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The overall goal of this project remains to explore the toxic effects of physical and chemical carcinogens on the immature mammary gland as compared to these effects on the young adult mammary gland using a rat model. During the third grant year we have: 1) completed all comparative cytotoxicity studies showing that the immature mammary gland is more sensitive to radiation and NMU but not DMBA, 2) adapted the "Big Blue" mutagenesis assay to the mammary gland and produced preliminary data suggesting the immature gland is more susceptible to NMU mutagenesis, 3) completed a radiation carcinogenesis study and set up an NMU carcinogenesis study, 4) adapted the comet assay to primary mammary cells, and 5) identified numerous genes that are either up or down regulated in immature mammary gland. We are currently analyzing members of these libraries. We feel that these studies will help mechanistically define the epidemiological observation in women which suggests that the immature mammary gland is more susceptible to environmental carcinogens than is the adult gland.

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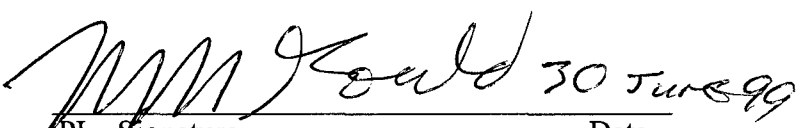

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INTRODUCTION

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 have 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 (TGFα) which signal through the same receptor. However, it is suggested on the basis of cellular and glandular distribution that only TGFα 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, TGFα 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 TGFα 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 TGFα then this would suggest new pharmacologic breast cancer prevention approaches using either TGFα or preferably non-peptide small molecules with TGFα 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 scopal 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 TGFα 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?

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Year Three: Progress 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 in year 3 (current) and will be prepared for publication on the first half of year 4.

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 AD_{50} (1). An AD_{50} 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 (2,3). Clonogen survival following carcinogen exposure is estimated by dividing the value of the AD_{50} of the control, non-treated group by the AD_{50} of the treated group.

Results

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

	Control		NMU		DMBA	
F344 donor mammary gland	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.

The research for this aim has been completed. We are currently writing a manuscript to submit for publication regarding these results.

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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.

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 during this update 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. 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.

Figure 1 (p. 14) 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 Figure 1) and non-injected control (0 in 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.

Figure 2 (p.14) 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 (Figure 3, p.15). 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. However, the small sample size of this pilot study must be taken into consideration.

Thus, at the end of this update period, the Big Blue transgenic mutagenesis system has been established for the detection of NMU-induced mutations in rat mammary epithelial cells. Preliminary data indicate that immature rat mammary epithelial cells are more susceptible to the mutagenic effects of NMU than mature rat mammary epithelial cells.

Because the pilot experiment was successful, future studies of age-differential mutagenesis will continue to be conducted with NMU as the mutagenic agent instead of ionizing radiation. First, a larger experiment to determine the optimal expression period will be conducted. 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. The lower dose will be included to determine whether mutagenic effects can be detected in rat mammary epithelial cells at a dose that will produce less gross toxicity than the higher dose. After the expression period of 1, 3, or 5 weeks of housing under standard conditions, the rats will be sacrificed. Primary RMECs will be collected and genomic DNA will be isolated

Genomic DNA will be packaged and plated according to Stratagene's instructions (2, 3). 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. Mutants will be sequenced and 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.

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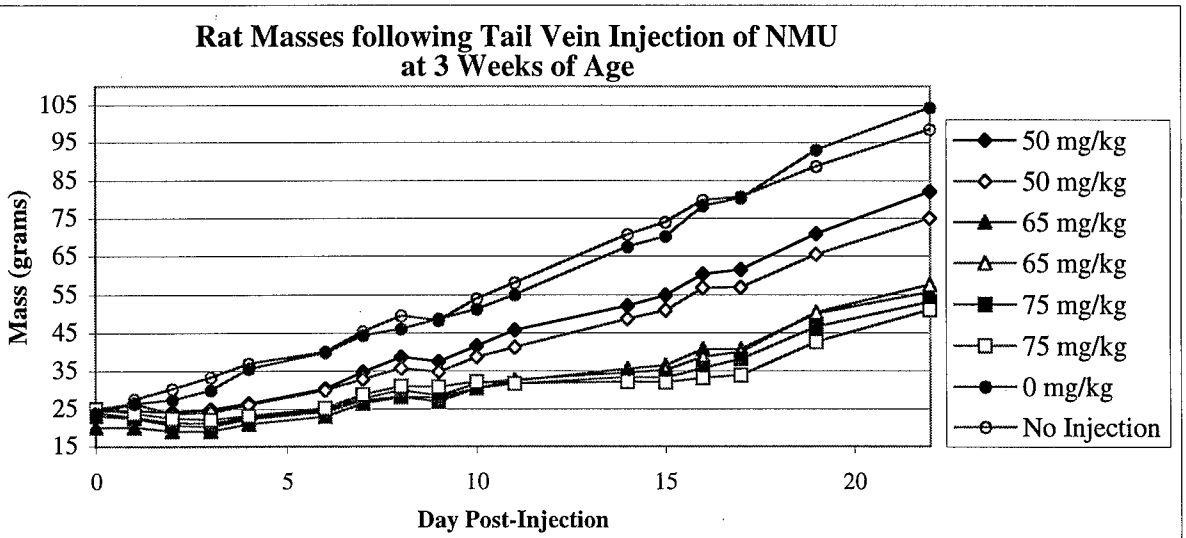
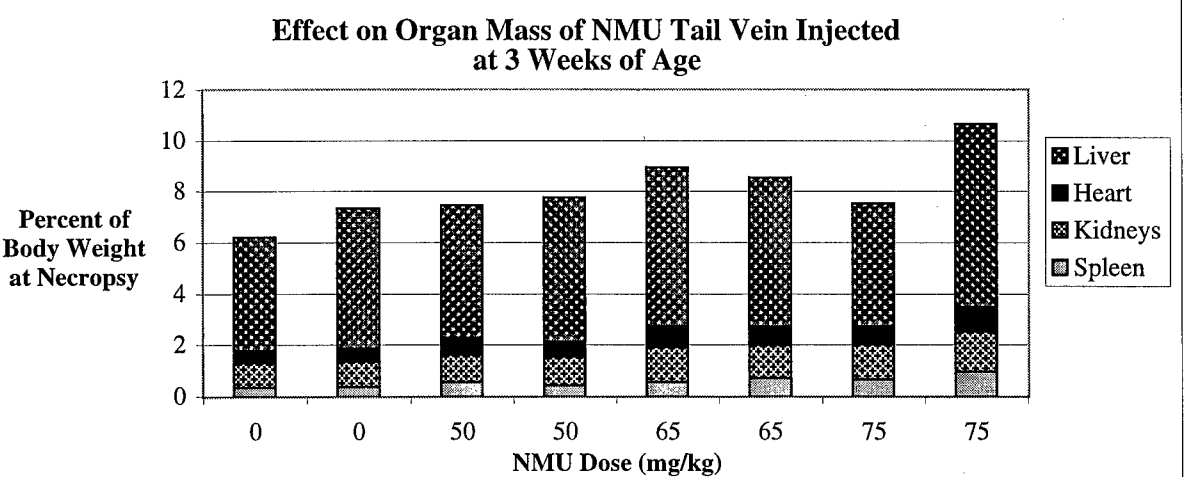
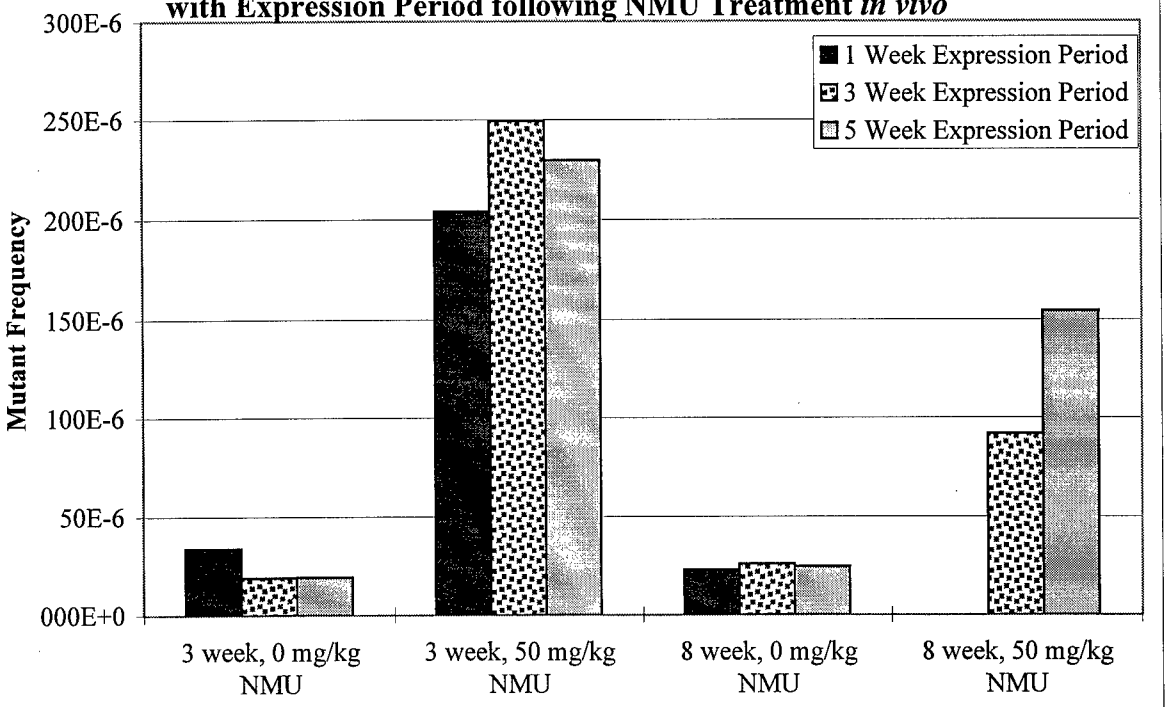


