ABSTRACT

JEFFERSON, WENDY NOBLE. Neonatal Exposure to the Phytoestrogen, Genistein Alters Ovarian Differentiation and Development. (under the direction of Gerald LeBlanc and Retha Newbold.)

Genistein, the primary phytoestrogen in soy, was investigated for potential adverse effects on the developing female reproductive system with particular focus on the ovary. Mice were treated with genistein at doses that span the range of human exposure including vegetarian mothers during pregnancy and lactation to infants on soy based infant formulas. Neonatal genistein exposure caused the formation of multi-oocyte follicles (MOFs) in the ovary. This effect is mediated by ERβ as mice lacking this receptor do not develop MOFs while mice lacking ERα do. Further study of genistein's effects on the ovary revealed inhibition of neonatal oocyte nest breakdown; oocytes were still attached by intercellular bridges and the normal progression of apoptosis was attenuated. Mechanistic studies of MOF formation revealed alterations in cell adhesion molecules. In addition, genistein is not unique in its ability to cause ovarian disruption; other environmental estrogens caused MOFs as well as altered cell adhesion molecule expression. Further, these effects appear to be exacerbated by preferential binding to ERβ. Assessment of reproductive function showed that mice treated with genistein (0.5 and 5 mg/kg) showed signs of early reproductive senescence while mice treated with genistein (50 mg/kg) exhibited infertility characterized by fewer, smaller, implantation sites as well as reabsorptions; ovaries from these mice had reduced numbers of corpora lutea. Stimulation with exogenous gonadotropins restored ovulation, suggesting problems with the hypothalamic-gonadal axis. These data taken together demonstrate that neonatal exposure to genistein at environmentally relevant doses causes adverse effects on the developing reproductive system and in particular on the ovary.

NEONATAL EXPOSURE TO THE PHYTOESTROGEN, GENISTEIN ALTERS OVARIAN DIFFERENTIATION AND DEVELOPMENT

by

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I dedicate this work to …

My husband, Chris, for his limitless support
and encouragement of my chosen path.
He took special care of our two wonderful children
so I would have time to finish this
long, yet rewarding journey.

and

My two children, Tori and Tanner
for their understanding and patience.

and

My Mom and Dad for all of their encouragement all of my life.
They gave me the gift of believing in myself and that
I could do anything in life that I set my mind to.

BIOGRAPHY

I was born in Hampton, Virginia, the first of two daughters of Amelia Keech and Ernest Richard Noble; my given name was Wendy Cheryl Noble. My sister, Heidi Gayle, was born 2 ½ years later while we lived in Belhaven, North Carolina where I spent my childhood. My mother says that she named me after Wendy in the story about Peter Pan and my sister after the novel, "Heidi". I guess she really liked to read as she still does today. Growing up in the small rural town of Belhaven on the east coast of North Carolina was a slow, easy way of life. My father was an electrical engineer by trade but a fisherman by heart. We spent many weekends on the boat that he built with his own two hands bringing in the tremendous bounty of the sea. Living in farm country, we also had a large garden where we grew our own vegetables. My sister and I helped keep the weeds to a minimum and harvested the beans, tomatoes, cucumbers, squash and just about anything else you could grow in a garden. It was during this time that I learned to appreciate nature and all that is has to offer and that we should actively protect it. I knew from a very early age that I wanted to be a scientist. I also knew which college I was going to attend, North Carolina State University of course, my father's alma mater. As planned, I graduated from John A. Wilkinson High School in Belhaven and set off for the "big city" of Raleigh to attend N.C. State to study science. Before I get too far, I left out one small detail, my soon to be husband Chris. I met him during high school as he grew up in another small town close to mine and we were instant friends. When I left for Raleigh, there was really only one thing that I decided I couldn't live without and that was him. Two years later, we were married and although my parent's feared that I would drop out of college and never go back, the opposite happened, I graduated with a double major, a B.S. in Biochemistry and a B.A. in Chemistry, imagine that. The summer

before I graduated, I decided that I needed some experience in the real world if I had any hope of finding a job when I finished. I noticed a small announcement on the career counselor's door about a summer job at the National Institute of Environmental Health Sciences so I applied thinking I had no chance. Sure enough I got an interview the next week with one of the most wonderful people I have ever met, Retha Newbold. It just so happened that she also grew up on the east coast of North Carolina as I did so we had a lot in common and she offered me the job for the summer. That moment changed my life forever and I will always be grateful that she took a chance on a small town girl with nothing to offer except a smile and the promise of hard work. I guess it worked out since I have been in her lab for the last sixteen years and counting. When I finished college, I applied for graduate school but alas, I did not get in. I was very disappointed because I knew that I wanted to know more but that wasn't in the cards for the moment. I think Retha somehow knew that graduate school was in my future so she convinced me to take a few graduate courses in the mean time. I really enjoyed them but I had something more important to do first, my first child was born during a snowy January, Tori Nicole and three and a half years later her little brother came along during a very hot July, Tanner Bryant. I continued to balance a career and a family but school was just too much so it would have to wait a little while longer. By then, I knew that I wanted to go back to school and seek a higher degree. In the fall of 2000, I was accepted into the PhD program in the Department of Environmental and Molecular Toxicology at North Carolina State and the rest is history. Five years later, I feel that I have learned a lot but most of all I have learned that I can do anything that I set my mind to – I guess my parents were right all along.

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I am also indebted to Elizabeth Padilla-Banks (Lisa) for taking over many of the day-to-day tasks of the laboratory so I could go back to school. I would also like to thank her for her hard work on many of the projects presented in this body of work. She has always helped me to see the light at the end of the tunnel and I hope to be able to repay her with the same optimism she gave me as she goes back to school herself.

I would like to thank John Couse in Ken Korach's lab for the collaborations using transgenic mouse models in some of the studies presented herein. I would also like to thank John for our many conversations about science and life and his unique approach to science that always makes me think "outside of the box".

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1. INTRODUCTION

1.1 Developmental Effects of Estrogens

It has been well known for many years that compounds with estrogenic activity can cause deleterious effects on the developing reproductive system of both males and females if exposure occurs during critical time periods [1-8]. The potent, synthetic estrogen, diethylstilbestrol (DES) is the most well studied compound, causing adverse effects on the reproductive tract of rodents as well as humans [3, 6, 9-11]. DES was given to pregnant women from the 1940s until the 1970s to prevent threatened miscarriage, but it was also given to women experiencing a normal pregnancy. Worldwide estimates suggest that between 2 and 8 million pregnant women were exposed to DES resulting in millions of humans being exposed to DES *in utero*. In the early 1970s, an association was made between a very rare clear cell carcinoma of the vagina in young women and *in utero* exposure to DES. Thereafter, the drug was withdrawn for use during pregnancy but by then, millions of people had been exposed to this potent estrogen. To study the adverse effects of this drug, many research laboratories developed rodent models to replicate and predict potential adverse outcomes in the human population [3, 9, 12, 13]. For example, the murine model showed that mice treated prenatally with DES developed uterine and vaginal cancer in females, testicular cancer in males, as well as malformations of the reproductive tract and reduced fertility in both males and females . Studies with the DES-exposed human population revealed that many prenatally exposed women had structural abnormalities of the reproductive tract as well as poor reproductive outcome, similar to those reported in mice [6]. Thus, the mouse model both confirmed as well as predicted the human outcomes. A comparison of

the similarities between the abnormalities following prenatal exposure in humans and rodents is shown in Table 1.1, summarized from Newbold et al., 1995 [14]. In addition to adverse effects in the first generation, there have been studies showing that these effects are transmitted to subsequent generations of both male and female descendents [15-18]. While the effect on subsequent generations in humans is still undetermined, it is an area of research that needs further attention. Although DES is no longer commercially available, public and scientific concern remains regarding exposure to other estrogenic chemicals in our environment and whether these chemicals cause similar adverse consequences as DES on the developing reproductive system.

1.2 Environmental Endocrine Disruptors

There are over 80,000 chemicals in commercial use around the world today and many of the chemicals in our environment have been shown to have endocrine disrupting activity by both *in vivo* and *in vitro* assays [4, 5]. An endocrine disrupting chemical (EDC) is any chemical that interferes with normal function of the endocrine system. These include but are not limited to estrogens and anti-estrogens, androgens and anti-androgens, progestins, and hormones with thyroid like activity or gonadotropin activity. These chemicals have diverse chemical structures and are used for many different purposes including pesticides, non-ionic surfactants, plasticizers and plastic coatings, dietary supplements, and cosmetics. Naturally occurring sources include plants and mold.

Studies suggest that environmental exposure to some of these compounds is adversely affecting wildlife populations [5]. For example, the alligator population in

Lake Apopka, Florida has declined over the last few decades and studies have shown that there are alterations in male reproductive structures including microphallus [19, 20]. This has been attributed to exposure of the eggs to high levels of the DDT metabolite, DDE, resulting from a chemical spill in the lake decades earlier [19, 20]. There have been reports of frog deformities, including limb multiplicity, in the lakes of Minnesota [21]. These effects have been reproduced in the laboratory using water from these lakes and is presumed to be caused by an environmental agent; perhaps a retinoic acid mimetic compound [22]. Still other reports describe white perch from the Great Lakes to have a high incidence of intersex gonads which is thought to be from exposure to an estrogenic endocrine disruptor [23].

A large body of evidence from laboratory studies that show endocrine disruption of many chemicals found in our environment. Although most of these studies use doses in excess of what environmental exposures might be, the effects can be profound. Several studies have shown the impact of the fungicide, vinclozolin on male sexual development if given during critical periods of sex differentiation [24, 25]. This chemical has been shown to act as an anti-androgen, blocking the normal effects of testosterone on male sexual differentiation, leaving the animals anatomically feminized and infertile at higher doses. A recent report describes epigenetic trans-generational effects on male fertility following prenatal exposure to these chemicals [26]. Male rats exposed to vinclozolin prenatally exhibit decreased spermatogenic capacity as well as infertility and these effects are transmitted to males of subsequent generations [26, 27].

There is also some sparse evidence in humans that endocrine disruption is occurring. There have been reports of reduced sperm counts and increased male

reproductive tract disorders over the last half century; this is thought to be the result of estrogenic or anti-androgenic endocrine disruptors but this remains controversial [28-30]. A link between blood levels of DDE, a metabolite of the pesticide DDT, and breast cancer was shown by an epidemiology study [31]. Further, a recent report describes an association between decreased anogenital distance (AGD) in human male infants and increased prenatal phthalate exposure [32]. Since phthalates have been shown to cause similar effects as vinclozolin and other anti-androgens on male sexual differentiation and reproductive function in laboratory animals including decreased AGD [33], there is certainly precedence for endocrine disrupting effects in humans.

The class of the endocrine disrupting chemicals (EDCs) that has been studied the most is the estrogens. Assays have been in place for many decades to determine estrogenic activity including the "gold standard" rodent uterotropic bioassay. The level of estrogenic activity of EDCs is also quite variable; some achieve an estrogenic response similar in magnitude to the natural hormone, 17β-estradiol only requiring a higher dose, while other EDCs show only weak activity even at very high doses [34-39]. While the estrogenic activity of a number of compounds is now known, their ability to elicit similar developmental effects as the model environmental estrogen DES is largely unknown. A few environmental estrogens that have been fairly well-studied for developmental effects include the pesticides *o,p'*-DDT and methoxychlor. Both of these compounds exhibit persistent estrus and reduced fertility if exposure occurs during development similar to DES [8, 40]. Although DDT was banned by the EPA in the United States in 1972, this chemical persists in our environment; methoxychor is still widely used so environmental exposures to both of these compounds still occur. Further, studies show that there is an

estrogenic compound found in dental sealants and in the linings of metal cans, bisphenol A (BPA), which can causes effects on the developing reproductive system of females if exposure occurs perinatally [41-43]. This chemical is widely used in our environment and the effects of these exposures on humans and wildlife are currently unknown.

1.3 Estrogen Receptor Mediated Mechanism of Hormone Action

Estrogens are known to regulate multiple cell functions in target tissues, including growth and differentiation via nuclear receptor-mediated pathways. Furthermore, aberrant temporal or over-stimulation of the estrogen signaling pathway during development has long been known to result in multiple long-term abnormalities in the reproductive tract, including neoplasia . Until recently, the majority of estrogen actions were thought to be mediated via a single form of nuclear estrogen receptor (ER). However, the discovery of a second ER, termed ERβ (to differentiate from the original, now termed ERα), has prompted a reevaluation of the physiology and toxicology of the estrogen signaling system.

The biological significance of two ER subtypes remains unclear but may explain the selective and divergent actions of estrogens that occur in various target tissues. Whereas ERα appears to be the predominant ER form in the Müllerian-derived structures of the female reproductive tract [44-48], easily detectable levels of both ERs are present in the gonads of both sexes [49-51]. In the reproductive tract of late gestational and neonatal female mice, ERα immunoreactivity is localized to the stromal and epithelial cells of the uterus and the interstitium of the ovary, whereas ERβ immunoreactivity is limited to the ovarian granulosa cells, with little to no detectable expression in the uterus

[49, 50, 52]. Other investigators have also reported the presence of ERβ in the human ovary, predominantly in the granulosa cells [53, 54]. No detectable immunoreactivity for ERβ has been found in oocytes, despite previous descriptions of detectable ERα transcripts in mouse and human oocytes [55, 56]. The divergent expression patterns of ERα and ERβ in the developing and adult reproductive tract indicate the complexity of the estrogen signaling system and suggest the two receptors likely play different physiological roles.

Another layer of complexity has been recently realized with differential binding affinities between the two receptors. For example, 17β-estradiol (E_2) exhibits a similar *in vitro* binding affinity for both ERs, while several synthetic and naturally occurring xenoestrogens exhibit a binding preference for one of the two receptors although this binding is not typically as strong as E_2 [36, 37, 57, 58]. For example, it takes twenty times as much of the phytoestrogen genistein to elicit similar binding affinity to ERα as E_2 [36]. In addition, some chemicals have a higher binding affinity to either ERα or ERβ; genistein exhibits preferential binding to ERβ over ERα. In fact, genistein binds to ERβ 20-80 times better than to ERα [36, 37, 57].

The diverging transcriptional activation of the two receptors when bound to different ligands as well as the importance of cellular context also add complexity to the mechanism of estrogen action [47, 59-63]. For example, *in vitro* transcriptional activation assays have shown 4-hydroxytamoxifen to be a partial agonist when bound to ERα, but a full antagonist when interacting with ERβ [63]. Compounds with these differential effects between the two receptors are now known as selective estrogen receptor modulators (SERMs). SERMs are a class of chemicals that take advantage of

the plasticity of the binding pocket of the estrogen receptors (α and β) and modulate the surface conformation in such a way as to affect the subsequent binding of co-factors necessary for transcription [64]. Tamoxifen was the first SERM described *in vivo* mainly due to its antagonistic activity in the mammary gland and agonistic activity in bone and uterus [65]. There are now second generation SERMs developed specifically for their selective activity in a particular tissue without effects in other tissues; raloxifene is one example [65, 66]. Recent studies have shown the molecular basis for distinct SERM biocharacter. Several factors determine the activity of a particular compound within a particular cell; 1) ERα or ERβ selectivity 2) induction of different conformation of the receptor 3) selective interaction or recruitment of co-regulators 4) cellular context or target promoter. For example, genistein prefers to bind to ERβ and it induces a different conformation of ERβ compared to E_2 as determined by crystallography [67]. This different conformation most likely contributes to receptor's ability to preferentially recruit specific co-regulators. This has been shown *in vitro* using a Glutathione S-Transferase (GST) pull down assay; ERβ bound to genistein recruits transcription intermediary factor 2 (TIF2, an ER co-activator) 12,000 times better than when genistein is bound to ERα [68]. The nature of the target promoter can also influence the end result; for example, E_2 bound to ERα is a full agonist on a thymidine kinase promoter *in vitro* but shows no activity at all when E_2 is bound to ERβ [69]. A schematic representation of the molecular mechanisms involved in ER mediated signaling is shown in Figure 1.1.

Taken together, a very complex, multi-faceted mechanism of estrogen receptor mediated estrogen action is predicted for endocrine disrupting chemicals. There would most likely be differential effects based on the chemical being tested, the binding

preference of either ERα or ERβ of that chemical, the target tissue of interest and even the target gene of interest. Studying differential effects of environmental estrogens compared to the historical studies using DES or the natural hormone E_2 is an emerging field of research.

1.4 Phytoestrogens

Since phytoestrogens have been shown to bind to both ERα and ERβ and to have estrogenic activity *in vitro* as well as *in vivo* assays, concern has risen over human exposure to these estrogenic chemicals. Phytoestrogens are found in high levels in soy products known to be present in the human diet and are likely substantial components of vegetarian diets; therefore, human fetuses may be exposed to these compounds during *in utero* development. Furthermore, childhood exposure to high levels phytoestrogens may occur through soy-based infant formulas and soy-based foods that are often specifically marketed for children [70, 71]. In fact, isoflavone concentrations in soy-based infant formulas can far exceed amounts found in the average adult vegetarian diet in the United States [72]. It is estimated that infants consuming a diet of soy-based formulas are exposed to 6-9 mg/kg/day of genistein, whereas adults consuming a diet modest in soy isoflavones are exposed to approximately 1 mg/kg/day [72]. Soybeans also have an extremely variable isoflavone content depending on variety and environmental conditions such as growing season and location [73]; the USDA reports highly variable amounts of genistein in soy products [74]. This could lead to even higher levels of genistein exposure than expected in particular lots of soy-based products.

The adverse effects of phytoestrogens on fertility have been known for years since sheep grazing on red clover exhibit infertility; this is thought to be due to estrogenic substances found in clover [7]. Another study showed that captive cheetahs exhibited reduced fertility while on soy based diets containing very high levels of phytoestrogens; replacement of soy protein with chicken protein restored fertility [75]. Therefore, these examples show that phytoestrogens exist in our environment at concentrations high enough to be active and have adverse effects.

Over the last few years, public and scientific interest in phytoestrogens, like genistein, has increased because of its proposed beneficial effects. Currently, there are conflicting results on developmental exposure to genistein suggesting some beneficial effects but also adverse effects depending on the timing of exposure, dose level and endpoints examined. For example, two studies report that exposure to genistein prenatally prevents carcinogen-induced mammary gland cancer in rats [76, 77] while another study shows an increase in mammary gland cancer if the developmental window of exposure is shifted to neonatal life [78]. Other investigations report improved cholesterol synthesis rates of human infants consuming soy-based formulas [79]. Recent studies from our laboratory show that neonatal exposure to genistein leads to the induction of uterine adenocarcinoma in mice [80], similar to that previously described following neonatal DES exposure. Additional suggestions for adverse effects of genistein can be seen in an epidemiology study which shows that pregnant women consuming a vegetarian diet during pregnancy have male offspring with an increased incidence of hypospadias; this could be due to high maternal levels of soy isoflavones [81]. Further, an epidemiology of health outcomes in young adults who were fed soy

based formulas as infants reported an increase in use of allergy medicines in both men and women, and longer menstrual bleeding and more discomfort during the menstrual cycle in women [82, 83]. Therefore, the adverse effects of developmental exposure to genistein remain of concern.

1.5 Hypotheses

Since genistein has been shown to have estrogenic activity and it preferentially binds to ERβ over ERα, my research focuses on the developmental effects of this compound on the ovary. The rationale for this is the following: 1) the ovary expresses ERβ throughout development; 2) ERβ is the predominant receptor subtype expressed in the ovary; 2) genistein preferentially binds to ERβ; and 4) previous work with other estrogens has shown developmental effects in the ovary. Therefore, I hypothesized that the ovary should be a particularly sensitive target tissue for adverse developmental effects of genistein. The potential adverse effects of genistein have important human health implications since human fetuses and neonates are exposed to high levels of genistein during development.

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Table 1.1 Comparative Developmental Effects of DES in Mice and Humans

Type of Abnormality	**Male Offspring**	**Female Offspring**
Functional	Subfertility / Infertility	Subfertility / Infertility
	Decreased Sperm Counts	Poor Reproductive Outcome
Structural	Microphallus	Abnormal Structure
	Hypospadias	-oviduct
	Retained Testis	-uterus
	Retained Mullerian Remnants	-cervix
		-vagina
		Paraovarian Cysts of mesonephric origin
Cellular	Inflammation	Lesions
	Epididymal Cysts	-ovary
	Tumors	-oviduct
	-testis	-uterus
	-prostate	-vagina
	-seminal vesicle	Uterine Fibroids
		Vaginal Adenosis
		Vaginal Adenocarcinoma
		Mammary Tumors

A more comprehensive table can be found in Newbold et al., 1995 [14].

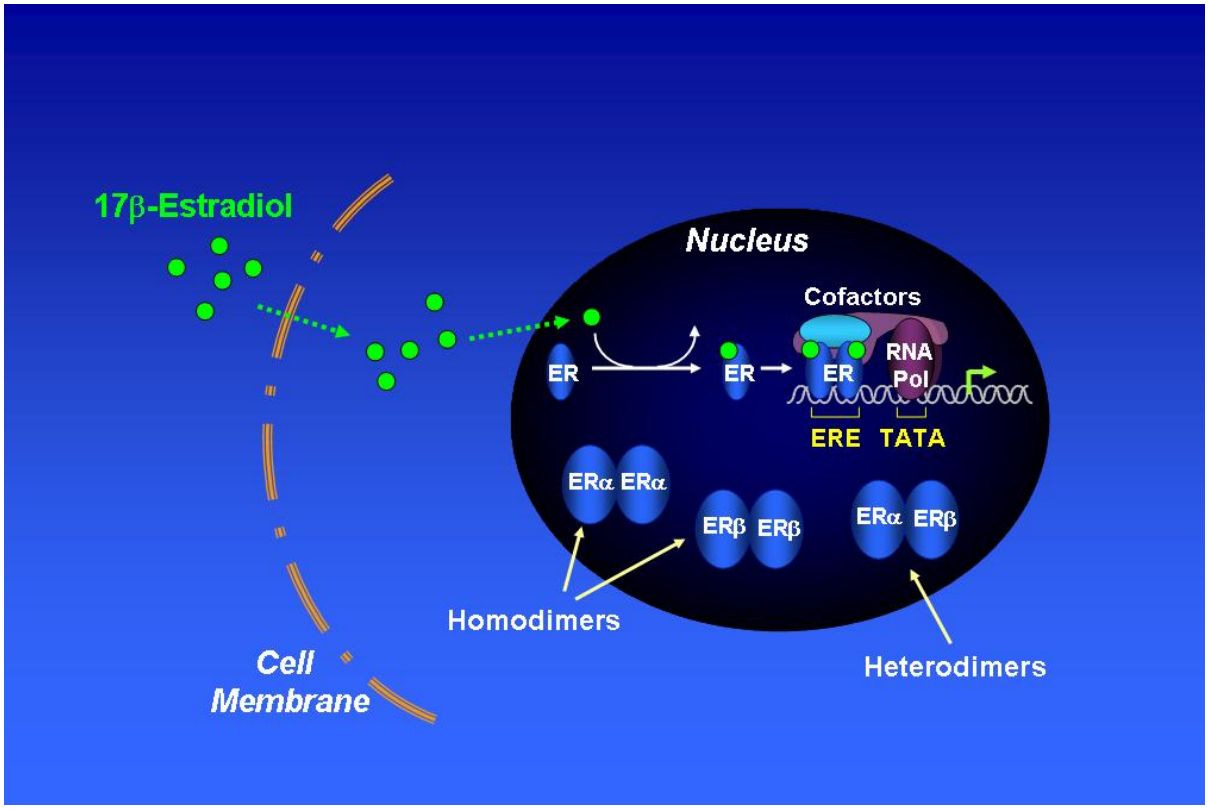

Figure 1.1 Molecular mechanisms of estrogen receptor mediated actions.

2. Neonatal Exposure to Genistein Induces Estrogen Receptor-α Expression and Multi-Oocyte Follicles in the Maturing Mouse Ovary: Evidence for ERβ-Mediated and Non-Estrogenic Actions

2.1 Abstract

Outbred CD-1 mice were treated neonatally on days 1-5 with the phytoestrogen, genistein (1, 10, or 100 μg/pup/day) and ovaries collected on days 5, 12, and 19. Ribonuclease protection assay analysis of ovarian mRNA showed that ERβ predominated over ERα in controls and increased with age. Genistein treatment did not alter ERβ expression, however, ERα was increased on days 5 and 12. ERβ was immunolocalized in granulosa cells while ERα was immunolocalized in interstitial and thecal cells. Genistein treatment caused a dramatic increase in ERα in granulosa cells. Genistein treated βERKO mice showed a similar induction of ERα as seen in CD-1 mice suggesting that ERβ does not mediate this effect. Similar ERα induction in granulosa cells was seen in CD-1 mice treated with lavendustin A, a tyrosine kinase inhibitor that has no known estrogenic actions, suggesting this property of genistein may be responsible. As a functional analysis, genistein treated mice were superovulated and number of oocytes counted. A statistically significant increase in the number of oocytes ovulated was seen in the lowest dose, while a decrease was seen in the two higher doses. This increase in ovulatory capacity in the low dose coincided with increased ERα expression. Histological evaluations on day 19 revealed a dose related increase in multi-

oocyte follicles (MOF) in genistein-treated mice. Tyrosine kinase inhibition was apparently not responsible for MOF since they were not present in lavendustin treated mice, however ERβ must play a role since mice lacking ERβ showed no MOF. These data taken together, demonstrate alterations in the ovary following neonatal exposure to genistein. Given that human infants are exposed to high levels of genistein in soy-based foods, this study indicates that the effects of such exposure on the developing reproductive tract warrant further investigation.

