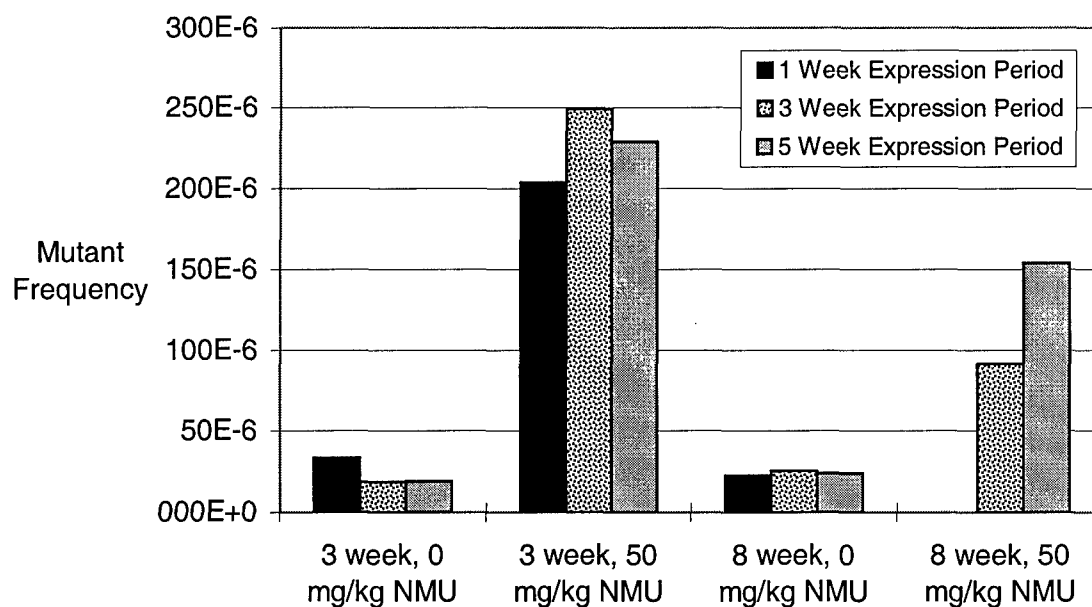


Effect on Organ Mass of NMU Tail Vein Injected at 3 Weeks of Age



Aim 2 - Figure 2. The effect of NMU on specific organs is displayed. Organ masses are normalized by dividing by the rat's body mass at time of necropsy. The same rats were used to produce Aim 2 - Figures 1 and 2.

**Rat Mammary Epithelial Cell Mutant Frequencies
with Expression Period following NMU Treatment *in vivo***



Aim 2 - Figure 3. Mutant frequencies from a preliminary Big Blue assay are shown. NMU induces mutants in MECs from both mature and immature rats. Note the higher mutant frequencies after all expression periods in the immature MECs.

Aim 3: Is the immature gland more sensitive than the mature gland to the scopol carcinogenic effect of radiation?

The purpose of this aim is to determine if the immature rat mammary gland in an intact 3 week old F344 rat is more sensitive to radiation-induced carcinogenesis than is the mature rat mammary gland in an intact 8 week old F344 rat.

Dosimetry: Anesthetized rats were irradiated with 6 Mev electrons from a Clinac 2300 medical linear accelerator. The rats were laid supine on the treatment couch and placed into a collimated radiation field from the bottom of their ears to the anus. A 1.5 cm thick slab of bolus (tissue equivalent material) was placed over their skin to reduce the range of the electrons into the body and to protect the ovaries. The top surface of the bolus was set at 100cm from the target of the accelerator.

Anesthetized intact rats were laid supine on a flat surface and measurements were taken (maximum length from rat's chin to anus, maximum width across rat and average distance from skin surface of rat to surface at location of ovaries). These measurements were taken for several rats in each age group. The mammary glands were assumed to extend to a depth of 0.5cm beneath the skin surface. The machine was programmed to deliver 2.5 Gy at a 2 cm depth. With the 1.5 cm bolus in place, this is 0.5 cm depth beneath the rat's skin. Because the dose decreases with depth, the tissue toward the skin received a higher dose. The maximum dose to the rat was calculated to be 3.2 Gy at the skin surface. The ovaries of the smallest rat (the smallest rat's ovaries would receive the highest dose) would receive a calculated dose of 12% of 2.5 Gy, or 0.3 Gy. To verify the dose received, two animals from each age group were irradiated as above after TLD's were inserted at 6 locations/rat (RB and LB, RD and LD, one at each ovary). The TLD's were read using a Victoreen Thermoluminescence Dosimeter Reader (Model 2800), normalized to a calibration standard exposed at the same time as the animals and the resulting micro Coulombs were converted into Gy (dose received).

Aim 3 - Table I. Calculation of irradiation dose to mammary glands and ovaries using TLDs.

Rat of Age x	Location of TLD	mCoulombs (average)	Dose in Gy (average)
3 week old	RB	5.95	3.23
3 week old	LB	5.85	3.18
3 week old	RD	5.59	3.04
3 week old	LD	NR	NR
3 week old	R ovary	NR	NR
3 week old	L ovary	3.61	1.95
8 week old	RB	4.14	2.25
8 week old	LB	4.84	2.63
8 week old	RD	4.25	2.31
8 week old	LD	4.56	2.48
8 week old	R ovary	2.38	1.29
8 week old	L ovary	1.87	1.02

NR=Not Reportable, TLD's were not readable due to moisture absorbed

Highest dose to mammary gland measured was 3.23 Gy, highest dose calculated was 3.2 Gy.

Highest dose to ovaries measured was 1.95 Gy, highest dose calculated was .3 Gy.

Results:

The following table lists the palpation/histopathological results for the tumors removed from the rats at 1 year post-treatment for the experimental groups.

Aim 3 - Table II. Mammary tumors identified in rats at 1 year post-irradiation.

		<u>untreated</u> <u>controls</u>	<u>8 week old</u> <u>~2.5 Gy</u>	<u>3 week old</u> <u>~2.5 Gy</u>
	# animals/group	36	31	31
carcinoma	total # tumors/group	0	2	0
adenoma	total # tumors/group	0	0	0
fibroadenoma	total # tumors/group	0	4	0
hyperplasia	total # tumors/group	0	0	0
carcinosarcoma	total # tumors/group	0	0	0

Due to the lack of tumors developing in the irradiated rats, it was speculated that the irradiation dose may have destroyed ovarian function in the rats. We checked all the rats for estrous cycling status over a three week period and it appeared that all the rats were going through normal estrous cycling. It was then speculated that the irradiation dose was not great enough to induced mammary tumors without some type of hormonal promotion. At one year post-irradiation (9/98), all the rats were thoroughly palpated. All tumors identified by palpation were removed for histopathological analysis. All animals then underwent hormonal manipulation, involving the removal of the adrenal glands and grafting of an isologous pituitary and slow release estron capsule in the spleen to reduce circulating levels of glucocorticoids and increase levels of prolactin respectively. Unfortunately, the animals did not tolerate the surgery well and many died within 3-4 days after the surgical procedure. Due to decreasing health of the remaining animals, all rats were necropsied on 11/5/98, approximately two months following hormonal manipulation. The following table shows the tumors which were collected at this time, including those removed prior to the surgery.

Aim 3 - Table III. Mammary tumors identified in the irradiated rats that survived hormonal manipulation surgery.

		<u>untreated</u> <u>controls</u>	<u>8 week old</u> <u>~2.5 Gy</u>	<u>3 week old</u> <u>~2.5 Gy</u>
	# animals/group	22	19	9
carcinoma	total # tumors/group	0	10	0
adenoma	total # tumors/group	0	2	0
fibroadenoma	total # tumors/group	0	6	1
hyperplasia	total # tumors/group	0	1	0
carcinosarcoma	total # tumors/group	0	1	0

From this data it may be concluded that the rats irradiated at 3 weeks of age were more likely to die following the hormonal manipulation surgery. In addition, the 3 week old irradiated rats which did survive did not develop any carcinomas as compared to the 8 week old irradiated rats.

