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INTRODUCTION:

In utero exposures to estrogen or estrogen mimics such may alter later breast cancer risk. Some of these estrogen-responsive pathways utilized during fetal development, are re-employed at times of tissue remodeling or wound healing during adulthood. These signal transduction systems effect proliferation, differentiation and apoptosis which in turn may affect later breast cancer risk. The heavy metal cadmium potently binds to and activates the estrogen receptor, having a half life in the mammalian body of over 30 years. Previous studies have shown that *in utero* exposure to cadmium at the levels present in some human environments accelerated puberty onset and altered some of the indicators of mammary gland development in rats. In this study we sought to determine whether *in utero* exposure to low doses of dietary cadmium altered puberty-related body and mammary gland development and ultimately breast cancer risk.

To test this possibility, we exposed pregnant rat dams to a diet similar to the human in fat content, 30% and very low doses of cadmium, 0.075 or 0.15 mg/kg feed cadmium throughout pregnancy. The effects on (i) birth weight, (ii) postnatal weight development, (iii) vaginal opening/puberty onset, (iv) mammary gland development, and (v) DMBA-induced mammary tumorigenesis were investigated. After parturition, all rats were switched to AIN93 laboratory chow. Birth-weight was not affected by fetal cadmium exposure, but the higher cadmium dose induced a long-lasting increase in postnatal body weight that was first detected on postnatal day 5 ($p < 0.04$), and it accelerated vaginal opening ($p < 0.03$). Final mammary tumor incidence was highest in the higher cadmium group (80% of rats developed tumors) and lowest in the lower cadmium group (56% tumor incidence) ($p < 0.001$); 73% of the control rats developed mammary tumors. These findings indicate that *in utero* exposure to 0.15 mg cadmium per kg feed via maternal diet increases postnatal weight development and induces earlier puberty onset. Exposure to a lower dose of dietary cadmium, 0.075 mg/kg feed, slightly decreased weight during puberty and significantly reduced susceptibility to later mammary carcinogenesis, when compared to rats exposed to the 0.15 mg/kg feed cadmium containing diet.

OBJECTIVES:

Objective 1: Determine whether *in utero* exposure to cadmium changes the patterns of proliferation and differentiation of mammary epithelial cells.

Objective 2: Determine whether *in utero* exposure to cadmium alters the arcuate nucleus, the center of hypothalamic control of both puberty on-set and appetite regulation and the mechanism by which this occurs.

Objective 3: Determine if *in utero* exposure to cadmium increases the risk of breast cancer.

Objective 1: Determine whether *in utero* exposure to cadmium changes the patterns of proliferation and differentiation of mammary epithelial cells.

Task 1: Determine the whether *in utero* exposure to cadmium alters the proliferation and differentiation of the epithelial cells of the mammary gland (Year 1, Months 6-18)

I. Indications of differences in gross morphology at days 28 and 50

a. Day 28 Pre-pubertal Mammary Gland Analysis: Terminal end buds (TEBs) end bud numbers and qualitative epithelial density

1. Day 28 TEBs

A statistically significant difference existed between the low dose cadmium (averaged TEB number 45.5, SEM $\pm$ 3.71) and both estrogen (averaged TEB number 82, SEM $\pm$ 4.24, $p < 0.027$, One-Way ANOVA) or high dose cadmium (averaged TEB number 78.8, SEM $\pm$ 7.63, $p < 0.047$, One-Way ANOVA) exposed animals.

However there was no significant difference between control (averaged TEB number 59.6, SEM ± 10.26) and high dose, or estrogen exposed offspring. The power of this test was 0.75.

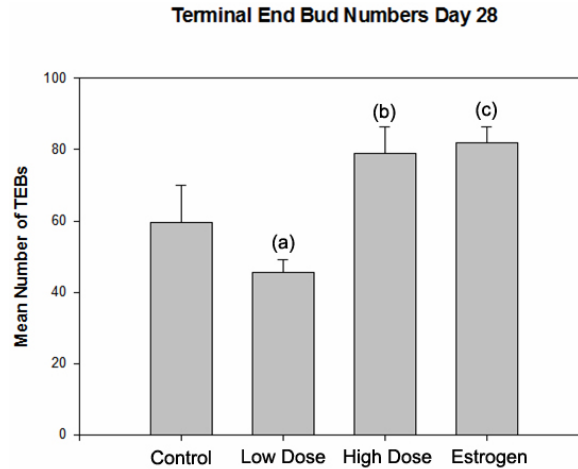


Figure 1. Terminal end bud numbers were counted from carmine-alum stained whole mounts. TEB numbers were analyzed using SigmaStat. Low dose treatment group (a) was significantly less than either the high dose cadmium (b) or estrogen (c) exposed off spring. (One-Way ANOVA based on group affiliation, $a < b$, $p < 0.027$, and $a < c$, $p < 0.047$).

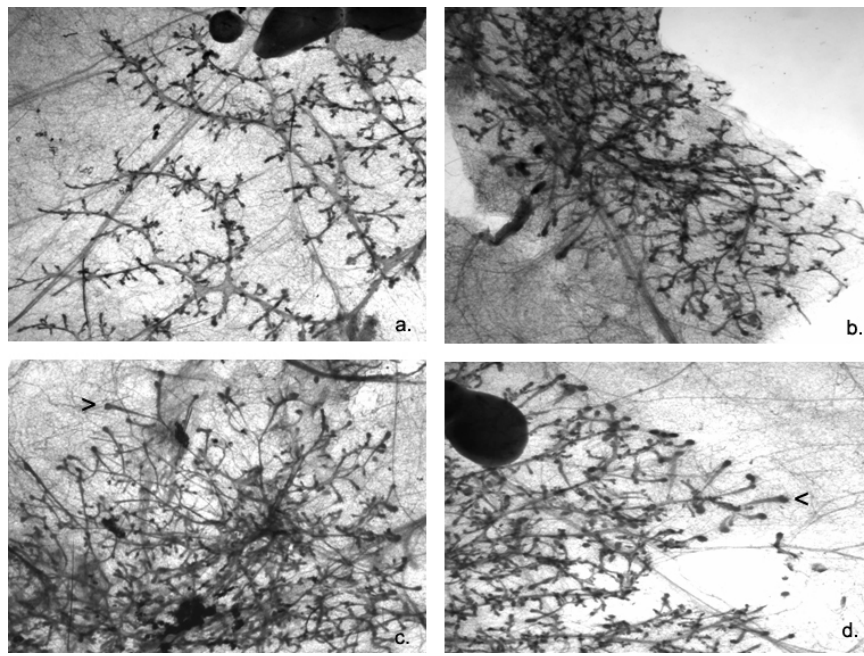


Figure 2 : Qualitative differences among, (a) control, (b) low dose cadmium (0.075 mg/kg), (c) high dose cadmium (0.15 mg/kg) and (d) estrogen exposed rats, at post-natal day 28. Pre-pubertal differences in epithelial density and terminal end bud numbers. In (a) control group, the epithelial branching appeared to be much lower than in all other groups. Notice that (b) the low dose group was more densely branched with a lower number of terminal end buds. However both (c) the high dose group and (d) the estrogen treated group had increased terminal end bud number, with a denser epithelia than seen in the control group.

b. **Post-puberty, Day 50 Gross and fine morphology: Grading Estrus Status, Epithelial Density, TEB number, Allometric Growth, Proliferation and Apoptotic Ratios**

1. **Grading Estrus Status by Duct and Lobule Type**

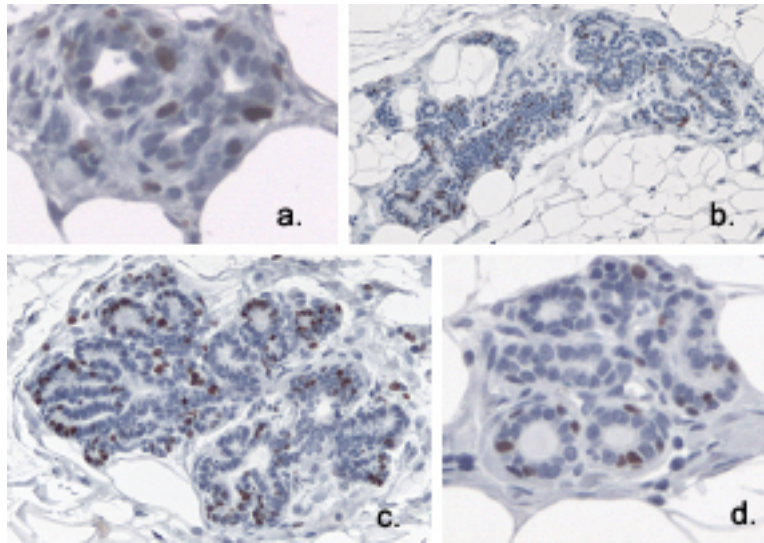


Figure 3. Examples of Estrus Staging based on Duct and Lobule Type: a) pro-estrus, b) estrus, c) metestrus and d) diestrus. Criteria for each stage were based on published standards (Schedin et al., 2000). Structures were stained for Ki67, a marker of cellular proliferation. Counterstain was with Harris mercury-free hematoxylin. Proliferation and apoptosis vary significantly in untreated Sprague Dawley female rats depending on estrus stage. These can be periods of epithelial tissue expansion, such as metestrus (similar to pregnancy and lactation) or retraction, such as diestrus 1 or 2 (similar to involution).