2.2 Introduction

Increased awareness of the resulting consequences of human and wildlife exposure to natural and synthetic estrogens has inspired the emerging field of environmental endocrine disruption. Several laboratories, including our own, have characterized the teratogenic and carcinogenic effects of gestational and neonatal exposure to 17β-estradiol and the synthetic estrogen, diethylstilbestrol (DES), in both rodents and humans [1-3]. In more recent years, similar investigations of less potent but more ubiquitous estrogenic substances, namely the plant derived phytoestrogens, have been initiated. Perhaps the impetus for these investigations is the awareness that phytoestrogens, such as genistein and coumestrol, are known to be present in the human diet and are likely substantial components of vegetarian diets. Furthermore, childhood exposure to high levels of genistein and other phytoestrogens may occur through soy-based infant formulas and foods that are often specifically marketed for children [4, 5]. In fact isoflavone concentrations in commercially available soy-based infant formulas can

far exceed amounts found in the average adult vegetarian diet in the United States [6]. It is estimated that infants consuming a diet of soy-based formulas are exposed to 6-9 mg/kg/day of soy isoflavones, over 65% of which is genistein, whereas adults consuming a diet modest in soy isoflavones may be exposed to approximately 1 mg/kg/day [6]. Still, scientific data supporting the benefits of dietary phytoestrogens are insufficient. Reported beneficial effects of phytoestrogen exposure include a reduction in the risk of DMBA-induced mammary cancer in rats [7] and a lowering of cholesterol in humans [8]. Equally lacking are convincing laboratory studies describing possible detrimental effects of phytoestrogen exposure. However, we have recently reported that neonatal exposure to genistein leads to the induction of uterine adenocarcinoma in mice [9], similar to effects previously described following neonatal DES exposure. Hilakivi-Clarke et al. have also described an increased incidence of mammary tumors in female rats following prenatal exposure to genistein [10]. In humans, the possible detrimental effects of a vegetarian diet consumed during pregnancy include increased incidence of hypospadias in male offspring, perhaps due to abnormally high maternal levels of soy isoflavone [11]. Therefore, the effects of either accidental or intended exposure to dietary phytoestrogens are of growing public health concern.

Estrogens are known to regulate multiple cell functions in target tissues, including growth and differentiation via nuclear receptor-mediated pathways. Furthermore, aberrant temporal or over-stimulation of the estrogen signaling pathway during development has long been known to result in multiple long-term abnormalities in the reproductive tract, including neoplasia [1-3]. Until recently, the majority of estrogen

actions were thought to be mediated via a single form of nuclear estrogen receptor (ER). However, the discovery of a second ER, termed ERβ (to differentiate from the original, now termed ERα), has prompted a reevaluation of the physiology and toxicology of the estrogen signaling system. The biological significance of two ER subtypes remains unclear but may explain the selective and diverging actions of estrogens that occur in various target tissues. Whereas ERα appears to be the predominant ER form in the Müllerian-derived structures of the female reproductive tract [12-15], easily detectable levels of both ERs are present in the gonads of both sexes [16-18]. In the reproductive tract of late gestational and neonatal mice, ERα immunoreactivity is localized to the epithelial cells of the uterus and the interstitium of the ovary [17, 19], whereas ERβ immunoreactivity is limited to the ovarian granulosa cells, with little to no detectable expression in the uterus [18]. Others have also reported the presence of ERβ in the human ovary [20, 21]. No detectable immunoreactivity for either ER has been reported in oocytes, despite previous descriptions of detectable ERα transcripts in mouse and human oocytes [22, 23]. The divergent expression patterns of ERα and ERβ in the developing and adult reproductive tract indicate the complexity of the estrogen signaling system and suggest the two receptors likely play different physiological roles. This is further supported by the diverging transactivational activities and varied binding affinities of the two receptors for different ligands [24, 25]. For example, *in vitro* transactivational assays have shown 4-hydroxytamoxifen to be a partial agonist when bound to ERα, but a full antagonist when interacting with ERβ [26]. Furthermore, 17β-estradiol exhibits a similar *in vitro* binding affinity for both ERs, whereas several synthetic and naturally

occurring xenoestrogens exhibit a binding preference for one of the two receptors [24, 25].

Genistein has long been recognized to have significant estrogenic activity in both *in vivo* and *in vitro* assays, including the induction of estrogen-regulated genes, hypertrophy, and cell proliferation in the rodent uterus [27, 28]. Furthermore, ERβ has been shown to exert a 20-fold higher relative binding affinity for genistein than does ERα [25]. A similar binding preference for ERβ has been reported for additional phytoestrogens, including coumestrol and naringenin [25]. However, the role of ERα and ERβ and the extent to which each contributes to the actions and possible toxicity of genistein exposure has only recently come under investigation [24, 25]. An additional confounding factor unique to the study of genistein is its well-described ability to inhibit tyrosine-specific kinases. This property of genistein was first described in 1987, in which Akiyama et al. showed *in vitro* that genistein was able to inhibit the tyrosine-kinase activity of the epidermal growth factor (EGF) receptor via competitive inhibition for ATP binding [29]. Since this report, genistein is now marketed as a reagent to effectively inhibit tyrosine-specific protein kinases in laboratory studies [30]. More recently, genistein's ability to inhibit tyrosine-specific kinases has been shown to be independent of its ER-mediated hormonal activities *in vitro* [31, 32].

Herein, we describe a syndrome of effects of neonatal genistein exposure on the maturing CD-1 mouse ovary that may be categorized as follows: a biochemical effect as the induction of ectopic expression of ERα in granulosa cells; a morphological effect as the induction of multi-oocyte follicles (MOF) in the ovary; and a functional effect as the

altered ovarian response to superovulation treatment. Furthermore, using the gene-targeted ERα-null (αERKO) and ERβ-null (βERKO) mice as well as the non-estrogenic tyrosine-kinase inhibitor (lavendustin A), we were able to differentiate the estrogenic and tyrosine-kinase inhibitory properties of genistein and ascribe these properties to different ultimate effects in the ovary.

2.3 Materials and Methods

Animals

All animal procedures complied with an approved NIEHS/NIH animal care protocol. Adult CD-1 [Crl: CD-1 (ICR) BR] mice were obtained from Charles River Breeding Laboratories (Raleigh, NC) and bred to male mice of the same strain in the breeding facility at the National Institute of Environmental Health Sciences (NIEHS; Research Triangle Park, NC). Vaginal plug detection was considered day 0 of pregnancy. Pregnant mice were housed under controlled lighting (12 h light and 12 h dark) and temperature (21-22C) conditions. Mice were provided with NIH 31 laboratory mouse chow and fresh water *ad libitum*. Pregnant mice delivered their young at 19 days of gestation at which time female pups were pooled together and redistributed to 8 females per dam. Pups were treated on days 1-5 with genistein (Sigma Chemical Company, St. Louis, MO) by subcutaneous injection at doses of 1, 10, or 100 μg/pup/day in corn oil (day of birth = day 1). These doses are approximately 0.5, 5, or 50 mg/kg/day (estimating 2 grams/pup). All CD-1 experiments consisted of 16 mice per treatment group per age. A second group of CD-1 mice were generated as stated above and treated

with the tyrosine kinase inhibitor, lavendustin A (Sigma Chemical Company), at doses of 1 or 10 μg/pup/day (0.5 or 5 mg/kg/day) on days 1-5 (8 mice per group). The doses of lavendustin A chosen for this study were based on a study that showed tyrosine kinase inhibtion *in vivo* at a dose of 1.3 mg/kg/day [33].

The estrogen receptor-α knockout (αERKO) mice on a background of C57BL6 and ERβ knockout (βERKO) mice on a background of C57BL6/129J have been described previously [34] and were obtained from the breeding colony at NIEHS. Heterozygous breeding pairs from both knockout lines were used to generate pups for treatment. On the day of birth, female pups of each line were pooled together and redistributed to foster CD-1 mothers to avoid differences in maternal behaviors between the two ERKO lines. Pups were treated with genistein as described above.

In all experiments, mice were sacrificed on either day 5, 12 or 19 (except ERKO mice were sacrificed on day 19 only). For RNA or protein extraction, the reproductive tract plus gonads were collected into cold PBS, the ovaries carefully dissected from oviducts, frozen on dry ice, and pooled together per treatment group. Approximately 8 mice per treatment were pooled on days 5 and 12 and 4 mice per treatment were pooled on day 19. For histological and immunohistochemical evaluations, the reproductive tract and gonads were dissected and immediately fixed in buffered formalin for 6 h at 4C, transferred to 70% ethyl alcohol at 4C, followed by routine paraffin embedding.

RNA Isolation and Ribonuclease Protection Assay (RPA)

Total RNA was extracted from each sample of pooled tissue using TRIZOL reagent (GIBCO-BRL, Grand Island, NY) according to the manufacturer's protocol. The concentration of each preparation was determined by A_{260} measurement and the integrity determined by visualization following 1% agarose gel electrophoresis. All RNA preparations were stored at -70C until further use.

The generation and use of the riboprobes used in this study have been previously described [16]. Antisense riboprobes were generated from linearized templates using the Maxiscript reagents (Ambion, Austin, TX), the appropriate RNA polymerase (T3 or T7) and the incorporation of [^{32}P]-CTP (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's protocol. The full-length mouse ERα antisense riboprobe was 490 nucleotides (nt) and produced a specific protected fragment of 366 nt [16]. The mouse ERβ antisense riboprobe was 318 nt in full length and generated a protected fragment of 262 nt [16]. An antisense riboprobe specific for mouse cyclophilin, used to equate loading among lanes, was generated from the template pTRI-Cyclophilin (Ambion) at a full-length of 165 nt and produced a protected fragment of 103 nt.

All RPA reactions consisted of 5 x 10^4 cpm of each of the three probes, 10 μg sample RNA, and yeast tRNA (for a final total RNA equal to 25 μg), which were mixed and ethanol precipitated at -70C for a minimum of 3 h to a maximum of overnight. The resulting pellets were then processed through the RPA using the Hybspeed RPA reagents (Ambion) according to the manufacturer's protocol. Final analysis of protected

fragments was carried out by electrophoresis on a 1.5 mm thick, 6% bis-acrylamide/8.3 M urea/1X TBE gel (NOVEX) which was then fixed, dried and exposed to a phosphorimager screen followed by exposure to x-ray film. Quantitation of the protected fragments from each sample was carried out using a Molecular Dynamics PhosphoImager Storm 860 and ImageQuant software (Sunnyvale, CA).

Protein Isolation and ERα Western Blotting

Nuclear protein was isolated from samples of pooled ovaries collected at 19 days of age using the N-PER kit (Pierce) and then sample concentration determined using the BCA kit (Pierce) according to the manufacturer's protocol. For western blot analysis, a total of 25 μg per sample was electrophoresed on a 10% Bis-Tris gel (NOVEX) and transferred to nitrocellulose. The gel was then stained with Simply blue (Invitrogen) to insure the efficiency of the transfer. The blot was then washed in Tris-buffered saline with 1% Tween-20 (TBS-T) at pH 7.4, blocked with 10% bovine serum albumin (BSA) for 30 min at room temperature, and then allowed to incubate with anti-ERα antibody (Sigma, catalog #E1396) at a dilution of 1:5000 for 1 hour at room temperature. Specific immunoreactivity was then detected using the enhanced chemiluminescence detection reagents (Amersham) according to the manufacturer's protocol. Following detection, the blot was then stained with India ink to insure equal loading and efficient transfer.

Estrogen Receptor Immunohistochemistry

Ovarian tissues were embedded in paraffin and serially cut in 4 μm sections. Tissue sections from a minimum of four mice per treatment for each time point were randomly selected and immunostained for ERα and ERβ. For ERα immunodetection, tissue sections were deparaffinized in xylene, hydrated in a series of graded ethanols, and washed in 1X Automation Buffer (AB; Fisher Scientific, Norcross, GA). Sections were then treated with 3% hydrogen peroxide to eliminate endogenous peroxidase. Following washes in AB, sections were placed in coplin jars in citrate buffer pH 8.0 (Biocare Medical, Walnut Creek, CA) and placed into a decloaker (Biocare Medical) at a setting of 5 min for antigen retrieval. Following the antigen retrieval step, sections were rinsed with dH_20 and then treated according to the instructions provided with the mouse on mouse kit (M.O.M. kit; Vector Laboratories, Burlingame, CA). Briefly, sections were incubated in blocking solution for 1 hour at room temperature. Sections were rinsed with tris buffered saline (TBS, pH 7.4) and then incubated with anti-mouse ERα (Oncogene Science, Manhasset, NY) diluted 1:250 in diluent from the kit for 30 min. Negative controls were run on adjacent tissue sections with preimmune serum or without the primary antibody (buffer only). Sections were then incubated with biotinylated goat anti-mouse and ABC following the instructions in the kit. Visualization of the peroxidase was carried out by covering the sections with diaminobenzidine (DAB; Sigma Chemical Company) at 0.5 mg/ml in AB containing 0.01% H_2O_2 for 10 min. Sections were rinsed in distilled water, counterstained with hematoxylin, dehydrated in a graded series of ethanols, xylenes, and coverslipped for evaluation by light microscopy.

Adjacent ovarian tissue sections were also immunostained for ERβ using techniques previously described [18]. Briefly, sections were deparaffinized, rehydrated, endogenous peroxidase eliminated, and antigen retrieval performed as described for ERα above. Following the antigen retrieval step, tissue sections were rinsed with water and then AB, pH 6.8. All AB used for the detection of ERβ is at pH 6.8 (lower pH was found to enhance the signal of this protein). Sections were then incubated with 10% bovine serum albumin (BSA) in AB for 20 min, then incubated overnight at 4C using rabbit anti-mouse ERβ at a dilution of 1:200 in AB, pH 6.8 (Oncogene Science). Negative controls of adjacent tissue sections were incubated with preimmune serum at the same dilution or no primary antibody (buffer only). Sections were then rinsed and incubated with biotinylated goat anti-rabbit (Vector Laboratories) at a concentration of 1:500 for one hour followed by ExtrAvidin Peroxidase (Sigma) for 30 min. Protein detection was visualized with DAB as described for ERα. Sections were rinsed in distilled water, counterstained with hematoxylin, dehydrated, and coverslipped and evaluated using light microscopy.

Morphological Evaluation

Morphological evaluations of ovarian sections were carried out on ovaries from animals of each treatment group at 19 days of age. For each animal, three sections (6 μm) were prepared from both ovaries from different depths and stained with hematoxylin and eosin according to standard laboratory procedures [35]. Ovaries from eight mice per treatment group were analyzed for the presence of multi-oocyte follicles (MOFs) and

atretic follicles by light microscopy. The presence of one MOF in a single ovarian section categorized a mouse as positive for MOFs. The number of atretic follicles was reported as the number per ovary section.

Superovulation Study

As a functional analysis of the ovary, the ovulatory capacity of neonatally genistein-treated mice was determined using methods previously described [36]. CD-1 mice treated neonatally with genistein as described above were weaned at 21 days of age. On day 22, all mice received a single s.c. injection of 2.2 IU pregnant mares' serum gonadotropin (PMSG) (Sigma) followed 48-52 h later with 3.2 IU human chorionic gonadotropin (hCG) (Sigma). The animals were then euthanized 16-20 h after the hCG injection and the oviduct removed to M-2 medium (Specialty Media, Lavallette, NJ) supplemented with 0.3% hyaluronidase (Sigma). The oocyte/cumulus mass was surgically extracted from the oviduct and the oocytes were counted after enzymatic disassociation from the surrounding cumulus. The data shown are the sum of the two experiments, each of which included 8 mice per treatment group.

2.4 Results

Genistein induction of ERα in the maturing ovary

Fig. 2.1 shows the results of RPAs for ERα and ERβ mRNA in the ovaries of untreated prepubertal mice, indicating that ERβ transcripts were the predominant receptor transcript expressed in the ovaries at all ages examined, with a pronounced increase

occurring between 5 and 12 days of age. ERα mRNA was also detectable at all ages examined but exhibit relatively stable levels on days 5 and 12 and an apparent decrease at 19 days of age. However, at 5 days of age, mice exposed to the lowest dose of genistein (Gen-1) exhibited an almost 3-fold increase in the level of ERα mRNA; this pattern was no longer apparent in similarly treated mice at 12 and 19 days of age (Fig. 2.2A). A similar increase in ERα mRNA levels was observed in the middle genistein group (Gen 10), with levels peaking at 12 days of age. ERβ mRNA showed a similar expression pattern as that of ERα on day 5 with increases in the Gen-1 and Gen-10 dose group; this increase was no longer apparent at 12 and 19 days of age (Fig. 2.2A). The highest genistein treatment group (Gen-100) showed a decrease in ERα and ERβ mRNA at 5 days of age when compared to controls (Fig. 2.2B) but this difference also became less apparent by 12 and 19 days of age (Fig. 2.2B).

To correlate the observed changes in ER transcript levels to changes in protein levels, western blot and immunohistochemistical (IHC) analysis for ERα and IHC for ERβ were carried out on day 19. In agreement with the genistein-induced increase in ERα mRNA levels shown in Fig. 2.2, a correlating increase in ERα protein in the ovary was indicated by western blot (Fig. 2.3A). As expected, ERα immunoreactivity was easily detectable and localized to the interstitial and thecal cells and not detectable in granulosa cells of the ovary in control mice (Fig. 2.3B and 2.3C). While ERα was localized in the interstitial and thecal cells of genistein treated mice as in controls, IHC demonstrated a striking induction of expression of ERα in the granulosa cells (Fig. 2.3D-

2.3G). Once again, as with the mRNA, the strongest induction of ERα protein was observed in the Gen 1 and 10 dose groups (Fig. 2.3D and 2.3E). The highest treatment group (Gen-100) also showed ERα expression in the granulosa cells but the pattern of staining appeared to be more mosaic with some cells staining darker than others (Fig. 2.3H).

IHC detection of ERβ in control and genistein treated mice indicated strong expression in the granulosa cells at all ages examined, in agreement with previous reports (Fig. 2.4A and 2.4B). With the exception of a slight increase in ERβ immunoreactivity in the Gen 1 group (Fig. 2.4C and 2.4D), no obvious changes in the localization of ERβ were observed.

Genistein-induction of ERα in granulosa cells is not ERβ-mediated

As described earlier, genistein is known to possess both ER-mediated estrogenic activity and ER-independent tyrosine-kinase inhibitory properties. To gain insight into the pathway by which genistein elicits ectopic induction of ERα in the granulosa cells of the maturing ovary, we carried out two experiments. The first study involved the treatment and evaluation of mice lacking either ERα (αERKO) or ERβ (βERKO), to determine if this effect was due to the estrogenic properties of genistein and if so, which receptor is involved. Wild type (C57BL/6), αERKO and βERKO animals were exposed to genistein as neonates and evaluated at 19 days of age similar to those studies on CD-1 mice described above. The C57BL/6 wild type and βERKO mice treated with vehicle

exhibited the same ER expression pattern as the CD-1 controls described earlier, i.e. ERα being localized to the interstitial and thecal cells and ERβ being limited to the granulosa cells (data not shown). Also in agreement with the results in the CD-1 mice, wild-type C57BL/6 mice exposed to genistein exhibited the induction of ERα in the granulosa cells (Fig. 2.5A). Interestingly, genistein-induction of ERα in the granulosa cells was preserved in the βERKO mice (Fig. 2.5B), indicating that this effect of genistein exposure is not dependent on the presence of functional ERβ. Because αERKO mice lack immunoreactivity for ERα [37], we are not able to evaluate the role that ERα may have in this genistein effect; however, we can report that neonatal genistein exposure had no effect on ERβ expression in the granulosa cells of the αERKO ovary.

With evidence that genistein-induction of ERα in granulosa cells was not ERβ-dependent, a second study was undertaken to evaluate the possibility that this effect may be attributed to the tyrosine-kinase inhibitory action of genistein. In this experiment, neonatal CD-1 mice were exposed to either genistein as before, or lavendustin A, a specific tyrosine-kinase inhibitor with no documented estrogenic activity [31, 33]. Ovaries from these mice were collected and evaluated at 19 days of age for ERα immunoreactivity. As shown in Fig. 2.5 (C, D), ovaries from mice exposed to the lower dose of lavendustin A exhibited no apparent differences in ERα expression or localization when compared to those of control treated animals. In contrast, ovaries from animals exposed to the high dose of lavendustin A exhibited an appreciable induction of ERα immunoreactivity in the granulosa cells, although not as robust as that observed in

the genistein-treated mice described above (Fig. 2.5D). The lavendustin A treatment had no apparent effect on ERβ levels in the ovary (data not shown). Therefore, the genistein induction of ERα in granulosa cells is independent of functional ERβ and may be in part attributed to the tyrosine-kinase inhibitory property of genistein.

Genistein induction of multi-oocyte follicles (MOF) in the ovary

Ovaries from genistein-exposed mice exhibited no obvious gross changes compared to those of control treated animals. However, microscopic evaluation indicated a remarkable induction of multi-oocyte follicles (MOF) at 19 days of age in animals exposed to genistein as neonates (Table 2.1). In contrast, among a total of 8 control mice, no MOFs were observed at any age examined. Furthermore, a dose-related increase in the incidence of MOF was observed, with 75% of those females exposed to the highest dose of genistein exhibiting at least one MOF. Along with a dose-related increase in the number of animals exhibiting MOF was a corresponding increase in the number of MOF per ovary (Table 2.1). In fact, an ovary of the Gen 100 group exhibited 8 MOFs in a single section (not shown). An example of a section of one of the genistein treated ovaries can be seen in Fig. 6 which shows a number of MOFs in the same section as well as example of a tri-ovular follicle. Also observed in genistein-exposed animals at 19 days of age was an increased incidence in atretic intermediate and large follicles in the Gen 10 group compared to controls (4.5 ± 0.4 per ovary section in controls vs. 9.1 ± 1.0 in Gen-10).

The multi-oocyte follicles most often possessed two distinct germ cells but animals exposed to the highest genistein dose exhibited MOF with as many as 4 oocytes in a single follicle. The oocytes of the MOF were consistently similar in size and each appeared relatively healthy when viewed by light microscopy. The MOF appeared most similar to preantral stage follicles, possessing multiple layers of granulosa cells and an intact theca. No indications of a disruption in the basement membrane of the MOF were observed.

Genistein induction of multi-oocyte follicles (MOF) in the ovary is ERβ-mediated

To gain insight into the pathway by which genistein exposure results in the appearance of MOF in the ovary, we once again evaluated ovaries from genistein exposed αERKO and βERKO mice as well as CD-1 mice exposed to lavendustin A. In contrast to the CD-1 mice, a small percentage of wild-type C57BL/6 mice treated with vehicle only exhibited MOF, indicating this to be an underlying phenotype in this strain as previously reported [38]. However, increasing doses of neonatal genistein exposure led to similar increases in the occurrence of MOF in the wild type C57BL/6, with animals of the Gen 10 and Gen 100 groups exhibiting an 82% and 100% incidence, respectively (Table 2.1). Also similar to the results in the CD-1 was an increased frequency of MOF per ovary with the higher genistein doses, as one C57BL/6 female of the Gen 50,000 group exhibited 10 MOF in a single ovarian section. A similar dose-response to genistein-induction of MOF was exhibited in the exposed αERKO mice, indicating this effect of genistein to be independent of functional ERα. In contrast, the ovaries of

genistein-exposed βERKO mice indicated a dramatic decrease in the incidence of MOF, with only 1 of a total of 12 treated mice exhibiting at least one MOF. Females exposed to either dose of the tyrosine-kinase inhibitor, lavendustin A, exhibited no incidence of MOF. Therefore, the induction of MOF by neonatal genistein exposure appears to be due to an ERβ-mediated mechanism rather than inhibition of tyrosine-specific kinases.

Evaluation of genistein on ovarian function by superovulation

To gain some insight as to the effect that neonatal genistein exposure may have on the functional capacity of the ovary, control and neonatally exposed mice of 22-23 days of age were superovulated and evaluated for oocyte yield. As shown in Table 2.2, we observed divergent effects of genistein exposure among the 3 doses used. Whereas females exposed to the two highest doses of genistein exhibited a below normal oocyte yield (not statistically significant from controls), females exposed to the lowest dose of genistein exhibited a statistically significant increase ($p<0.05$) in the number of ovulated oocytes. Table 2.2 shows the combined results of two separate superovulation trials, both of which produced similar results.

2.5 Discussion

In the current study, we have evaluated a series of effects of neonatal genistein exposure on the maturing mouse ovary that may be categorized as follows: a biochemical effect as the stimulation of ectopic expression of ERα in granulosa cells; a morphological effect as the induction of MOF in the ovary; and a functional effect as the altered ovarian

response to superovulation treatment. Furthermore, through the use of the ERKO mice and the non-estrogenic tyrosine-kinase inhibitor, lavendustin A, we have determined that these effects rely on more than one response mechanism of genistein. Whereas the induction of MOF in the ovary appears to be an ERβ-mediated effect of genistein, the stimulation of ectopic ERα expression in the granulosa cells may be attributed to tyrosine-kinase inhibitory actions of genistein. Furthermore, the two effects of neonatal genistein exposure on the ovary are not directly related as illustrated by the ability of genistein to induce MOF in the αERKO ovary.