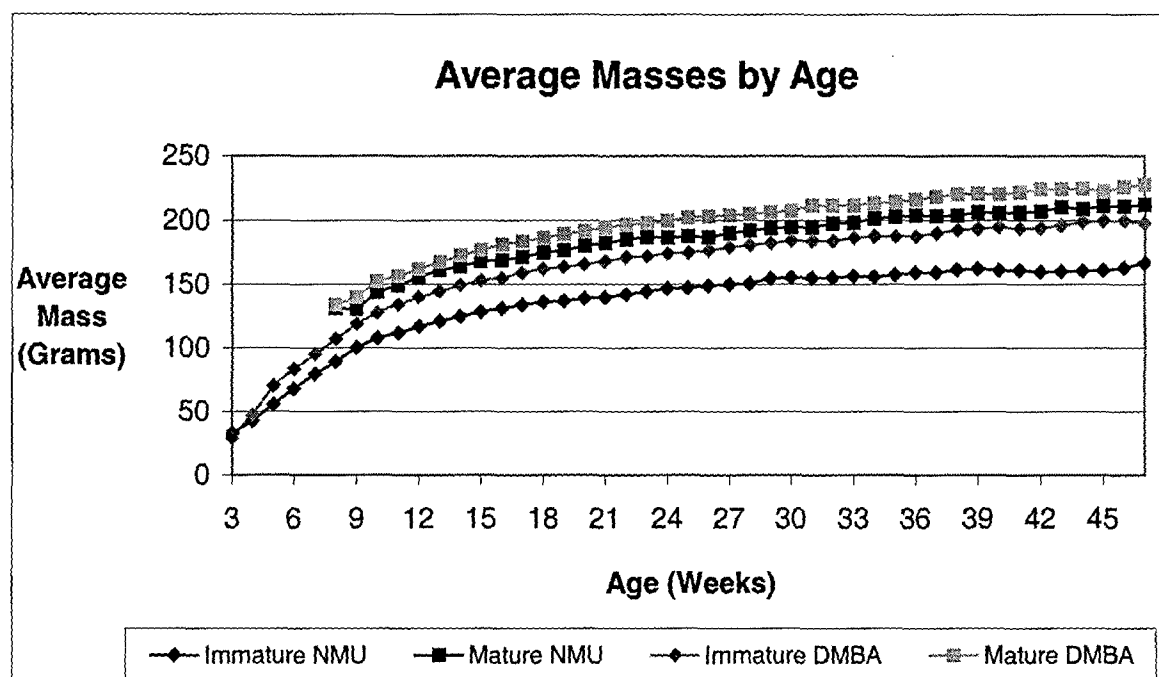
Because work conducted under Aim 1 revealed that N-nitroso-N-methylurea (NMU) produced age-differential survival but dimethylbenz(a)anthracene (DMBA) did not, the carcinogenicity studies of Aim 3 were expanded to include the two chemical carcinogens. Immature Fischer 344 rats were bred in house, and mature Fischer 344 rats were purchased and allowed to acclimate to the animal care facility. The immature and mature Fischer 344 rats were treated with NMU by tail vein or dimethylbenz(a)anthracene (DMBA) by oral gavage, both at 50 mg/kg. A total of thirty rats were treated per group. Initially, rats were handled and their masses recorded twice per week in order to acclimate the rats to palpation. Thereafter, the rats were palpated and their masses recorded weekly.

Aim 3 - Figure 1 displays the masses of the rats throughout the experiment. As shown, the animals treated with NMU show reduced masses compared to the DMBA-treated rats, with a more pronounced effect in immature than mature rats.

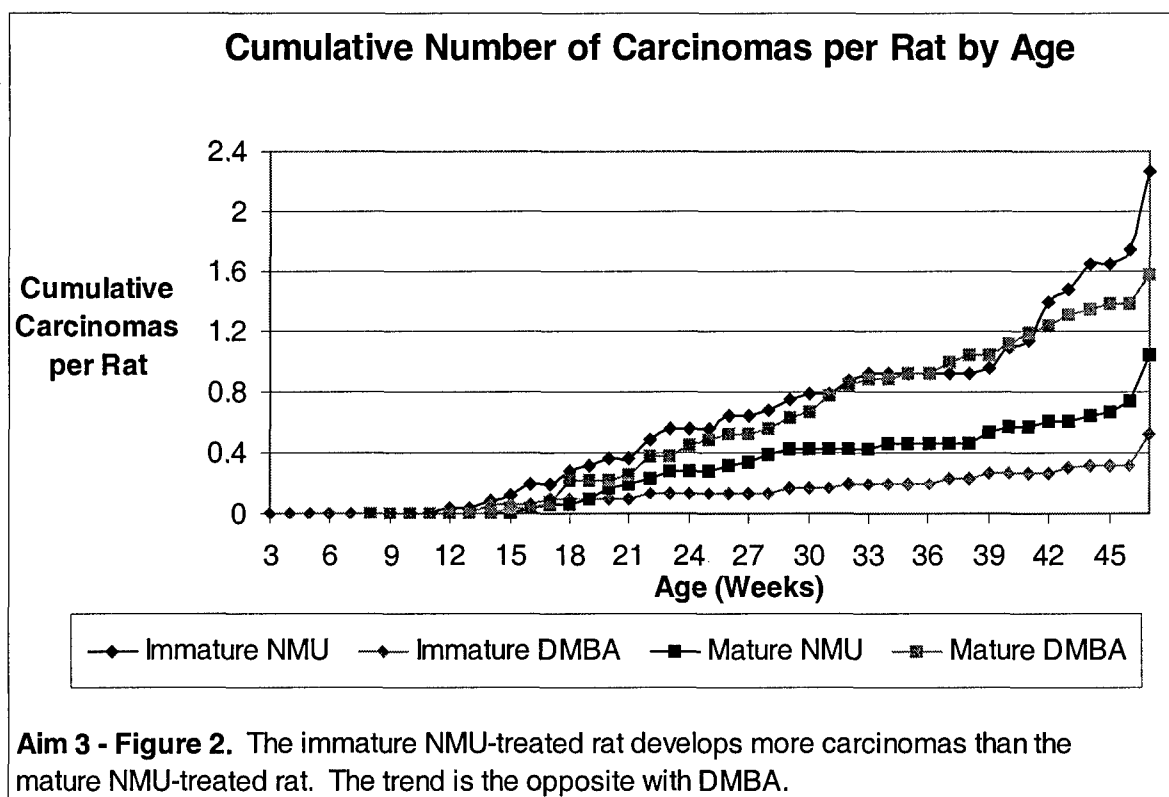
Aim 3 - Figure 2 displays the cumulative number of carcinomas per rat according to age. As shown, the group with the greatest number of carcinomas per rat was the immature NMU group, with the immature DMBA group accumulating the lowest tumor burden. By the end of the experiment, the immature NMU group had an average of 2.3 carcinomas per rat, while the mature NMU group had an average of 1.0 carcinomas per rat. The trend was the opposite with DMBA; immature rats only had 0.5 tumors per rat compared to the adults, which had 1.6 carcinomas per rat. With NMU, the immature rats developed greater than twice the number of tumors per rat as the mature rats.

Aim 3 - Figure 3 displays the percentage of rats with carcinomas by age. Fifty percent of immature rats treated with NMU and mature rats treated with DMBA had carcinomas by 23 weeks post-treatment, which was at 26 and 31 weeks of age, respectively. Fifty percent of mature rats treated with NMU had carcinomas by 39 weeks post-treatment, which was at 47 weeks of age. By the end of the experiment, only 41.4 percent of immature rats treated with DMBA had carcinomas. The latency, then, is much shorter in immature NMU-treated rats than mature NMU-treated rats.