2. **Epithelial Density**

Epithelial density is considered one of the strongest known biomarkers of increased breast cancer risk in humans. This may be inversely related to body weight and menopausal status and has been found in human to be positively related to estrogenic environment *in utero* (Cerhan et al., 2005; Li et al., 2005). In my study of Female Sprague Dawley rats, the average density of the epithelial tree was compared to the density of the fat pad. Electronic photomicrographs were obtained using a stereomicroscope, bright-field light setting with a 0.5X lens attached to a digital camera. Electronic photomicrographs were then analyzed using ImageJ. The procedure was as follows: about a 1 cm square was drawn within the epithelial tree and density measured. The same square was then moved to a fat pad location which did not have any epithelial tree and density measured. A ratio between the two was then obtained. For each whole-mount this was done for seven different field views. Epithelial density of each treatment group was compared to the control group by One-Way ANOVA on Ranks. *Estrogen exposed offspring* had a significantly increased epithelial density ($p < 0.05$) compared to the low dose treatment group, but there was no statistically significant difference in density between control, low dose or high dose treatment groups. Medians were as follows: control (1.592, 25%=1.327, 75%=1.747), low dose (1.539, 25%=1.34, 75%=1.796), high dose (1.561, 25%=1.381, 75%=1.850) and estrogen (1.719, 25%=1.545, 75%=2.156).

The epithelial density also differed significantly by estrus status. Measurement of effect of estrus status on epithelial density was analyzed against group affiliation by Two-Way ANOVA. During metestrus, the epithelial density (Mean 1.922, SEM ± 0.0634) was significantly higher than during diestrus-1 (Mean 1.552, SEM ± 0.0853 , $p < 0.005$) or diestrus-2 (Mean 1.534, SEM ± 0.0766 , $p < 0.001$) regardless of treatment group. Mean $\pm$ SEM density values during estrus were as follows: estrus (Mean 1.646, SEM ± 0.0627) and pro-estrus (Mean 1.619, SEM ± 0.121) were not significantly altered compared to metestrus or diestrus 1 or 2 stages. Interestingly, both during diestrus-1 and diestrus-2 in the rat model, levels of progesterone are significantly

decreased. They are at base-line levels during metestrus. However this effect may yet be related to estrogens. It is known that estrogen can accentuate progesterone related proliferation of the epithelial cells of the mammary gland fat pad so this finding may be significant in regards to delayed effects of hormonal estrogens levels on Sprague-Dawley Rat mammary gland physiology during estrus cycling.

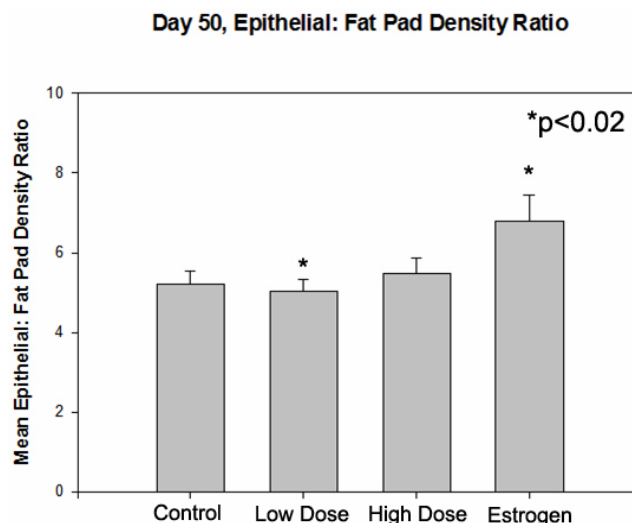


Figure 4. Epithelial density to fat pad density at post-natal day 50. Epithelial Density varied between groups at Day 50, with the low dose group having the lowest epithelial density and the estrogen exposed group having the highest epithelial density. In humans, exposure to excess estrogens during pre-natal development has also been found to increase epithelial density, and this may be correlated to later breast cancer risk (Li et al., 2005). The power of this test by One-Way ANOVA, controlling for group affiliation alone, was 0.75. When data are analyzed by Two-Way ANOVA based both on group affiliation and estrus status, there is no significant difference based on group affiliation, but there are significant differences based on estrus status (see below).

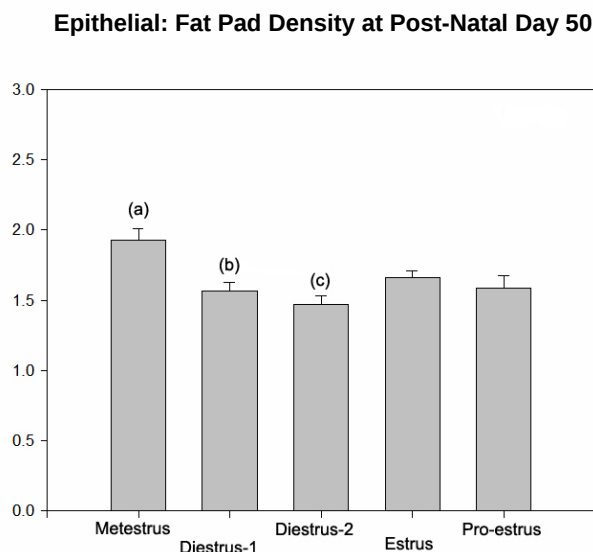


Figure 5: Epithelial Density at Day 50 varied by estrus status independent of treatment group. The most significant difference was between metestrus (a) and diestrus-1 (b) ($p<0.001$) or metestrus (a) and diestrus-2 (c) ($p<0.005$), Two-Way ANOVA, controlling for treatment group. The power of this test was 0.963.

3. Day 50 TEBs

The average terminal end bud number in the control group was 20, low dose group 23, and estrogen and high dose groups had an average of 27 (n=5 per group). Thus, there was a 25% decrease in terminal end bud number in the low dose and control groups versus high and estrogen groups. However, this was not a statistically significant difference between groups by One-Way ANOVA ($p < 0.420$). These negative findings should be cautiously interpreted though because in this data set sample size may not have been large enough (was very much below a desired power of 0.8, with a power of 0.58) to accurately test differences in terminal end bud number. It is usually assumed at this age that a lower number of primary end buds indicates higher rates of differentiation of the end structures responsible for invasion of the fat pad during development, namely TEBs. In this study we utilized several markers of epithelial differentiation and development which are altered in a statistically significant pattern for the low dose cadmium treatment group. If terminal end bud number is deemed to be important, future studies will use 6-8 offspring per group per developmental time-point to remedy the problem of statistical study power. However, again, a group of n=5 provided significant study power for other endpoints examined both in whole mounts, immunohistochemical staining and *in situ* TUNEL assay.

4. Day 50 Allometric growth indicators: Length of epithelial tree compared to total mammary gland parenchyma

Allometric growth refers to the phenomenon that at certain times during development some body parts will grow at a faster rate than other body parts or the body as a whole. In this instance, during puberty, the mammary gland fat pad grows at a much slower rate than the mammary gland epithelial tree, thus displaying allometric growth. Allometric growth generally occurs during specific periods of mammary gland development namely: fetal development and puberty.

Measurement of allometric growth was obtained by first measuring distance from primary lymph node to end of the fat pad (total mammary gland parenchyma) and comparing that length to distance from primary lymph node to end of epithelial tree (mammary gland epithelia). This gives an indication of the ratio of growth of the mammary epithelial tree compared to the length of the total mammary gland parenchyma. Puberty is a time of mammary gland *cell growth, differentiation and remodeling*. Thus alterations in allometric growth can be considered **indirect measurements** of mammary gland growth and cellular differentiation.

It might be assumed that the higher the percentage of fat pad filled with epithelial tree, the more developed the mammary gland. In this instance an average of 71% of the length of the fat pad was filled with the mammary epithelial tree in **low dose cadmium group** compared to an average of 53% in the **high dose group** ($p < 0.005$). Control group had an average of 65% and estrogen group 67%. These differences in allometric growth were inversely related to differences in later breast cancer incidence.