In agreement with previous reports, we have shown that transcripts for both ERα and ERβ are detectable in the prepubertal mouse ovary, and expression of each is limited to the thecal/interstitial and granulosa cells, respectively [17, 18]. Furthermore, ERβ is clearly the predominant form expressed during the neonatal period and increases more than two-fold between the ages of 5 and 12 days, similar to findings in the rat ovary [39], whereas ERα expression in the ovary remains low and relatively stable during this same period in both mice (as show herein) and rats [39]. Genistein exposure had no discernable effect on ERβ expression levels or pattern in the maturing ovary. These findings are similar to that of Ikeda et al. in which neonatal treatment of mice with estradiol benzoate also had no effect on ERβ expression [40]. In contrast, we observed a large induction of ERα expression in the granulosa cells following neonatal genistein exposure. Interestingly, the greatest rise in ERα mRNA levels was the 3-fold increase seen at day 5 (Gen 1 dose), within 24 hours of the final genistein treatment, whereas the

peak level of ERα immunoreactivity in the granulosa cells was not observed until 19 days of age. Such a delay between the appearance of mRNA and detectable protein has been shown for other transcripts during oogenesis and spermatogenesis [41, 42]. Our laboratory recently reported a similar delay in the ontogeny of ERβ in the developing testis of the mouse [18]. Interestingly, genistein-induction of ERα expression in the granulosa cells exhibited an inverse dose-response in that the most abundant expression of ERα was observed in the ovaries of mice exposed to the lowest dose of neonatal genistein. However, this dose-response relationship is not atypical in the study of xenoestrogen effects, as similar findings of low-dose effects on other reproductive parameters following prenatal or neonatal exposure to estrogenic compounds such as DES, bisphenol A, and methoxychlor have also been reported [43-46].

The mechanism by which neonatal genistein exposure results in the stimulation of ERα expression in the granulosa cells of the ovary remains to be fully elucidated. Transcriptional regulation of the ERα gene is not well understood [47]. Recent studies have shown that regulatory sequences of the human and rodent ERα genes are highly complex units, possessing as many as seven exons in the 5'-untranslated sequences [47]. Kos et al. has recently reported that the mouse ERα gene is transcribed from at least 5 distinct promoters, all of which result in transcripts encoding the same functional 66 kDa protein [48]. In addition, several studies have reported positive auto-regulation of the ERα promoter via ligand-bound ERα *in vitro* [49-51], and have shown that multiple partial estrogen response elements (EREs) within the human ERα promoter allow for ER binding and up-regulation of receptor expression [49]. Therefore, it is plausible that the

genistein induction of ERα expression in granulosa cells observed here is mediated via the activation of ERα on its own promoter, or perhaps via ERβ acting upon the ERα promoter. However, neither 17β-estradiol nor DES has been reported to cause a similar induction of ERα expression in granulosa cells of the maturing ovary, arguing against the possibility of a direct receptor-mediated effect of genistein on the ERα promoter. Furthermore, the ovaries of the maturing βERKO female were also susceptible to genistein-induction of ERα expression in the granulosa cells, indicating the underlying mechanism is not dependent on functional ERβ. Because the αERKO mice by definition lack functional ERα, we are not able to evaluate the role that ERα may have in auto-regulation.

Based on our findings that the induction of ERα in granulosa cells was unique to neonatal genistein exposure, we hypothesized that this effect may be due to genistein's tyrosine-kinase inhibitory properties. We therefore treated neonatal females with lavendustin A, a specific tyrosine-kinase inhibitor that possesses no known estrogenic activity. Interestingly, treatments with lavendustin A also resulted in the induction of ERα expression in the granulosa cells, although the level of ERα immunoreactivity was not as robust as that seen following genistein treatment. The reasons for the less dramatic effect of lavendustin A are unknown at this time. Since the inhibitory actions of both genistein and lavendustin A on tyrosine-specific kinases have been mostly characterized under *in vitro* conditions, it is difficult to determine the optimum dose required to achieve a similar effect *in vivo* for either compound. Certainly, the pharmacokinetics of the drugs

and therefore the active amounts delivered to the ovary are likely to differ. Furthermore, because genistein has been shown to preferentially bind ERβ, it is possible that this interaction may allow for a high concentration of genistein within the granulosa cells of the ovary, endowing ERβ with the role of chaperone for genistein rather than an active partner. Nonetheless, the induction of ERα expression in the granulosa cells of the prepubertal ovary by both genistein and the non-estrogenic lavendustin A strongly indicates this effect to be related to the inhibition of tyrosine-specific kinases within these cells.

As discussed, ERα expression in ovarian granulosa cells is low to undetectable during folliculogenesis in the mature ovary. However, ERα expression rapidly increases following ovulation in luteinizing granulosa cells that are undergoing terminal differentiation to form the corpus luteum (CL) with a coincidental decrease in ERβ [14, 52]. These data indicate that ERα is the predominant receptor form involved in mediating estrogen actions in the rodent CL. Therefore, a possible mechanism for the induction of ectopic expression of ERα in granulosa cells by genistein may be via the stimulation of signaling pathways involved in the normal induction of ERα expression in the CL. Recent studies have shown that the induction of ERα expression in rat CL and in primary cultures of luteinizing cells is highly dependent upon prolactin signaling via the prolactin-receptor [52, 53]. However, Frasor et al. has recently shown that prolactin induction of ERα expression in luteinizing granulosa cells occurs via the intracellular Jak2/Stat5 pathway, which is highly dependent upon a cascade of tyrosine-

phosphorylation events [53]. Therefore, it might be expected that genistein would in fact block prolactin signaling via its ability to specifically inhibit tyrosine kinase activity. Still, alternate prolactin signaling pathways involving Src kinases, MAP-kinases and protein kinase C, have been reported [54] and may not be as susceptible to inhibition by genistein or lavendustin A.

An indirect mechanism by which neonatal genistein exposure may alter prolactin signaling in the ovary may be via the induction of alterations in the hypothalamic-pituitary axis. Although the pituitary lactotroph is a well-characterized estrogen target cell, the neuroendocrine mechanisms that regulate lactotroph function do not fully mature until after birth in the rodent [55], making these tissues highly susceptible to insults from xenoestrogen exposure. Neonatal exposure of female mice to DES has been shown to result in hyperprolactinemia in the weeks just following treatment [56, 57]. More recently, Khurana et al. has shown that rats exposed to low doses of the xenoestrogens, octylphenol and bisphenol A, also exhibit hyperprolactinemia as early as 25 days of age [58]. Although similar *in vivo* data for genistein has not yet been reported, Stahl et al. recently demonstrated that genistein is able to stimulate cell proliferation and prolactin synthesis in PR1 cells, a pituitary-tumor derived cell line [59]. These same studies showed that genistein was as potent as estradiol in stimulating prolactin synthesis and this action of both hormones was inhibited by the complete ER-antagonist, ICI-182,780, strongly indicating a common ER-mediated mechanism [59]. Therefore, it is conceivable that neonatal genistein exposure in the mouse has resulted in premature increases in

serum prolactin levels, which in turn may have a luteotropic action on the granulosa cells of the ovary, including the induction of ERα expression.

An interesting consequence of the induction of ERα in the granulosa cells following neonatal genistein treatment is the presence of both ER forms within the same cell type. This is in contrast to the natural expression pattern exhibited by most tissues in which ERα and ERβ are limited to separate compartments of a tissue, with the exception of certain regions of the brain [60]. Therefore, this unique effect of genistein resulting in co-expression of both ER forms introduces the possibility of ER-heterodimer actions within the cell. ER-heterodimer formation has been shown to be possible for both the human [61] and mouse [62] receptor forms *in vitro*. These studies invariably agree that the ER-heterodimer behaves as a positive transcription factor *in vitro*, most often exhibiting transactivational activity that is reduced relative to the ERα homodimer but above that of the ERβ homodimer [61, 62]. However, the existence of the ER-heterodimer complex and its role in estrogen signaling *in vivo* remains to be substantiated. Perhaps the model described in this paper could be used as a unique opportunity to study the *in vivo* consequences of ERα/ERβ heterodimers and the possible alternate gene transcription produced through these complexes as opposed to the respective homodimers.

Another observed effect of neonatal genistein exposure was the induction of multi-oocyte follicles (MOF) in the ovary. This effect exhibited the more typical dose-response curve, in contrast to the induction in ERα expression discussed above, suggesting that the underlying mechanisms likely differ. It has long been recognized that

pre- and neonatal exposure to DES also results in the appearance of MOF in the mouse [38, 63, 64], yet the mechanism remains unclear. Iguchi et al. demonstrated the neonatal ovary to be uniquely susceptible to this effect of DES relative to the adult ovary; and this effect was specific to estrogen action and not induced by similar treatments with progesterone or testosterone, when combined with an aromatase-inhibitor [65, 66]. Furthermore, this effect of DES appears to be directly on the ovary, as neonatal ovaries exposed to DES in culture and then transplanted to adult hosts exhibit MOF [66]. In this study we have furthered these findings by demonstrating that ovaries of βERKO females are completely resistant to this effect of genistein, while αERKO mice exhibited an incidence of MOF comparable to wild-type mice following genistein exposure. The lack of MOF in animals treated with lavendustin A indicates that inhibition of tyrosine-specific kinases does not likely play a role in this effect of genistein. Therefore, these data strongly indicate that ERβ is critical to this action of genistein on the maturing mouse ovary.

Interestingly, MOF in the mouse ovary does not appear to be unique to estrogen exposure. Recent descriptions of mice lacking GDF-9 or BMP-15 [67], both oocyte-secreted growth factors, or preliminary data from mice lacking IRS-1 [68], which is a member of the insulin and IGF signaling pathway, exhibit an increased incidence of MOF. Also, mice overexpressing the inhibin-a gene [69] exhibit an increased incidence of MOF. Therefore, the mechanisms underlying the development of MOF are obviously complex and involve multiple pathways. It is likely that MOF are the result of a failure of primary follicular cells to separate primordial oocytes during the initial stages of

follicle organization. Perhaps genistein acts via ERβ to alter the proper granulosa cell response to the oocyte-secreted growth factors mentioned above or may alter the expression of proteases or adhesive proteins involved in oocyte-granulosa cell interaction. Although reported incidence of MOF in humans ranges from 24% [70] to 85% [71] depending on the study, both reports agree that the proportion of total follicles in the human ovary that are multi-oocyte is less than 1%.

The final effect of neonatal genistein exposure on the maturing mouse ovary observed in this study was found following superovulation of exposed females with exogenous gonadotropins. Interestingly, mice treated with the lowest dose of genistein as neonates exhibited an increased ovulatory capacity compared to untreated controls, whereas animals treated with higher doses yielded a reduced number of ovulated oocytes. The underlying mechanism for this bi-phasic effect of genistein is unclear. Because both the αERKO and βERKO models exhibit innate reductions in ovulatory efficiency following superovulation [34], these animals were not included in this portion of the study. It is interesting however to note that the genistein dose which induced the greatest levels of ERα expression in the granulosa cells was also that which produced the highest number of oocytes following superovulation. Estrogen action is known to be an anti-atretic factor in the ovary [72] and clearly facilitates ovulation as demonstrated by the ERKO mice. Therefore, perhaps the elevated expression of ERα within the granulosa cells has allowed for a greater number of follicles available for ovulation during the gonadotropin treatment. Iguchi et al. showed that superovulation of mice following neonatal exposure to DES resulted in a decrease in the number of MOF in the ovary,

speculating that perhaps a portion of the MOF were ovulated although assays to determine this were not carried out. The possibility exists that greater number of oocytes were present in the oviduct of the Gen 1 group because of the rupture of MOF, however, our studies herein indicated that females exposed to the higher doses of genistein actually possessed a greater number of MOF but a reduction in oocyte yield following superovulation which suggests this is not a likely possibility.

Since mice exposed to the lowest dose of genistein are ovulating more oocytes following stimulation, it is possible that these animals exhibit a premature depletion of oocytes, resulting in reduced fertility earlier in life. In fact, our laboratory has shown that mice exposed to low doses of DES prenatally exhibit an increased number of CL at 2 months of age with a subsequent decrease by 6 months of age relative to controls [35]. Studies are currently underway in our laboratory to determine the effects of neonatal genistein exposure on fertility and the possibility that these mice will exhibit infertility at an earlier age than their control counterparts.

In summary, we have shown that neonatal genistein exposure produces multiple effects on the morphology and function of the mouse ovary. Furthermore, we have begun to elucidate the mechanisms by which genistein elicits such effects. The ectopic induction of ERα expression in the granulosa cells of the ovary appears to be associated more with the tyrosine-kinase inhibitory properties of genistein rather than its estrogen actions, although indirect effects secondary to estrogenization of the hypothalamic-pituitary axis cannot be ruled out. In contrast, the induction of MOF in the ovary, which appears to be a direct effect and unrelated to the changes in ERα expression, is dependent

on the presence of functional ERβ within the ovary. Future investigations into the mechanisms of the diverse effects of genistein will prove invaluable in evaluating the possible effects of phytoestrogens on reproductive function.

2.6 References

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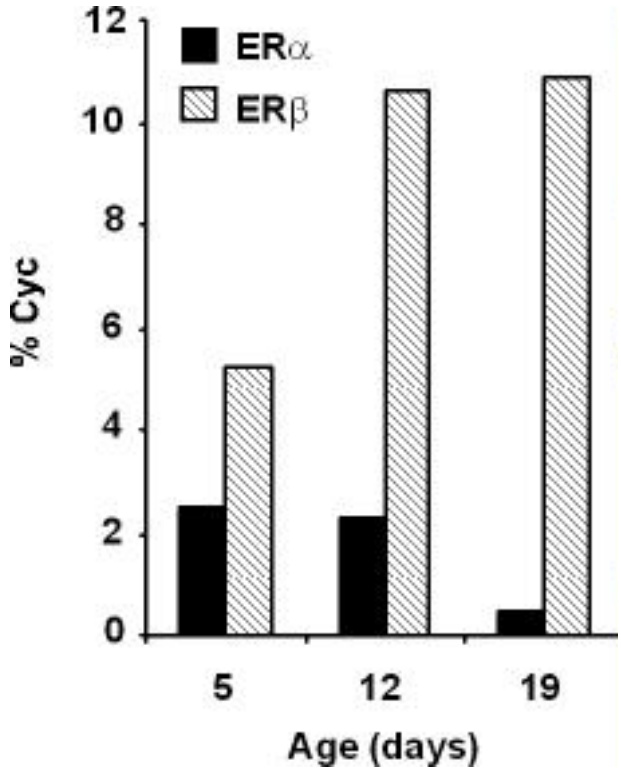

Figure 2.1: RPA results of ERα and ERβ in the ovary in control mice throughout development. Total ovarian RNA from mice 5, 12, and 19 days of age was assayed for ER expression by RPA. ERα is expressed on day 5 and decreases over the next two time points. ERβ is highly expressed in the ovary all ages examined and shows an increase with age. Note, ERβ is the predominant ER subtype in the ovary at all ages examined. Each sample was normalized to cyc and the data is presented as %cyc.

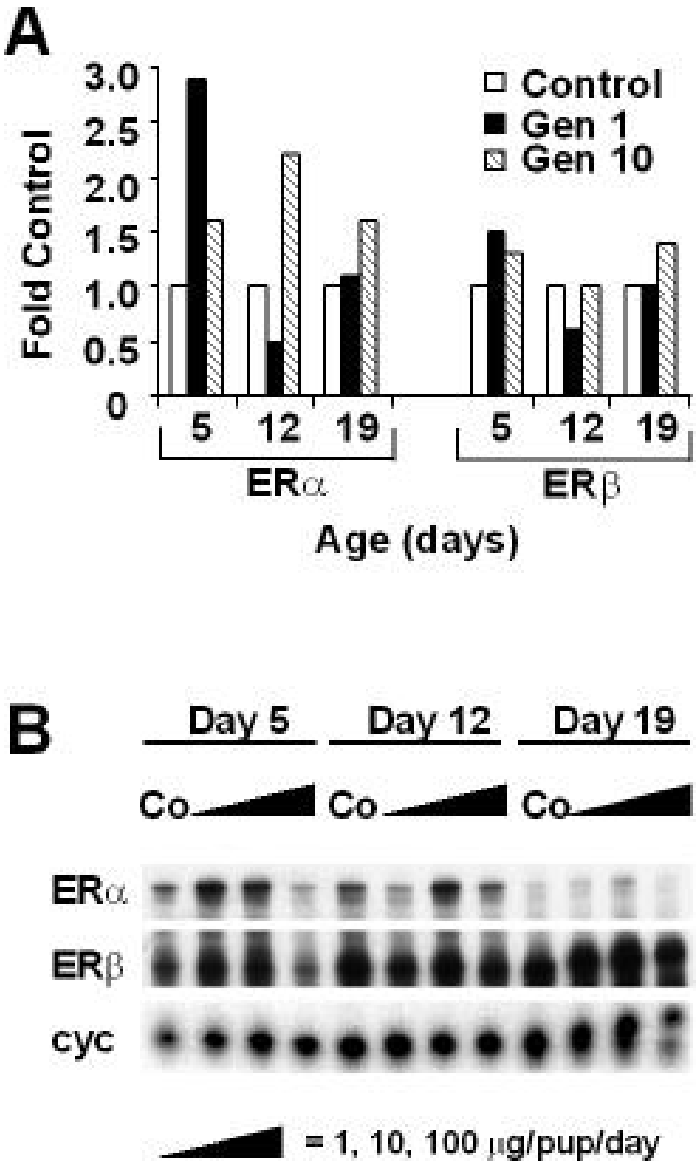

Figure 2.2: RPA results of ERα and ERβ mRNA in the ovary throughout development following neonatal genistein exposure. Total ovarian RNA from vehicle and genistein-treated mice of 5, 12, and 19 days of age was assayed for ER expression by RPA. Each sample was normalized to cyc as a % then fold control was calculated by using the normalized value of ERα or ERβ divided by its own age-matched control value. There is ERα and ERβ mRNA present in all samples. There is an increase in ERα mRNA on day 5 following the lowest dose of genistein and an increase in ERα on day 12 following Gen 10 treatment (panel A). ERβ appears to be increased at 5 days of age in the Gen 1 and Gen 10 groups, but this increase is less apparent by 12 and 19 days of age. At the highest dose of genistein (Gen 100) ERα and ERβ appear to be decreased at 5 days of age but this difference is also less apparent at 12 and 19 days of age (panel B).

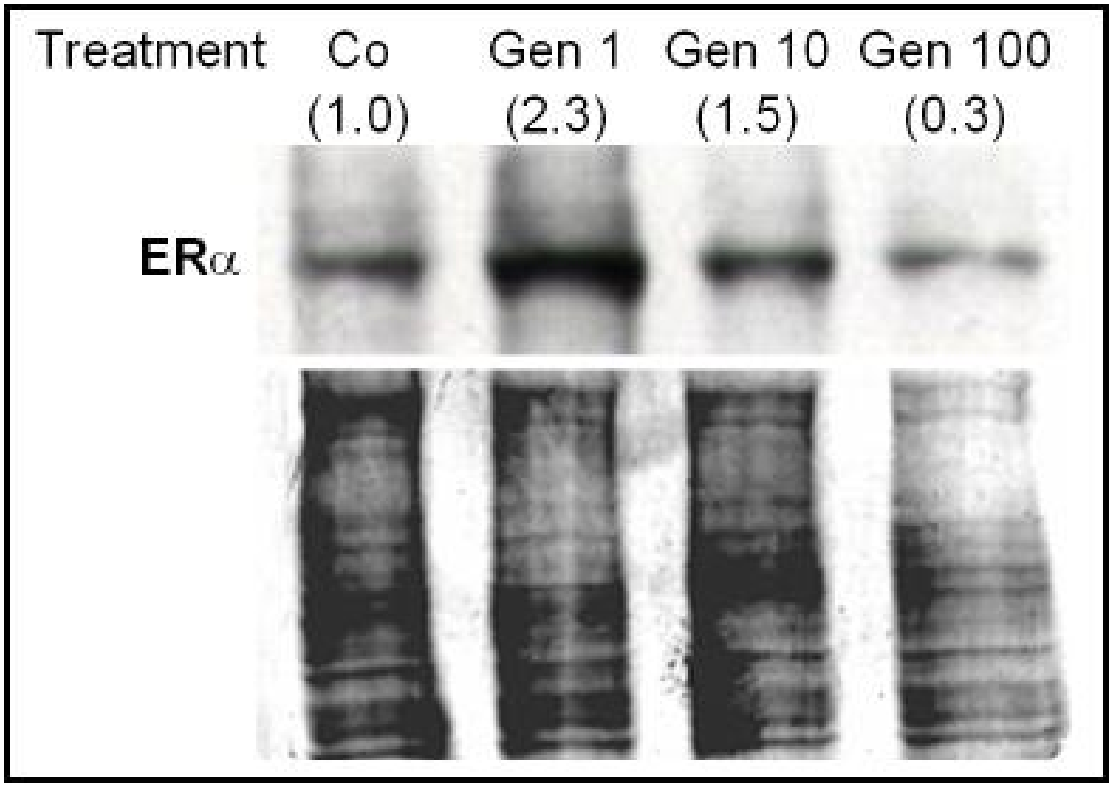

Figure 2.3A: ERα protein expression in CD-1 day 19 ovaries following neonatal exposure to genistein. Panel A: ERα Western blot of nuclear protein from ovaries collected at 19 days of age. There is an increase in ERα protein in the ovary following the lowest dose of genistein (Lane 2) compared to controls (Lane 1). There is also an apparent decrease in ERα in the highest dose of genistein (Lane 4) compared to controls. Lane 1 – control, Lane 2 – Gen 1, Lane 3 – Gen 10, Lane 4 – Gen 100

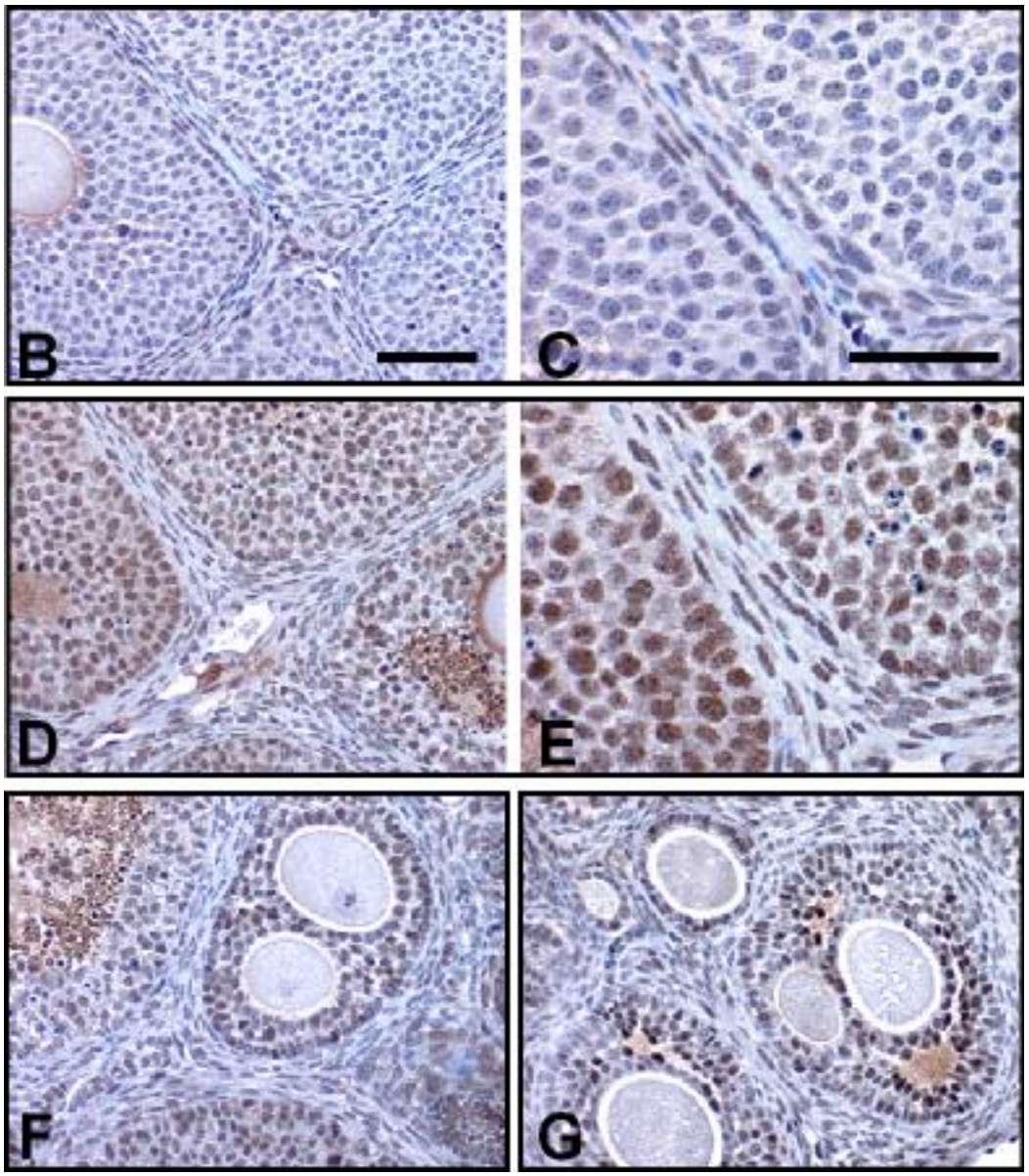

Figure 2.3B-G: ERα protein expression in CD-1 day 19 ovaries following neonatal exposure to genistein. Panel B-G: ERα immunohistochemistry on CD-1 day 19 ovaries following neonatal exposure to genistein. Panels B and C – control, panel D and E – Gen 1, panel F – Gen 10, panel G – Gen 100. ERα can be seen predominantly in the interstitial and thecal cells of the ovary in the control animals. For the genistein treated mice, ERα can be seen in the interstitial, thecal and granulosa cells. A multi-oocyte follicle can be seen in the Gen 10 and 100 photographs. Bar = 100 microns.