Aim 2, Figure 1. Each data point indicates the mass of one rat injected by tail vein at three weeks of age with the concentration of NMU indicated in the legend.



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 Figures 1 and 2.



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. Immature and mature control levels do not differ.

Aim 3: Is the immature gland more sensitive than the mature gland to the scopal 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.

		untreated controls	8 week old ~2.5 Gy	3 week old ~2.5 Gy
	# 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.

		untreated controls	8 week old ~2.5 Gy	3 week old ~2.5 Gy
	# 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.

The research for the long-term irradiation carcinogenesis experiment is now complete.

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 have been expanded to include the two chemical carcinogens.

Immature Fischer 344 rats were bred in house, and mature Fischer 344rats were purchased and allowed to acclimate to the animal care facility. Immature rats were treated with NMU by tail vein injection or DMBA by oral gavage at three weeks of age on one of two dosing dates one week apart. Mature rats were treated with NMU by tail vein injection or DMBA by oral gavage at eight weeks of age on one of two dosing dates one week apart. Both carcinogens were administered at 50 mg/kg. NMU was dissolved in 0.9% saline solution acidified to pH 5.0 with acetic acid, and DMBA was dissolved in sesame oil. A total of 30 rats (31 for immature DMBA rats) per group were treated. Initially, rats were handled and their masses recorded twice a week in order to acclimate the animals to palpation. Beginning three or four weeks after treatment (depending on dosing date), the animals have been palpated and their masses recorded weekly. Animals treated with NMU show reduced masses compared to those dosed with DMBA. The effect is more marked in the immature rats than in the mature rats. Tumor data are expected within six months.

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.

At the beginning of the update period, the neutral and alkaline comet assays were well established for the analysis of ionizing radiation-induced DNA double and single strand breaks. Trypsinization conditions were beginning to be determined for the first experiments using primary mammary epithelial cells isolated from immature and mature rats.

In the last update, it was proposed that the work conducted during this update period would further optimize 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 shown in Figure 1 (p.22) illustrate that currently used conditions 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 as shown in Figure 2 (p.22), 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. Figure 3 (p.22) illustrates the dose-response curve seen with mature and immature cells analyzed using the neutral comet assay. There does not appear to be a difference in double stranded DNA breaks immediately following ionizing radiation. Repair times up to 2 hours do not reveal any differences between immature and mature cells under the conditions currently in use. Further optimization of neutral conditions and expanded repair studies (later time points will be included) will be conducted in the next update period.

Alkaline comet assays have also been performed on primary RMECs isolated from immature and mature Fischer rats and treated *in vitro* with NMU. A dose-response curve has been generated for RMECs treated in this way as seen in Figure 4 (p.23). Figure 5 (p.23) demonstrates the 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. Another possible explanation is that the mature cells repair the DNA methylation using methylguanine methyltransferase, while the immature cells repair the damage using base or nucleotide excision repair, which would produce many small DNA fragments. These possibilities will be further investigated in the next update period.

Therefore, at the end of this update period, the alkaline comet assay has been optimized for the detection of single strand DNA breaks in primary rat mammary epithelial cells irradiated in culture. The neutral assay is nearing complete optimization. Preliminary data from both neutral

and alkaline assays indicate that there is no difference between immature and mature rat mammary epithelial cell DNA strand breaks or their repair in short term repair assays. However, preliminary data indicate that there is a difference in the processing of NMU-induced single strand breaks or alkali-labile lesions between immature and mature primary rat mammary epithelial cells. Future studies will include further optimization of neutral comet assay conditions, expanded repair studies of double strand breaks induced by ionizing radiation, and further study of the difference in handling of NMU-induced lesions in primary rat mammary epithelial cells.

The alkaline comet assay will be performed the day after plating as described with the noted differences (1, 2). Medium will be drawn off the cells and fresh complete hormonal medium (CHM) containing 0.1 mM NMU will be added to plates of cells in a staggered start fashion to ensure that all cells are treated for exactly 30 minutes at 37 C. For repair studies, NMU-containing CHM will be aspirated and the cells will be rinsed twice with Ca^{++}/Mg^{++} - free phosphate buffered saline (PBS), and fresh CHM (without NMU) will be added. The cells will be allowed to incubate for the appropriate time after which the cells will be trypsinized. For analysis without repair, the CHM containing NMU will be aspirated; the cells will be rinsed twice with Ca^{++}/Mg^{++} - free PBS and the cells will be trypsinized. CHM will be added to stop the trypsinization and cells will be counted.