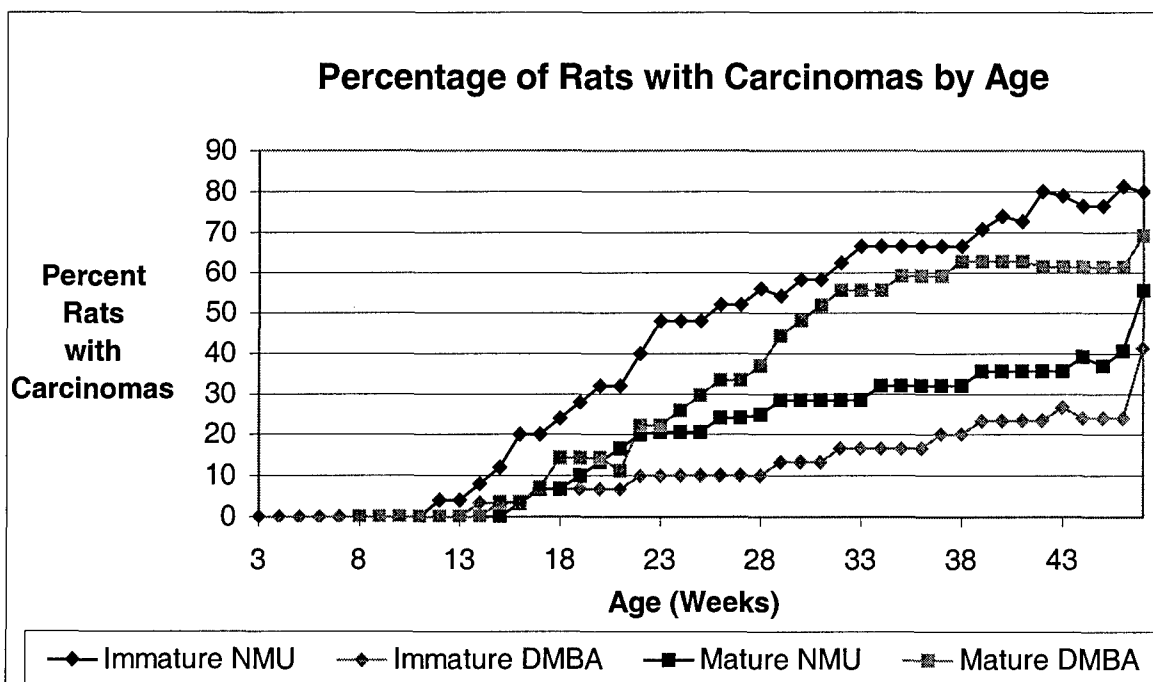
Thus, both of these parameters indicate that the immature RMEC is more susceptible to the carcinogenic effects of NMU – but not DMBA – than the mature RMEC. This correlates with the increased susceptibility of the immature RMEC to the cytolethal effects of NMU.



Aim 3 - Figure 1. Immature rats treated with NMU display the greatest mass effect.



Aim 3 - Figure 2. The immature NMU-treated rat develops more carcinomas than the mature NMU-treated rat. The trend is the opposite with DMBA.



Aim 3 - Figure 3. Tumors arise first in the immature NMU-treated rat.

Aim 4: Does irradiation of the immature gland (in contrast to the mature gland) result in a) more extensive DNA damage, b) more poorly repaired damage, or c) a greater induction of apoptotic cell death?

The purpose of this aim is to determine whether the age-differential survival of the irradiated rat mammary epithelial cells (RMECs) is due to differing amounts of DNA strand single or double strand breaks induced in the RMECs or the repair of such damage.

Methods and Materials

The alkaline comet assay has been successfully performed using human mammary epithelial cells (17) and to detect alkylation damage (18-21). Primary rat mammary epithelial cells were isolated from Fischer 344 rats as described for the treatment in culture limiting dilution *in vivo* transplantation assay as described in Aim 1.

The alkaline comet assay was performed the day after plating as described with the noted differences (17,18). Medium was drawn off the cells and fresh complete hormonal medium (CHM) containing 0.1 mM NMU was added to triplicate plates of cells in a staggered start fashion to ensure that all cells were treated for exactly 30 minutes at 37 C. For repair studies, NMU-containing CHM were aspirated and the cells were rinsed twice with $\text{Ca}^{++}/\text{Mg}^{++}$ - free phosphate buffered saline (PBS), and fresh CHM (without NMU) was added. The cells were allowed to incubate for the appropriate time after which the cells were trypsinized. For analysis without repair, the CHM containing NMU was aspirated; the cells were rinsed twice with $\text{Ca}^{++}/\text{Mg}^{++}$ - free PBS and the cells were trypsinized. CHM was added to stop the trypsinization and cells were counted.

To 200 μL of cells at 80,000 cells/mL was added 1.5 mL of 1% low melting point agarose held at 40° C. The cells were spread on fully frosted glass slides precoated with 200 μL of the same agarose. After gelling in the dark, the slides were lysed. The slides were transferred to alkaline unwinding/electrophoresis buffer at room temperature in the dark for 20 minutes then placed in fresh unwinding/electrophoresis buffer in the electrophoresis unit for 20 minutes. Slides were electrophoresed at 20 V, 300 mA for 50 minutes, after which they were rinsed in water for 5 minutes before being stained with 1:20,000 SYBR Gold at room temperature for 20 minutes. The slides were rinsed in water for five minutes before being stored. The slides were analyzed using the fluorescein filter on a fluorescence microscope and tail moments were analyzed by NelmsComet software.

Results

The neutral and alkaline comet assays were well established for the analysis of ionizing radiation-induced DNA double and single strand breaks (17,22). Trypsinization conditions were determined for the first experiments using primary mammary epithelial cells isolated from immature and mature rats. We next optimized the conditions of the alkaline and neutral comet assays performed on primary RMECs isolated from immature and mature Fischer rats and irradiated in culture.

Alkaline conditions were optimized first because of their simplicity relative to the neutral

assay. Results illustrate that the conditions used can readily distinguish between control and irradiated cells. There was no discernible difference in median tail moments between mature and immature cells either immediately following irradiation or after incubation following irradiation., indicating no difference between immature and mature mammary epithelial cells in single strand DNA breaks or alkali labile sites after treatment with ionizing radiation.

Since the alkaline conditions were optimized, the neutral conditions were next optimized. It is the neutral assay that will provide insight about the potentially cytotoxic DNA double strand breaks. There did not appear to be a difference in double stranded DNA breaks immediately following ionizing radiation. Repair times up to 2 hours did not reveal any differences between immature and mature cells under the conditions currently in use.

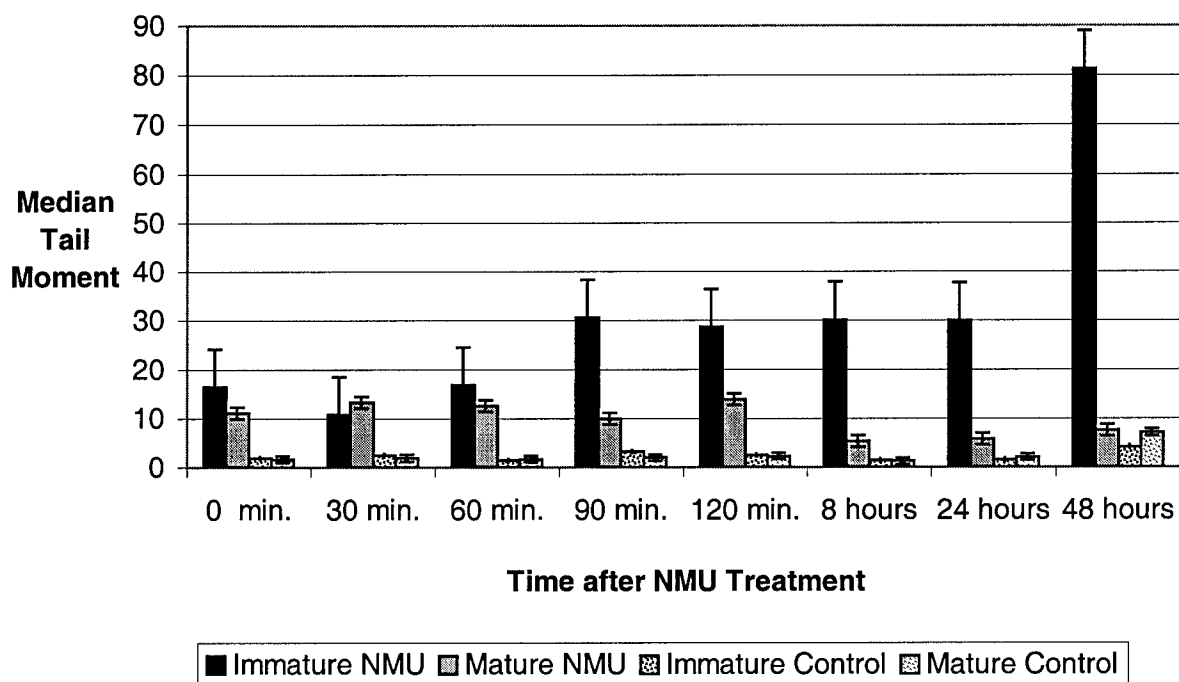
Alkaline comet assays were also performed on primary RMECs isolated from immature and mature Fischer rats and treated *in vitro* with NMU. A dose-response curve was generated for RMECs treated in this way. We also demonstrated an age-related difference in median tail moments following exposure to NMU and further incubation. Beginning at 90 minutes following removal of NMU, the immature cells have an increased median tail moment that persists throughout the duration of the experiment. In fact, there is another increase at 48 hours, the time at which the mature cells have reached median tail moment values similar to untreated cells, suggesting that the mature cells have by 48 hours repaired the NMU-induced single strand breaks or alkali labile sites.. These differences could be due to differential apoptosis occurring in the immature cells.