5. Immunohistochemical staining for proliferation (Ki67) and apoptotic markers (TUNEL)

Immunohistochemistry was performed on all samples obtained at Day 28 and Day 50 to examine the effects of in utero exposure to cadmium on the proliferation of epithelial cells. Results are summarized in Table 1 below, which also summarizes apoptosis (TUNEL) findings. As indicated in this task, epithelial cell proliferation in tumor tissue was also examined for Ki67 index and its overall index was 0.32, thus 32% of the cells stained positively for cell cycle indicator, Ki67. As a negative control, involuting mammary gland was stained for Ki67, and proliferation rates were found to be low (10%). The methods for performing these tests are described in the attached abstract, in the appendix section, entitled *In utero exposure to Cadmium, and Breast Cancer Risk in Female Sprague Dawley Rats*, presented at the Annual American Association for Cancer Research meeting, 2007 in Los Angeles, CA. For summarized data, see Table 1 on the following page.

Day 28 Female Offspring					
In utero exposure Treatment Group	Control: 30% fat + vehicle	Low Dose: 30% fat + 0.075 mg CdCl₂/kg Feed	High Dose: 30% fat + 0.150 mg CdCl₂/kg Feed	Estrogen Control: 30% fat + excess estrogen	Statistical Significance
Average Ki67 Indices by Mammary gland Structure (n=5 animals per group, 700-1500 cells per structure for each animal)	TEBs: 0.384, SEM ±0.0285 Lobules: 0.0856, SEM ±0.0338 Ducts: 0.113, SEM±0.0324	TEBs: 0.415, SEM ±0.0300 Lobules: 0.137, SEM ±0.0355 Ducts: 0.129, SEM±0.0355	TEBs: 0.322 SEM ±0.0300 Lobules: 0.147, SEM±0.0317 Ducts: 0.112, SEM±0.0338	TEBs: 0.454, SEM±0.0311 Lobules: 0.146, SEM±0.0346 Ducts: 0.246, SEM±0.0346	p<0.005 estrogen vs. high dose and control via Two-Way ANOVA; proliferation in the terminal end buds was most significantly altered.
Day 50 Female Offspring					
In utero exposure Treatment Group	Control: 30% fat + vehicle	Low Dose: 30% fat + 0.075 mg CdCl₂/kg Feed	High Dose: 30% fat + 0.150 mg CdCl₂/kg Feed	Estrogen Control: 30% fat + excess estrogen	Statistical Significance
Average Ki67 Indices by Mammary gland Structure (n=5 animals per group, 700-1500 cells per structure for each animal)	TEBs: 0.439, SEM±0.0397 Lobules: 0.322, SEM±0.0405 Ducts: 0.315, SEM±0.0390	TEBs: 0.444, SEM±0.0390 Lobules: 0.353, SEM±0.0390 Ducts: 0.334, SEM±0.0413	TEBs: 0.233, SEM±0.0358 Lobules: 0.114, SEM±0.0358 Ducts: 0.108, SEM±0.0390	TEBs: 0.389, SEM±0.0364 Lobules: 0.215, SEM±0.0376 Ducts: 0.191, SEM±0.0376	p<0.001 by Two Way ANOVA controlling for estrus status, with significant differences between control/low dose groups vs. high dose/estrogen groups.
Average Apoptotic Index by TUNEL	TEBs: 0.296, SEM±0.0378 Lobules: 0.316, SEM±0.0392 Ducts: 0.298, SEM±0.0354	TEBs: 0.104, SEM±0.0419 Lobules: 0.306, SEM±0.0438 Ducts: 0.220, SEM±0.0486	TEBs: 0.503, SEM±0.0496 Lobules: 0.472, SEM±0.0506 Ducts: 0.455, SEM±0.0517	TEBs: 0.327, SEM±0.0438 Lobules: 0.405, SEM±0.0496 Ducts: 0.286, SEM±0.0453	p<0.001 by Two-Way ANOVA controlling for estrus status or structure. Significant differences existed between control and both low and high dose groups. Control and Estrogen treated groups did not differ significantly.
Estrus Status of Animals in Treatment group	N=3, estrus, N=2, pro-estrus	N=2, diestrus 1, N=2 diestrus 2, N=1 metestrus	N=2, metestrus, N=1 estrus, N=2 diestrus 1	N=1, diestrus 1, N=2 estrus, N=2 metestrus	No significant differences in proliferation were due to estrus status alone.

Table 1: Ki67 staining was done for each of day 28 and day 50 samples. There was a significant difference at day 28 between estrogen and low dose groups versus high dose and control groups when data were analyzed based on mammary gland structure (Two-Way ANOVA, p<0.005). However by day 50 the proliferation rate of the low dose groups had significantly decreased and thus low dose and control proliferation rates were significantly lower than the high dose and estrogen proliferation rates. No significant differences were due to estrus status.

6. Day 50 Proliferation: Apoptosis Ratios

In the low dose group, the group with the lowest tumor incidence, the PAR index was highest, indicating that the balance of proliferating or differentiating cells was higher than those undergoing apoptosis. The PAR ratio was significantly altered in the low dose group versus all other groups regardless of structure (Two-Way ANOVA, $p < 0.008$). Ki67 index was used here because it is well established that PCNA is part of a DNA repair mechanism, and thus it is questionable as to whether cells expressing PCNA will complete cell division. It is important to note that proliferation is a net result of the process of asymmetric and symmetric cell division, both important components of differentiation. However this does not give a direct indicator of cellular differentiation but rather whether or not the mammary epithelial is undergoing a net cellular expansion (more proliferation than apoptosis) or a net retraction (such as involution when remodeling much go on to achieve a state similar to virgin gland). Another parameter to consider is that TUNEL in situ analysis probes for DNA strand breaks. It does not always per se indicate that the cell is undergoing apoptosis; however most cells with significant numbers of DNA strand breaks do undergo cell death.

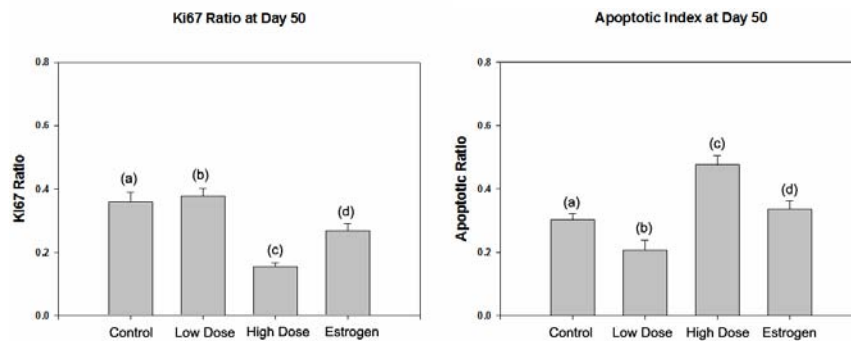


Figure 6. Ki67 (proliferation) and Apoptotic Index at Day 50. Animals were sacrificed n=5 per group on post-natal day 50. Paraffin embedded sections were immunohistochemical stained for Ki67, a marker of cell proliferation or in situ probed by TUNEL for indications of apoptosis. In the case of Ki67, there were significant differences in overall proliferation between (b) the low dose group and (c) the high dose group ($p < 0.05$). In the case of Apoptotic index, there were significant differences between (b) the low dose group and (c) the high dose group, ($p < 0.05$).

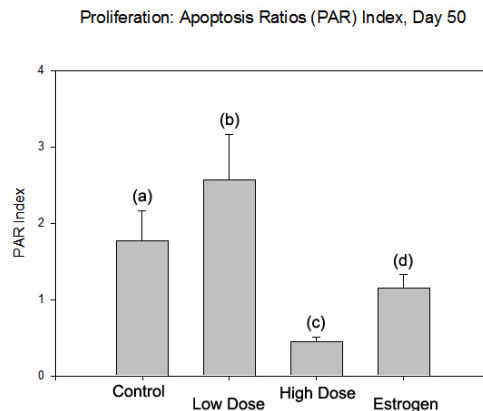


Figure 7: PAR index at Day 50, an indication of mammary gland growth. A ratio of proliferation to apoptosis was taken to distinguish the overall rate of either growth or retraction of the mammary gland epithelia. There existed a significant difference between (b) the low dose group and (c) the high dose group, ($p < 0.05$).