At this point the cells will be treated and the alkaline comet assay will be performed as for irradiated cells. To 200 _L of cells at 80,000 cells/mL will be added 1.5 mL of 1% low melting point agarose held at 40 C. The cells will be spread on fully frosted glass slides precoated with 200 _L of the same agarose. After gelling in the dark, the slides will be lysed. The cells will be lysed and their DNA allowed to unwind. Slides will be electrophoresed, rinsed and stained with 1:20,000 SYBR Gold. The slides will be rinsed in water for five minutes before being stored. The slides will be analyzed using the fluorescein filter on a fluorescence microscope and tail moments will be analyzed by NelmsComet software.

Although the comet assay has been successfully performed in the laboratory, other methods, such as sucrose gradients and the alkaline elution assay are available to answer similar questions. Additionally, NMU adduct levels could be determined directly.

To further characterize the differences detected in preliminary experiments, the assay will be performed as above, but O^6-benzylguanine will be added to the cells when plated and will be present in the CHM at all times to ensure complete inhibition of MGMT after preliminary studies to determine the appropriate concentration to use (3-5).

If the hypothesis that immature cells repair O^6-methylguanine by nucleotide or base excision repair but mature cells repair O^6-methylguanine using MGMT, then there should be no effect of addition of the inhibitor in the immature cells but an increase in tail moment of the mature cells.

Since the increased tail moments seen in the immature cells could be due to apoptosis, apoptotic indices will be determined. The acridine orange/Ethidium bromide staining and Annexin V assays will be performed on immature and mature cells.

The enzymatic activity of methylguanine methyltransferase in immature and mature rat mammary epithelial cells treated with NMU will be determined as described (6). Briefly, lysates of RMECs treated in culture with NMU will be prepared and added to a reaction mixture containing ^3H-methylated DNA enriched with O^6-methylguanine. After incubation, protein will be recovered. Precipitated DNA will be hydrolyzed and liberated O^6-methylguanine will be quantitated by scintillation counting.

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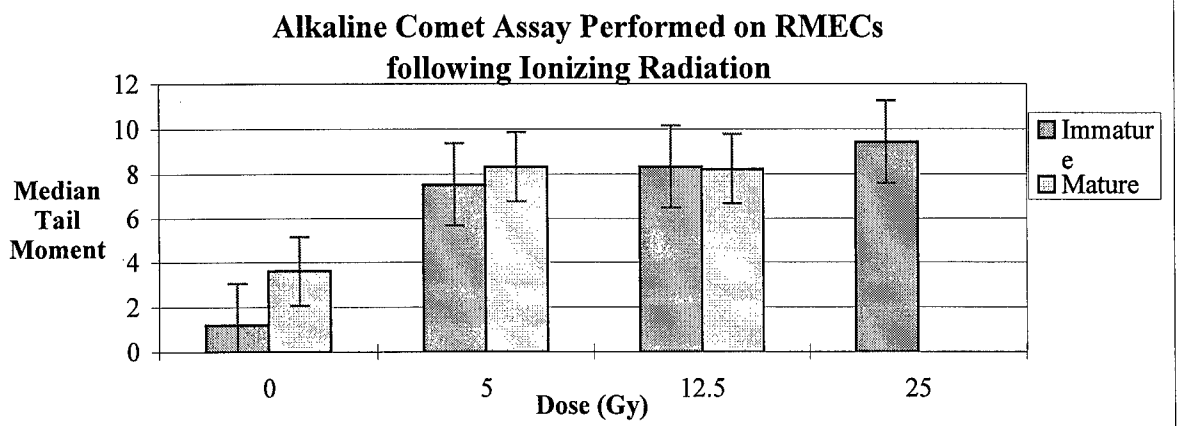
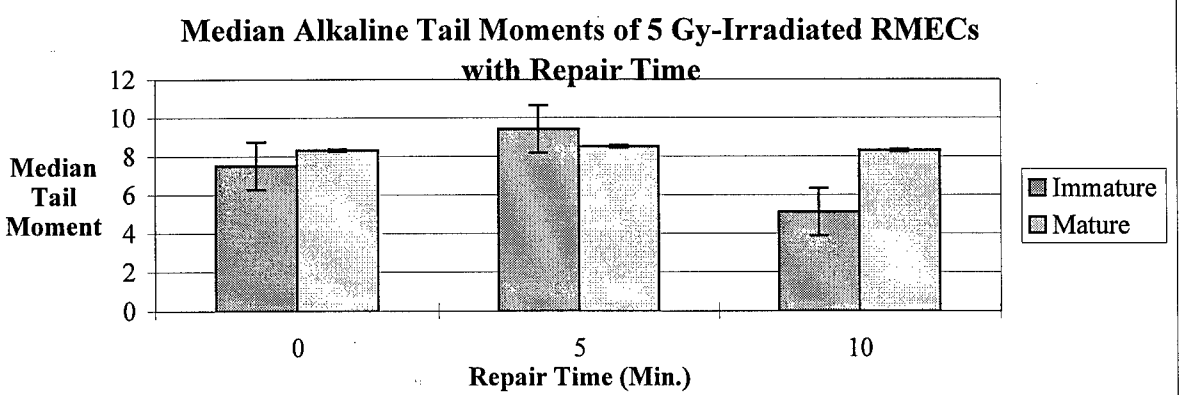
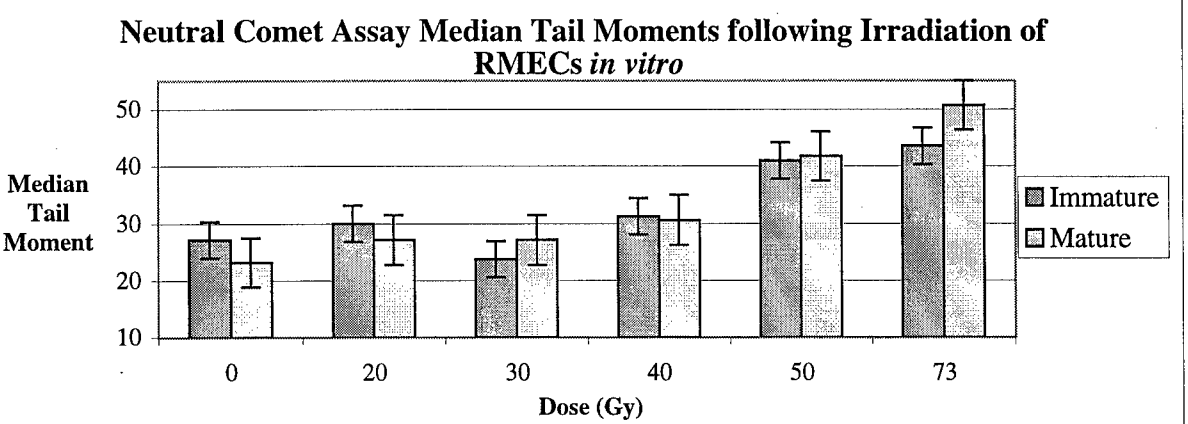