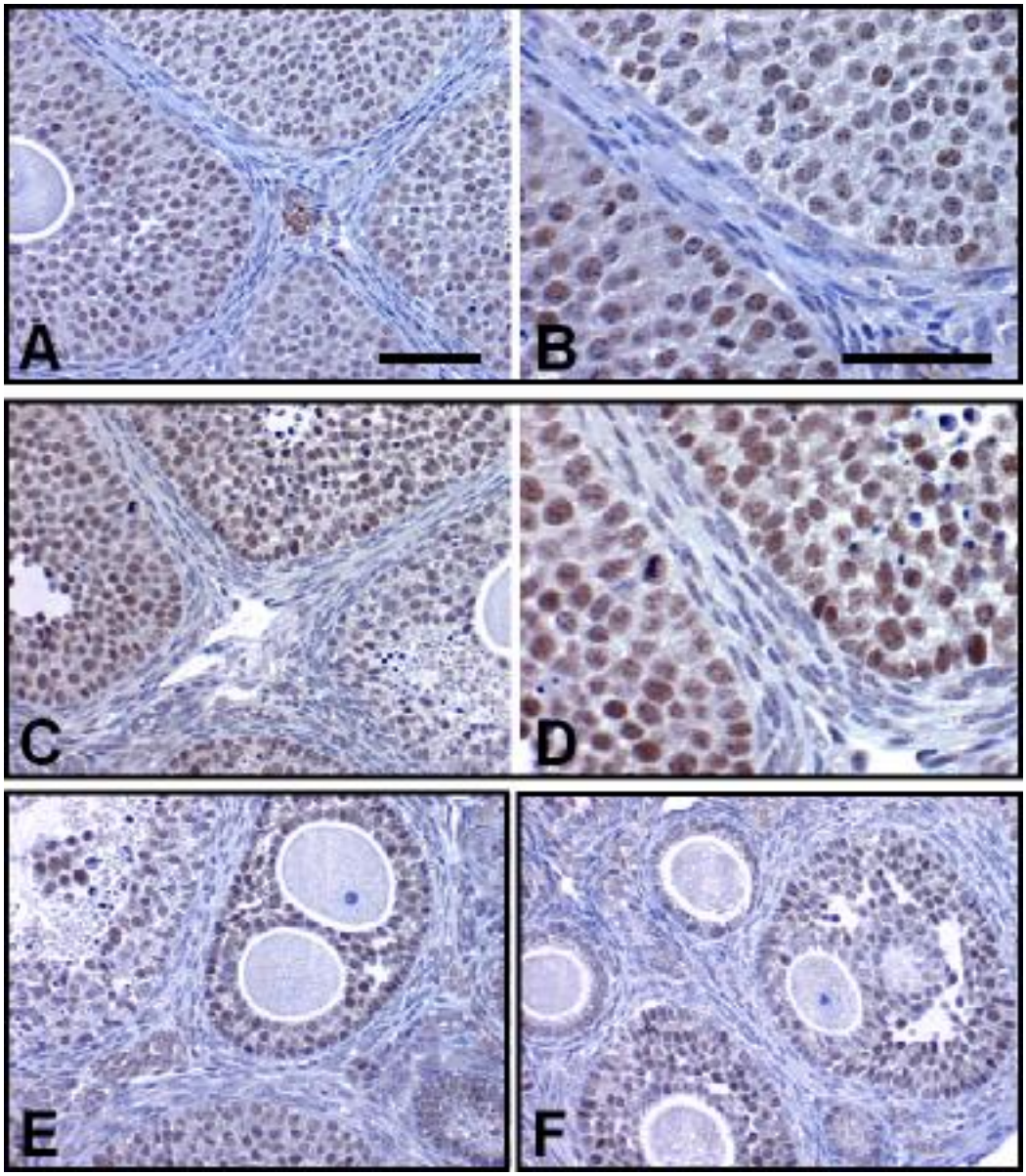

Figure 2.4: ERβ immunohistochemistry on CD-1 day 19 ovaries following neonatal exposure to genistein. Panels A and B – control, panels C and D – Gen 1, panel E – Gen 10, panel F – Gen 100. ERβ can be seen predominantly in the granulosa cells of the ovary in the control animals. For the genistein treated mice, an increase in ERβ can be seen in the granulosa cells. A multi-oocyte follicle can be seen in the Gen 10 and 100 photographs. Bar = 100 microns.

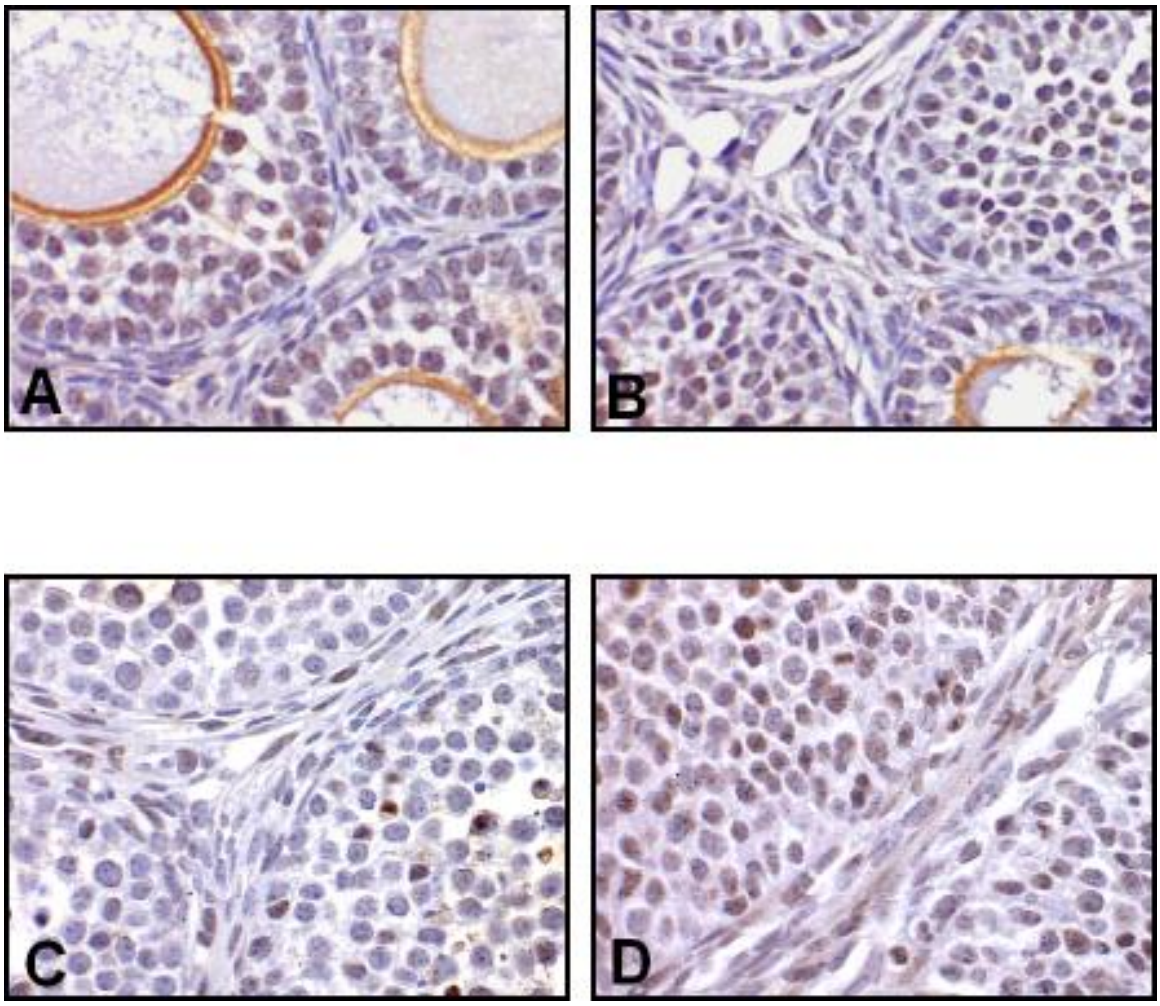

Figure 2.5: ERα immunohistochemistry at 19 days of age in genistein treated βERKO mice and lavendustin A treated CD-1 mice. Panel A – Wild type Gen 10, Panel B – βERKO Gen 10, Panel C – Lavendustin 1, Panel D – Lavendustin 10. Note the localization of ERα in the granulosa cells in all panels.

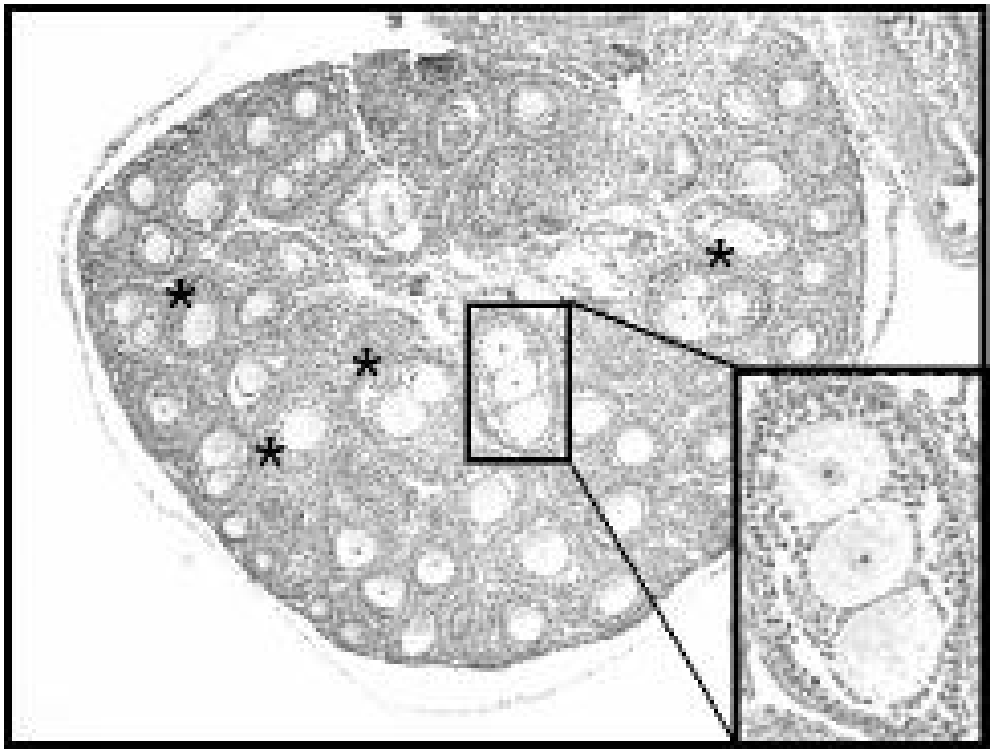

Figure 2.6: Morphology of multi-oocyte follicles following neonatal genistein treatment. Shown is a representative ovary (C57/Bl6) at 19 days of age following neonatal treatment with genistein 100 μg/pup/day, illustrating the presence of several MOFs (arrows). Shown in the inset is an example of three oocytes within a single follicle, with the nuclei of two of these being clearly visible.

Table 2.1: Incidence of multi-oocyte follicles following neonatal treatment with genistein or lavendustin A.

		Genistein			Lavendustin	
Genotype	**Vehicle**	**1**	**10**	**100**	**1**	**10**
Wild Type CD-1	0/8 (0)	1/8 (2)	2/8 (4)	6/8 (8)	0/8 (0)	0/8 (0)
Wild Type C57BL/6	1/11 (1)	1/11 (1)	9/11 (3)	11/11 (10)	nd	nd
αERKO	1/3 (1)	2/4 (1)	4/6 (4)	nd	nd	nd
βERKO	1/2 (1)	0/4 (0)	0/5 (0)	1/3 (2)	nd	nd

Mice were treated as described in Materials and Methods and the ovaries collected at 19 days of age examined by light microscopy. Data represents the number of mice that demonstrated at least one multi-oocyte follicle in any section examined.

Number in parenthesis is the highest number of multi-oocyte follicles observed in a single section from that treatment group.

nd = not determined.

Table 2.2: Number of oocytes ovulated following superovulation in mice treated neonatally with Genistein.

Treatment (μg/pup/day)	**N**	**Number of Oocytes**
Control	15	23.2 ± 2.8
Gen 1	16	33.9 ± 3.3*
Gen 10	14	17.9 ± 1.4
Gen 100	16	16.5 ± 1.8

Mice were treated neonatally on days 1-5 with genistein at doses of 1, 10, or 100 μg/pup/day. At 22 days of age, mice were treated with PMS and HCG as described in the Materials and Methods section. Mice were sacrificed 16-20 hrs following the last injection, ovaries/oviducts collected and incubated with hyaluronidase, oviducts were then ruptured and oocytes counted. A minimum of 14 mice was counted per dose.

* $p<0.05$ when compared to controls using Dunnett's test.

3. Adverse Effects on Female Development and Reproduction in CD-1 Mice Following Neonatal Exposure to the Phytoestrogen Genistein at Environmentally Relevant Doses

3.1 Abstract

Outbred female CD-1 mice were treated with genistein (Gen), the primary phytoestrogen in soy, by subcutaneous injections on neonatal days 1-5 at doses of 0.5, 5 or 50 mg/kg/day (Gen-0.5, Gen-5 and Gen-50). Day of vaginal opening was observed in mice treated with Gen and compared to controls and although there were some differences, they were not statistically significant. Gen-treated mice had prolonged estrous cycles with a dose and age related increase in severity of abnormal cycles. Females treated with Gen-0.5 or Gen-5 bred to control males at 2, 4 and 6 months showed statistically significant decreases in the number of live pups over time with increasing dose; at 6 months, 60% of the females in the Gen-0.5 group and 40% in the Gen-5 group delivered live pups compared to 100% of controls. Mice treated with Gen-50 did not deliver live pups. Interestingly, at 2 months, >60% of the mice treated with Gen-50 were fertile as determined by uterine implantation sites but pregnancy was not maintained; pregnancy loss was characterized by fewer, smaller implantation sites and increased reabsorptions. Mice treated with lower doses of Gen had increased numbers of corpora lutea compared to controls while mice treated with the highest dose had decreased numbers; however, superovulation with eCG/hCG yielded similar numbers of oocytes as controls. Serum levels of progesterone, estradiol and testosterone were similar between Gen-treated and control mice when measured prior to puberty and during pregnancy. In summary, neonatal treatment with Gen caused

abnormal estrous cycles, altered ovarian function, early reproductive senescence, and subfertility/infertility at environmentally relevant doses.

3.2 Introduction

Exposure to estrogenic substances during critical periods of development can have adverse consequences on differentiating reproductive systems of rodents and humans [1]. The most well known example is the synthetic estrogen, diethylstilbestrol (DES) that has been documented to cause benign and malignant reproductive tract abnormalities in prenatally-exposed males and females of several species [1]. In particular, malformations of the female reproductive tract, alterations in the onset of puberty, alterations in estrous cyclicity, subfertility/infertility, and reproductive tract lesions have been reported in experimental animals and humans [1-4]. Since numerous chemicals in our environment possess estrogenic activity [5-7], the possibility exists that some of these chemicals may disrupt normal processes of development, differentiation, and subsequent function of the reproductive system similar to the adverse effects caused by DES [8].

Phytoestrogens are a group of naturally occurring compounds that have been reported to cause fertility problems in animals [9-13]. Of particular concern is genistein (Gen), the major phytoestrogen in soy products [14], that has potent estrogenic activity both *in vitro* and *in vivo* [15-18]. Human fetuses and neonates can be exposed to high levels of Gen if their mothers consume excessive amounts of soy [19] or if they are given soy-based formulas and other soy products marketed specifically for children [14, 20, 21]. The concentrations of Gen and other isoflavones found in some of these soy-based products far exceed the amount found in an adult diet; one study estimates that infants fed soy-based formulas consume approximately 6-9

mg/kg/day of Gen compared to 1 mg/kg/day for an adult vegetarian [14]. Soybeans also have an extremely variable isoflavone content depending on variety and environmental conditions such as growing season and location [22] and the USDA reports variable amounts of Gen in various soy products [23].

Over the last few years, public and scientific interest in phytoestrogens, like Gen, has increased because of its proposed beneficial effects. Currently, there are mixed results on developmental exposure to Gen suggesting some beneficial effects but also adverse effects depending on the timing of exposure, dose level and endpoints examined. For example, two studies report that exposure to Gen prenatally prevents carcinogen-induced mammary gland cancer in rats [24, 25] while another study shows an increase in mammary gland cancer if the developmental window of exposure is shifted to neonatal life [26]. Other investigations report improved cholesterol synthesis rates of human infants consuming soy-based formulas [27]. Vegetarian diets containing high levels of soy during pregnancy have been associated with increased incidence of hypospadias in the male offspring [28]. Further, an epidemiology of health outcomes in young adults who were fed soy based infant formulas reported an increase in more frequent use of allergy medicines in both men and women and longer menstrual bleeding and more discomfort during the menstrual cycle in women [29, 30]. So the adverse effects of developmental exposure to Gen remain of concern.

A recent study from our laboratory has shown that neonatal exposure to Gen at a dose of 50 mg/kg/day on days 1-5 leads to an increased incidence of uterine adenocarcinoma in mice later in life; the incidence of uterine tumors in Gen-treated mice (35%) was similar to the incidence found in mice treated with an equal estrogenic dose of DES [0.001 mg/kg/day (31%)] [31]. Although Gen was administered as subcutaneous injections, the levels of Gen used in our

studies produced circulating serum levels similar to the range of that found in infants consuming soy-based formulas [32]. Therefore, the dose of Gen to the target tissue was comparable between subcutaneous injections and oral exposures. Similar findings have also been reported by Lewis et al., in neonatal rats using a dose of 40 mg/kg/day [33]. Further studies have shown adverse effects on the developing rat following Gen exposure including brain function, estrous cyclicity and reproductive behavior [34, 35]. Studies using other phytoestrogens including coumestrol [9, 36], daidzein [13, 35] and red clover [10, 12] have also demonstrated disruptions in reproduction and/or reproductive endpoints, supporting the concept that phytoestrogens, although weaker than DES or 17β-estradiol, can cause adverse effects on the developing reproductive tract [7]. Also, some of these effects may not be apparent until later in life similar to those caused by DES [1].

To gain further understanding into the mechanism by which phytoestrogens can interfere with development and reproduction, a recent study in our laboratory examined the effects of Gen on the developing ovary following neonatal exposure to doses of 0.5, 5 and 50 mg/kg/day. Effects on the ovary included alterations in morphology with the presence of multi-oocyte follicles (MOFs) as well as alterations in function as determined by ovulatory capacity [37]. The formation of MOFs involves the actions of estrogen receptor (ER) β as *Esr2*-null (bERKO) mice do not exhibit Genistein-induced MOFs [37].

The doses of Gen used in our studies were chosen to span the range of human exposure levels and estrogen activity. Previously, we have shown that Gen at a dose of 50 mg/kg/day is estrogenic in the mouse uterotropic bioassay in a 5 day old neonate and an immature mouse, and this dose is equal in estrogenic activity to DES 0.001 mg/kg/day [7, 31]. Therefore, we chose Gen 50 mg/kg/day as our highest dose and used two lower doses of Gen to examine lower level

effects. These doses span the range to which vegetarian mothers are exposed during pregnancy and lactation, and to which infants are exposed on soy-based formulas [14, 19, 20, 28].

To further study the effects on the developing murine reproductive system, our current study examines the effects of neonatal Gen exposure on attainment of puberty and subsequent fertility including ovarian function, estrous cyclicity and pregnancy outcome over time.

3.3 Materials and Methods

Animals and Neonatal Treatment

Adult CD-1 [Crl:CD-1 (ICR) BR] mice were obtained from Charles River Breeding Laboratories (Raleigh, NC) and bred to male mice of the same strain in the breeding facility at the National Institute of Environmental Health Sciences (NIEHS; Research Triangle Park, NC). Vaginal plug detection was considered day 0 of pregnancy. Pregnant mice were housed under controlled lighting (12 h light and 12 h dark) and temperature (21-22° C) conditions. Mice were housed in polysulfone, ventilated cages (Technoplast, Inc., Exton, PA) and provided with NIH-31 laboratory mouse chow and fresh water *ad libitum*. All animal procedures complied with an approved NIEHS/NIH animal care protocol.

Pregnant mice delivered their young on day 19 of gestation, pups were separated according to sex and then randomly standardized to 8 female pups per litter (a minimum of three litters are represented in each standardized litter); male pups were used in another experiment. Female pups were then assigned to a dose group and treated on days 1-5 with Gen (Sigma, St. Louis, MO) by subcutaneous injection at doses of 0.5, 5 or 50 mg/kg/day dissolved in corn oil or left untreated as controls; these treatment groups are referred to throughout the remainder of the study as Gen-0.5,

Gen-5, or Gen-50. (These doses are approximately 1, 10, or 100 μg/pup/day, respectively.) Mice were weaned at 22 days of age and housed 4 per cage.

Assessment of Puberty and Estrous Cyclicity

At weaning, mice (16 per group) treated with Gen (Gen-0.5, Gen-5 or Gen 50) and controls were checked daily until vaginal opening was observed. These mice were then allowed to age to 2 months of age (after establishing regular cycles) and monitored for 2 weeks for estrous cyclicity by taking daily vaginal smears. Smears were stained with hematoxylin and eosin (H & E) and evaluated for the stage of the estrous cycle. These mice were then allowed to age to 6 months and daily vaginal smears were taken again for 2 weeks, stained with H & E and evaluated.

Fertility Assessments

Control female mice or those treated with Gen-0.5 or Gen-5 mg/kg/day were allowed to age to 2, 4 and 6 months of age (8 mice per treatment group) and bred to proven control 2 month-old male mice of the same strain. The females were housed with males overnight and checked the following morning for the presence of a vaginal plug. Females that were vaginal plug positive were removed, single housed until delivery of pups or until it was apparent they were not pregnant. Breeding continued for two consecutive weeks at each time period (2, 4 and 6 months of age) to allow for the maximal possibility of pregnancy. Females delivered their young and pups were sexed and counted. The same group of females was tested at each time point.

In a separate experiment, the fertility of Gen-50 female mice compared to controls was tested as described above. At 2-3 months of age, control and Gen-50 treated females (8 per group) were bred to proven control males for a two-week period and allowed to deliver their pups. None of the mice treated with Gen-50 delivered live pups. A repeat of this study using another 8 mice per group confirmed that Gen-50 treatment resulted in no live births so this dose was not further tested at 4 and 6 months of age.

Implantation and Pregnancy Assessment of the Gen-50 Mice

Since mice treated neonatally with Gen 50 mg/kg did not deliver live pups, implantation defects and pregnancy loss were further assessed. At 2 months of age, control and Gen-50 female mice were bred to proven control males of the same strain (64 controls and 64 Gen-50 females). Female mice were housed overnight with males and checked for a vaginal plug the following morning. The day of vaginal plug positive was recorded as day 0 of gestation and females were then single housed. The reproductive tract was collected on pregnancy days 6, 8 or 10 and the total number of visible implantation sites in the uterus was determined. Half of the plug positive mice in each group were assessed for reabsorption sites on pregnancy days 6, 8 or 10 by collecting the uteri and soaking them in 2% NaOH in PBS for 1 hour. Blood was collected from the remaining plug positive mice in each group on pregnancy days 6, 8 or 10 to determine hormone levels of progesterone, estradiol and testosterone. [The plug positive mice were split into two groups, one for implantation site counts and one for blood collection, because the two procedures could not be performed in the same mouse due to inability to visualize implantation sites in mice that were bled (unpublished observations)]. Ovaries were also collected from pregnant mice on pregnancy days 6, 8 or 10 and fixed in 10% neutral buffered formalin, cut at 6

microns and stained with hematoxylin and eosin to determine the number of corpora lutea (CLs) per mouse. The number of CLs was determined by counting the number of CLs in both ovaries from three sections per ovary. The averages from each mouse were combined across the treatment group and the mean ± s.e.m. determined for the group.

Ovarian Function

Ovaries were collected from 6 week or 4 month-old control and Gen-treated mice (Gen-0.5, Gen-5 and Gen-50), fixed in 10% formalin, and processed for histological evaluation (8 mice per treatment group per age). The number of CLs per mouse was determined as described above and the data for the group is expressed as the mean ± s.e.m.