SUMMARY OF MAMMARY GLAND DEVELOPMENT RESULTS

Many of the indicators of significant differences between groups were between the low dose cadmium group and either control, high dose and/or estrogen treated groups. These results can be interpreted in two ways: a) the enhanced allometric growth and mammary gland cellular expansion (PAR index) in the low dose group was due to a slight delay in pubertal on-set and the glands had not completed their normal pubertal development or b) the allometric growth and PAR index represented indirectly an acceleration of growth and differentiation of the mammary gland epithelia and thus a fat pad with enhanced signs of maturity and a longer epithelial tree. The second interpretation is supported by the PAR index or proliferation-to-apoptosis ratio index. PAR index indicates the relative rate of proliferation versus apoptosis in the gland, and thus the overall state of the gland in regards to remodeling. The offspring exposed to a low dose of cadmium *in utero* however had an extremely high PAR index, indicating a net expansion of their mammary glands regardless of estrus stage. Remodeling which occurs during estrus cycle and during pregnancy, lactation and involution involves a balance of proliferation, differentiation and apoptosis. Whilst a tumor may experience enhanced proliferation with little apoptosis, it is most likely not experiencing enhanced differentiation of the cancer “stem” cells. However in non-cancerous epithelia, an enhanced PAR index may indicate increased differentiation, especially as asymmetric cell division is a form of net cell proliferation. Also there is no evidence either for or contrary to the theory that cancer stem cells do “differentiate” but may be “stuck” in a symmetrical division state, and thus their increased proliferation is not apposed by appropriate differentiation or apoptotic competency. Especially because of the fact that apoptosis may be considered the ultimate state of differentiation, and signal transduction is compromised in most tumor tissue, thus leading to a lack of competency to apoptosis.

This study’s results indicate that estrogens or estrogenic endocrine disruptors can influence the allometric growth and development of mammary gland in offspring exposed *in utero* but that the effect is dose dependent. Whether or not these effects are directly influenced by estrogen receptor activity through signal transduction or indirectly influenced by imprinting of important signal transduction pathways remains to be determined. Other possibilities also exist such as signal transduction through growth factors or other hormonal receptors. A few of these will be discussed further.

In regards to tumor growth factors, many estrogen-inducible growth factors exist and their expression may be influenced by the local mammary gland hormonal milieu. It is unknown whether estrogen, apart from its mitogenic effects, can also up-regulate pathways which promote paracrine or autocrine induced differentiation, thus providing indirectly eventual competency for cell death. For these reasons, TGF β 3 was chosen for further examination in this study. It has dual roles in oncogenesis and embryogenesis, is both an autocrine and paracrine growth factor, and contains an estrogen response element in its promoter region. More detail will be provided in this document under Task 2 Summary.

II. Indicators of overall body development: birth weight, timing of puberty on-set, body weight during peri-puberty (day 28), puberty (day 35), and post-puberty (day 50), and uterine development (days 28 and 50)

a) Birth Weight and body weight during pre-pubertal, pubertal and post-pubertal development

There was no significant differences in birth weight between groups by Kruskal Wallace One-Way ANOVA on Ranks, ($p = 0.229$), with an median weight of animals (in milligrams) as follows: Control, 6.513 mg,

25%=6.140, 75%=6.825 mg, low dose 6.618 mg, 25%=6.358, 75%=7.544, high dose 7.173 mg, 25%=6.272, 75%=10.856, and estrogen 7.173 mg, 25%=6.272, 75%=10.856. There were significant differences in body weight during peri-puberty (post-natal day 35) between the high dose cadmium group and all other groups. However this difference disappeared post-pubertally (post-natal day 50) and was not correlated with increased breast cancer susceptibility.

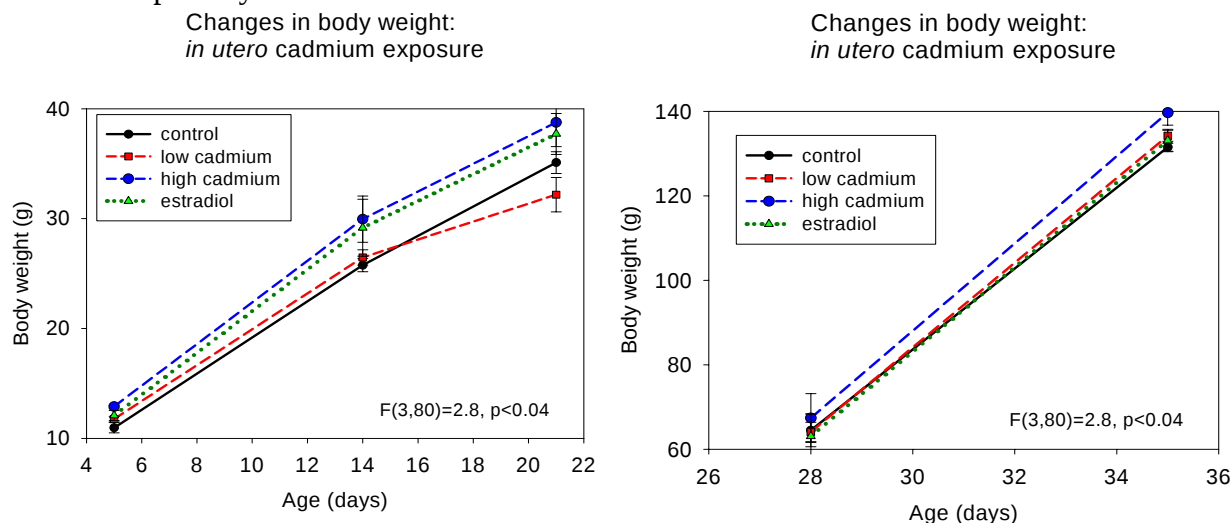


Figure 8: Body Weight during Development.

b) Timing of puberty-onset

Puberty on-set was accelerated in a statistically significant manner in the higher dose cadmium group, compared to all other groups, Log rank 8.4, $p<0.04$. However this was not correlated to increased breast cancer risk. Instead a low dose exposure to cadmium, which was not significant, did not alter puberty onset, and instead gave a significant decrease in tumor incidence (56% versus 73 or 80% for control and high dose cadmium groups). See Tumor Data in Section III for more details. Range of puberty onset for groups was as follows: control (31-38 days), low dose cadmium (26-39 days), high dose cadmium (26-37 days), estrogen (31-38 days).

c) Indicators of Uterine Development

i. Day 28 Uterine wet weight

No difference between groups of uterine wet weight ($p<0.439$) or offspring's body weight ($p<0.7$). The average uterine wet weight for each group at day 28 was: control 64.8 mg ($SEM\pm0.0047$); low dose 55.4 mg ($SEM\pm0.0025$); high dose 61 mg ($SEM\pm0.0059$); estrogen exposed 63.16 mg ($SEM\pm0.00393$). The power of this test was 0.050.

ii. Day 50 Uterine thickness

Uterine thickness was graded on animal sacrifice by histological analysis of a trained physician. The grading assignments were as follows: Thin=1, Thin/Thick=1.5, Medium=2, and Thick=3. No significant difference in uterine thickness (by Two-way ANOVA accounting for estrus status, $p<0.495$) or body weight (Two-way ANOVA between groups, $p<0.942$) were found which could not be due to random sampling variability. The uterine wet thickness averages were as follows: control means 1.875, SEM $\pm$ 0.529; low dose mean 1.875, SEM $\pm$ 0.847; high dose mean 2.375, SEM $\pm$ 0.770; estrogen mean 1.875, SEM $\pm$ 0.728.

Objective 3: Determine if *in utero* exposure to cadmium increases the risk of breast cancer.

Task 3: Determine whether *in utero* exposure to cadmium alters the risk of breast cancer (Year 1 and 2, Months 9-24)

III. *In utero* exposure to cadmium and breast cancer susceptibility:

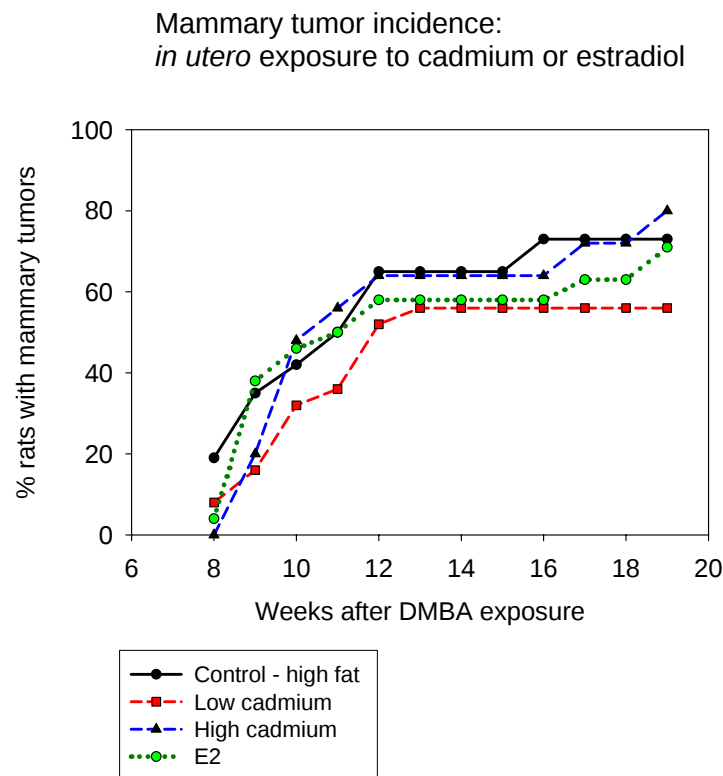


Figure 9: Tumor incidence, latency and multiplicity and proliferation: Although there was a significant difference between groups in tumor incidence ($p<0.001$, all groups relative to low dose group which was significantly reduced incidence of tumors). In this instance the overall multiplicity and tumor latency were not strongly affected. Initially the control group has the largest percentage of tumors at week 8 after DMBA administration, but by week 10 the only significant differences were between the low cadmium group and all other groups.