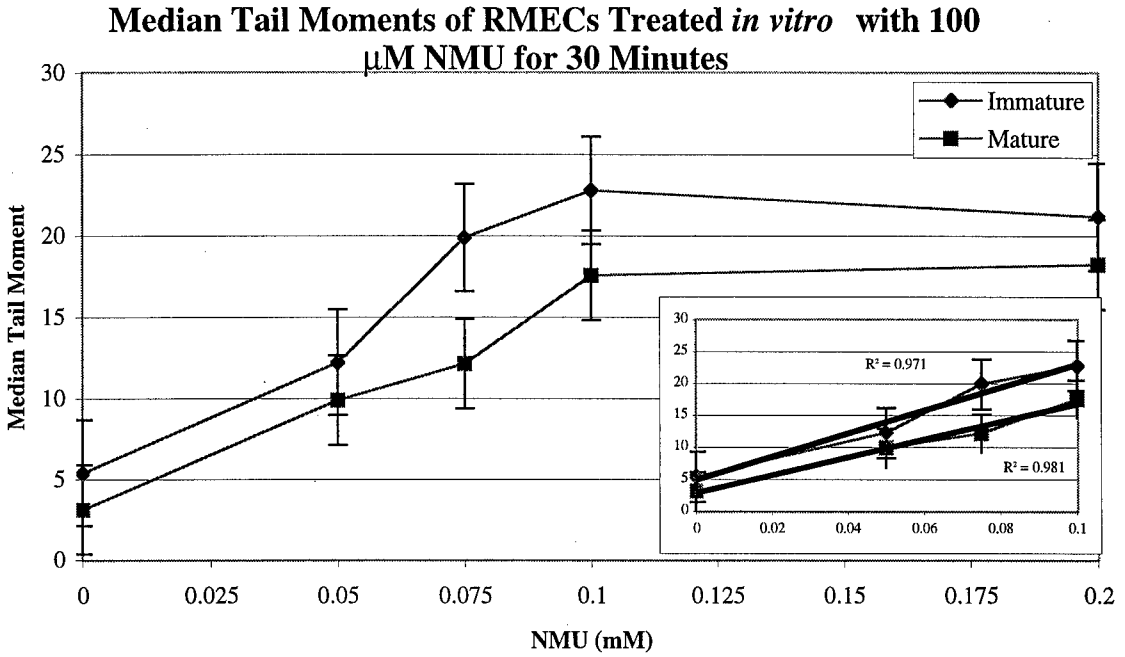
Aim 4, Figure 1. Median tail moments were calculated from the 50 comets analyzed per condition. Error bars represent the standard error.



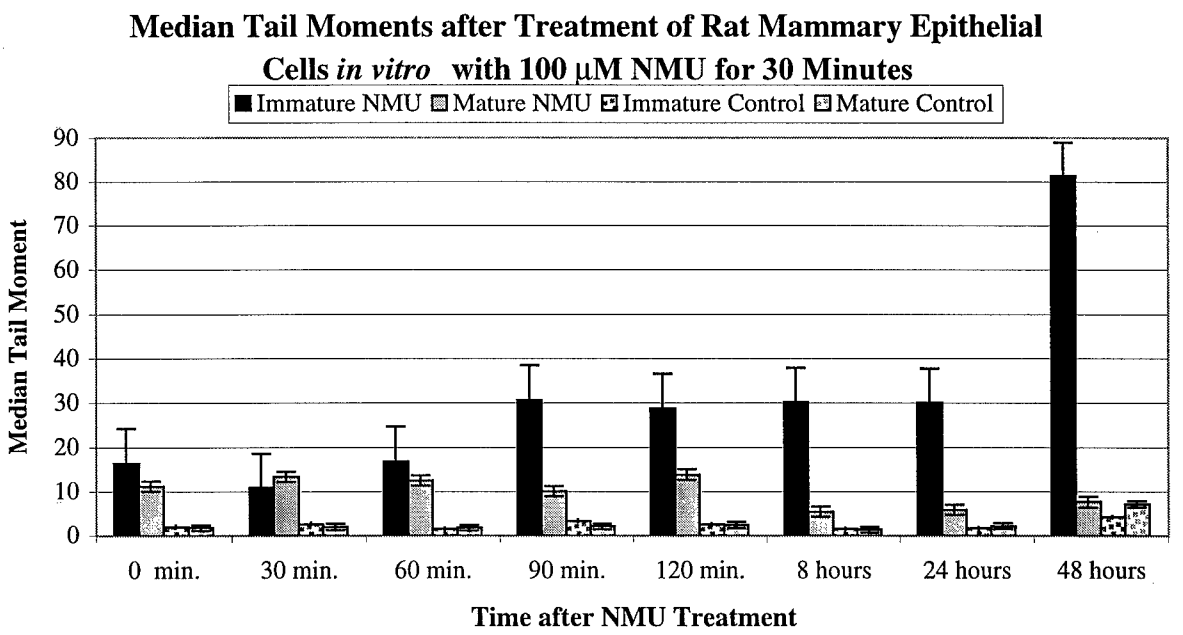
Aim 4, Figure 2. Median tail moments were calculated from the 50 comets analyzed per condition. Error bars represent the standard error.



Aim 4, Figure 3. Median tail moments were calculated from 50 comets/condition. Error bars represent standard error. Other experiments conducted only with immature cells indicate that the dose-response is linear through 125 Gy, after which there is a plateau.



Aim 4, Figure 4. Median tail moments are calculated from 50 comets/condition. The inset displays the linearity of the dose-response; symbols are the same as for the main graph.



Aim 4, Figure 5. Median tail moments from 50 comets/condition are shown. Error bars are standard errors.

Aim 5: Is the lack of TGFα 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 (TGFα) 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 TGFα, while the mature gland does. The basis for this assumption is a publication reporting that TGFα 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 TGFα 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 TGFα mRNA may not indicate the absence of TGFα protein, since Smith, *et al.* could not detect TGFα mRNA in RNA isolated from lactating rat mammary gland, where TGFα activity was found (1989). Also, McAndrew *et al.*(1994) report that the level of TGFα mRNA doesn't necessarily directly relate to the amount of protein, since they found a decrease in immunoreactive TGFα in the pregnant rat mammary gland, but others reported an increase in TGFα mRNA expression at that stage (Liscia *et al.* 1990 and Snedeker *et al.* 1991). McAndrew *et al.* reported no difference in the immunolocalization of TGFα in the mammary glands of immature (21 day old) and mature (50 day old, the oldest rats studied) female Ludwig Wistar OLA rats (1994). The 50 kDa immunoreactive TGFα 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 TGFα than the epithelial cells of the older rats, although the myoepithelial staining was stronger in the 21-day and 50-day old rats.

On one hand, the murine gland appears to age-differentially express TGFα mRNA. On the other hand, the Ludwig Wistar OLA 21- and 50- day old rats express the same amount of protein. However, the rats used in the present study will be Fischer 344 rats, which might differ from the Ludwig Wistar OLA rat. This discrepancy must be reconciled before serious work can begin on this aim.

Toward that end, a TGFα immunohistochemistry system kit has been purchased, and optimal primary antibody concentrations have been determined for staining both immature and mature rat mammary glands. Mammary glands from mature and immature irradiated (because the slides are available) and control rats will be analyzed by immunohistochemistry for TGFα. If age-differential staining is observed, then work will continue with this aim. If not, a logical candidate gene identified in Aim 6 will be further studied under Aim 5.

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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?

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

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.