As shown in Aim 4 - Figure 1, there are no differences in single strand breaks immediately following treatment in culture with 0.1 mM NMU. However, there are subsequent differences. While treated mature RMECs experience a reduction or constant levels of single strand DNA breaks with time after treatment, immature RMECs experience an increase in single strand DNA breaks beginning approximately two hours following treatment. These increased breaks persist throughout the assay period.

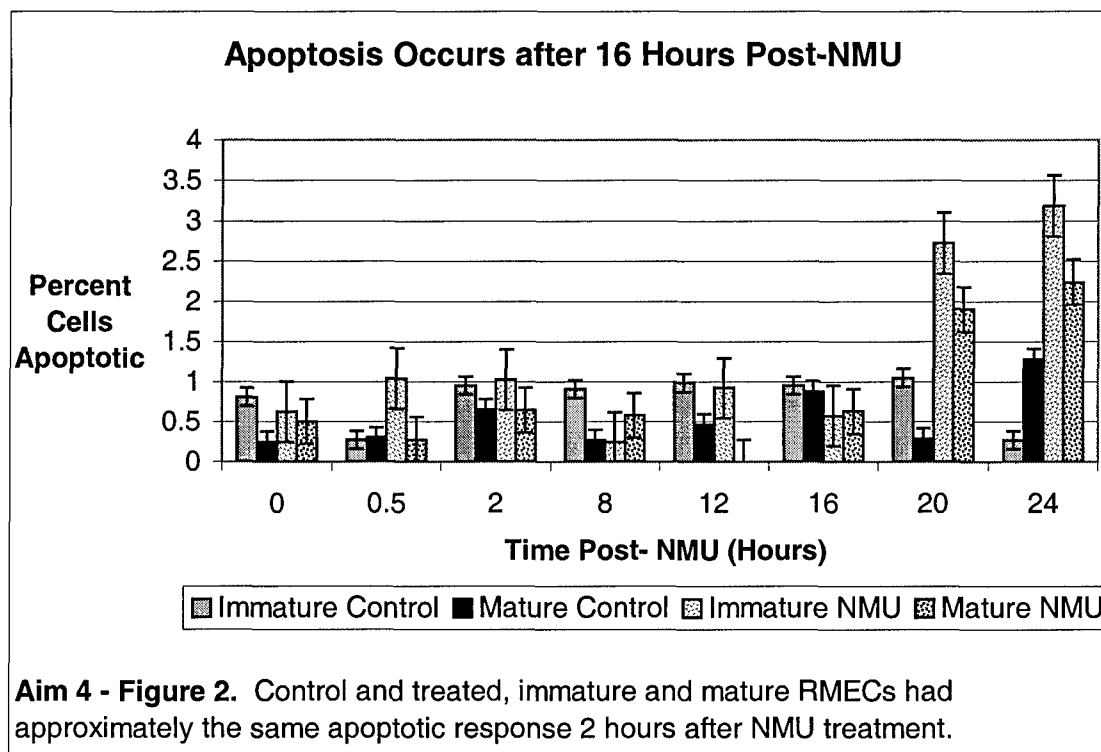
In order to assess age-related differences in apoptosis, immature and mature RMECs were treated in primary culture with NMU and scored for apoptosis using Hoescht staining. Chromatin condensation was the primary morphological characteristic diagnostic of apoptotic cells. As shown in Aim 4 - Figure 2, NMU does not induce apoptosis until between 16 and 20 hours post-treatment. By 20 hours, the immature cells showed greater levels of apoptosis than mature RMECs. To narrow this time of apoptosis induction, another time course experiment was performed. As shown in Aim 4 - Figure 3, apoptosis induction occurs between 16 and 17 hours post-NMU and is greater in immature than in mature cells.

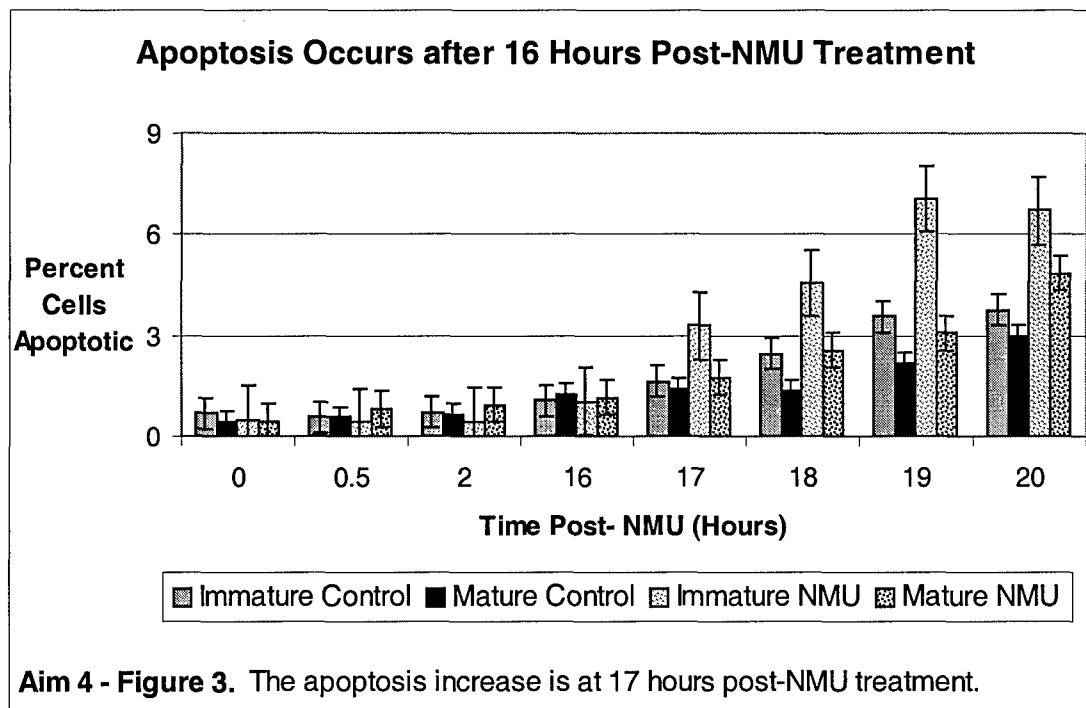
Thus, the increased tail moments exhibited by the immature RMECs indicate that while the immature cells do not appear inherently more susceptible to the DNA-damaging effects of NMU, the immature cells do process the DNA damage differently than the mature cells. This effect is not due to apoptotic differences, which do manifest later with immature RMECs displaying greater levels of apoptosis.

**Median Tail Moments after Treatment of Rat Mammary Epithelial Cells
in vitro with 100 mM NMU for 30 Minutes**



Aim 4 - Figure 1. Median tail moments from 50 comets/condition are shown. Error bars are standard errors.





Aim 5: Is the lack of TGF α production by cells of the immature mammary gland related to increased sensitivity to radiation induced cell killing?

The purpose of this aim is to determine if the absence of transforming growth factor α (TGF α) in the immature mammary gland plays a role in the increased sensitivity to ionizing radiation as compared to that of the mature gland. This aim is predicated on the assumption that the immature mammary gland does not produce TGF α , while the mature gland does. The basis for this assumption is a publication reporting that TGF α mRNA was not detected in the immature mouse mammary gland, but it was in the mature gland.