Objective 2: Determine whether *in utero* exposure to cadmium alters the arcuate nucleus, the center of hypothalamic control of both puberty on-set and appetite regulation and the mechanism by which this occurs.

Task 2: Determine whether *in utero* exposure to cadmium alters the arcuate nucleus, the center of hypothalamic control of both p 2 and 3, Month 1

Vaginal opening: *in utero* cadmium exposure

sm by which this occurs (Year

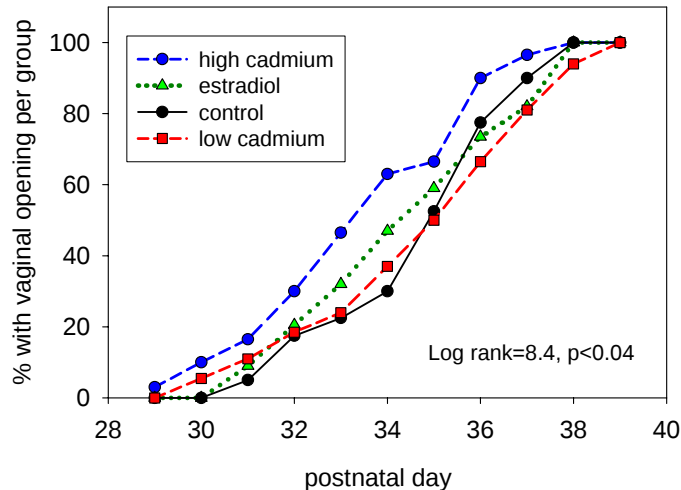


Figure 10: Puberty onset as indicated by vaginal opening. Determination of pubertal on-set was done by monitoring offspring continuously. There was a significant acceleration of pubertal on-set in the higher dose group; however this was not correlated to increased tumor incidence.

a) Female rats will be sacrificed at the 10 time points, including puberty through post-puberty.

This task has been accomplished with additional time-points relevant to mammary gland development. Developmental time-points included: embryonic day 18; late pregnancy, day 18; post natal days 5, 10, 21 (pre-puberty), 35 (puberty) and 50 (post-pubertal); lactation (24 hrs post parturition); involution (72 hrs post weaning). An estrogen-inducible growth factor (as determined *in vitro* studies in mammary epithelial cells, and *in vivo* studies in other tissue), has been described by immunohistochemistry based on both developmental time point and tissue type. Staining exhibits more than one pattern and expression level depending on that state of the mammary gland, age and tissue type. Interestingly the percentage of positive staining found in the neonatal mammary anlagen (0.12%) of Sprague Dawley rats was similar to levels found in tumor tissue (0.19%).

Further studies on *in utero* exposures to estrogen, development and signal transduction in rodent models are suggested in the Section entitled: Conclusions based on data from Objectives 1, 2 and 3 and Rational for a Modification of Objective 2 and Tasks. The data I present here is preliminary. Statistical analysis of further data such as TGFβ3 counts for positive cells or changes in expression patterns by hormonal milieu are in the process of being confirmed, and more data may need to be gathered for appropriate power to determine statistical significance.

AGE/CONDITION	HORMONAL STATE	STAINING PATTERNS APPARENT:				
		SECRETORY	CYTOSOLIC	PERI-NUCLEAR	NUCLEAR EVEN	GRADIENT FIELD
<i>PND 50</i>	Estrus, sudden ↓E2, ↑P	X	X	X	X	X
	Metestrus, baseline levels of E2, P		X	X	X	
	Diestrus I, ↓E2, ↓P	X	X			
	Diestrus II, ↑E2, ↓P		X	X	X	X
	Pro-estrus, ↑E2, ↑P, ↑LH		X	X		
<i>Lactating</i>	↓E2 and ↓P	X	X			
<i>Involuting</i>	↑E2 promotes involution		X	X	X	X
<i>Gestation Day 18-21</i>	↑E2 and ↑P	X	X	X		
<i>Tumor Tissue</i>	May be sensitive or refractive	X		X		

Table 2: Staining patterns of TGFβ3 by hormonal state or estrus status. Animals obtained on a developmental time-point continuum were sacrificed and tissue processed for immunohistochemistry. The expression patterns of TGFβ3 were observed on seven fields from 4 untreated animals. When less than 4 animals were available, serial sections was examined. Some of these staining patterns are also visible in peri-pubertal animals at post-natal day 35.

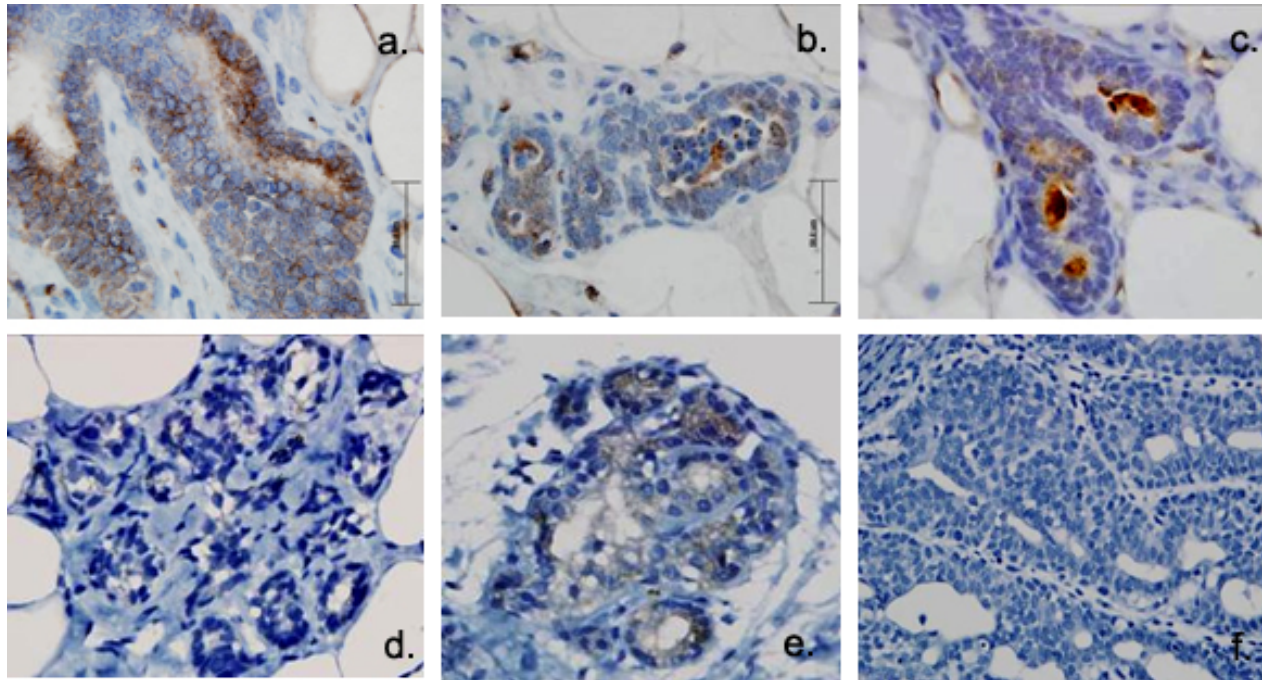


Figure 11: Examples of variation of TGF β 3 staining patterns in Day 50, nulliparous, unexposed mammary gland (a-c), and mammary gland with tumor (d-e). Notice neoplastic lobules adjacent to tumor tissue (d) lobules distal to tumor tissue (e) and lack of expression in ductal carcinoma *in situ* tumor tissue (f). As controls, both an IgG against the animal in which the primary antibody was raised and a peptide competition assay were performed.