In our last year report, we have demonstrated the successful application of PCR-select subtraction method in cloning the differentially expressed sequences in 3-wk and 8-wk F344 mammary glands. Among the cDNAs being characterized, several clones encoded the α-casein and κ-casein protein. Casein genes are markers for mammary differentiation (1, 2), their expression in rat mammary glands are known to associate with sexual maturation. A third sequence encoding the lipopolysacchride binding protein (LBP) was also identified by using both reverse northern dot blotting. The differential expression of LBP in 3-wk and 8-wk mammary was confirmed by ribonuclease protection assay. We have previously reported that approximately 300 8-wk subtraction clones were isolated, 96 of them were analyzed by reverse northern blotting and selectively sequenced. In this report, we focused on the characterization of 3-wk and 8-wk PCR-Select subtraction cDNA clones

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 acdified water ad libitum. The rat were housed under a 12 hour light and 12 hour dark cycle.

RNA Isolation and cDNA Synthesis

Lower mammary glands (glands D, E and F) were collected from 3-week and 8-week olds 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. Poly(A)+ RNA was then prepared from the total RNA using RNeasy kits (QIAGEN). Double-stranded cDNA (ds cDNA) was synthesized from poly(A)+ RNA using the SuperScript Choice System (Life Technologies). Tester and driver ds cDNAs are prepared from two mRNA samples under comparison.

Subtraction cloning of differentially expressed sequences

The differentially expressed sequences in 3-wk and 8-wk cDNAs were subtracted, amplified and subcloned using the PCR-Select cDNA subtraction kit (CLONTECH Laboratory, Inc.) as shown in Figure 1 (p.33). The manufacturer's procedures were followed with minor modifications. During the second PCR, subpopulation nested PCR primers (Table I) were used to replace the nested PCR primer 2R . Twenty seven thermal cycles were performed for the primary PCR and 19 to 23 cycles for the secondary PCR with other parameters specified by manufacturer's protocols. The secondary PCR products from subtracted 3-wk and 8-wk F344 mammary gland cDNA were separated on 5% polyacrylamide gel and visualized by Fluoroimager after staining with SYBR Gold nucleic acid gel stain (Molecular Probes) or by silver staining (3).

Aim 6 - Table I. Subpopulation primers for the second PCR

Subpopulation primer	Sequence*
Nested PCR primer 2R/A	AGCGTGGTCGCGGCCGAGGT**A**
Nested PCR primer 2R/C	AGCGTGGTCGCGGCCGAGGT**C**
Nested PCR primer 2R/G	AGCGTGGTCGCGGCCGAGGT**G**
Nested PCR primer 2R/T	AGCGTGGTCGCGGCCGAGGT**T**
Nested PCR primer 2R/ACA	AGCGTGGTCGCGGCCGAGGT**ACA**
Nested PCR primer 2R/ACC	AGCGTGGTCGCGGCCGAGGT**ACC**
Nested PCR primer 2R/ACG	AGCGTGGTCGCGGCCGAGGT**ACG**
Nested PCR primer 2R/ACT	AGCGTGGTCGCGGCCGAGGT**ACT**

* subpopulation Nested PCR primer 2R/ differs from primer 2R by containing additional nucleotide(s) variant at 3' end.

Subcloning and characterizing the subtracted cDNA sequences

To subclone the 3-wk subtraction clones, silver stained bands from a duplicate polyacrylamide gel (Fig. 2, p.34) were disected and eluted in TE buffer, followed by PCR-reamplification using nested PCR-primer P1 and nested PCR-primer R2 under following conditions: 98°C for 1 min, followed by 35 cycle of 95°C for 1 min, 55°C for 45 sec, 72°C for 4 min, and completed by 72°C for 5 min. PCR products from individual bands were subcloned into pCRII vector using the TA cloning kit (InVitrogen).. Recombinant subclones were identified by PCR screening and selected clones were sequenced by cycle sequencing. The 8-wk subtracted sequences were subloned directly from entire PCR product mixture of each primer set into the pCRII vector. During the updating period, more potentially regulated clones identified by reverse northern blotting (Fig 3, p.35) were further investigated by using ribonuclease protection assay (RPA) using RPAII kit (Ambion). The remaining 8-wk subtraction clones were sequenced by a cycle sequencing method.

The cycle sequencing was carried out using the Big Dye reagent under following conditions: 95°C

for 3 min, followed by 40 cycles of 94°C for 30 sec, 45°C for 30 sec and 52°C for 4 min, and completed by 72°C for 7 min. The sequencing samples were deciphered in Perkin-Elmer Sequencer 377 (Norwalk, CT) Identities of the subtraction clones were analyzed by searching the GenBank database using the NCBI's sequence similarity search tool BLAST. Clones with different signal levels in reverse northern dot blot were further examined by RPA.

Results and Discussion

Different patterns were observed between PCR-amplified subtracted 3-wk and 8-wk cDNAs (Fig. 2, p.34). The isolation of α-casein and κ-casein cDNA confirmed the previous observation and the successfulness of the PCR-select subtraction procedure. Several clones have demonstrated differential expression between 3-wk and 8-wk mammary glands by (Fig. 3. p.35). In addition to lipopolysaccharide binding protein (LBP), α-casein and κ-casein, two additional differentially expressed clones were identified as selenoprotein P, stearyl-CoA desaturase, respectively. The differential expression of these mRNAs were further confirmed by RPA, they were expressed 3-fold hgher in 8-wk mammary gland (Fig. 4, p.36). It has been suggested that LPB play a role in the host immune responses to injury and infection, however, the mechanism remained to be characterized (4). Selenoprotein P functions to protect the plasma membrane from oxidative damage (5). The stearyl-CoA desaturase plays an important role in fat cell metabolism (6).

Seventy five 3-wk subtracted sequences were subcloned from silver-stained gel. All these 3-wk clones and remaining 8-wk clones isolated previously were sequenced. Their identities revealed by database search were summarized in Table II and Table III.

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

#*	Identity/Homology (# of multiple independent clones)
1	rat lactate dehydrogenase-A (2)
2	rat cytochrome oxydase subunit I cDNA
3	rat H-rev107
4	rat cytochrome oxidase subunit I
5	rat mRNA for protein disulfide isomerase
6	rat class I beta-tubulin cDNA
7	mouse mRNA for osteoblast specific factor 2 (OSF-2) (4)
8	mouse Nedd5 mRNA for septin (2)
9	mouse fibroblast growth factor inducible gene 14 (FIN14) mRNA
10	mouse TIMP-gene for metalloproteinase-3 tissue inhibitor
11	mouse alpha-2-macroglobulin (AM2) receptor
12	mouse SCF complex protein cul-1 mRNA (2)
13	mouse mRNA for cmplement subcomponent C1Q alpha-chain (2)
14	mouse 30kDa adipocyte complement-related protein Acrp30 mRNA
15	mouse transcription factor C1
16	human clone 25191 GTP-specific succinyl-CoA Synthetase
17	human ATP synthase
18	human placental protein 15
19	rat EST** AI030806
20	rat EST AI044121
21	rat EST AI059513
22	rat ovary EST AI407805 (2)
23	rat EST AI535463 (2)
24	rat EST AI556018.1
25	mouse embryo EST AA000847
26	mouse embryo EST AA288883
27	mouse mammary gland EST AA764413 (2)
28	mouse macrophage EST AA798026 (2)
29	mouse mammary gland EST AA832670 (2)
30	mouse mammary gland EST AA967067 (3)
31	mouse mammary gland EST AI226339 (2)
32	human mRNA for KIAA0767 protein (3)
33-35	Independent novel sequences