The ovulatory capacity of neonatally Gen treated mice was determined using methods previously described [38]. At 4 months of age, all mice received a single subcutaneous injection of 2.2 IU pregnant mares' serum gonadotropin (eCG; Sigma) followed 48-52 h later with 3.2 IU human chorionic gonadotropin (hCG; Sigma). The animals were then euthanized by CO_2 16-20 h after hCG injection and oviducts placed in M-2 medium (Specialty Media, Lavallette, NJ) supplemented with 0.3% hyaluronidase (Sigma). The oocyte/cumulus mass was surgically extracted from the oviduct and oocytes were counted after enzymatic disassociation from the surrounding cumulus. This experiment was carried out twice with each set including 8 mice per treatment group. Data from the two experiments were averaged together and are presented as mean ± s.e.m.

Serum Hormone Levels

To determine the baseline of circulating serum levels of progesterone and estradiol, serum measurements were taken prior to puberty before the onset of estrous cyclicity to avoid the variations of the cycle. Mice treated neonatally with Gen-0.5, Gen-5, Gen-50 or controls were euthanized by CO_2 at 19 days of age (8 mice per group) and blood collected from the caudal vena cava using a hypodermic needle and 1 cc syringe. Since the amount of blood obtained was limited due to the size of the animals, some samples were pooled to perform the assay. Blood was also collected during pregnancy from control and Gen-50 mice as described for prepubertal mice and assayed to determine circulating levels of progesterone, estradiol and testosterone.

All blood samples were centrifuged at 8,000 rpm at 4°C for 10 min; serum was isolated and frozen at –70C until further analysis. Serum levels of progesterone, estradiol and testosterone were measured using respective kits (Diagnostic Systems Laboratory, Webster, TX) according to the manufacturer's instructions.

Statistical Analysis

When examining continuous outcomes (day of vaginal opening, litter size, number of implantation sites, corpora lutea, and serum hormone measurements) an initial ANOVA was performed to determine differences and then each treatment group was compared to control by Dunnett's test using SAS. For serum hormone measurements at 19 days of age, where pooled samples were required, weighted analyses were done with the weights reflecting the pool size. For litter sizes at 2, 4 and 6 months of age, where the same animals were used at all three time points, a simultaneous analysis at all ages was also done; effects of age and dose were included

in the analysis. For hormones during pregnancy, since variance increased with the mean, analysis was done on a log scale.

When examining categorical outcomes (estrous cycle and pregnancy rates), Fisher exact tests were used to compare across treatment groups. For the pregnancy rate at 2, 4 and 6 months of age an analysis of trend was also performed using Cochran-Armitage test. Statistical significance was determined at $p<0.05$ for all tests.

3.4 Results

Genistein Effects on Puberty

Vaginal opening, used as an indicator of puberty, was determined following neonatal treatment with Gen (Table 1). Three out of 16 (19%) mice treated with Gen-0.5 had vaginal opening before their age matched controls with the earliest vaginal opening occurring at day 26 compared to day 29 for the controls. Two out of 16 (13%) mice from the Gen-5 and 1 out of 16 (6%) mice treated with Gen-50 had vaginal opening on day 28, one day earlier than controls. Mice from the Gen-50 group showed a general delay in vaginal opening when compared to controls with only 1 out of 16 (6%) of the Gen-50 mice exhibiting vaginal opening at 30 days of age compared to 6 out 15 (40%) of the controls. All mice in all treatment groups exhibited vaginal opening by 37 days of age. The average day of vaginal opening was calculated for each group and compared to controls using Dunnett's test; there were no statistically significant differences between control and Gen-treated mice (Table 3.1).

Mice treated with Gen-50 had an intense reddening of the area surrounding the vagina. This reddened area was present at weaning and before vaginal opening and remained throughout

puberty and into adulthood. Mice from the other two doses of Gen did not exhibit this abnormality.

Although some mice treated with Gen-0.5 and Gen-5 had early vaginal opening and some Gen-50 mice had delayed vaginal opening, circulating serum levels of estradiol and progesterone at 19 days of age just prior to puberty were not statistically different from controls by Dunnett's test (Table 3.2).

Genistein Effects on Estrous Cyclicity

At 2 months of age, all control mice [8/8 (100%)] exhibited normal estrous cycles but Gen-treated mice had altered cycles (Table 3.3; Figure 3.1A). Two out of 8 (25%) mice treated with Gen-0.5 had extended diestrus and 1/8 (13%) mice had extended estrus. Four out of 8 (50%) mice treated with Gen-5 had extended diestrus and 3 out of 8 (38%) had extended estrus. Mice treated with Gen-50 exhibited extended estrus [6/8 (75%)]; in fact, one mouse [1/8 (13%)] was in persistent estrus having cornified epithelium in all of its vaginal smears.

At 6 months of age, similar patterns of abnormal estrous cyclicity were observed in the Gen-treated mice as at 2 months but there was an increase in severity of altered cycles (Table 3.3; Figure 3.1B). In Gen-0.5 treated mice, 1/8 (13%) had extended estrus. Mice in the Gen-5 treatment group had extended diestrus [4/8 (50)], extended estrus [2/8 (25%)] and one demonstrated persistent estrus [1/8 (13%)]. Over half of the mice treated with Gen-50 were in persistent estrus [5/8 (63%)] indicating increased severity and higher incidence over time; in addition, there were 1/8 (13%) in extended diestrus and 2/8 (25%) in extended estrus. The

estrous cycle patterns for each mouse are illustrated in Figure 3.1A and 3.1B. The time spent in a stimulated (estrogenized) portion of the cycle is shown in black; the Gen-50 group shows increased black areas compared to controls. Differences among the doses in the distribution across categories are highly significant at 2 and 6 months using Fisher exact test ($p<0.01$).

Fertility Assessments

No difference was observed in the number of plug positive mice in any of the Gen-treated groups compared to controls at 2, 4, and 6 months of age (Table 3.4). There were significantly less pregnant mice in the Gen-treated groups at 2 months of age using the Fisher exact test ($p<0.05$) when the Gen-50 group was included; this group did not did not have any litters. In addition, a trend test showed a statistically significant decrease in the number of mice with litters at 2 and 6 months of age with increasing dose as determined by Cochran-Armitage test ($p<0.05$; Table 3.4); the significance of reduced numbers of mice with litters remains even when the Gen-50 group was excluded from the analysis. This effect is most pronounced at 6 months of age; fewer plug positive mice had litters in the Gen-0.5 group (60%) and even less in the Gen-5 group (40%) than their age matched controls (100%). This is consistent with early reproductive senescence.

The number of live pups in Gen-treated groups did not differ from the number of live pups in control groups at any age using Dunnett's test, looking at each time separately. However, looking at all ages simultaneously, the mice in the Gen-5 treatment group had significantly lower numbers of pups than controls while the Gen-0.5 treatment group was not statistically different (Table 3.4).

Implantation and Pregnancy Loss Assessment of the Gen-50 Mice

Since mice treated neonatally with the highest dose of Gen did not deliver live pups, pregnancy loss was furthered studied in this treatment group. There was no difference between control and Gen-50 mice in the number of mice that were plug-positive following mating [control 43/64 (67%); Gen 43/64(67%)]. Examination of uterine contents during the course of pregnancy showed that on pregnancy day 6, 8/13 (62%) of the Gen-treated plug positive mice had visible implantation sites compared to 16/18 (89%) of the controls (Figure 3.2A). In addition, the total number of implantation sites was less in the Gen-treated group (Figure 3.2B) and the ones that were present appeared to be smaller in size compared to the controls; there were no apparent reabsorptions at this stage of pregnancy. On pregnancy day 8, the outcome was similar to day 6 but with a lower number of Gen-treated plug positive mice showing signs of implantation sites [7/19 (37%)] compared with controls [18/19 (95%); Figure 3.2A]. In addition to fewer and smaller visible implantation sites (Figure 3.2B) as compared to controls, 3 mice had apparent reabsorptions (13 sites total). A representative example of a control uterus with implantation sites on day 8 of pregnancy can be seen in Figure 2C (a). Also seen in Figure 3.2C are 3 examples of uteri from plug positive Gen-treated mice on pregnancy day 8 (b, c, and d). Note the smaller size of the implantation sites in two of the Gen-treated examples (b and c) compared to control (a) as well as reabsorption sites in one example (c); the third uterus is from a mouse that had no visible implantation sites (d). By pregnancy day 10, there were still fewer Gen-treated mice with visible implantation sites [5/11 (45%)] compared to controls [6/6 (100%)] as well as fewer and smaller implantation sites (Figure 3.2A and 3.2B).

Statistical analysis was performed on the pregnancy rate using Fisher exact tests and the number of implantation sites using Dunnett's test (Figure 3.2A and 3.2B). Gen treatment significantly reduced the number of mice that were pregnant on day 8 and day 10 ($p<0.05$). Gen treatment also significantly reduced the number of implantation sites compared to controls on all days of pregnancy examined ($p<0.05$). (Non-pregnant mice were not included in the analysis of the average number of implantation sites per mouse.)

CLs were counted in ovaries from control and Gen-50 mice on pregnancy days 6, 8 and 10 (Figure 3). Of mice that were pregnant, Gen-treated mice had far fewer CLs than their control counterparts at all stages of pregnancy examined reaching statistical significance on day 6 and 8 using Dunnett's test ($p<0.05$). As expected, Gen-treated mice with no implantation sites had even fewer CLs than their pregnant counterparts (statistically significant at all three time points; Figure 3.3).

Serum hormone levels during pregnancy are shown in Figure 3.4. There were no statistically significant differences between the control and Gen-treated pregnant mice in the levels of progesterone at any stage of pregnancy examined despite the fact that there were fewer CLs in ovaries of the Gen-treated mice; however, the Gen-treated non-pregnant mice demonstrated significantly less progesterone compared to control pregnant mice on day 6 and day 8 (Figure 3.4A). There were also no significant differences in estradiol levels between treatment groups (Figure 3.4B), however, there was a large range in estradiol levels in the Gen-treated mice (pregnant and non-pregnant) compared to control mice on pregnancy day 6 (control pregnant 61.5-106.2; Gen pregnant 62.8-353.5; Gen non-pregnant 41.0-464.2 ng/ml) suggesting increased serum estradiol levels in some Gen-treated mice, however, this does not appear to depend on pregnancy outcome. There was also no significant difference in testosterone levels

between control pregnant and Gen-treated pregnant mice; Gen-treated non-pregnant mice had significantly less testosterone than control pregnant mice on day 8 (Figure 3.4C).

Ovarian Function

To examine the early reproductive senescence seen at Gen-0.5 and Gen-5, ovarian function was studied in all treatment groups. At 6 weeks of age, there were no statistically significant differences in the numbers of CLs in any of the Gen treatment groups compared to controls using Dunnett's test at $p<0.5$, however, 2/8 (25%) mice in the Gen-50 group did not have any CLs (Table 3.5) indicating that some mice were more severely affected than others.

At 4 months of age, there were more CLs in the Gen-0.5 and Gen-5 mice as compared to controls (significant in the Gen-5 group using Dunnett's test at $p<0.05$) suggesting that under their own hormonal cues, more oocytes are ovulated (Table 3.5). In sharp contrast, the Gen-50 mice did not have any CLs (also significant at $p<0.05$) indicating they do not ovulate under their own hormonal cues at this age suggesting an increase in severity over time.

To determine if these mice were capable of ovulation, oocytes were collected and counted from mice that were superovulated with eCG and hCG (Table 3.5). All Gen-treated mice had similar numbers of ovulated oocytes compared to controls suggesting that these mice are capable of ovulation following exogenous stimulation even though mice treated with the high dose of Gen do not appear to ovulate under their own hormonal cues.

3.5 Discussion

This study found that neonatal exposure of mice to Gen at environmentally relevant doses caused abnormal estrous cyclicity, altered ovarian function, early reproductive senescence, and subfertility/infertility. The relevance of our findings to human health and environmental exposure levels is a matter of concern. It is well known that humans are exposed *in utero* to varying levels of phytoestrogens with higher levels in vegetarian mothers who eat soy foods [19, 28]; human infants are also exposed to phytoestrogens in breast milk from vegetarian mothers [20, 39]. However, infants exposed to high levels of phytoestrogens (6-9 mg/kg/day) like Gen in soy based infant formulas and other soy products [14, 39, 40] are of most concern.

Although humans are mainly exposed to Gen by an oral route of exposure, data from our laboratory have demonstrated that serum levels of Gen in female mice following neonatal treatment with Gen-50 average 6.8 ± 1.4 μM [29] compared to human infants on soy based infant formulas who have circulating levels ranging from 1-5 μM [14, 32]. This study also showed high circulating levels of the aglycone form of Gen (~2 μM) which is 10-fold higher than an adult rat following similar exposure [41]; the aglycone form has been previously shown to exhibit ER binding activity [42]. The higher fraction of aglycone form of Gen has also been shown in a perinatal rat [43] supporting the idea that Gen glucuronidation is lower during the neonatal period compared to adulthood, most likely due to lower UDP glucuronosyltransferase (UGT) activities in neonatal mice [44, 45]. The ontogeny of many human UGT isoforms is similar to the pattern of rodent development with lower activity in the neonatal period [46, 47]. Although the serum circulating levels of the aglycone form of Gen in the neonatal infant is not known, an elevated fraction compared to adults similar to what is seen in neonatal rodents, seems likely. Data from

another laboratory using orally dosed neonatal rat pups with Gen at similar doses used in our study showed similar adverse effects including MOFs in the ovary and reduced female fertility [48]. Another study exposing Sprague-Dawley rats during pregnancy and lactation to Gen in the diet showed a reduction in the percentage of female mice delivering live pups (controls, 9/10; Gen 1250 ppm, 5/10) [49]. All of these data taken together strongly suggest that Gen exhibits similar results on the female reproductive system regardless of the route of exposure (oral or subcutaneous) or the species examined (rat or mouse).

We have previously addressed the role of diet used in our studies since the NIH-31 lab chow contains low levels of phytoestrogens. This diet contains approximately 98 μg/g of Gen and daidzein which is about 16.7 mg/kg/day for a 30 g mouse [50]. It has been shown that mice exposed to Gen at a dose of 16 mg/kg orally during lactation have a serum circulating level of genistein of 1.8 μg/ml but the level found in the milk was only 0.04 μg/ml which is 45 times less. Therefore, the amount of Gen that is consumed by the mother from the diet would result in very low exposure to the pups [33] and is far below the treatment levels used in this study. Further, control and Gen-treated mice were all fed the same diet. Therefore, any contribution of phytoestrogens from the diet was minimal in causing the effects observed in this study.

The current study indicates that there are problems with female reproductive development and function in mice exposed neonatally to Gen. There were no apparent differences in serum hormone levels in immature Gen-treated mice compared to controls indicating no inherent differences in circulating levels of progesterone, estradiol or testosterone between the treatment groups prior to puberty. While there were slight alterations in the onset of puberty, with lower doses advancing vaginal opening and higher doses delaying it, the mean day of vaginal opening was not statistically different between the groups. Although we did not show

a significant difference in the mean age of vaginal opening in the current study, others have shown differences, particularly in the rat model, supporting the possibility that puberty may be altered following developmental exposure to Gen depending on the time of exposure and the species examines. For example, Nikaido, et al [51] showed an earlier onset of puberty in mice following prenatal exposure to several environmental estrogens including Gen at a low dose of 0.5 mg/kg [51]. Levy, et al. [52] showed a delay in vaginal opening following prenatal exposure of rats to Gen at a dose of 5 mg/kg and Kouiki, et al. [35] showed an advanced time of vaginal opening in rats treated neonatally with Gen at a dose of 1 mg/kg.

Data from the current study also showed alterations in the estrous cycle of mice following neonatal exposure to Gen at all doses examined with extended cycle length being the most common finding. Others have shown similar estrous cycle alterations in other model systems including the study by Nikaido showing several environmental estrogens including Gen, resveratrol, zearalenone, and bisphenol A given during pregnancy caused extended estrous cycles [51]. Kouiki, et al. also showed irregular estrous cycles following neonatal exposure of rats to Gen with prolonged periods in estrus [51]. Interestingly, alterations in estrous cyclicity were exacerbated over time with more mice exhibiting persistent estrus at 6 months compared to 2 months of age, not only in the high dose but in the lower doses as well. This altered estrous cyclicity in the lower doses may in part explain the early reproductive senescence seen in these mice, particularly at 6 months of age.

Our current study also clearly demonstrates that the ovary is adversely affected by neonatal exposure to Gen. Although all Gen-treated mice ovulate under exogenous hormonal influence, the ovulation rate was much lower in the highest dose of Gen under their own hormonal cues as evidenced by fewer CLs at 2 months and during pregnancy, and by the absence

of CLs at 4 months of age. In addition, the lower doses of Gen treatment also resulted in alterations in ovarian function with more CLs than their control counterparts at 4 months of age. This enhanced ovulation rate is similar to what was seen at 26 days of age following superovulation in a previous study in our laboratory [37]. This may also in part explain the early reproductive senescence seen in the two lower doses of Gen treatment at 6 months of age. Since more oocytes are ovulated earlier, there may be a decrease in the number of oocytes available for fertilization at later time points. This effect is not unique to Gen since earlier studies in our laboratory using DES showed similar effects; low doses of prenatal and/or neonatal exposure to DES causes enhanced ovulation rates and early reproductive senescence further suggesting that ovulation of too many oocytes early in life may lead to lower fertility rates later in life [2]. It has been reported that aged mice do not typically exhaust their total complement of oocytes, however they exhibit characteristics of reproductive senescence including lowered responsiveness of the pituitary to estradiol, gradual loss of ovulatory function, decreased fertility and smaller litter sizes [53-57] but this occurs much later in life than 6 months of age which was observed following neonatal Gen exposure. Early reproductive senescence could be important for human reproductive health since more and more women are waiting longer to become pregnant [58].

One possible explanation of enhanced ovulation rates seen in lower doses was proposed by Faber, et al [59]. That study showed that neonatal exposure of rats to low doses of Gen (0.01 mg/kg) was associated with an increased pituitary response to GnRH producing higher levels of LH [59]. Mice treated with lower doses of Gen used in this study may be hyper-responsive to GnRH stimulation leading to enhanced ovulation rates which we have shown previously in younger mice [37] and again in older mice in this study. In addition, Faber et al., showed that

higher doses of Gen were associated with decreased pituitary responsiveness [59] which may explain the lower number of CLs at 2 months of age and the lack of CLs at 4 months of age observed herein. Altered pituitary responsiveness later in life could also account for the early reproductive senescence observed in mice treated with lower doses used in our study. Further investigation of Gen's effects on the hypothalamic-gonadal axis is currently underway in our laboratory.

The current study also shows that Gen adversely affects pregnancy outcome as mice exposed to the highest dose of Gen did not deliver live pups and mice exposed to lower doses of Gen showed signs of reduced fertility with age. While some of the mice treated with Gen-50 were able to become pregnant at 2 months of age, they were unable to carry these litters to term. The Gen-50 treated mice that became pregnant had fewer implantation sites that may be, in part, explained by the lower ovulation rate exhibited by these mice under their own hormonal cues. In addition to having fewer implantation sites, the implantation sites were much smaller than the implantation sites from controls at the same gestational age. There are several possibilities why this may have occurred. One explanation is that the lower number of CLs seen in the Gen-50 treated mice could have led to inability to support pregnancy due to lower levels of circulating progesterone as progesterone is essential for maintenance of pregnancy. However, we have shown that serum circulating levels of progesterone as well as estradiol and testosterone were similar in Gen-treated and control pregnant mice. Another possibility is that the uterus was unable to support pregnancy. The observation that the embryos implanted suggests that there was some capacity of the uterus to function properly. Another possibility is that the oocyte itself is of poor quality. We have shown previously that the development of the ovary and ovarian follicle were altered following neonatal Gen treatment [37]. Ovaries of Gen-treated mice

contained multi-oocyte follicles (MOFs) at 19 days of age, a phenotype not often seen in control CD-1 mice. This phenotype may be a marker for altered development of the ovary leading to oocytes that are of poor quality. In fact, a paper by Iguchi, et al., using neonatal DES treatment, showed that oocytes derived from single oocyte follicles were far more likely to be fertilized *in vitro* than oocytes derived from multi-oocyte follicles, suggesting that these oocytes are less competent [60]. Since neonatal Gen treatment causes an increase in MOFs [37], perhaps less of the ovulated oocytes are capable of being fertilized. We are currently investigating specific uterine and ovarian defects using embryo transplantation experiments.

In summary, our data demonstrate that neonatal exposure to Gen has deleterious effects on the developing murine reproductive system and can have long-term consequences on fertility at environmentally relevant doses. While there are certainly adverse effects on the reproductive system, this appears to be a multi-faceted problem as these mice have altered estrus cycles, altered ovarian function and lower pregnancy rates. While the most severe effects were seen at a dose of 50 mg/kg with lack of ovarian function and inability to carry pups to term, there were also adverse consequences to reproduction seen at the Gen 0.5 and 5 mg/kg treatment groups with altered ovarian function, extended estrous cycles and early reproductive senescence. Additional studies are warranted in human infants who are exposed to high levels of Gen during development before concluding that such exposure is safe.

3.6 References

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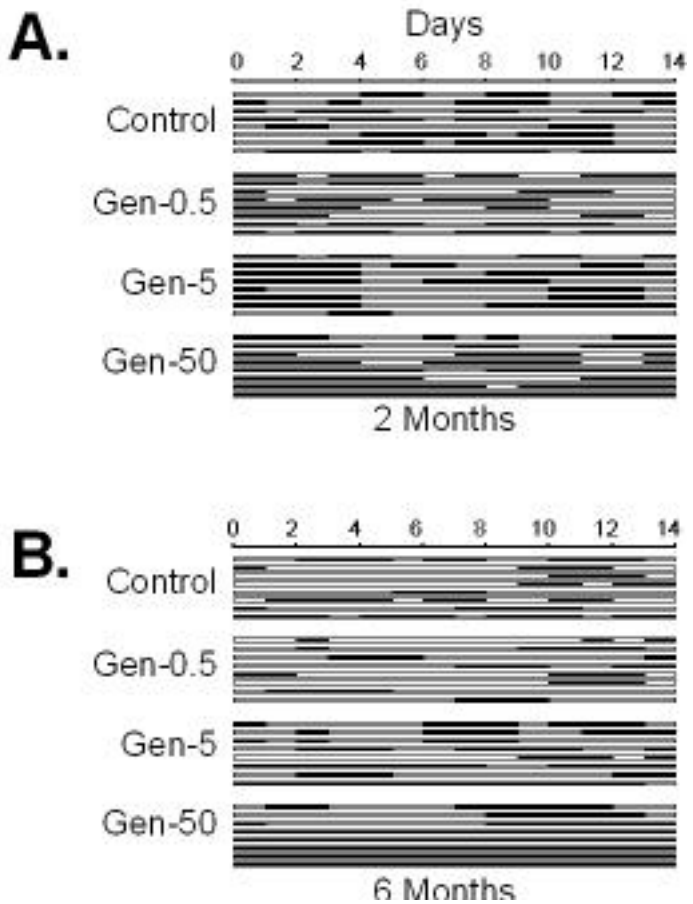

Figure 3.1: Altered estrous cycles in mice treated neonatally with Gen compared to controls.

Panel A: Patterns of estrous cyclicity in individual mice at 2 months of age. The dark bars represent proestrus, estrus or metestrus phase of the cycle. The open bars represent diestrus.

Panel B: Patterns of estrous cyclicity in individual mice at 6 months of age. Dark bars and open bars are the same as for 2 month data in Panel A. Note the increase in periods of time spent in stimulated phases of the cycle (dark areas) with increasing dose.

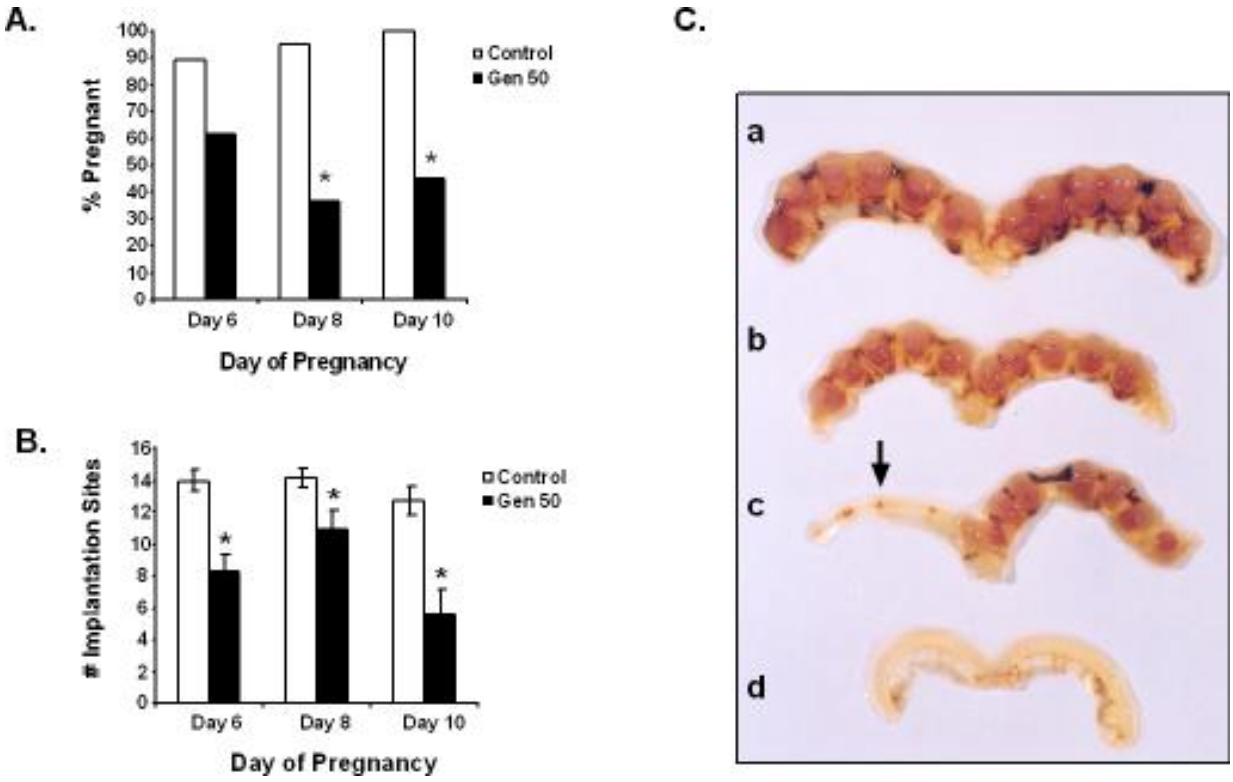

Figure 3.2: Pregnancy assessment in control and Gen-50 treated mice.