For further explanation and future plans, see section below: Conclusions based on data from Objectives 1, 2 and 3 and Rational for a Modification of Objective 2 and Tasks

CONCLUSIONS BASED ON DATA FROM OBJECTIVES 1, 2 AND 3 AND RATIONAL FOR A MODIFICATION OF OBJECTIVE 2 AND TASKS

The mammary gland morphometry in this study indicated that some endpoints were altered depending on treatment group, estrus status or dietary cadmium dosage. The epithelial density data demonstrate that although there is a cyclic change in mammary gland density during estrus and that although an *in utero* exposure to estrogen can alter later adult mammary gland epithelial density, dietary cadmium apparently has little to no effect. This was in contrast to earlier studies which did indicate a significant change in epithelial density based on exposure to cadmium. However the levels of dietary cadmium employed in this study (0.075 mg/kg of feed and 0.15 mg/kg of feed) were much lower those used in a previous study which found significant differences (Johnson et al., 2003). That study unquestionably demonstrated that cadmium does in deed mimic some of the effects of estrogens in the mammary gland and uterus of Sprague Dawley Rats. They did not however administer dietary cadmium continuously throughout the *in uteri* development of the mammary gland as their goal was not to do a dietary study. Instead it was an exposure study which utilized *in utero* doses of cadmium (0.5 or 5 mg/kg of maternal body weight) given only by injection on two gestational days, 12 and 17. The study I present here is different because it does mimic, in the Sprague-Dawley rodent model, a method by which humans may accumulate or be affected by cadmium, i.e. by dietary exposure. In fact other studies have demonstrated that later breast cancer risk can be altered with even lower doses of cadmium contained in dietary components such as flaxseed (Khan et al., 2007).

Another fact to keep in mind is that cadmium binds to the androgen receptor with a dissociation constant or K_D value of 2.8×10^{-10} (Martin et al., 2002). Thus cadmium may amplify the effects of the natural ligand, testosterone, which is a metabolite of dehydroepiandrosterone (DHEA). Incidentally DHEA been correlated with positive outcomes in breast cancer in humans, and the human breast also contains androgen receptors (Labrie, 2006). The dissociation constant here indicates the likelihood that cadmium will dissociate from the androgen receptor. When compared to the natural ligand (testosterone) with a dissociation constant of 1.19×10^{-10} , cadmium is only a weak competitor. Nonetheless cadmium's effects on androgen receptor binding are seen both *in vitro* in cell culture and *in vivo* in the Sprague Dawley Rat model (the tissue studied was prostate) (Martin et al., 2002; Wilson, 1985). The dissociation of cadmium for the estrogen receptor ER- α is $K_D = 1 \times 10^{-10}$ and thus preferential binding of cadmium is almost 3-fold higher for the estrogen receptor as compared to the androgen receptor. At low doses though, it is possible that endocrine disruption and physiological changes observed *in vivo* may be due to a shift in the balance of its combined estrogen and androgen receptor signal transduction stimulation. Further work could be done in this area, however the synergistic effects of androgen and estrogen stimulation *in vitro* in cell culture or *in vivo* on the mammary gland as it relates to breast cancer risk is not well-defined. The goal of this grant was to study the estrogenic, not androgenic effects on offspring exposed *in utero* to low doses (0.075 or 0.150 mg/kg of maternal body weight) of dietary cadmium. Thus for the rest of this study, a single hormonal stimulant, i.e., estrogen, will be utilized to examine the effect of *in utero* low doses of estrogens on mammary gland, body development, and other indicators of breast cancer risk.

Another goal of this study was to examine effects of *in utero* exposure to an estrogenic compound on mammary gland development and associated growth factors with both the potential for estrogen inducibility and dual roles in oncogenesis. For this reason, expression patterns of TGF β 3 were examined further both developmentally in untreated female Sprague Dawley Rats and between treatment groups (control, low dose cadmium, higher dose cadmium and estrogen) at pre-pubertal (21 or 28 Days) and post-pubertal (50 Days) time points. TGF β 3 contains an estrogen response element half-site in its promoter region and has been

demonstrated to be estrogen responsive *in vitro* in MCF-7 cells and *in vivo* in bone using a rodent model. It is also hypothesized to be a protective molecule whose expression is up-regulated post-parity in Sprague-Dawley Rat (Blakely et al., 2006; D'Cruz et al., 2002). My initial work does suggest that the patterns of expression in mammary gland are influenced by the hormonal state. As well it appears that endocrine disruptors can alter normal expression patterns (data not presented). However more data should be gathered to determine whether this is statically significant.

Upon examination, tumor tissue was found to contain exceptionally low amounts of TGF β 3 expression, comparable to that found in very early developmental state of the neonate mammary anlagen. Whether *in uteri* exposure to estrogen or estrogen mimics directly alters TGF β 3 expression, or whether this is an indirect phenomenon through fetal imprinting, is also an area for further study. Regardless of precise mechanisms, these initial results indicate that *in utero* exposure to estrogenic endocrine disruptor, cadmium, does have the potential to alter TGF β 3 expression in the mammary gland. Its direct response to estrogen in the mammary gland is unknown. For this reason, estrogen responsiveness will also be confirmed *in vivo* in the mammary gland in Sprague Dawley rodent model, by utilizing ovariectomized animals that have either an estrogen or vehicle (usually cholesterol) 30-day release pellet implanted.

As an initial basis for further study, I have established the normal expression patterns of TGF β 3 during Sprague- Dawley mammary gland development on days E18, PD5, PD10, PD21, PD35, PD50, post-weaning involution at 72 hrs, lactation for 24 hrs and late pregnancy (gestational day 18). Many of these time periods have different hormonal milieus, and because the expression patterns of TGF β 3 vary by hormonal state of the mammary gland, it suggests that this growth factor does alter its expression patterns in a hormone responsive manner. See Table 2 for a summary of this data. The developmental time-points studied are periods of quiescence (PD5 and 10), growth (mid-pregnancy and estrus) or differentiation (late pregnancy, early lactation and finally involution) in the mammary gland. The early developmental expression patterns are still in the process of being examined. Importantly, my preliminary data collected on tissue from a prior study of female animals treated *in utero* with estrogen indicates that overall, lower levels of TGF β 3 are expressed in the mammary epithelia of exposed versus unexposed offspring. This study will be repeated in female Sprague-Dawley offspring with two different doses of estrogen to determine whether a dependency on dose exists and which epithelial structures may be most strongly affected. The offspring exposed to a low dose (1 mg/kg of maternal body weight) or high dose (10 mg/kg of maternal body weight) of estrogen *in utero* will be sacrificed at post-natal days 28 (pre-puberty), and 50 (post-puberty) with an n=6 animals per time-point. Endpoints such as body weight during development, and puberty-onset will be monitored along with the examination of mammary gland morphometry.

Finally, because TGF β 3 signal transduction has not been fully characterized during fetal mammary gland development, it warrants further study. This may be accomplished utilizing a validated, sygeneic mouse model, FVB/N and *in vitro* assays in mouse mammary gland cells such as EpH4/K6. As an initial *in vitro* investigation of effects of microenvironment on differentiation of EpH4/K6 cell phenotypes, I have completed a short ultrastructural study utilizing electron microscopy. My co-authors and I will submit this study to Tissue and Cell this summer. Recently I had the opportunity to present some of my work to Dr. Salomon, Head of the Tumor Growth Factor Section in the Laboratory of Mammary Gland Biology and Tumorigenesis, at NCI, NIH. After our meeting he invited me to do a portion of my thesis work in his laboratory. Dr. Salomon is an expert in mammary gland development, tumor growth factors and their signal transduction partners (Normanno et al., 2005; Panico et al., 1996; Salomon and Lewis, 2004; Strizzi et al., 2005). Thus, the murine and cell culture studies related to TGF β 3 will be performed under Dr. Salomon's auspices at the Laboratory of Mammary Gland Biology and Tumorigenesis, the National Cancer Institute, NIH. This division is headed by Dr. Barbara Vonderhaar. Dr. Vonderhaar is an expert in hormonal environment influences on mouse model mammary gland growth and development, especially the effects of prolactin (Hovey et al., 2003; Hovey et al., 2002),

estrogens and progesterone (Atwood et al., 2000; Hovey et al., 2005). Their contact information is located in the appendix. The work on in utero exposure studies in Sprague Dawley Rat, both for estrogen dependency and development in the rat model, will be performed at Georgetown University. My progress through my thesis program and overall dissertation work will be guided by my thesis advisor at Georgetown University.

AREAS OF FUTURE STUDY RELEVANT TO BREAST CANCER PREVENTION AND TREATMENT

This DOD, BCRP Training Grant, may lead to new area of research. Namely, study of the effects of estrogen or estrogen mimics on local autocrine, paracrine and morphogenic tumor growth factors. Finding these relationships, dose dependency, changes due to timing of exposure and mechanisms will help to establish how estrogens and xenoestrogen exposures may influence later breast cancer risk. This could lead to new therapeutic regimens which help reduce the likelihood of breast cancer or mortality from it.