The assumption that the immature rat mammary gland does not produce TGF α was based on a study of murine mammary glands. However, other more recent studies on the rat mammary gland contradict this assumption. First, the failure to detect TGF α mRNA may not indicate the absence of TGF α protein, since Smith, *et al.* could not detect TGF α mRNA in RNA isolated from lactating rat mammary gland, where TGF α activity was found (23). Also, McAndrew *et al.* report that the level of TGF α mRNA doesn't necessarily directly relate to the amount of protein, since they found a decrease in immunoreactive TGF α in the pregnant rat mammary gland, but others reported an increase in TGF α mRNA expression at that stage (10,24,25). McAndrew *et al.* reported no difference in the immunolocalization of TGF α in the mammary glands of immature (21 day old) and mature (50 day old, the oldest rats studied) female Ludwig Wistar OLA rats (24). The 50 kDa immunoreactive TGF α was found in both the growing terminal end buds and alveolar buds, with the most staining found in the myoepithelial cells. In fact, the epithelial ductal cells of 1- and 6-day old rats stained more strongly for TGF α than the epithelial cells of the older rats, although the myoepithelial staining was stronger in the 21-day and 50-day old rats. Due to the findings listed above, this aim was not pursued.

Aim 6: How is the spectrum of gene expression in the immature and mature mammary glands different with regard to genes which could directly or indirectly confer altered DNA repair capacity?

The purpose of this study is to investigate difference of gene expression spectrum in the immature and mature mammary glands, and identify genes which could directly or indirectly confer altered cellular recovery capacity following cytotoxic and genotoxic, such as radiation-induced, damage.

Subtraction cloning genes differentially expressed in mammary glands from 3-wk and 8-wk old F344 rats

We have completed the sequencing of those 3-wk and 8-wk mammary cDNA clones isolated by PCR-select subtraction method. Thirty five 3-wk and one hundred seventeen 8-wk non-redundant cDNA sequences were identified (Aim 6 - Tables I and II). Half of the isolated sequences were either identical to known rat sequences or highly homologous to sequences from other species. Two fifths of the sequences showed identity or homology to the expressed sequence tags (EST). About one tenth of the sequences were novel with little or no homologous to sequences in the database. The sequences hit by NCBI's BLAST were expressed in a wide variety of tissues, ranging from the brain, heart, liver, lung, kidney, macrophage, ovary, skin, spleen, embryo and mammary gland. Among these sequences, several mouse ESTs were isolated from mammary gland. Beta-casein and κ -casein cDNAs were identified among the 8-wk cDNA clones. Casein genes are markers for mammary differentiation (26,27), their expression in rat mammary glands are known to associate with sexual maturation. The functions or putative functions of the encoded proteins range from structure protein such as beta-tubulin and gelsolin, to signal transduction component such as GTP-binding protein G- α -8 subunit, to cell cycle regulator such as cyclin D1. A selection of 3-wk and 8-wk clones were further characterized by ribonuclease protection assay (RPA). Recently, Affymetrix released a set of rat Genome Genechip. We have analyzed the gene expression patterns in 3-wk and 8-wk F344 mammary glands using Affymetrix rat Genome GeneChip. In this report, we focused on the characterization of selected 3-wk and 8-wk subtraction cDNA clones by RPA and gene expression analysis using Affymetrix rat Genome Genechip.

Aim 6 - Table I. Clones Potentially Over-expressed in 3-wk Old F344 Rat Mammary Gland

#*	Identity/Homology (# of multiple independent clones)	Accession No.
1	rat lactate dehydrogenase-A (2)	X01964
2	rat cytochrome oxydase subunit I cDNA	S79304
3	rat H-rev107	X76453
4	rat R-reggie-1.1 (plasmamembrane associated protein)	U64999
5	rat mRNA for protein disulfide isomerase X20918	
6	rat class I beta-tubulin cDNA	AB011679
7	mouse mRNA for osteoblast specific factor 2 (OSF-2) (4)	D13664
8	mouse Nedd5 mRNA for septin (2)	D49382.2
9	mouse fibroblast growth factor inducible gene 14 (FIN14) mRNA	U42386

10	mouse TIMP-gene for metalloproteinase-3 tissue inhibitor	Z30970
11	mouse alpha-2-macroglobulin (AM2) receptor	X67469
12	mouse SCF complex protein cul-1 mRNA (2)	AF083216
13	mouse mRNA for complement subcomponent C1q alpha-chain (2)	X58861
14	mouse 30kDa adipocyte complement-related protein Acrp30 mRNA	U37222
15	mouse transcription factor C1	U53925
16	mouse GTP-specific succinyl-CoA Synthetase β subunit	AF171077
17	human mitochondrial ATP synthase subunit C (9) isoform 3 (ATP5G3)	U09813.1, NM001689.1
18	human placental protein 15	X07315
19	human breast cancer-associated gene 1 protein (BCG1)	AF126181.1
20	rat EST** AI030806	
21	rat EST AI044121	
22	rat EST AI059513	
23	rat ovary EST AI407805 (2)	
24	rat EST AI535463 (2)	
25	rat EST AI556018.1	
26	mouse embryo EST AA000847	
27	mouse embryo EST AA288883	
28	mouse mammary gland EST AA764413 (2)	
29	mouse macrophage EST AA798026 (2)	
30	mouse mammary gland EST AA832670 (2)	
31	mouse mammary gland EST AA967067 (3)	
32	mouse mammary gland EST AI226339 (2)	
33	human mRNA for KIAA0767 protein (3)	
34-35	Independent novel sequences	

* Sequences subcloned from silver-stained secondary PCR product after re-amplification.

** EST, expressed sequence tags

Aim 6 - Table II. Clones Potentially Over-Expressed in 8-wk Old F344 Rat Mammary Gland

#	Identity/Homology (# of multiple clones)	Accession No.	#	Identity/Homology (# of multiple clones)	Accession No.
1	rat GTP-BP G- α -8 subunit (2)	M17525	58	rat EST AW142333	
2	rat MHC I RT1.C/E	X16976	59	rat EST* AA500532	
3	rat lipopolysacchride-BP (LBP)	L32132	60	rat EST AA874832 (3)	
4	rat putative RNA binding protein gene	U87598	61	rat EST AA925126	
5	rat GSH S-transferase (3)	J03752	62	rat EST AA964307	
6	rat α -casein	J00710	63	rat EST AI029606	
7	rat GEF-2 mRNA	AA003515	64	rat EST AI602058.1	
8	rat PI3-kinase Sreg p85 (2)	U50412	65	rat EST AW252437 (4)	
9	rat Stearyl-CoA desaturase (7)	J02585	66	rat EST AI713213 (3)	
10	rat ATP synthas (γ -3)	L19927	67	rat EST AI574984	
11	rat mitochondrial cytochrome c oxidase	X14848	68	rat EST AI575716.1	
12	rat κ -casein mRNA (3)	K02598	69	rat EST AW527890	