Panel A: % of pregnant mice as indicated by visible implantation sites following vaginal plug positive.

Panel B: Average number of implantation sites per pregnant mouse ± s.e.m. Asterisks indicate statistical significance from control using Fisher exact test for percent pregnant and Dunnett's test for the number of embryos with $p<0.05$.

Panel C: Photograph of representative uteri collected on day 8 of pregnancy and stained for 1 hour with 2% NaOH. a) control pregnant uterus; b) Gen-50 pregnant uterus; note the smaller size of the implantation sites in the uterus from the Gen-treated mouse compared to the control; c) Gen-50 pregnant uterus; note the reabsorption sites in this mouse (arrow); d) Gen-50 uterus with no apparent implantation sites.

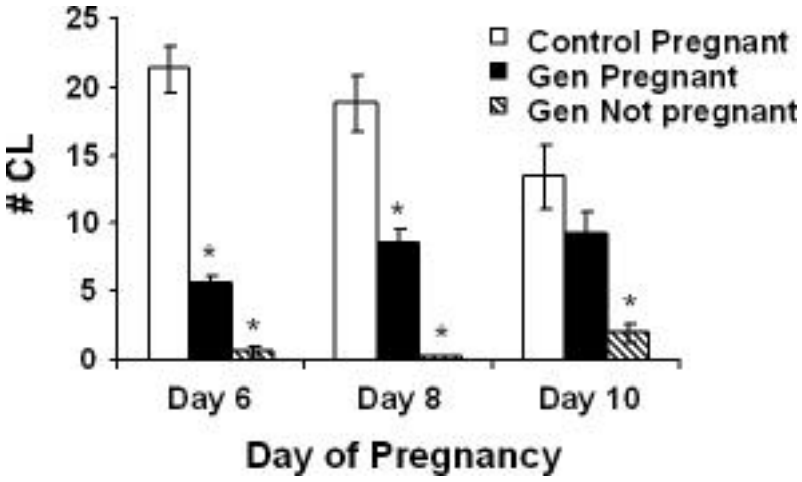

Figure 3.3: The number of corpora lutea (CLs) in control and Gen-50 treated mice. Average number of CLs per mouse is plotted at 6, 8 and 10 days of pregnancy ± s.e.m. CLs were determined from mice that were pregnant in control and Gen-treated mice. In addition, CLs were determined in Gen-treated mice with no apparent implantation sites. Asterisk indicates statistical significance compared to other groups in that time point using Dunnett's test ($p<0.05$).

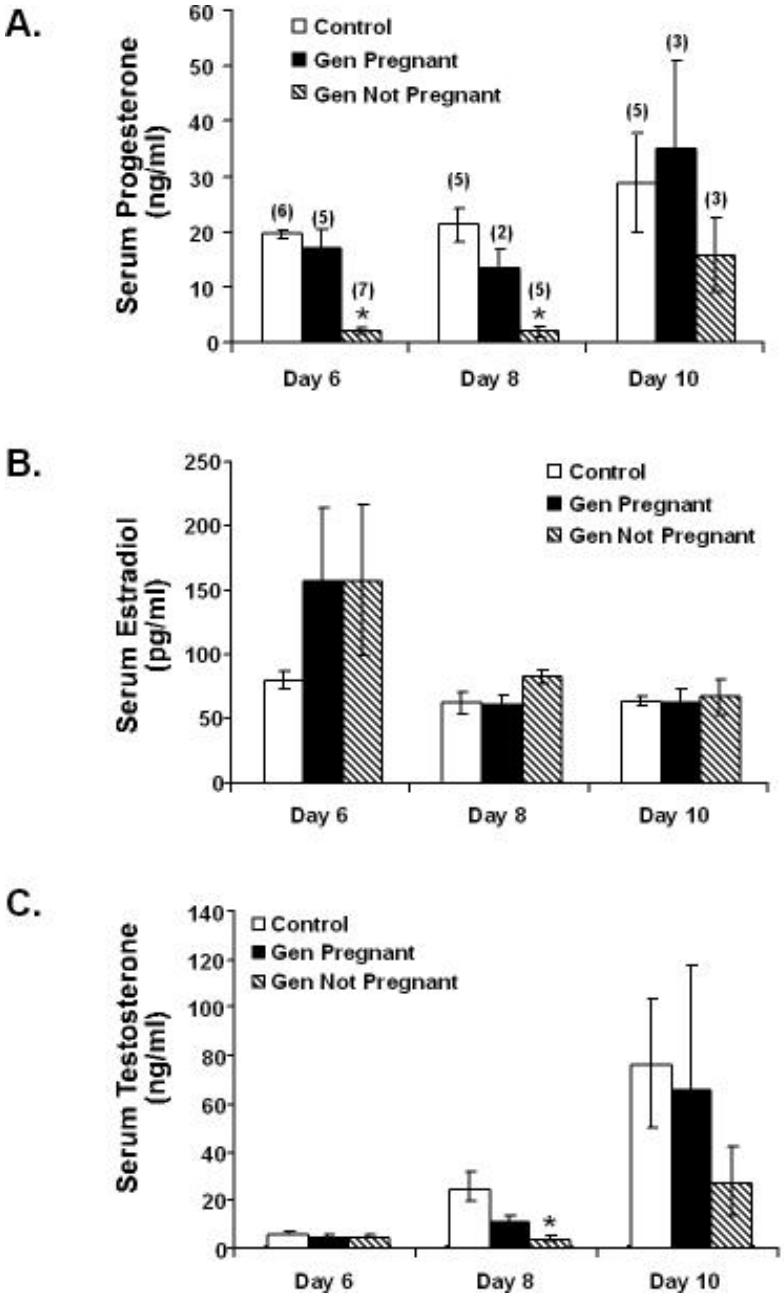

Figure 3.4: Serum hormone measurements in mice treated neonatally with Gen-50 during several stages of pregnancy. Average serum hormone levels are plotted for each treatment group ± s.e.m. The number of mice from each treatment group and time point is shown in parenthesis above each bar in panel A and these numbers apply to Panel B and Panel C. Panel A: Progesterone (ng/ml); Panel B: Estradiol (pg/ml); and Panel C: Testosterone (ng/ml). Asterisk denotes statistical significance compared to controls at the same stage of pregnancy using Dunnett's test ($p<0.05$).

Table 3.1: Day of vaginal opening for mice treated neonatally with Gen compared to controls.

			Age (Days)											
Treatment[a]	**n**	**Average**[b]	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**	**34**	**35**	**36**	**37**
Control	**15**	31.3 ± 0.6				4	2	5		2			2	
Gen-0.5	**16**	31.1 ± 0.7	1	1	1		3	3	2	3	1			1
Gen-5	**16**	30.9 ± 0.4			2		7		3	4				
Gen-50	**16**	31.6 ± 0.4			1			9	1	4	1			

[a] Mice were treated on days 1-5 with Gen 0.5, 5 or 50 mg/kg or left untreated as controls. Mice were observed from 22 days of age at weaning until vaginal opening occurred. The numbers are not cumulative but rather represent the number of mice newly recorded in each group each day.

[b] Average day of vaginal opening for each treatment group. There are no statistically significant differences between control and Gen-treated groups using Dunnett's test at $p<0.05$.

Table 3.2. Serum hormone measurements in prepubertal mice following neonatal genistein treatment.

Treatment	**n**	**Serum P_4**	**n**	**Serum E_2**
Control	3	2.1 ± 0.3	2	25.7 ± 1.3
Gen 0.5	8	1.9 ± 0.3	3	22.5 ± 1.5
Gen 5	5	1.5 ± 0.2	2	21.7 ± 4.2
Gen 50	8	1.8 ± 0.3	2	16.5 ± 4.6

Mice were treated neonatally on days 1-5 with Genistein 0.5, 5 or 50 mg/kg or left untreated for controls. At 19 days of age, female mice (N=8 per group) were sacrificed and blood collected. Serum was assayed for progesterone (P_4) and estradiol (E_2) as detailed in the materials and methods section. Since there was a very small volume to analyze from each mouse, some samples were pooled to be able to perform the assay. The number of pooled or individual samples is shown for each assay (n). The numbers reported are the weighted means for that treatment group ± s.e.m.; there were no statistically significant differences between treatment groups for P_4 or E_2 using Dunnett's test ($p<0.05$).

Table 3.3. Estrous cyclicity following neonatal exposure to genistein.

	2 Months of Age			6 Months of Age		
	Extended	Extended	Persistent	Extended	Extended	Persistent
Treatment[a]	Diestrus[b]	Estrus[b]	Estrus[c]	Diestrus[b]	Estrus[b]	Estrus[c]
Control	0/8	0/8	0/8	5/8	0/8	0/8
Gen-0.5*	2/8	1/8	0/8	5/8	1/8	0/8
Gen-5*	4/8	3/8	0/8	4/8	2/8	1/8
Gen-50*	0/8	6/8	1/8	1/8	2/8	5/8

[a] Mice were treated neonatally with Genistein 0.5, 5, or 50 mg/kg or left untreated as controls. At 2 and 6 months of age, vaginal smears were obtained for 14 consecutive days to determine cyclicity from 8 mice in each treatment group. The stage of the cycle was recorded each day for each mouse and the number of days in each stage was determined. The numbers shown in the table are the number of mice in each category for each treatment group.

[b] If the number of days in a particular stage exceeded 3 days, the mouse was considered to have an extended cycle. However, all of the mice that are shown in extended diestrus did have at least one cycle.

[c] If the mouse never showed entry into and out of the estrous cycle and the vaginal smears contained cornified epithelium in the vaginal smears, the mouse was considered in persistent estrus.

* Statistical analysis performed on this data using the Fisher exact test suggests that differences among the doses in the way the animals are distributed across the categories in both age groups are significant ($p<0.05$).

Table 3.4. Effects of neonatal genistein on fertility and the course of pregnancy outcome.

Age	Treatment[a]	# Plug positive[b]	% Pregnant[c]	Average # Live Pups[d]
2 Months	Control	6/8	6/6 (100)	15.2 ± 0.8
	Gen-0.5	6/8	6/6 (100)	13.2 ± 0.9
	Gen-5	8/8	6/8 (75)	11.5 ± 1.6
	Gen-50[e]	16/16	0/16 (0)	0
4 Months	Control	6/8	6/6 (100)	12.8 ± 1.8
	Gen-0.5	4/8	4/4 (100)	12.5 ± 1.6
	Gen-5	8/8	7/8 (88)	10.0 ± 2.4
	Gen-50	-	-	-
6 Months	Control	7/8	7/7 (100)	13.7 ± 1.4
	Gen-0.5	5/8	3/5 (60)	9.3 ± 2.2
	Gen-5	5/8	2/5 (40)	8.5 ± 2.5
	Gen-50	-	-	-

[a] Mice were treated neonatally on days 1-5 with Gen 0, 0.5, 5, or 50 mg/kg or left untreated as controls. At 2, 4, and 6 months of age, female mice were bred to control males for 3 consecutive days. A total of 8 mice per treatment group were bred for each time point except for Gen-50 where 16 (2 groups of 8 mice each) mice were bred at 2 months of age. Presence of vaginal plug was determined to be day 0 of pregnancy. Plug positive mice were single housed and allowed to deliver their young.
[b] The number of plug positive mice is shown out of the number of mice that were bred.
[c] % Pregnant is the number of mice that delivered live pups following vaginal plug positive. Number in parenthesis is %.
[d] The number of live pups was counted for each mouse that delivered live pups. Average number of live pups was determined for each treatment group; this did not include mice that were not pregnant.
[e] Mice treated with Gen 50 mg/kg on days 1-5 are shown in the 2 month group. None of these mice delivered live pups following 2 consecutive weeks of breeding.
* Using the Fisher Exact test there were significantly lower % pregnant mice in the Gen treated groups compared to control at 2 months of age. Using a Cochran- Armitage test for trend, there is a statistically significant trend for lower pregnancy rates as the dose of Gen increases at 2 and 6 months of age at $p<0.05$. This significance remains even if the test is performed without the Gen-50 dose.
** Using Dunnett's test, looking at each time separately, the treated groups did not differ from the control groups at any age. However, looking at all ages simultaneously, the mice in the Gen-5 treatment group had significantly lower numbers of pups than controls while the Gen-0.5 treatment group was not different.

Table 3.5: Effects of neonatal genistein on ovarian function.

6 Weeks		
Treatment[a]	# mice with CL	Average # CL
Control	5/5 (100)	6.0 ± 0.7
Gen-0.5	6/6 (100)	3.7 ± 0.5
Gen-5	8/8 (100)	5.5 ± 0.8
Gen-50	6/8 (75)	3.4 ± 0.9
4 Months		
Treatment[a]	# mice with CL	Average # CL
Control	7/7 (100)	9.2 ± 1.3
Gen-0.5	8/8 (100)	13.4 ± 2.0
Gen-5	8/8 (100)	18.0 ± 1.9*
Gen-50	0/8 (0)	0.0 ± 0.0*
Stimulated with eCG / hCG[b]		
4 months		
Treatment[a]	n	# oocytes ovulated
Control	14	15.4 ± 1.9
Gen-0.5	15	19.1 ± 1.9
Gen-5	14	18.0 ± 2.0
Gen-50	16	15.3 ± 1.7

[a] Mice were treated neoantally with Gen 0, 0.5, 5 or 50 mg/kg or left untreated as controls; ovaries were collected at 6 weeks and 4 months of age. (n=number of mice with adequate sections). The numbers are the averages from each mouse averaged across the treatment group ± s.e.m.

[b] At 4 months of age, mice were treated with eCG and hCG to stimulate ovulation (see Materials and Methods for details). The number of oocytes were counted per mouse and averaged across treatment groups. n=number of mice with ovulated oocytes. The data are the sum of two experiments.

* Statistically significant using Dunnett's test at $p<0.05$.

4. Neonatal Genistein Treatment Alters Ovarian Differentiation in the Mouse: Inhibition of Oocyte Nest Breakdown and Increased Oocyte Survival

4.1 Abstract

Early in ovarian differentiation, female mouse germ cells develop in clusters called oocyte nests or germline cysts. After birth, mouse germ cell nests break down into individual oocytes that are surrounded by somatic pre-granulosa cells to form primordial follicles. Previously, we have shown that mice treated neonatally with genistein, the primary soy phytoestrogen, have multi-oocyte follicles (MOFs); an effect apparently mediated by estrogen receptor-β. To determine if genistein treatment leads to MOFs by inhibiting breakdown of oocyte nests, mice were treated neonatally with genistein (50 mg/kg/day) on days 1-5 and the differentiation of the ovary compared with untreated controls. Mice treated with genistein had fewer single oocytes and a higher percentage of oocytes not enclosed in follicles. Oocytes from genistein treated mice exhibited intercellular bridges at 4 days of age, long after disappearing in controls by 2 days of age. There was also an increase in the number of oocytes that survived during the nest breakdown period and fewer oocytes undergoing apoptosis on neonatal day 3 in genistein treated mice as determined by poly ADP ribose polymerase (PARP) and deoxynucleotidyl transferase mediated deoxyuridine triphospate nick end-labeling (TUNEL). These data taken together suggest that genistein exposure during development alters ovarian differentiation by inhibiting oocyte nest breakdown and attenuating oocyte cell death.

4.2 Introduction

During normal ovarian differentiation, female germ cells are found as clusters or nests of cells. Recently these nests were found to have characteristics of germline cysts found in female invertebrates and almost all male animal species [1, 2]. Cysts are formed when oogonia undergo a series of incomplete cell divisions shortly after their formation resulting in clusters of connected cells that subsequently enter meiosis and are called oocytes [1]. After birth, mouse germline cysts or nests break down into individual oocytes (nest breakdown) that become surrounded by somatic pre-granulosa cells to form primordial follicles [3]. During the process of nest breakdown, a subset of oocytes in each nest dies with only a third of the initial number of oocytes surviving [3]. The key observation that a subpopulation of oocytes of the nest dies suggests that there are two cell types in the germ cell nests: 1) cells that survive, differentiate and become functional oocytes, and 2) cells that support or protect the oocytes and eventually die.

Selective cell death of some oocytes in the nest may involve regulation of cell survival and/or programmed cell death pathways. Steroid hormones have been implicated in the regulation of cell death in many tissues [4-10]. In the adult mammalian ovary, where many follicles undergo atresia (cell death), estrogen has been shown to protect granulosa cells from death [11]. In contrast, in the nervous system, there is evidence that estrogen promotes cell death [12]. It is currently not known if estrogen treatment regulates the number of oocytes that die during nest breakdown.

Estrogenic compounds can induce a variety of effects on reproductive organs including abnormal morphology and neoplasia. One effect is the appearance of

multioocyte follicles (MOFs) in the adult ovary [13]. In contrast to normal ovaries, ovaries from adult female mice treated as neonates with the major endogenous estrogen, 17β-estradiol (E_2) or the synthetic estrogen diethylstilbestrol (DES), have an increased occurrence of follicles with more than one oocyte [14-16]. Although this effect of estrogenic compounds has been known for many years, the origin of MOFs remains unknown. One possibility is that estrogen treatment during the time of neonatal ovarian differentiation results in oocyte nests that persist and become surrounded by granulosa cells resulting in MOFs in the adult ovary. Alternatively, nest breakdown may occur normally but the migration of granulosa cells around individual oocytes may be affected. If MOFs are the result of incomplete nest breakdown induced by estrogens, other chemicals that exert estrogenic activity could also cause this effect.

Genistein, a soybean phytoestrogen, has previously been shown to have estrogenic activity in both *in vitro* and *in vivo* studies in mice and rats [17-21]. The estrogenic activity of this compound is of concern because human fetuses and infants can be exposed to genistein during critical periods of development through soy consumption of mothers during pregnancy and lactation [22] and through soy-based infant formulas and other soy products that children consume [23-25]. The concentrations of genistein and other isoflavones found in some of these soy-based products can far exceed the amount found in an adult diet. In fact, infants on soy-based infant formulas consume approximately 6-9 mg/kg/day of genistein compared to 1 mg/kg/day in an adult vegetarian [24]. Neonatal exposure to genistein at a dose of 50 mg/kg/day leads to an increased incidence of uterine adenocarcinoma in mice later in life [26]. In addition, the incidence of uterine tumors in the genistein treated mice (35%) was similar to the

incidence in mice given an equal estrogenic dose of DES, 0.001 mg/kg/day (31%) [26]. The levels of genistein used in that study were subsequently shown to produce serum-circulating levels of genistein in mice similar to those found in infants consuming soy-based infant formulas [27]. Another study using rats supported these data by showing that a dose of 40 mg/kg/day also gave a serum circulating level of genistein similar to what is found in infants on soy-based formulas [28]. Recent studies have shown that developmental exposure to genistein can cause alterations in the development of the female reproductive tract of the rodent including altered estrous cyclicity, altered ovarian function, subfertility and infertility [29-32].

The effects of genistein on the developing ovary following neonatal exposure at doses of 0.5, 5 and 50 mg/kg/day included the presence of MOFs similar to those reported following neonatal exposure to E_2 or DES [14-16, 33]. Another study exposing rats orally during perinatal life also showed the presence of MOFs supporting genistein's ability to alter ovarian morphology in another species [34]. Genistein has tyrosine kinase inhibitory activity in addition to its estrogenic activity [35]. However, mice treated with lavendustin (a tyrosine kinase inhibitor without estrogenic activity) did not develop MOFs, eliminating genistein's tyrosine kinase inhibitory action as a possible mode of action in forming MOFs [33]. Transgenic mouse models lacking estrogen receptor (ER) α or β have demonstrated that these receptor subtypes are the primary mediators of estrogen activity in both reproductive and non-reproductive tissues [36, 37]. Transgenic mice lacking ERα developed MOFs following neonatal genistein treatment, but mice lacking ERβ did not show this phenotype, suggesting the effect of genistein is mediated specifically through ERβ [33]. In addition, mice lacking ERβ have been previously

shown to have reduced fertility, more atretic follicles, and fewer corpora lutea, suggesting that some of the oocytes may not be healthy, and more oocytes are dying instead of being ovulated [36, 37]. ERβ is expressed in ovarian granulosa cells of adult mice and has been detected as early as day 1 in neonatal mice [38, 39]. All of these data taken together suggest that ERβ may play a critical role in normal ovarian differentiation and that chemicals with estrogenic activity, particularly those that bind preferentially to ERβ, may disrupt this process.

Previous studies of the effect of genistein on the ovary have examined immature and adult rodents but nothing is known about the effects on younger mice. To further study the mechanisms involved in the formation of MOFs and possible disruption of the development of the ovary, the current study examines the effects of neonatal genistein treatment on ovarian differentiation including oocyte nest breakdown, primordial follicle assembly and development.

4.3 Materials and Methods

Animals and Neonatal Treatment

Adult CD-1 [Crl:CD-1 (ICR) BR] mice were obtained from Charles River Breeding Laboratories (Raleigh, NC) and bred to male mice of the same strain in the breeding facility at the National Institute of Environmental Health Sciences (NIEHS; Research Triangle Park, NC). Vaginal plug detection was considered day 0.5 of pregnancy. Pregnant mice were housed under controlled lighting (12 h light and 12 h dark) and temperature (21-22 ^{o}C) conditions. Mice were housed in polysulfone, ventilated cages (Technoplast, Inc., Exton, PA) and provided with NIH 31 laboratory

mouse chow and fresh water *ad libitum*. The diet has been previously analyzed for genistein content [40]. All animal procedures complied with an approved NIEHS/NIH animal care protocol and the Syracuse University Institutional Animal Care and Use Committee.

Pregnant mice delivered pups at 19.5 days of gestation; pups were separated according to sex, pooled together and then randomly standardized to 8 female pups per litter. Male pups were used in another experiment. Female pups were treated on days 1-5 with genistein (Sigma Chemical Company, St Louis, MO) by subcutaneous injection at 50 mg/kg/day in corn oil (approximately 100 μg/pup/day) or left untreated as controls. This dose and route of exposure of genistein has been previously shown in our laboratory to induce a high incidence of MOFs and to produce serum circulating levels of genistein of 6.8± 1.4 μM [26, 33]; this level is similar to that seen in infants consuming soy based infant formulas, 1-5 μM [24, 27].

Whole Mount Immunohistochemistry and Fluorescence Microscopy

Mice treated as described above were sacrificed on days 2, 3, 4, 5 and 6 by decapitation (8 mice per treatment group per age). These ages were chosen because nest breakdown and primordial follicle assembly occur during the first several days after birth [3]. The reproductive tract was removed and the ovaries were carefully dissected away from the remainder of the reproductive tract in cold PBS. Ovaries were fixed in 5% EM grade paraformaldehyde in PBS for 1 hour followed by several washes in 5% bovine serum albumin (BSA), 0.1% Triton X-100 in PBS. Whole ovaries were immunostained as previously described [1, 3, 41]. The Stat3 (C20) antibody (Santa Cruz Biotechnology,

La Jolla, CA) was used at a dilution of 1:500 [41]. Propidium iodide or Toto-3 (Molecular Probes, now part of Invitrogen, Carlsbad, CA) was used to label nuclei. As a negative control, immunohistochemistry was performed without primary antibody (data not shown). Samples were imaged on a Zeiss Pascal Confocal microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY).

Analysis of Oocyte Nest Breakdown, Primordial Follicle Assembly and Follicle Development

Whole ovaries labeled with the germ cell marker, Stat3 as described above, were examined for percent single oocytes relative to the total number of oocytes to assess oocyte nest breakdown as previously described [1, 3]. Stat3 has been shown to be a specific marker for germ cells [41]. Briefly, the number of individual oocytes relative to the number of oocytes in nests was determined by examining two regions per ovary. For each region, a single confocal section was examined. In addition, for each of these regions, a stack of ten sections, one micron apart centered around the single section was obtained. This stack of sections was used to determine if oocytes in the center section were associated with oocyte nests above or below the plane of focus; 7-8 mice per treatment group per age were analyzed. For primordial follicle assembly and development, the number of each type of follicle per region was determined by examining 4 representative, confocal sections at least 20 μm apart and determining the total number of each type of follicle per region. Two regions were examined per ovary and there were 7-8 mice per treatment group. For primordial follicle assembly, oocytes were considered unassembled if granulosa cells did not completely surround them (see

Figure 1A). Follicles were classified as followed: primordial (oocyte surrounded by several granulosa cells with flattened nuclei, see Figure 1B), primary (oocyte surrounded by one layer of granulosa cells with cuboidal nuclei, see Figure 1C), or secondary (oocyte surrounded by more than one layer of granulosa cells).