ABSTRACTS AND PUBLICATIONS RESULTING FROM THIS GRANT

Webster JD, Khan G, Martin MB, Hilakivi-Clarke L. *In utero exposure to Cadmium and Mammary Gland Cancer Risk in Female Rats*. AACR Annual Meeting, Los Angeles California, 2007

Webster JD, Chapman GB and Hilakivi-Clarke L. *An ultrastructural characterization of the EpH4/K6 murine mammary gland cell line grown under varied micro-environments in 3-dimensional cell culture*. In preparation for submission to Tissue and Cell, 2007

Webster JD, Khan G, Hilakivi-Clarke L. *In Utero Exposure to Low Doses of Dietary Cadmium alters Mammary Gland Development and Transforming Growth Factor Beta-3 Expression Patterns in Sprague Dawley Rat*. In preparation.

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APPENDIX

I. Contact information for Drs. Salomon and Vonderhaar.

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II. AACR Letter Size PDF of Poster Presented at 2007 Annual Meeting in Los Angeles CA.

See next page.

In utero exposure to Cadmium and Mammary Gland Cancer Risk in Female Rats

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Abstract

The heavy metal cadmium, an endocrine disruptor, potently binds to and activates the estrogen receptor. Our previous studies have shown that *in utero* exposure to cadmium at the levels present in human environment accelerated puberty onset and altered mammary gland development in rats, suggesting that it might increase susceptibility to develop mammary cancers. To test this possibility, we exposed pregnant rat dams to high fat diets containing 0, 0.075 or 0.15 mg/kg feed cadmium throughout pregnancy and determined the effect on (i) birth weight, (ii) postnatal weight development, (iii) vaginal opening/puberty onset, (iv) mammary gland development, and (v) DMBA-induced mammary tumorigenesis. After parturition, all rats were switched to AIN93 laboratory chow. Birth-weight was not affected by fetal cadmium exposure, but the higher cadmium dose induced a long-lasting increase in postnatal body weight that was first detected on postnatal day 5 ($p<0.04$), and it accelerated vaginal opening ($p<0.03$). Final mammary tumor incidence was highest in the higher cadmium group (80% of rats developed tumors) and lowest in the lower cadmium group (56% tumor incidence) ($p<0.001$); 73% of the control rats developed mammary tumors. These findings indicate that *in utero* exposure to 0.15 mg cadmium per kg feed via maternal diet increases postnatal weight development and induces earlier puberty onset. However, it does not increase susceptibility to development of mammary tumors.

Introduction

In utero exposures to estrogen mimics such as cadmium may alter later breast cancer risk. Cadmium has a higher affinity for the estrogen receptor than the receptor's natural ligand, estradiol, and may alter fetal developmental pathways normally mediated by estrogens. Some of these estrogen-responsive pathways affected by fetal estrogenic exposure may be re-employed at times of mammary gland tissue remodeling during estrus cycling in adulthood. This remodeling employs signal transduction systems effecting proliferation, differentiation and apoptosis, and must be precisely and accurately controlled to avoid unregulated tissue growth or tumor formation.

Methods

Treatment groups: Pregnant Sprague-Dawley dams were exposed to corn oil vehicle, 0.075 or 0.15 mg/kg feed cadmium throughout pregnancy. For the estrogen treatment group, daily injections of 0.05 mL began gestational day 20. These treatments were in the context of 30% dietary fat, similar to that of a human diet. At birth all offspring were maintained on AIN93 chow (16% fat). **Measurement of developmental endpoints:** Body weight and monitoring for vaginal opening (an indication of puberty on-set) were performed during development. **Mammary tissue collection:** At pre-pubertal day 28 and, post pubertal day 50, animals were sacrificed (n=5 per group). Mammary tissue was collected for whole mount (4th inguinal gland), immunohistochemistry (5th inguinal gland), protein and mRNA expression (2nd gland). Tissue collected for immunohistochemistry was fixed in 10% formalin, washed in PBS, dehydrated in 70% ethanol, and paraffin embedded. Slide sections were 4 μ m thick. **Collection of Uteri:** Upon sacrifice offspring uteri were collected, weighed, graded by thickness and fixed in 10% formalin for later embedding and sectioning. **Proliferation analysis:** Slides were stained using a rabbit monoclonal antibody to Ki67 (Abcam ab16667-500), utilizing DAKO Envision Kit for Rabbit (DAKO, K4002). Imaging was performed using Olympus Scanning microscope BX100, ImagePro software. Positive and negative nuclei were counted using ImageJ (NIH). **Carmines Stain of Whole Mount Mammary Gland:** NIH protocol at mammary.nih.gov **Mammary gland epithelial to fat pad ratio:** calculated by measuring from primary lymph node to end of epithelial tree and primary lymph node to end of fat pad in carmine stained whole mount mammary gland. **Day 50 determination of estrus stage:** Estrus status was graded for each animal using morphological criteria of Schedin et. al. **Tumor multiplicity and latency:** Per group, 19-24 rats were monitored for tumors for 20 weeks post DMBA challenge. **Data Analysis:** ANOVA Test using SigmaStat.

Results

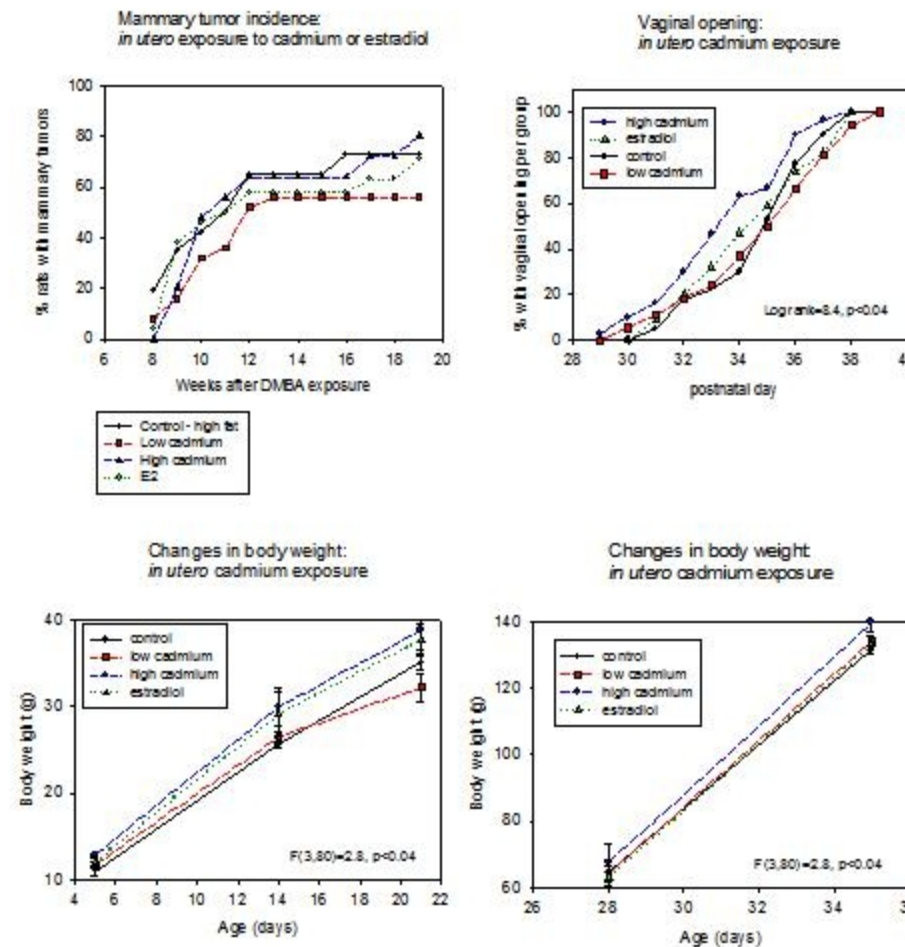


Figure 1. Tumor incidence and developmental changes due to *in utero* exposure to cadmium. Mammary Tumor incidence was significantly lowered in offspring of dams exposed to low dose cadmium (0.075 g/kg of feed) during gestation ($p<0.001$). Offspring exposed *in utero* to high dose cadmium (0.15 g/kg of feed cadmium) experienced accelerated pubertal on-set as compared to control, estradiol and low dose groups (Log rank=8.4, $p<0.04$). There was a long-lasting increase in body weight in the high dose group ($p<0.04$). However neither increased body weight nor accelerated pubertal onset were correlated with increased mammary tumor incidence in the high dose cadmium group compared to other groups.