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

** EST, expressed sequence tags

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

#	Identity/Homology (# of multiple clones)	#	Identity/Homology (# of multiple clones)
1	rat GTP-BP G-α-8 subunit (2)	57	human putative Cu^{++}-transporting P-type ATPase
2	rat MHC I RT1.C/E	58	human rab3-GAP regulatory domain (2)
3	rat lipopolysacchride-BP (LBP)	59	human PCA clone DJ0740D02 7p14
4	rat putative RNA binding protein gene	60	human mRNA for Pex protein
5	rat GSH S-transferase (3)	61	human KIAA0682 protein mRNA
6	rat α-casein	62	human KIAA0105 gene (3)
7	rat GEF-2human DAP-kinase mRNA	63	human DAP-kinase
8	rat PI3-kinase Sreg p85 (2)	64	human chromosome 14q32 clone BAC2B24
9	rat Stearyl-CoA desaturase (7)	65	human angio-associated migratory cell protein, AAMP mRNA
10	rat ATP synthas (γ-3)	66	human angio-associated migratory cell protein (AAMP) mRNA, fragment II
11	rat mitochondrial cytochrome c oxidase		
12	rat κ-casein mRNA (3)	67	rat EST* AA500532
13	rat vimentin cDNA (2)	68	rat liver EST AA800355
14	rat amphotenin	69	rat spleen EST AA801434
15	rat CD14	70	rat kidney EST AA817882
16	rat oxytocin receptor (OTR) (2)	71	rat EST AA874832 (3)
17	rat ceruloplasmin, Cu^{++}binding protein (2)	72	rat EST AA925126
18	rat putative guanylate-BP	73	rat EST AA964307
19	rat transferrin (6)	74	rat lung EST AI009650
20	rat Peptidylglycine α-amidating monooxygenase	75	rat EST AI029606
21	homologous Vitamin D-BP gene, exon 2 (2)	76	rat Kidney EST AI045201
22	rat AKAP95, kinase A anchor protein	77	rat liver EST AI169729
23	rat Cyclin D1	78	rat ovary EST AI176665
24	rat ryudocan=heparan sulfate proteoglycan core protein (2)	79	rat ovary EST AI177091 (7)
25	rat partial brain mRNA (clone sap33f)	80	rat kidney EST AI232974
26	rat X chromosome-linked phosphoglycerate kinase	81	rat ovary EST AI236402
27	rat mitochondrial cytochrome c oxidase s.u. III	82	rat ovary EST AI236798
28	rat plasma glutathione peroxidase (2)	83	rat placental EST AI237848 (2)
29	rat glucose-dependent insulinotropic polypeptide receptor (2)	84	rat ovary EST AI408502 (2)
30	rat glucose-dependent insulinotropic polypeptide receptor, different fragment	85	rat heart EST AI410810
31	3' homologous to the rat epithelial cell transmembrane Ag precursor	86	rat ovary EST AI497797
32	rat Xlas, GTP-binding protein (G-α-8) mRNA	87	rat EST AI574984
33	rat cytoplasmic dynein heavy chain (MAP1C)	88	rat EST AI575716.1
34	rat clone N27 mRNA, MNU indeuced mammary tumor	89	rat embryo EST AI600100 (3)
35	rat MHC class 1 RT1, Aw3 protein	90	rat EST AI602058.1
36	rat RK/IF-1 mRNA (I-kappa-B a chain)	91	rat EST AI602912 (4)
37	rat tissue factor protein mRNA	92	rat EST AI713213
38	rat cathepsin H mRNA	93	mouse embryo EST AA008689
39	rat GTS-P gene, encoding placental-type glutathione S-transferase	94	mouse kidney EST AA106453
40	rat proteasome subunit RC6-1 (2)	95	mouse EST AA183632
41	rat ribosomal protein L10a	96	mouse EST AA185231
42	mouse occludin mRNA	97	mouse EST AA261654
43	mouse Mfg-2 mRNA	98	mouse EST AA435303
44	mouse NKx-5.1 homeobox	99	mouse mammary gland EST AA611141
45	mouse gelsolin gene cDNA (3)	100	mouse skin EST AA840274
46	mouse GDP-dissociation inhibitor mRNA	101	mouse mammary gland EST AA959536 (7)
47	mouse MHC region containing the Q of the class I	102	mouse liver EST AI529140
48	mouse CDV-1R protein mRNA	103	mouse EST AI587910.1
49	mouse putative transcriptional factor mRNA	104	mouse EST AI605638
50	mouse complement C4 mRNA	105	mouse EST AI626967
51	mouse cyclin D-interacting myb-like protein (Dmp1)	106	mouse EST W81852

52	mouse Wnt-4 mRNA (2)	107	human EST AA344414
53	mouse putative guanylate binding protein mRNA, 3' untranslated region	108	human EST AA904725
54	mouse cDNA for ADAMTS-1		
55	human rab11a GTPase (3)	109-120	Independent novel clones
56	human mitochondrial ATP synthase s.u.9		

* EST, expressed sequence tags

More than half of the isolated sequences were either identical to known rat sequences or highly homologous to sequences from other species. One third 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 fetched 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. 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 these clones are currently under investigation by RPA.

Conclusions

Multiple differentially expressed sequences were isolated from subtracted 8-wk F344 cDNA library by a PCR-select subtraction method. This indicated the subtraction procedure is successful in isolating differentially expressed sequences presented in two comparing populations. Two out of five cDNA clones characterized encode the milk proteins α-casein and κ-casein, implicating that more clones isolated in this study will be physiologically relevant to the maturation of mammary gland. More than two hundred independent sequences have been cycle sequenced. Of these sequences, 140 showed identity or homology to sequences in GenBank database. This study will provide a handful markers for the molecular anatomy of mammary gland during its maturation process.