Determination of Germ Cell Number

The number of oocytes per region was determined by counting the number of oocytes in 4 representative confocal sections at least 20 μm apart and determining the total number of oocytes per region. Two regions were examined per ovary. Ovaries from 7-8 mice per treatment group were analyzed on postnatal days 2, 4 and 6.

Transmission Electron Microscopy

Ovaries from control and genistein-treated mice at 4 days of age were dissected in cold PBS, fixed in 2.5% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA) in Jones' Phosphate Buffer (65 mM NaCl, 2.5 mM KCl, 3.5 mM NaH_2PO_4, 1.5 mM Na_2HPO_4, pH 7.2) and processed for transmission electron microscopy at the State University of New York Upstate Medical University Pathology department EM facility. Samples were post-fixed in 1% OsO_4, dehydrated through an ethanol series, equilibrated in propylene oxide, and embedded in Araldite 502 (Electron Microscopy Sciences, Fort Washington, PA). Thin sections were stained with uranyl acetate and lead citrate. For each data point, four sections from one ovary, each 20 microns apart were examined. The samples were analyzed with a FEI Tecnai BioTwin 12 transmission electron microscope and images recorded with a AMT Advantage Plus CCD camera.

Cell Death Assessment

For poly (ADP ribose) polymerase (PARP) labeling, whole mount immunohistochemistry was performed on ovaries collected from control and genistein-treated mice on days 1-6 (6-8 mice per treatment group per age). Whole ovaries were stained sequentially, first with Stat3 antibody followed by anti-rabbit Alexa 568 secondary antibody to label all oocytes (as described above) and then with antibody against the apoptosis specific cleaved form of PARP directly labeled with Alexa 488 at a dilution of 1:100 (New England Biolabs, Ipswich, MA). The number of PARP positive oocytes was determined as a percent of the total number of oocytes in 4 representative, confocal sections in each mouse ovary at least 20 μm apart.

For terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL), ovaries were collected from control and genistein-treated mice on days 2, 3 and 4 and fixed in cold 10% neutral buffered formalin (4 mice per treatment group). Tissues were changed to cold 70% ethanol, embedded in paraffin and sectioned at 6 μm. Sections were deparaffinized, rehydrated and detection of apoptotic cells was carried out using the TUNEL apoptosis detection kit following the instructions provided in the kit (Upstate, Lake Placid, NY) with the following exceptions. The secondary antibody used was ExtrAvidin Peroxidase (Sigma Chemical Company) at a dilution of 1:50 for 30 min at room temperature followed by NovaRED following the kit instructions (Vector Laboratories, Burlingame, CA). The sections were then counterstained with hematoxylin, dehydrated and coverslipped. A minimum of two non-adjacent sections from 3 mice per age per treatment group were stained and counted

using this method. The percentage of apoptotic oocytes was determined, averaged per mouse and then averaged for each treatment group for each age.

Statistical Analysis

A two-way ANOVA was conducted using main effects of hormone and day on percentage follicle type, percentage single oocytes, number of oocytes, percentage TUNEL positive cells and percentage PARP positive oocytes. PROC GLM of SAS 9.1 (SAS Institute Inc., Cary, NC) was used to calculate the least-squares means and test specific hypotheses for hormone effects on each day. A level of $p < 0.05$ was considered significant.

4.4 Results

Primordial Follicle Assembly was Disrupted Following Neonatal Genistein Treatment

To examine the effects of neonatal genistein treatment on primordial follicle assembly and development, we determined the number of unassembled oocytes and primordial and primary follicles in genistein-treated and control mice at 4 days of age using ovary whole mounts immunostained with Stat3 and propidium iodide. The percent of unassembled follicles was significantly higher in the genistein-treated mice (73.4 ± 3.7%) compared to controls (56.7 ± 2.9%) while the percent of primordial and primary follicles was significantly lower in the genistein-treated mice (Figure 4.1). Thus, primordial follicle assembly was disrupted in genistein-treated mice.

Genistein Treatment Inhibits Oocyte Nest Breakdown

Although the rate of primordial follicle assembly was reduced following genistein treatment, it was not clear if this was due to a problem of granulosa cell migration around the oocytes or a deficiency in oocyte nest breakdown. To determine this, once again we examined ovary whole mounts immunostained for Stat-3 and propidium iodide. Figure 4.2 shows a day 4 control ovary (panel A-C) and a day 4 genistein-treated ovary (panel D-F). The control ovaries had a very high percentage of single oocytes (44%) similar to previously reported data [1, 3]. In contrast, the genistein-treated ovaries had fewer single oocytes (21.2%) and several large oocyte nests were still apparent. A summary of the percentage of single oocytes in each treatment group can be seen in Figure 4.3 and Table 4.1. There were significantly fewer single oocytes at 4, 5 and 6 days of age following neonatal genistein treatment; the largest difference was at 6 days of age where 57% of oocytes are single in control ovaries and only 36% of oocytes in the genistein-treated group were single. While the percentage of single oocytes is decreased in treated ovaries the overall number of oocytes increases due to decreased apoptosis. However, not only the percentage but the actual number of single oocytes in the treated group decreased. These results support the idea that genistein inhibits the process of oocyte nest breakdown.

To determine if the oocytes were still connected by intercellular bridges (as germline cysts would be) we used transmission electron microscopy to measure the frequency of intercellular bridges connecting oocytes. In a previous study the frequency of bridges declined rapidly in control CD-1 mice shortly after birth becoming virtually undetectable by 3 days of age [1, 3]. We did not detect any intercellular bridges in

control mice at 4 days of age in this study (0 out of 325 oocytes). However, in genistein-treated mice, the frequency of bridges was 0.5% (3 out of 633 oocytes) supporting the idea that genistein treatment is inhibiting nest breakdown. Examples of electron micrographs from a day 1 control ovary (Figure 4A) and a day 4 genistein-treated ovary (Figure 4B) are shown.

Neonatal Oocyte Death was Reduced with Genistein Treatment

Using ovaries whole mount immunostained for Stat3 and propidium iodide, we counted the total number of oocytes on PND 2, 4 and 6 (Figure 4.5). We found significantly more oocytes at PND 4 and 6 in treated mice supporting the idea that genistein treatment increases oocyte survival. We counted oocytes in representative regions of ovaries, therefore a change in ovary size would affect the total number of oocytes. However, we did not observe any differences in ovary size with genistein treatment that might impact the overall number of oocytes.

Since genistein treatment led to increased oocyte numbers, we wanted to determine if genistein affected oocyte programmed cell death. To assess apoptosis, the percentage of PARP positive and TUNEL positive oocytes were compared between control and genistein-treated mice throughout the treatment period. Representative ovary sections immunostained with PARP are shown in Figure 4.6B (day 3 control) and Figure 4.6C (day 3 genistein-treated) and representative ovary sections stained for TUNEL are shown in Figure 4.6E (day 3 control) and Figure 4.6F (day 3 genistein treated). A summary of the percentage of PARP positive cells at 2, 3 and 4 days of age are shown in Figure 4.6A and the percentage of TUNEL positive cells at 2, 3 and 4 days of age are

shown in Figure 4.6D. There were differences between control and genistein-treated ovaries using either method to detect cell death. The most significant difference was at 3 days of age where there was a significant decrease in the percentage of oocytes undergoing apoptosis following neonatal genistein treatment as assessed by both PARP and TUNEL. In contrast, there was a significant increase in apoptosis using the TUNEL method at 2 days of age, however, this was not observed using PARP labeling. This suggests that genistein may initially induce or speed up the process of cell death of some oocytes. Since TUNEL and PARP measure apoptosis at different stages, this may explain the discrepancy between the two and further supports the idea that this effect is transient, only occurring for a brief time directly following the first treatment. These data, taken together with the overall increase in oocyte numbers at 4 and 6 days of age (Figure 4.5), support the idea that genistein treatment influences oocyte survival.

4.5 Discussion

Previous studies have shown that neonatal exposure to the phytoestrogen genistein alters ovarian morphology later in life including the presence of MOFs [33]. Two possibilities for the process by which these abnormal follicles formed were that the granulosa cells improperly enclosed more than one oocyte or the process of oocyte nest breakdown was disrupted. Therefore, we examined the differentiation of the ovary during the time of neonatal genistein treatment. The data from this study show that there are a much higher percentage of unassembled oocytes following genistein treatment as well as many fewer single oocyte follicles. In addition, there were still intercellular bridges connecting the oocytes in the genistein-treated mice while none were apparent in

the control mice by 4 days of age. This indicates that MOFs resulted from incomplete breakdown of oocyte nests leaving the pre-granulosa cells multiple oocytes to surround during the differentiation process.

The mechanism by which oocyte nest breakdown normally occurs is not fully understood. In addition, the mechanism by which genistein disrupts this process is not known although ERβ has been implicated as having a role [33]. We propose that during normal ovarian differentiation, exposure of fetal oocytes to maternal estrogen keeps the oocytes in nests. Shortly after birth, the level of estrogen drops and initiation of nest breakdown is triggered (Figure 4.7). Therefore, nest breakdown is inhibited when neonatal oocytes are exposed to estrogenic compounds such as genistein. Previous work from others on the presence of MOFs following specific windows of exposure to DES supports this hypothesis; mice treated during the first few days after birth develop MOFs while mice treated later than day 10 do not [16]. In addition, mice treated prenatally with DES also had fewer MOFs than mice treated neonatally [42]. This work was also replicated *in vitro* by the same laboratory suggesting that neonatal mouse ovaries are highly susceptible to forming MOFs in the presence of exogenous estrogens while other stages of development are less sensitive [15, 16]. These data support our hypothesis that estrogen is responsible for maintaining oocyte nests early in development, but after oocyte nest breakdown has occurred, MOFs can no longer be formed. The fact that prenatal exposure to DES also does not induce many MOFs (although other abnormalities occur), also suggests that exogenous estrogen during pregnancy only mimics the normal process of inhibiting oocyte nest breakdown during this period of development.

Neonatal treatment with testosterone and progesterone has also been found to result in increased MOFs [16, 43]. For testosterone, the effect is likely due to its conversion to estrogen because inhibition of this conversion process suppresses the effect of testosterone treatment [43]. In a recent study in rats, neonatal progesterone treatment was found to reduce primordial follicle assembly, while both progesterone and estrogen treatment reduced the primordial to primary follicle transition in the initial wave of folliculogenesis [44]; however, oocyte nest breakdown was not specifically examined. This study also showed reduced neonatal oocyte programmed cell death in progesterone treated mice. Together these data suggest that the steroid hormone balance may also be important for proper ovarian differentiation and development.

Recent work examining aromatase deficient mice also contributes to our knowledge of the effects of estrogens on the developing ovary. Aromatase is the enzyme necessary for the conversion of testosterone into estrogen. Thus, aromatase deficient mice lack endogenous estrogen. Adult female aromatase knock out mice have fewer primordial follicles than wild type animals and estrogen treatment of adults does not alleviate this effect suggesting that estrogen may play a role earlier, during the formation of primordial follicles which occurs neonatally [45].

In the ovaries of normal adult female mice, follicles consist of one oocyte surrounded by one or more layers of granulosa cells; follicles with more than one oocyte are rarely found (less than 1% in the Swiss strain; [46]). However, the percent of animals with MOFs as well as the percent MOFs per total follicles varies widely depending on mouse strain [15, 16]. These data suggest that genetic modifiers may play a role in ovarian differentiation and in turn the activity of these modifiers may be altered by

exogenous influences. MOFs have also been reported in 98% of human ovaries at a frequency of 0.6 to 2.44 % per ovary and up to 8 % of follicles collected for IVF [47, 48] . The MOFs observed in mice and humans have been postulated to be remnants of oocyte clusters that did not separate and become enclosed in follicles during neonatal primordial follicle assembly [15, 16, 47]. It is unclear whether genistein treatment alters oocyte quality. There is evidence in mice that oocytes derived from MOFs have a reduced fertilization rate [49] although in humans no difference has been found [48].

The molecular mechanisms by which genistein caused these effects on the developing ovary are just beginning to be elucidated. Previously, ERβ was found to be involved in the formation of MOFs present in immature mice treated with genistein [33]. In that study, immature mice lacking ERβ did not have MOFs when treated with genistein as neonates. This finding is consistent with the hypothesis that genistein is acting as an estrogen through ERβ to disrupt ovarian differentiation. ERβ has been detected in the ovary early in development by RT-PCR, Western blotting and immunohistochemistry [38, 39] suggesting the possibility that genistein acts directly on the ovary through ERβ. While ERβ is predominantly expressed in the granulosa cells later in development, the expression of ERβ in the oocytes themselves very early during development cannot be ruled out. In either case, genistein acts as an estrogen interacting with ERβ to send a signal that ultimately inhibits oocyte nest breakdown. Further evidence for estrogen's direct effects on the developing ovary comes from an *in vitro* study showing neonatal ovaries grown in culture and then transplanted into a host (under the kidney capsule) develop MOFs in the presence of estrogens [14].

Several other genes have been implicated in neonatal ovarian differentiation. For example, mice lacking bone morphogenetic factor 15 (BMP-15) or growth differentiation factor 9 (GDF-9) have an increased number of MOFs as well as other defects of ovarian differentiation [50]. Both proteins are members of the transforming growth factor β (TGFβ) superfamily; they are oocyte-secreted factors expressed early in ovarian differentiation [51]. In contrast, transgenic over-expression of another member of the TGFβ family inhibin-α disrupts normal ovarian development and ovaries exhibit MOFs [52] and Factor in the germline (Fig) α knock out mice lack the ability to form primordial follicles [53]. Therefore, it appears that there are many factors that contribute to proper differentiation of the ovary during the neonatal time period and disruption of any of these appears to cause permanent alterations in ovarian morphology and possibly function later in life.

Another point of regulation in ovarian differentiation is the natural process of oocyte cell death. During oocyte nest breakdown, approximately two thirds of the oocytes die by apoptosis [3]. We have shown in the current study that oocyte cell death is initially increased at 2 days of age using the TUNEL method but this appears to be transient since the PARP1 method did not show this difference. PARP1 measures apoptosis earlier in the process and by 24 hours after the first treatment, there is no difference in the number of oocytes entering apoptosis in the genistein treated ovaries compared to the controls. However, apoptosis is attenuated on day 3 following neonatal genistein-treatment by both detection methods and there is also an increase in the overall total number of oocytes. This suggests that cell survival and/or cell death pathways are altered following neonatal genistein treatment. In addition, estrogens have been shown to

alter survival usually inhibiting cell death and enhancing cell survival [11, 12]. In the developing ovary, compelling evidence from two existing transgenic mouse strains supports the idea that the B-cell lymphoma/leukemia-2 (Bcl-2) family of proteins may be involved in regulating apoptosis during oocyte nest breakdown. Mice over-expressing Bcl-2 (a cell survival gene) and mice lacking Bax (a cell death gene) both have increased numbers of oocytes during neonatal life [54, 55]. In addition, adult females with a targeted disruption of *bcl-2* have fewer oocytes and follicles with either a degenerating oocyte or no oocyte at all [56].

Over the last few years, research on phytoestrogens, like genistein, has increased. There are mixed results suggesting some beneficial effects as well as some adverse effects depending on the timing of exposure, dose level and endpoint examined. Some studies show that exposure to genistein early in life prevents carcinogen induced mammary gland cancer [57, 58] while others show increased mammary gland cancer occurs following treatment during specific developmental windows [59]. Others have shown improved cholesterol synthesis rates of human infants consuming soy-based formulas [60] . On the other hand, vegetarian diets usually contain high levels of soy and recent epidemiology reports have shown an association of a vegetarian diet during pregnancy with an increased incidence of hypospadias in the male offspring [61] , and an increase in autoimmune disease and the use of allergy medicines in children fed soy-based infant formulas [62] . In addition to genistein, neonatal exposure to another environmental estrogen, bisphenol A has been shown to cause MOFs in mice [42] . This finding further supports the idea that a compound's estrogenic activity can cause altered ovarian differentiation.

In conclusion, we have shown that genistein alters ovarian differentiation during neonatal development. Ovaries from neonatal mice treated with genistein have more oocytes not enclosed in follicles, more oocytes persisting in nests and retention of oocyte intercellular bridges. Retention of intercellular bridges between oocytes also demonstrates that genistein inhibits oocyte nest breakdown in neonatal mice. In addition, neonatal genistein treatment influenced oocyte survival as shown by decreased oocyte apoptosis and increased oocyte numbers. All of these data, taken together, show that ovarian differentiation is a complex and multi-faceted process. Disruption in any of these pathways can lead to alterations in the normal progression of ovarian development and subsequent normal ovarian function.

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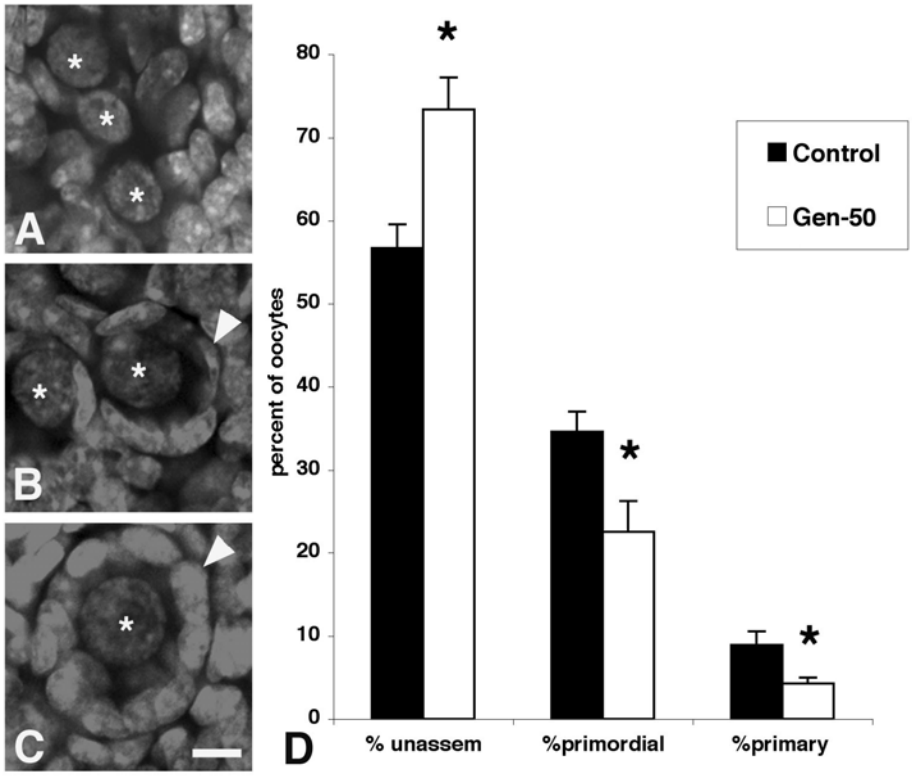

Figure 4.1 Analysis of primordial follicle assembly and follicle development following neonatal genistein treatment. Neonatal mice were injected with genistein or left untreated as controls. (A-C) Examples of follicle morphology at different stages of follicle development from PND 4 ovaries labeled with Toto-3 to visualize nuclei. (A) Three unassembled oocytes are shown with nuclei indicated by asterisks. (B) Two primordial follicles are shown with oocyte nuclei indicated by asterisks. Arrowhead indicates an associated granulosa cell with a flattened nucleus. (C) A primary follicle is shown with the oocyte nucleus indicated by an asterisk. Arrowhead indicates an associated granulosa cell with a cuboidal nucleus. Scale bar = 10 □m. (D) Percentage of unassembled, primordial and primary follicles is shown at postnatal day 4. Data are presented as the mean ± SEM from seven to eight ovaries. * indicates a significant different between control and genistein treated ovaries (one-tailed test, $p < 0.05$). $N = 7$ - 8 ovaries per group.

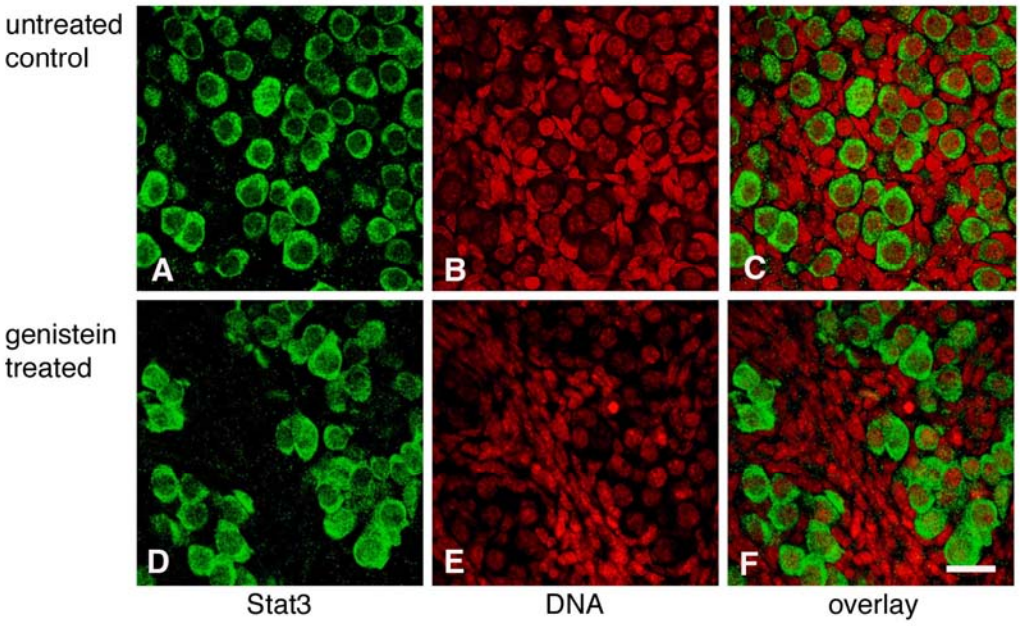

Figure 4.2 Oocytes in control and genistein treated mice visualized by confocal microscopy. (A-C) Confocal section of a control day 4 ovary labeled with Stat3 antibody to visualize oocytes (A), propidium iodide to visualize nuclei (B) and overlay of A and B (C). (D-F) confocal section of a Gen-treated day 4 ovary labeled with Stat3 antibody (D), propidium iodide (E) and overlay (F). Stat3 (green), propidium iodide (red). Scale bar = 20 μm.

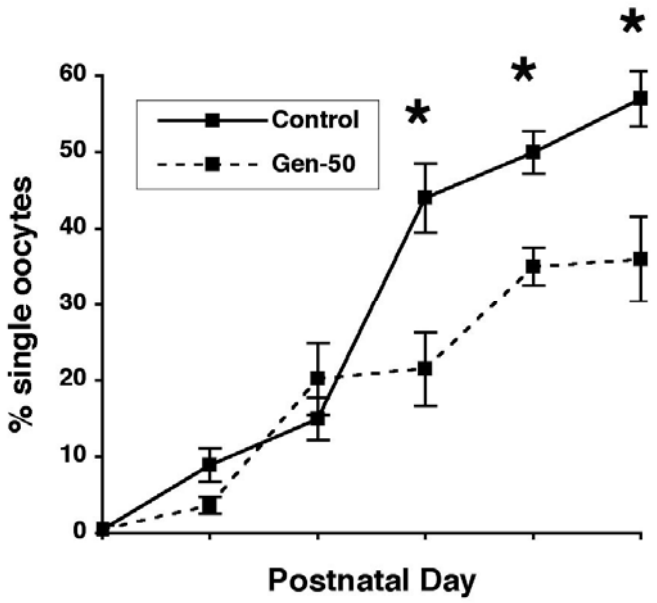

Figure 4.3 Genistein treatment inhibits oocyte nest breakdown. The percentage of single oocytes is plotted from postnatal day 1 to postnatal day 6 in control (—) and Gen-treated (----) mice. Data are presented as the mean ± SEM. * indicates a significant different between control and genistein treated ovaries (one-tailed test, $p < 0.01$). $N = 6$ - 8 ovaries per group.