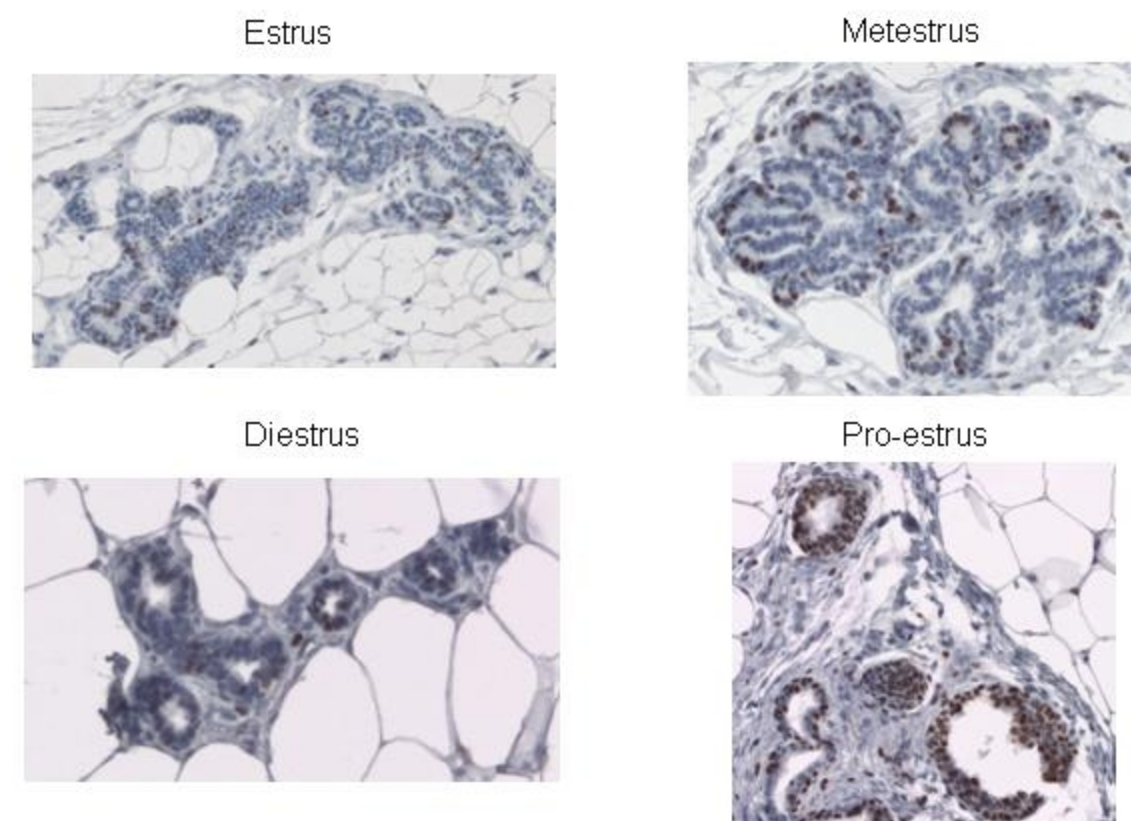


Figure 2. Estrus Staging in Day 50 sprague-dawley rat by lobule and duct type. Each estrus cycle throughout life, cells within the mammary gland epithelia are re-organized. This process involves proliferation, differentiation and apoptosis depending on cell type and location. In this study, cellular proliferation rates varied significantly between groups regardless of estrus stage. Photographed at 20X magnification, but some pictures are enlarged.



Figure 3. Ki67 Staining in Female Rat Offspring. Ki67 is expressed during all proliferative phases of cell cycle. 20X and 100X magnifications. Positive and negative nuclei were counted using ImageJ software. Counts were separated on the basis of morphological structure, i.e. terminal end bud, lobule or duct. Cell type may be distinguished at 100X magnification.

Day 28 Female Offspring					
<i>In utero</i> exposure Treatment Group	Control: 30% fat + vehicle	Low Dose: 30% fat + 0.075 mg CdCl ₂ /kg Feed	High Dose: 30% fat + 0.150 mg CdCl ₂ /kg Feed	Estrogen Control: 30% fat + excess estrogen	Statistical Significance
Epithelial tree: Fat pad ratio (n=5 per group)	0.65	0.71	0.53	0.67	$p<0.004$ Low dose vs. High dose; $p<0.025$ High dose vs. estrogen, One-Way ANOVA, Tukey Test
Average Ki67 Indices by Mammary gland Structure (n=5 animals per group, 700-1500 cells per structure for each animal)	TEBs: 0.42 Lobules: 0.08 Ducts: 0.10	TEBs: 0.39 Lobules: 0.14 Ducts: 0.18	TEBs: 0.33 Lobules: 0.16 Ducts: 0.14	TEBs: 0.35 Lobules: 0.15 Ducts: 0.27	$p<0.005$ estrogen and low dose vs. high dose and control via Two-Way ANOVA; Ductal proliferation was most significantly altered.
Uterine Wet Weight	Average (n=5): 64.8 mg	Average (n=4): 55.4 mg	Average (n=5): 61 mg	Average (n=5): 63.16 mg	No statistically significant difference.
Day 50 Female Offspring					
<i>In utero</i> exposure Treatment Group	Control: 30% fat + vehicle	Low Dose: 30% fat + 0.075 mg CdCl ₂ /kg Feed	High Dose: 30% fat + 0.150 mg CdCl ₂ /kg Feed	Estrogen Control: 30% fat + excess estrogen	Statistical Significance
Average Ki67 Indices by Mammary gland Structure (n=5 animals per group, 700-1500 cells per structure for each animal)	TEBs: 0.42 Lobules: 0.39 Ducts: 0.25	TEBs: 0.40 Lobules: 0.35 Ducts: 0.27	TEBs: 0.24 Lobules: 0.11 Ducts: 0.10	TEBs: 0.34 Lobules: 0.22 Ducts: 0.20	$p<0.001$ by Two Way ANOVA controlling for estrus status, with significant differences between control and low dose vs. high dose and estrogen groups.
Estrus Status of Animals in Treatment group	N=3, estrus, N=2, pro-estrus	N=2, diestrus 1, N=2 diestrus 2, N=1 metestrus	N=2, metestrus, N=1 estrus, N=2 diestrus 1	N=1, diestrus 1, N=2 estrus, N=2 metestrus	No significant differences in proliferation were due to estrus status alone.

Table 1. Markers of mammary gland development in female rat offspring. Day 28 Offspring. Epithelial tree: fat pad ratio was measured in lieu of mammary gland density, which is a significant marker of breast cancer risk in humans. Low dose cadmium treatment resulted in significantly increased mammary epithelial tree: fat pad ratio compared to other treatment groups ($p<0.004$, One-Way ANOVA). Mammary gland cellular proliferation as measured by Ki67 index, was significantly increased in offspring exposed to low dose cadmium or estrogen *in utero* ($p<0.005$ by Two-way ANOVA). Although there was a slight decrease in uterine wet-weight in the low dose group, this decrease was not statistically significant ($p<0.67$ by Two-way ANOVA accounting for total body weight). **Day 50 Offspring.** Average Ki67 index was significantly increased in control and low dose groups versus high dose and estrogen groups, regardless of estrus status ($p<0.001$ by Two-Way ANOVA, Tukey Test).

Summary

- Offspring exposed to a higher dose of cadmium *in utero* underwent puberty earlier, and sustained an increase in body weight, compared to control and low dose cadmium group. However, this did not significantly effect later mammary tumorigenesis.
- Post-pubertal mammary gland tissue undergoes cyclic re-organization of epithelia cell strata based on estrus stage. This remodeling process utilizes developmental pathways involved in proliferation, differentiation and apoptosis. These pathways must be carefully controlled to prevent tumorigenesis.
- Biomarkers of mammary gland development, Ki67 index (proliferation) and epithelial: fat pad ratio were altered in offspring exposed *in utero* to estrogen or high dose cadmium group and were age dependent.
- Tumor tissue contains cells similar to those found in healthy epithelium, but these cells are sometimes poorly differentiated, undergo uncontrolled proliferation, and fail to apoptosis when given appropriate cues. In the low dose cadmium group there was a *reduction* in tumor incidence. Whether there was also a concurrent increase in differentiation or apoptosis remains to be tested.
- The mechanisms by which low dose cadmium exposure *in utero* may enhance developmental pathways that are involved in suppression of tumorigenesis is yet to be determined.

Conclusion

It is possible that through an estrogen-mediated mechanism, perhaps imprinting, fetal exposure to cadmium effects the pathways involved in adult mammary gland tissue remodeling leading to altered breast cancer risk. Our study indicates that *in utero* exposure to cadmium can have a long term effect on body weight and accelerates puberty on-set, but that it does not increase later breast cancer risk. These effects and tumor incidence were dose dependent. In this case, a low dose *in utero* cadmium exposure was protective, while a higher dose exposure did not significantly increase breast cancer risk.

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