13	rat vimentin cDNA (2)	X62952	70	rat EST AW141446 (7)
14	rat amphoterin	M64986	71	rat brain EST AI227878
15	rat CD14	U51804	72	rat embryo EST AI600100 (4)
16	rat oxytocin receptor (OTR) (2)	U15280	73	rat embryo EST AI915496
17	rat ceruloplasmin, Cu ⁺⁺ binding protein (2)	L33869	74	rat heart EST AI410810
18	rat 16S rRNA	J01438	75	rat kidney EST AA817882
19	rat transferrin (6)	D38380	76	rat kidney EST AI232974
20	rat Peptidylglycine α -amidating monooxygenase	X59687	77	rat kidney EST AI045201
21	rat AKAP95, kinase A anchor protein	U01914	78	rat liver EST AA800355
22	rat Cyclin D1	U14014	79	rat liver EST AI169729
23	rat ryudocan=heparan sulfate proteoglycan core protein (2) S61868, M81786		80	rat ovary EST AI176435 (3)
24	rat partial brain mRNA (clone sap33f)	X94520	81	rat ovary EST AI176665
25	rat X chromosome-linked phosphoglycerate kinase	M31788	82	rat ovary EST AI177091 (7) = AW144280
26	rat mitochondrial cytochrome c oxidase s.u. III	M27315	83	rat ovary EST AI236402
27	rat plasma glutathione peroxidase (2)	D00680	84	rat ovary EST AI236798
28	rat glucose-dependent insulinotropic polypeptide receptor (2) AF050667		85	rat ovary EST AI408502 (2)
29	rat cytosolic malic enzyme	M30596	86	rat ovary EST AI497797
30	rat cytoplasmic dynein heavy chain (MAP1C)	L08505	87	rat ovary EST AI236798
31	rat clone N27 mRNA, MNU induced mammary tumor	U30789	88	rat ovary EST AI141995
32	rat growth potentiating factor	D42148	89	rat placental EST AI237848 (2), AI013888, AA998473
33	rat MHC class I RT1, Aw3 protein	L40363		rat spleen EST AA801434
34	rat RK/IF-1 mRNA (I-kappa-B α chain)	X63594	90	
35	rat tissue factor protein mRNA	U07619	91	mouse EST AA119054
36	rat cathepsin H mRNA	Y00768	92	mouse EST AW 763651 (6)
37	rat GTS-P gene, encoding placental-type glutathione S-transferase L29427		93	mouse EST AA183632 = AW495498
38	rat proteasome subunit RC6-1 (2)	D30804	94	mouse EST AA185231
39	rat ribosomal protein L10a	X93352	95	mouse EST AA435303
40	mouse occludin mRNA	U49185	96	mouse EST AI587910.1
41	mouse Mfg-2 mRNA	L16904	97	mouse EST AW702139
42	mouse NKx-5.1 homeobox	X75330	98	mouse EST AI626967
43	mouse gelsolin gene cDNA (3)	J04953	99	mouse EST W81852
44	mouse SPARC-related protein	AF070470	100	mouse EST AW258602
45	mouse GDP-dissociation inhibitor mRNA	L07918	101	mouse embryo EST AA008689
46	mouse CDV-1R protein mRNA	Y10495	102	mouse kidney EST AI790315
47	mouse putative transcriptional factor mRNA	AF091234	103	mouse kidney EST AA106453
48	mouse complement C4 mRNA	M11729	104	mouse liver EST AI529140
49	mouse cyclin D-interacting myb-like protein (Dmp1)	U70017	105	mouse mammary gland EST AA611141
50	mouse Wnt-4 mRNA (2)	M89797	106	mouse skin EST AA840274
51	mouse putative guanylate binding protein mRNA, 3' untranslated region M81128		107	human EST AA344414
52	mouse cDNA for ADAMTS-1	AB001735	108	human EST AA904725
53	mouse mRNA for Pex3 protein	AF152996	109	human EST AF038239
54	human mitochondrial ATP synthase s.u.9	U09813		
55	human putative Cu ⁺⁺ -transporting P-type ATPase NM_000052		110-118	Independent novel clones
56	human rab3-GAP regulatory domain (2)	AF004842		
57	human DAP-kinase	X76104		

* EST, expressed sequence tags

Materials and methods

Animals

Virus-free F344 female and male rats were obtained from Harlan Sprague-Darley, Inc. (Indianapolis, IN). The breeding of the rats to create 3 week old F344 female rats was performed at our facility. 8 week old F344 female rats were obtained directly from Sprague-Darley, Inc. All rats were provided with Teklad Lab Clox chow (Harlan Teklad, Madison, WI) and acidified water ad libitum. The rats were housed under a 12 hour light and 12 hour dark cycle.

RNA Isolation

Lower mammary glands (glands D, E and F) were collected from 3-week and 8-week old female F344 rats and immediately frozen in liquid nitrogen. To isolate the total RNA, 200 mg frozen tissue was homogenized in 4 ml RNazol B reagent (Tel-Test) with Polytron homogenizer at setting 8 for 30 seconds. The total RNA was extracted by following the manufacture procedure.

Ribonuclease protection assay of subtraction clones

Selected subtraction clones of the following categories were further analyzed by RPA:

- genes involved in signal transduction pathways
- genes encoding transcription factors
- genes involved in cell differentiation and/or proliferation
- genes related to tumor suppressor genes, oncogenes or mammary tumorigenesis
- genes involved in mammary development
- EST expressed in mammary gland in other species

Complementary DNA corresponding to those selected clones were RT-PCR amplified from F344 mammary gland total RNA and subcloned into pCRII TA cloning vector (Invitrogen), or the subtraction clones (*) were used directly for RPA probe synthesis. The oligonucleotide primers for constructing RPA probes were listed in Table III. ³²P-labeled ribonucleotide probes were synthesized using MAXIscript T7 kit (Ambion) and purified by denaturing PAGE. Ribonucleotide protection assay of mammary gland total RNA samples were performed using RPA III kit (Ambion) following the manufacturer's protocol. Protected probes were resolved on by denaturing PAGE and exposed to phosphorimager cassette (Molecular Dynamics). The signal intensities were analyzed with ImageQuant software (Molecular Dynamics).

Aim 6 - Table III. Oligonucleotide primers for constructing RPA probes

Clone	Forward primer	Reverse primer
Nedd5	5'-TCACCTTTGGACATGGA	5'-CTTCATCTGATTCTGCAT
EGF-induced gene 14	5'-TTCGGGGTAGTGTGAGCT	5'-CATCTGAGAATCTGGACA
TIMP-3	5'-GATGGTAAGGTGGTGACA	5'-GAAACTTGACACCCATCT
AM2 receptor	5'-TACACAGGCGATAAGTG	5'-CAGGAAGCCAGGTAGACA
H-rev 107	*	*
BCG1	5'-CCTTAGAGCCAACTGATG	5'-ACTCATCAGTAATGAGCT
rab11 GTPase	5'-ATGCTGGTAATACACAT	5'-ACCACTTGAGCATAA

NKx-5.1 homeobox	5'-GTCACCAGAGCTTCTGAG	5'-GAGTGTCCAGCAGGGTGA
Oxytocin receptor	5'-GTGCAGACATGACAGTA	5'-CTTTCACATAGACTGGCA
Cytosolic malic enzyme	5'-TAGATGACTGAGAGCTCA	5'-AGACAGAGTGTCTCCAA
Cyclin D1	5'-TACCTCATGTATCACCTA	5'-TGCACCAGAGACTCAGAA
gelsolin	5'-CCAAGCTCTACAAGGTCT	5'-ATCTTGGAGATGAAGTCA
GDP-dissociation inhibitor	*	*
rab3-GAP regulatory domain	5'-TCTCTGAAGAGTGTGAAG	5'-CTGTCCAGTTGAGGTCTT
glutathione transferase	5'-CCTTTACCTCCTATGCAA	
CDV-1 receptor protein	5'-TACACCCAAGAGGAGCTT	5'-ATCACACTCCTGGGTTAG
Dmp1 (cyclin D-interacting myb-like protein)	*	*
N27 (NMU-induced mammary tumor)	*	*
growth potentiating factor	5'-TACCGTGGATGCATGACT	5'-ACCTGCTCCAGTGTTGCA
kappa-casein	*	*
Wnt-4	*	*
I kappa-B	*	*
cathepsin H	5'-AACTGCTTGGCAGACAAAGG	5'-GAAGATTCTAGGTCCACATGG
homologous to mouse EST AA967067	5'-TGATACTCATGGACGTTGGAC	5'-GGAGGATTCCGTGGTATTGG
homologous to mouse EST AA764413	5'-TCAGTTACCAACCAGACCTG	5'-ACTGGATGGACTAGGTTGCTG
homologous to mouse EST AA959536	5'-CAGAAGTCCTGGGACTTTATG	5'-ACAATACCGGCACAGAGTTCC
homologous to mouse EST AA611141	5'-CTCAACTGGAACATGAGGAC	5'-AATCCTCTAACCTTGGAGGCT
homologous to human angio-associated migratory cell protein (AAMP)	5'-ACCTGTGTTGCCACCAACCAG	5'-AAGGACTCCACGGAGTTGGAC

* The subtraction clones were used as RPA probe constructs.