References

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Aim 6 - Figure 1

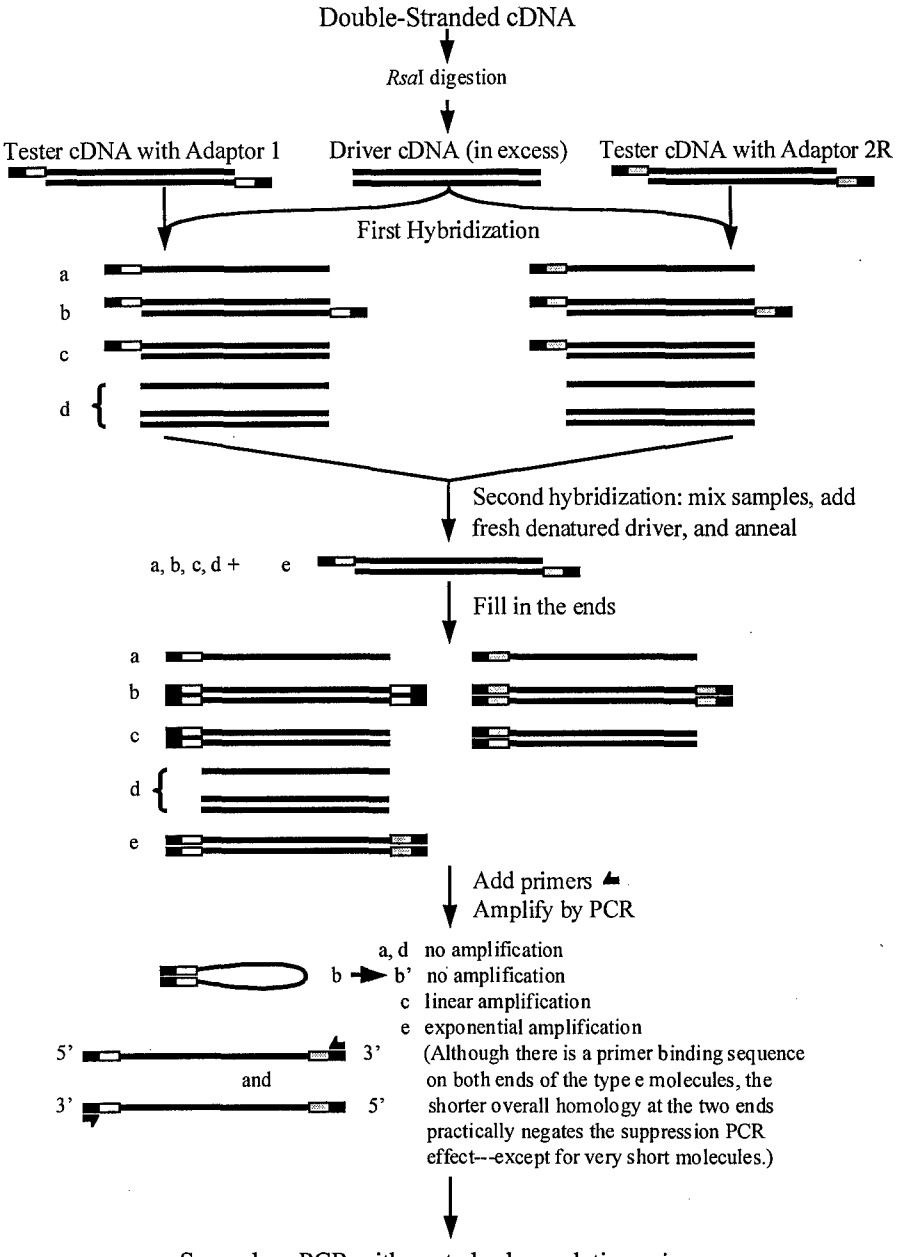

Fig. 1. Schematic diagram of PCR-select cDNA subtraction. The cDNA in which specific transcripts are to be found is defined "testes" and the reference is defined as "driver". Type **e** molecules are formed only if the sequence is up-regulated in the tester cDNA. Solid lines represent the outer part of the Adaptor 1 and 2R longer strands and corresponding PCR primer 1 sequence. Clear boxes represent the inner part of the Adaptor 1 and the corresponding nested PCR primer 1sequence; shaded boxes represent the inner part of Adaptor 2R and the corresponding nested primer 2R sequence.

Aim 6 - Figure 2

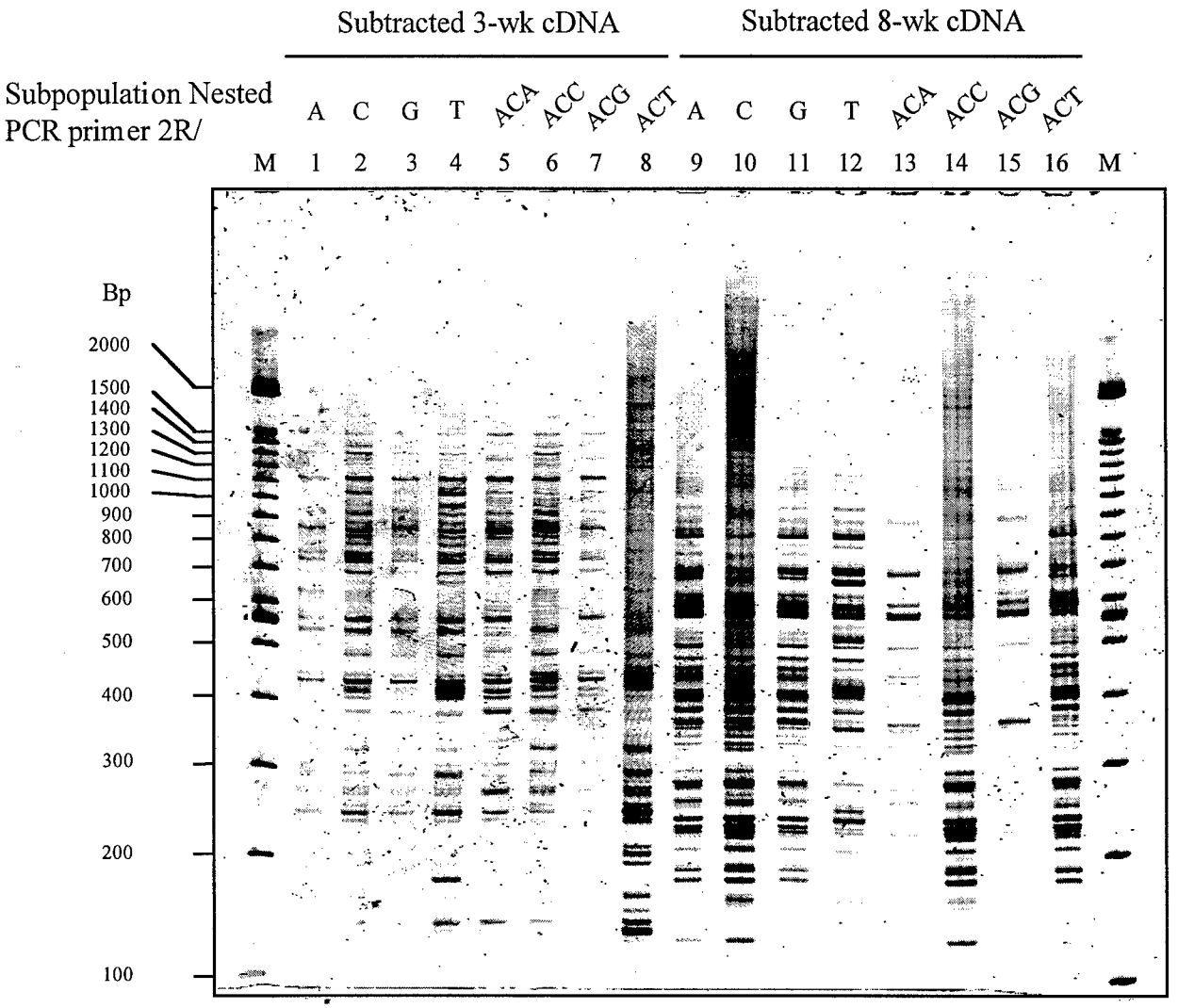

Fig. 2. Display of PCR-amplified subtractive libraries. The secondary PCR products of the subtracted mammary gland cDNAs were resolved by electrophoreses on 4% polyacrylamide gel. Nested PCR primer 1 and Nested PCR subpopulation reverse primer 2R/ (Table I) were used in the secondary PCR. The 3' end of each subppopularion primer 2R/ was indicated. Lanes 1 to 8, subtracted 3-wk cDNA samples; lanes 9-16, subtracted 8-wk cDNA samples.