Affymetrix GeneChip analysis of rat mammary gene expression profile

Equal amount of total RNA from several (usually 4) rats of same age group were pooled for cDNA synthesis. Double-stranded cDNA was synthesized with a cDNA synthesis kit (Life Technologies Superscript cDNA Synthesis System) by using an T7 promoter oligo-linked oligo(dT)₂₄ primer (Genset Corp.) for priming the first cDNA synthesis. The double-stranded cDNAs were used as template for *in vitro* transcription (BioArray High Yield RNA Transcript labeling Kit) in the presence of biotin-11-CTP and biotin-16-UTP (Enzo Diagnostics). Twenty µg of biotin-labeled cRNA product were fragmented into 35-100bp pieces at 94°C for 35 min in buffer [40 mM Tris:acetate (pH 8.1)/100 mM potassium acetate/30 mM magnesium acetate]. Fifteen µg fragmented biotin-labeled cRNA were then used to make a hybridization mix with Herring sperm DNA (0.1 mg/ml; Sigma) and Acetylated BSA (0.2 mg/ml; Life Technologies) in MES Hybridization Buffer [100 mM MES (pH6.6), 1 M NaCl, 20 mM EDTA, 0.01% Tween 20]. Four prekaryotic cRNA (1.5 pM BioB, 5 pM BioC, 25 pM BioD, and 100 pM Cre) samples were added as internal controls for hybridization efficiency as described by the manufacturer (Affymetrix). Hybridization mix (200 µl) was hybridized to the Rat Genome U34A GeneChip at 45°C for 16-20 hrs. Each GeneChip was washed (Affymetrix Fluidics Station) and scanned (Hewlett Packard, GeneArray scanner G2500A) according to procedures developed by

manufacturer (Affymetrix). Scanned output files were analyzed using GeneChip Analysis Suite 3.1 software (Affymetrix).

Results and Discussion

In addition to previously characterized subtraction clones, twenty eight more clones were further analyzed by RPA. Among them, eight clones demonstrated differential expression between 3-week and 8-week mammary glands (Aim 6 - Figure 1). H-rev 107, rat BCG1, gelsolin and glutathione S-transferase expressed at higher levels in 3-week mammary glands and oxytocin receptor growth potentiating factor, κ -casein and EST AA611141 in 8-week glands (Aim 6 - Table IV). H-rev 107 is a class II tumor suppressor gene that is homologous to human tazarotene-induced gene 3 (TIG-3). Increased TIG-3 expression is correlated with decreased proliferation (28). Rat BCG1 is homologous to a human sequence over-expressed in freshly isolated human breast cancer specimens (29). Gelsolin has been demonstrated to play a role in cytoskeleton restructuring during cell differentiation (30). Oxytocin receptor plays an important role in lactating. Recently, it has been suggested that oxytocin receptor may also play a role in mammary gland development (31). The growth potentiating factor has been shown to regulate vascular smooth muscle cell proliferation (32). The dramatic increased expression of κ -casein (27) in 8-wk mammary glands is consistent with the maturation of mammary glands. The identity and of EST homologous to mouse EST AA611141 is not clear.

Aim 6 -Table IV. Summary of ribonuclease protection assay

Gene	3-wk mamary	8-wk mammary
H-rev 107	3.32	1
BCG1	3.51	1
gelsolin	2.42	1
glutathione S-transferase	2.26	1
oxytocin receptor	1	4.25
growth potentiating factor	1	2.6
κ -casein	1	192
homolog of mouse EST AA611141	1	9.51

In order to identify differentially expressed genes in larger scale, we hybridized Affymetrix rat genome U34A GeneChips separately with cRNA targets derived from 3-week or 8-week F344 mammary gland RNAs. After hybridization, staining, washing and scanning, the signals on Genome chip were analyzed GeneChip Analysis Suite 3.1 software (Affymetrix). Differentially expressed sequences were summarized in Aim 6 - Table V. Probe sets with expression greater than 10 fold difference between 3-wk and 8-wk F344 mammary glands were summarized in Aim 6 - Table VI.

Aim 6 – Table V. Summary of differentially expressed sequences in 8-wk versus 3-wk F344 mammary glands analyzed by rat genome GeneChip

Fold change*	Number of sequences
≥ 50	2
< 50 and ≥ 20	5
< 20 and ≥ 10	7
< 10 and ≥ 5	24
< 5 and ≥ 3	65
< 3 and ≥ 2	136
≤ -2 and > -3	254
≤ -3 and > -5	143
≤ -5 and > -10	59
≤ -10 and > -20	18
≤ -20 and > -50	4
< -50	3

* Negative number in fold change indicates the expression -is suppressed in 8-wk mammary gland

Aim 6 – Table VI. Probe sets with greater than 10-fold changes in expression levels between 3-wk and 8-wk F344 mammary glands

Probe Set	Identity of Probe	Diff Call	Avg Diff Change	Fold Change
L08100	Rat glycyl 1 mRNA, complete cds	I*	27850	193.4
AA946503	EST202002 Normalized rat ovary, Bento Soares Rattus sp. cDNA clone ROVAR89 3' end, mRNA sequence	I	33686	81.8
J00711	rat beta-casein mrna	I	4545	43.9
X77932	R.norvegicus mRNA for sodium channel, beta subunit	I	4499	43.5
K02598	Rat kappa-casein mRNA, complete cds	I	27421	43.3
M60753	R.norvegicus catechol-O-methyltransferase mRNA, complete cds	I	2653	26.1
M93257	Rattus norvegicus catechol-O-methyltransferase mRNA, 3' flank	I	5514	20.2
M62781	Rat insulin-like growth factor binding protein 5 (IGFBP-5) mRNA, complete cds	I	1690	13.5
D26307	Rattus norvegicus jun-D gene, complete cds	I	1018	10.6
M98049	Rattus norvegicus pancreatitis-associated protein precursor (pap) mRNA, complete cds	I	1008	10.5
M14656	Rat osteopontin mRNA, complete cds	I	985	10.3
U31866	Rattus norvegicus Nclone10 mRNA	I	25935	10.1
AI229031	EST225726 Normalized rat brain, Bento Soares Rattus sp. cDNA clone RBRDD18 3' end, mRNA sequence	I	949	10.0
M32167	Rat glioma-derived vascular endothelial cell growth factor mRNA, complete cds	D**	-979	-10.2
X16933	Rat mRNA for hnRNP C protein, partial	D	-1678	-10.9
U93880	Rattus norvegicus insulin receptor substrate-3 (IRS-3) mRNA, complete cds	D	-1069	-11.1

AA893384	EST197187 Normalized rat kidney, Bento Soares Rattus sp. cDNA clone RKIBF74 3' end, mRNA sequence	D	-1129	-11.7
X64589	R.norvegicus mRNA for cyclin B	D	-1155	-11.9
AI639185	rx00841s Rat mixed-tissue library Rattus norvegicus cDNA clone rx00841 3', mRNA sequence	D	-1221	-12.5
AF082160	Rattus norvegicus GRIFIN mRNA, complete cds	D	-5074	-12.7
AF022136	Rattus norvegicus connexin 40 (GJA5) gene, partial cds	D	-1242	-12.7
K03242	Rat Schwann cell peripheral myelin (P-0) mRNA, complete cds	D	-1279	-13.1
AI236601	EST233163 Normalized rat ovary, Bento Soares Rattus sp. cDNA clone ROVDI22 3' end, mRNA sequence	D	-1320	-13.5
AA859740	Rattus norvegicus cDNA clone UI-R-E0-bx-b-06-0-UI 3', mRNA sequence	D	-1477	-15.0
X59864	Rat ASM15 gene	D	-23800	-15.2
U00964	Rattus norvegicus common salivary protein 1 mRNA, complete cds	D	-2232	-15.3
AI639525	Rat mixed-tissue library Rattus norvegicus cDNA clone rx01430 3', mRNA sequence	D	-4036	-16.8
S56464	HKII=hexokinase II [rats, epididymal fat pad, mRNA Partial	D	-1704	-17.1
J03588	Rat guanidinoacetate methyltransferase mRNA, complete cds	D	-1773	-17.8
L19180	Rat receptor-linked protein tyrosine phosphatase (PTP-P1) mRNA, complete cds	D	-1826	-18.3
AA800232	EST189729 Normalized rat heart, Bento Soares Rattus sp. cDNA clone RHEAM72 3' end, mRNA sequence	D	-2140	-21.2
X60328	R.norvegicus mRNA for cytosolic epoxide hydrolase	D	-2521	-24.8
U73030	attus norvegicus pituitary tumor transforming gene (PTTG) mRNA, complete cds	D	-2714	-26.6
L06040	Rattus norvegicus 12-lipoxygenase mRNA, complete cds	D	-5056	-48.8
AF036761	Rattus norvegicus stearyl-CoA desaturase 2 mRNA, partial cds	D	-6208	-59.7
S69383	12-lipoxygenase [rats, pineal glands, mRNA, 2216 nt]	D	-7476	-71.6
X17012	Rat IGFII gene for insulin-like growth factor II	D	-12693	-120.9

* I, increased expression in 8-wk F344 versus 3-wk mammary glands

** D, decreased expression in 8-wk F344 versus 3-wk mammary glands

Both beta-casein and kappa-casein expression levels were shown to increase more than 40 folds by GeneChip study. The expression fold change of kappa-casein is consistent with the RPA data (Aim 6 - Table IV). The difference in fold change is most likely due to the low basal levels in 3-wk mammary glands. The differentially expressed sequences identified by GeneChip were compared to those subtraction clones analyzed by RPA (Aim 6 - Table VII). Only a fraction of the rat sequences identified by subtraction cloning have been put on the rat Genome U34A GeneChip. Among them, glutathione S-transferase expression levels stay the same by GeneChip study. The GeneChip analysis of growth potentiating factor and lipopolysaccharide binding protein (LPS) were same as that by RPA. In general, the results were consistent between RPA and Genechip, especially when the fold changes were high. Genechip application is more suitable for high throughput screening than the subtraction hybridization; however, the subtraction method can compliment the genechip study to provide information of those novel sequences and/or sequences not yet available on the genechip.

Aim 6 – Table VII. Comparison of selected data by Genechip analysis to those by RPA

Gene	Fold change	
	RPA	Genechip
H-rev 107	-3.32*	NA
homolog to human BCG1	-3.51	NA
homolog to mouse gelsolin	-2.42	NA
glutathione S-transferase	-2.26	-1.0
oxytocin receptor	4.25	NA
growth potentiating factor	2.6	2.8
κ-casein	192	43.3
homolog of mouse EST AA611141	9.51	NA
lipopolysaccharide binding protein (8)	> 5.0	8.0

* Negative number indicate the decreased expression in 8-wk mammary glands.

Conclusions

Multiple differentially expressed sequences were isolated from subtracted 8-wk F344 cDNA library by a PCR-select subtraction method. The differential expression levels of selected clones have been verified by RPA. We have also identify a wider range of differentially expressed sequences by using Affymetrix rat Genome U34A GeneChip. Several sequences were commonly identified by both methods. We are currently preparing a manuscript of this study for publishing in a reviewed scientific journal.

Aim 6 - Fig. 1. Ribonuclease protection assay of selected subtraction clones

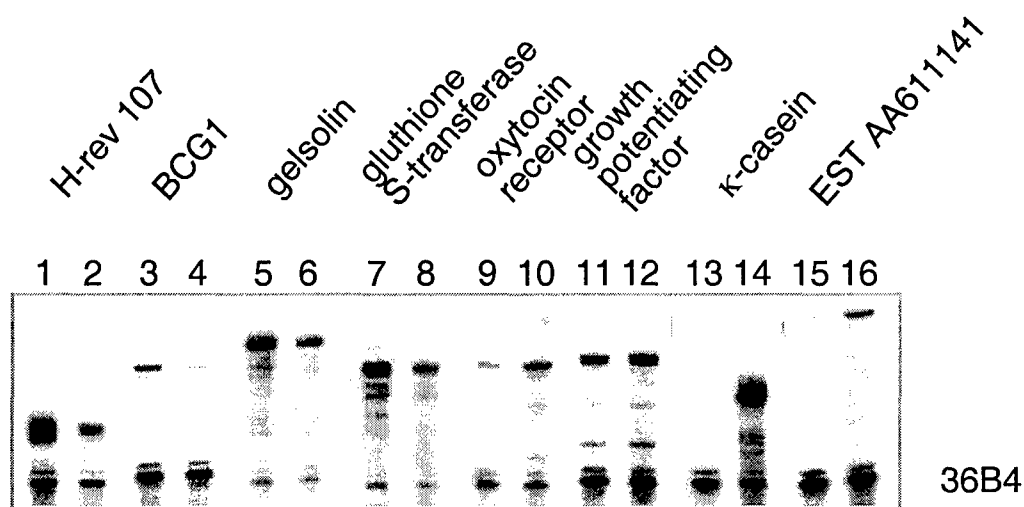


Fig. 1. Ribonuclease protection assay of mammary gland mRNA. Ten μ g of 3-wk and 8-wk mammary gland total RNA was hybridized to 32 P-labeled H-rev 107, BCG1, gelsolin, glutathione S-transferase, oxytocin receptor, growth potentiating factor, κ -casein, homolog of mouse EST AA611141 and 36B4 riboprobes and 42°C overnight. For control, yeast tRNA was added to each probe. After RNase A1/H digestion, the protected probes were resolved in 6% denaturing polyacrylamide gel at 225 volts for 1 hour in TBE buffer. The gel was then dried onto 3MM chromatography paper and exposed to phosphorimager cassette overnight and analyzed.

KEY RESEARCH ACCOMPLISHMENTS

- The immature mammary gland is more susceptible to cell killing by distinct classes of carcinogens. These include ionizing radiation and alkylating agents but not aromatic hydrocarbons.
- This increase in susceptibility is not due to initial DNA damage but is due to a diminished repair capacity.
- The increased susceptibility to cell killing of immature versus mature mammary cells extends both to specific locus mutagenesis and to an increased susceptibility to mammary carcinogenesis.
- The immature mammary gland when compared to the mature mammary gland has an overall distinct mammary gene expression profile as measured both by subtractive hybridization and DNA microchip array methods.

REPORTABLE OUTCOMES

Abstracts

J.D. Haag and M.N. Gould. Increased sensitivity to cell killing of immature rat mammary clonogenic cells following NMU but not DMBA exposure. Proceedings of the American Association for Cancer Research, 40:508, 1999.

K.-S. Chen, J.D. Haag and M.N. Gould. Subtractive screening of sequences differentially expressed in rat mammary glands of different developmental stages. Proceedings of the American Association for Cancer Research, 40:30, 1999.

K.-S. Chen and M.N. Gould. Screening and Characterizing Genes Differentially Expressed in Rat Mammary Glands of Different Developmental Stages. Proceedings of the American Association for Cancer Research, 41:579, 2000.

J.L. Ariazi and M.N. Gould. Increased Susceptibility of Immature Rat Mammary Epithelial Cells to Cytotoxicity and Mutagenesis by Selected Carcinogens. Proceedings of the Era of Hope Department of Defense Breast Cancer Research Program Meeting. 1:272, June 8-11, 2000.

Predoctoral Traineeship

Jennifer L. Ariazi. Mechanisms Underlying the Increased Susceptibility of the Immature Mammary Gland to Selected Carcinogens. BC990254 Started July 1, 2000 for 3 years.

CONCLUSIONS

Epidemiological studies strongly suggest that the immature breast of young women is the most susceptible developmental stage to breast cancer initiation. During the course of the investigations funded under this project, we have developed and validated an animal model to be used to better understand the mechanisms underlying this differential susceptibility; such knowledge will be critical to design and test prevention strategies for this age-specific risk

Specifically, we have shown that the immature mammary gland is differentially sensitive to cytotoxicity, mutagenesis and carcinogenesis. We have evidence to support the hypothesis that this increased susceptibility is carcinogen-class specific and is likely related to attenuated DNA repair capacity in the immature gland. In the future, we plan to detail the specific deficiencies of repair pathways in the immature mammary gland. Finally we plan to develop chemopreventive and population approaches to reduce the initiation of breast cancer in young women.

BIBLIOGRAPHY OF ALL PUBLICATIONS/MEETING ABSTRACTS

J.D. Haag and M.N. Gould. Increased sensitivity to cell killing of immature rat mammary clonogenic cells following NMU but not DMBA exposure. Proceedings of the American Association for Cancer Research, 40:508, 1999.

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LIST OF PERSONNEL (receiving pay from the research effort)

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