Aim 6 - Figure 3

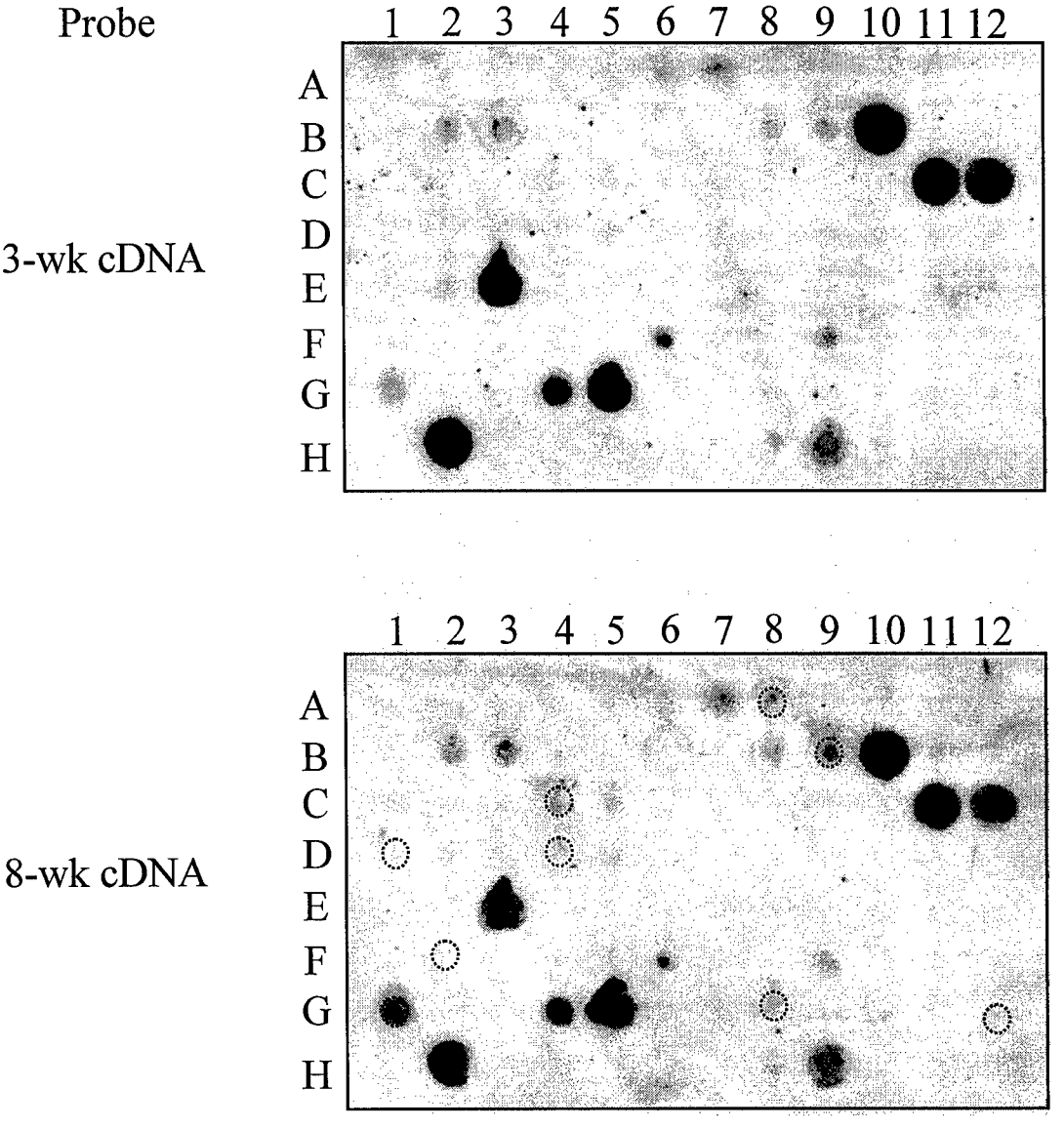

Fig. 3. Reverse northern blot. The PCR-amplified inserts of ninety six clones were blotted to duplicated Hybond-N membranes. Each membrane was hybridized with ^{32}P-labeled cDNA probe derived from the 3-wk and 8-wk F344 mammary gland mRNA, respectively. The hybridized membranes were washed under high strigent conditions and exposed to phosphoimager cassette. Clones with a signal ratio higher than 3 are highlighted by dashed circles. Four clones verified by sequencing are listed below: A8, lipopolysacchride binding protein (LBP); B9, α-casein; C4, stearyl-CoA desatursae; D4, κ-casein. 3.

Aim 6 - Figure 4

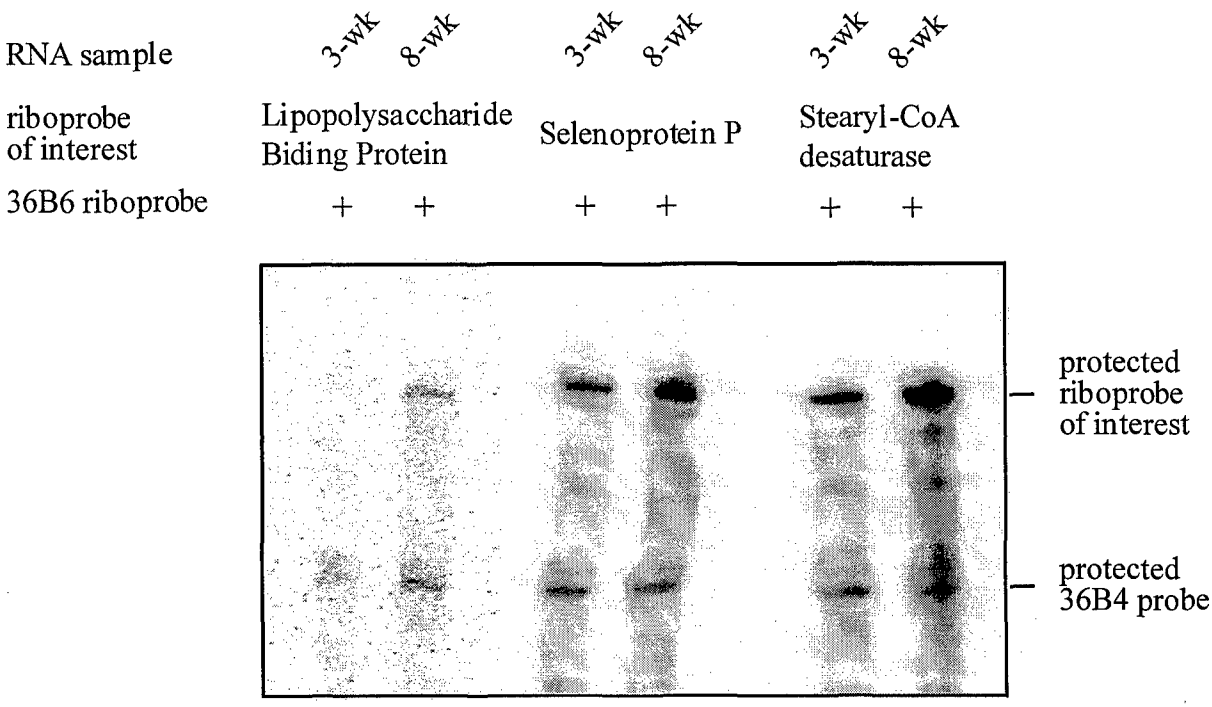

Fig. 4. Ribonuclease protection assay of mammary gland mRNA. Ten mg of 3-wk and 8-wk mammary gland total RNA was hybridized to ^{32}P-labeled LPB, selenoprotein P, stearyl-CoA desaturase and 36B4 riboprobes and incubated at 42°C overnight. For control, yeast tRNA was added to each probes. After RNase 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 chromotography paper and exposed to phosphoimager cassette overnight and analyzed.