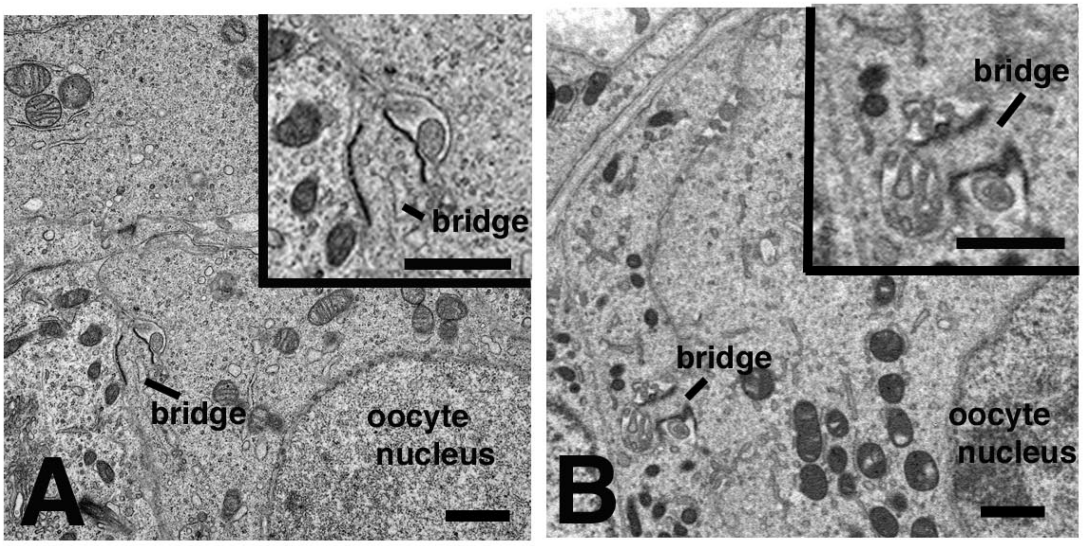

Figure 4.4 Persistence of intercellular bridges connecting oocytes in genistein treated mice. (A) Electron micrograph showing the presence of an intercellular bridge connecting two oocytes on neonatal day 1 (only one oocyte can be seen in this photograph). Inset is enlargement of a region of panel A to show further detail of the bridge. (B) Electron micrograph showing an intercellular bridge connecting two oocytes on neonatal day 4 following genistein treatment. Inset is enlargement of a region of panel B. Scale bars = 1 μm.

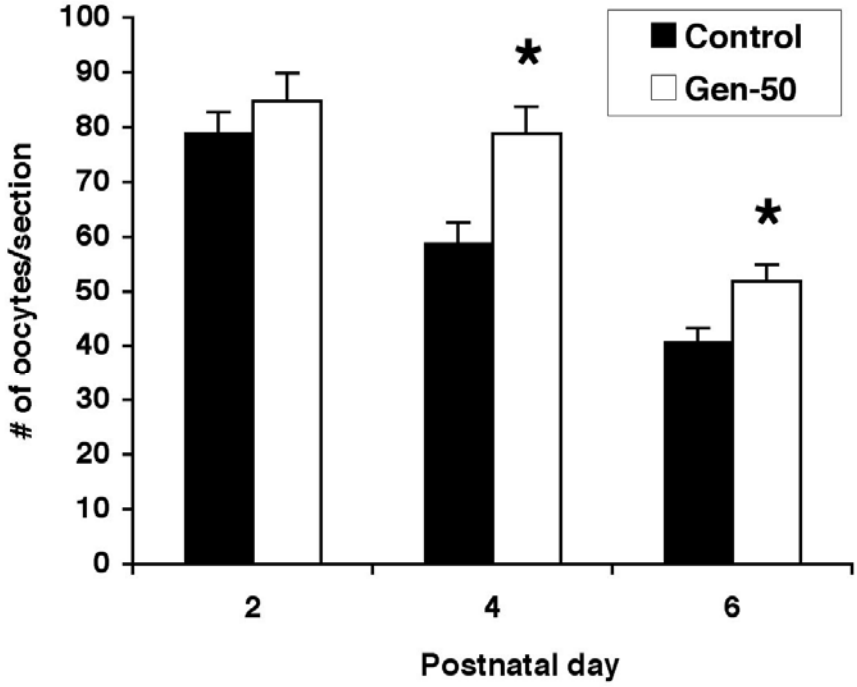

Figure 4.5 Numbers of oocytes per section in control and genistein treated mice on postnatal day 2, 4 and 6. Data are presented as the mean ± SEM. * indicates a significant different between control and genistein treated ovaries (one-tailed test, $p < 0.05$). $N = 6$ - 8 ovaries per group.

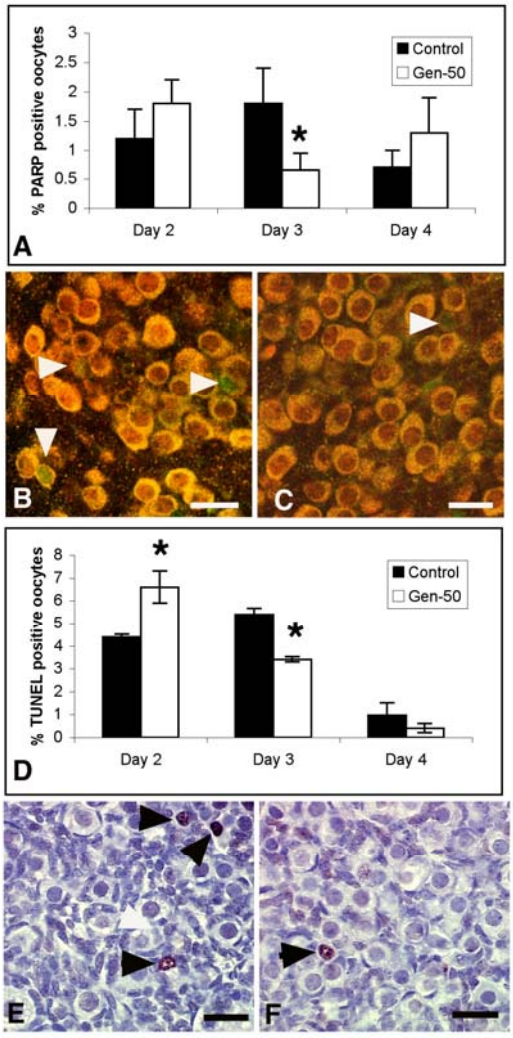

Figure 4.6 Decreased oocyte programmed cell death in genistein treated animals. (A, D) The percentage of apoptotic oocytes in representative microscope sections in control and treated animals detected by PARP antibody (A) and TUNEL assay (D). All apoptotic cells in the ovary were detected using the TUNEL method, while apoptotic germ cells were detected using an antibody for cleaved PARP in combination with the germ cell-specific antibody, Stat3. Data are presented as the mean ± SEM from eight ovaries per time point. * indicates a significant different between control and genistein treated ovaries (one-tailed test, $p < 0.05$). $N = 6$ - 8 ovaries per group for PARP analysis. $N = 3$ ovaries per group for TUNEL analysis. Detection of apoptosis in control (B, E) and genistein treated (C, F) ovaries. (B, C) Examples of dying germ cells (white arrowheads) detected using an antibody specific for cleaved PARP (green) and the germ cell-specific antibody Stat3 (yellow) in control (B) and treated (C) ovaries. (E, F) Examples of apoptosis detected in the ovary using the TUNEL method (brown) in control (E) and treated (F) ovaries. Scale bar = 20 μm.

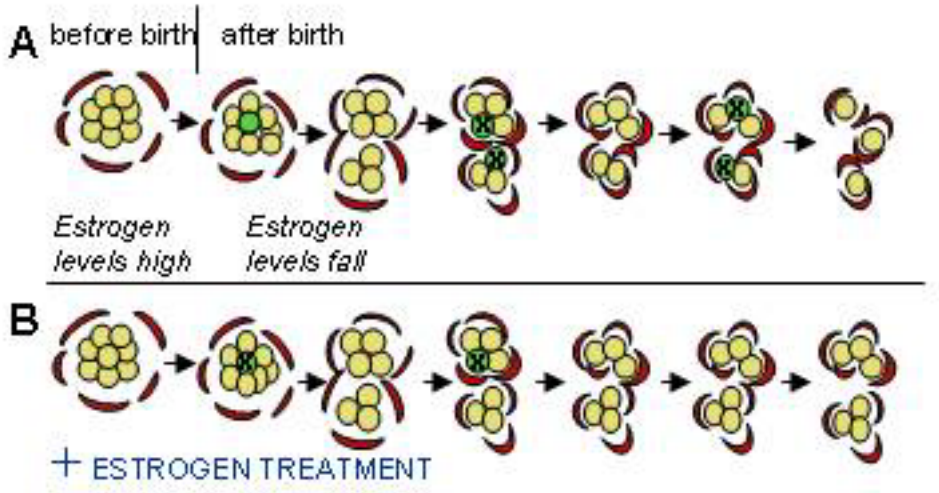

Figure 4.7 Schematic model of nest breakdown and estrogen signaling. (A) Maternal estrogens are at high levels prior to birth and nests are maintained. After birth, estrogen levels in the neonate drop quickly, some oocytes die and nests break apart. (B) Treatment with estrogens after birth inhibits oocyte nest breakdown and programmed cell death.

Table 4.1. Percentage of single oocytes, number of oocytes, percentage of TUNEL positive and percentage PARP positive oocytes with and without genistein treatment.

Age PND	% single oocytes		Oocytes/region		% TUNEL +		% PARP +	
	control	genistein	control	genistein	control	genistein	control	genistein
1	0.5 ± 0.5	0.5 ± 0.5	nd	nd	nd	nd	2.6 ± 0.5	2.6 ± 0.6
2	8.9 ± 2.2	3.7 ± 1.1	79.1 ± 3.6	87.4 ± 5.1	4.4 ± 0.1	6.6 ± 0.7	1.2 ± 0.6	1.8 ± 0.4
3	15.0 ± 2.8	20.2 ± 4.7	nd	nd	5.4 ± 0.3	3.4 ± 0.1	1.8 ± 0.5	0.7 ± 0.3
4	44.0 ± 4.5	21.5 ± 4.8	58.9 ± 3.7	78.7 ± 5.0	1.0 ± 0.5	0.4 ± 0.2	0.7 ± 0.3	1.3 ± 0.6
5	50.0 ± 2.8	35.0 ± 2.5	nd	nd	nd	nd	0.9 ± 0.3	1.3 ± 0.5
6	57.4 ± 3.6	36.7 ± 5.6	40.9 ± 2.3	51.8 ± 3.0	nd	nd	0.5 ± 0.3	0.7± 0.3

Numbers are the averages ± SEM. nd = not determined.

5. Neonatal Exposure to Environmental Estrogens Alters Ovarian Differentiation and Expression of Cell Adhesion Molecules: Preferential Binding to Estrogen Receptor β Determines Severity

5.1 Abstract

Neonatal exposure to the phytoestrogen genistein alters ovarian differentiation resulting in multi-oocyte follicles (MOFs). Further, we have shown that this effect is mediated through estrogen receptor (ER) β since mice lacking ERβ do not develop MOFs. We have also shown that genistein inhibits oocyte nest breakdown during ovarian differentiation. Neonatal CD-1 mice were treated with genistein (50 mg/kg) on days 1-5 or left untreated to further study the mechanisms involved in altered ovarian differentiation. At 19 days, ovarian RNA expression of cell adhesion molecules was compared using a cell adhesion molecule array; 32/96 genes were altered including catenins and cadherins. Western blotting of α-catenin, VE-cadherin and P-cadherin supported the array data. Additional mice were treated on days 1-5 with equal estrogenic doses of daidzein-250, coumestrol-2, nonylphenol-200, HPTE-200, DDT-200, bisphenol A (BPA)-250 or diethylstilbestrol (DES)-1 mg/kg to determine if other environmental estrogens could cause MOFs and altered cell adhesion molecule expression similar to genistein. A high incidence of MOFs was seen following genistein (6/6) and daidzein (5/7) treatment with > 5 MOFs/mouse; a medium incidence with nonylphenol (7/8), coumestrol (4/8) and HPTE (3/8) with < 3 MOF/mouse; and a low incidence with DES (1/8), BPA (2/8) and DDT (0/8). Compounds without estrogenic activity (ICI-10, 1/8;

DHT-1, 1/8; progesterone-10, 0/8) had few MOFs similar to controls (1/8). In summary, environmental estrogens alter ovarian differentiation; severity of these effects correlates with preferential ERβ binding suggesting knowledge of a chemical's estrogenic activity alone might not be sufficient to predict adverse effects in the ovary.

5.2 Introduction

Previous work has shown that chemicals with estrogenic activity alter reproductive tract tissues in both males and females if exposure occurs during critical periods of development (1-4). In particular, the potent synthetic estrogen, diethylstilbestrol (DES) has been shown to cause malformations of the reproductive tract, alterations in estrous cyclicity, subfertility/infertility and reproductive tract lesions including cancer in laboratory animals as well as humans if given perinatally .

There is a growing body of work showing that many environmental chemicals exhibit estrogenic activity both *in vivo* and *in vitro* (8-12). These compounds include but are not limited to phytoestrogens such as genistein, daidzein and coumestrol; placticizers such as bisphenol A; surfactants such as nonylphenol; and pesticides like DDT, methoxychlor, and its metabolite, HPTE. Some of these compounds have been shown to cause alterations in the reproductive tract similar to DES if exposure occurs during development (9, 13-20).

Many of the actions of estrogens are mediated through the estrogen receptor (ER). There are two major ER subtypes expressed in mammalian tissues, ERα and ERβ (21-24). ERα and ERβ are widely distributed throughout the body and both receptors are

expressed in most reproductive tract tissues (21, 23, 25-28). However, they are not usually expressed in the same cell type within the same tissue (27, 29, 30). There is also a difference in the abundance of one receptor versus the other within a tissue; for example, ERβ appears to be the predominant receptor expressed in the ovary (granulosa cells) while ERα appears to be the predominant receptor expressed in the uterus (28, 30). There are also some differences between species, as mice have little to no ERβ in the uterus while rats appear to have some level of expression (22, 30, 31).

In addition to the differences in tissue distribution of ERα and ERβ, there are also differences in a chemical's preference for one receptor over the other. Many environmental chemicals have been shown to bind to both receptors although this binding is not as strong as the endogenous ligand, 17β-estradiol (E_2) (8, 32-34). For example it takes twenty times as much genistein to elicit similar binding affinity to ERα as E_2 (8). In addition, some chemicals have a higher binding affinity to either ERα or ERβ. For example, genistein exhibits preferential binding to ERβ over ERα; in fact, genistein binds to ERβ 20-80 times better than to ERα (8, 32, 33). These differential effects in binding affinity could account for differential effects in tissues with varying levels of each receptor subtype, even at equal estrogenic doses since this is determined largely by uterotropic bioassay data where ERα is predominantly expressed.

Of particular interest are tissues with predominant ERβ expression such as the ovary. We have recently shown that genistein can alter normal ovarian differentiation if exposure occurs during neonatal life (35). During normal ovarian differentiation, female germ cells (oocytes) undergo a series of incomplete cell divisions shortly after their

arrival into the gonad, resulting in clusters called cysts or nests (36). After birth, mouse germ cell nests break down into individual oocytes surrounded by somatic pre-granulosa cells to form primordial follicles (37). However, following neonatal genistein treatment, oocyte nests maintain intercellular bridges and fail to breakdown, leading to the presence of multi-oocyte follicles (MOFs) later in life (26, 35). We have also shown that the formation of MOFs is mediated through ERβ as mice lacking this receptor do not develop MOFs following neonatal treatment with genistein (26). The presence of MOFs suggests that differentiation of the ovary did not occur properly (35). In addition, there is evidence that oocytes from MOFs do not fertilize as efficiently as oocytes from single oocytes follicles, suggesting that this alteration may lead to less competent oocytes (38).

The molecular mechanism involved in oocyte nest breakdown is not well understood. Since genistein inhibits the breakdown of oocyte nests, we hypothesized that cell adhesion molecules may be involved in this process and that genistein may alter the expression of these proteins. Several of these proteins have been identified in the developing ovary including E-cadherin, N-cadherin, P-cadherin (39-44), α-catenin as well as β-catenin (39-41) and integrins (45). Furthermore, E-cadherin and α-catenin have been shown to be regulated by estrogens as well as gonadotropins later in life (46-48). Therefore, genistein, acting as an estrogen, may disrupt the expression of cell adhesion molecules.

The current study was designed to determine if genistein is unique in its ability to alter ovarian differentiation by comparing the incidence of MOFs following neonatal exposure to genistein with several other environmental estrogens. To further elucidate

the mechanisms involved in altered ovarian differentiation, we determined possible alterations in cell adhesion molecule expression in the ovary following neonatal exposure to genistein as well as other environmental estrogens.

5.3 Materials and Methods

Animals

Adult CD-1 [Crl:CD-1 (ICR) BR] mice were obtained from Charles River Breeding Laboratories (Raleigh, NC) and bred to male mice of the same strain in the breeding facility at the National Institute of Environmental Health Sciences (NIEHS; Research Triangle Park, NC). Vaginal plug detection was considered day 0 of pregnancy. Pregnant mice were housed under controlled lighting (12 h light and 12 h dark) and temperature (21-22C) conditions. Mice were provided with NIH 31 laboratory mouse chow and fresh water *ad libitum*. All animal procedures complied with an approved NIEHS/NIH animal care protocol.

Pregnant mice delivered their young on day 19 of gestation, pups were separated according to sex, pooled together and then randomly standardized to 8 female pups per litter. Pups were treated on days 1-5 by subcutaneous injection of genistein (50 mg/kg) suspended in corn oil or left untreated as controls. Additional groups of female mice were treated neonatally with other compounds in corn oil listed in Table 5.1 at equal estrogenic doses (10, 11). Another group of mice were treated similarly on days 1-5 with several doses of DES dissolved in corn oil (0.01, 0.1 and 1 mg/kg). All mice were sacrificed at 19 days of age by CO_2 asphyxiation; ovaries were dissected carefully away from the oviduct

and either fixed in 10% neutral buffered formalin for histological evaluation or frozen on dry ice for Western blotting analysis or RNA isolation.

RNA Isolation and Genistein Expression Analysis

Ovaries were collected and frozen from mice treated with genistein 50 mg/kg or left untreated as controls (8 per treatment group). RNA was isolated using the RNeasy kit following the manufacturer's instructions (Qiagen, Valencia, CA); the quality of the RNA was checked on a 1% Agarose gel observing the 18S and 28S ribosomal RNA bands. RNA was pooled (8 mice per treatment group) and 5 μg of total RNA was used for gene expression profiling using the mouse adhesion and extracellular matrix molecules blot (MM-010) according to the instructions provided with the GEArray Q Series kit (SuperArray, Bethesda, MD). Briefly, the GEArray blot was pre-hybridized with the provided solution at 60C for 2 hours. RNA from control and genistein-treated ovaries was labeled with [α-^{32}P]-dCTP (10μCi/ml) and added to the pre-hybridizing membranes and incubated overnight at 60C. Following several washes provided in the kit, the membrane was exposed to a Phosphoimager for quantitative analysis.

Protein Isolation and Western blotting

Ovaries were collected and frozen from mice treated neonatally with compounds listed in Table 1 (4 mice per treatment group). Proteins were isolated using the N-PER kit following the manufacturer's instructions (Pierce, Rockford, IL). Protein concentrations were determined using the BCA protein assay following the manufacturer's instructions

(Pierce). Proteins (10 μg) were run on 10% Bis-Tris mini-gels (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose using the X-Cell II mini gel apparatus (Invitrogen). Blots were incubated with 3% hydrogen peroxide to block endogenous peroxidases and then with 10% BSA in Tris buffered saline (TBS, pH 7.4) with 1% Tween-20 (TBS-T) to block non-specific sites. Blots were then incubated with the primary antibody diluted in TBS-T for 2 hours at room temperature. Tissues were then incubated with appropriate secondary diluted in TBS-T for 30 min. All antibodies and dilutions are shown in Table 5.2. Blots were then incubated with West Dura reagent (Pierce) for 5 min and exposed to film for 30 sec to 1 min to detect immunoreactive bands. Films were scanned into an image analysis program (Image Pro 4.1, Media Cybernetics, Silver Spring, MD) to determine intensity and area of the bands. Blots were then stripped with Restore according the manufacturer's instructions (Pierce) and stained with Mem Code total protein stain (Pierce) to determine equal loading efficiency for each gel.

Immunohistochemistry

Ovaries from control and genistein treated mice (4 per group) were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at 6 μm. Tissues were deparaffinized in xylenes and hydrated in a graded series of alcohols. Antigen retrieval was performed using a decloaker and citrate buffer (Biocare Medical, Concord, CA). Non-specific peroxidases were blocked using 3% hydrogen peroxide followed by 10% BSA in TBS-T for 30 minutes. Primary antibodies were applied for 2 hours in TBS-T followed by appropriate secondary biotinylated antibodies. Sections were then incubated with

ExtrAvidin peroxidase (Sigma) diluted 1:50 for 30 min. Visualization of the proteins was carried out using the NovaRED kit (Vector). Sections were counterstained by hematoxylin, dehydrated and coverslipped. All antibodies and dilutions are shown in Table 5.2.

Ovarian Morphology

Ovaries were collected at 19 days of age from 8 mice per treatment group, however there were two groups of control mice run concurrently as the treatments were split into two weeks (16 total controls). Ovaries were fixed in 10% neutral buffered formalin, embedded in paraffin and sectioned at 6 microns. The number of MOFs was counted from 3 sections at 3 depths from both ovaries (9 sections per mouse) to determine the presence of MOF in each animal; some mice did not have sufficient ovarian tissue to analyze, thus excluded from the analysis. The total number of MOF seen in each animal (9 sections per mouse) was used to obtain the number of MOF per mouse. These numbers were then averaged across treatment groups and reported.

Statistical Analysis

Statistical analysis was performed on the image analysis data using JMP software (SAS, Cary, NC). ANOVA was performed followed by Dunnett's test with $p<0.05$ being reported as statistically significant.

5.4 Results

Gene Expression Analysis

The results of the SuperArray analysis are seen in Table 5.3. Of the 96 genes represented on this array, 32 showed an increase or decrease over control by a factor of 1.5 or more (Table 5.3). Of these, only 5 showed a decrease in expression following neonatal genistein treatment, while the other 27 showed an increase. The relative expression levels were categorized into low, medium and high expression levels (low <500; medium 500-2000; high >2000 counts). Several members of the cadherin, catenin and integrin families were altered following neonatal genistein treatment, while the matrix metalloproteinases (MMPs) were largely unaffected. Several genes (α-catenin, VE-cadherin, and P-cadherin) were selected for validation and follow up using Western blot analysis of these proteins.

Western Blotting of Selected Cell Adhesion Molecules

Western blots for α-catenin, VE-cadherin and P-cadherin in the cytoplasmic fractions from ovaries of control and genistein-50 treated mice can be seen in Figure 5.1. There was an increase in the levels of all of these proteins in the ovary following neonatal genistein treatment compared to controls. This data supports the array data.

Immunhistochemistry of Selected Cell Adhesion Molecules

Ovarian tissue sections from control and genistein treated ovaries immunostained for α-catenin, VE-cadherin and P-cadherin can be seen in Figure 5.2. The localization of α-catenin following neonatal genistein treatment appears to be predominantly around the

oocyte and in the granulosa cells with increased protein expression following neonatal genistein treatment compared to controls (Figure 5.2A-D). VE-cadherin appears to be limited to the vasculature, once again more highly expressed in the genistein treated ovaries compared to controls (Figure 5.2E-H). The expression of P-cadherin is predominantly around the oocytes and in the granulosa cells but there is also some expression in the interstitial cell compartment; there is an apparent increase following neonatal genistein treatment compared to controls (Figure 5.2I-L). A negative control for each of these antibodies (no primary antibody) is shown in the insets in Figure 5.2, panels C, G and K showing no non-specific immunostaining.

Ovarian Morphology

Mice treated neonatally with the compounds and doses listed in Table 5.1 were analyzed for the presence of MOFs at 19 days of age. A summary of this data can be seen in Table 5.4 (number of mice with adequate sections is also shown in this table). Control mice had a very low incidence of MOFs (1/16) as previously reported (26, 49). Mice treated neonatally with genistein showed the presence of MOF in all of the animals examined in this study (6/6), similar to our previously published data (26). The number of MOFs per mouse was also very high (5.3) following neonatal genistein treatment; included in this number were also two triple oocytes as well as 1 quadruple oocyte follicle. Neonatal daidzein treatment also caused a large percentage of mice to have MOFs (5/7) and the number of MOFs per mouse was similar to genistein (5.6) including

4 triple oocyte follicles. These two compounds were categorized as high inducers of MOF formation (Table 5.4).

Neonatal exposure to nonylphenol also caused most of the mice to have MOFs (7/8) but the total number of MOFs was much lower with the number of MOFs per mouse at 2.6 including 1 quadruple oocyte follicle and no triples. Neonatal exposure to coumestrol and HPTE caused similar numbers of mice to have MOFs (4/8 and 3/8, respectively) and similar numbers of MOFs per mouse as nonylphenol treatment (2.0 and 2.3 respectively). These three compounds were categorized as medium inducers of MOF formation (Table 5.4).

Neonatal exposure to Bisphenol A, DES and DDT did not cause large numbers of mice to have MOFs (2/8, 1/8, 1/8 and 0/8, respectively). They also did not cause high numbers of MOFs per mouse (1.0, 1.0, 2.0 and 0, respectively). These four compounds were categorized as low inducers of MOF formation (Table 5.4).

Three additional compounds without estrogenic activity were examined as negative controls including DHT, Progesterone and the anti-estrogen, ICI. As expected, none of these compounds caused more MOFs than the controls in this study (ICI, 1/8; DHT, 1/8; Progesterone, 0/8)

Table 5.4 also shows the previously published relative binding affinities (RBA) for all of the estrogenic compounds used in this study for both mouse and human if the data were available (8, 32-34). This data shows the wide range of binding preference for one receptor over the other with genistein showing an almost 90 times greater preference for mouse ERβ and DES showing a 10 times preference for mouse ERα (32). This data

also shows the binding preference of one receptor over the other generally holds true in both humans and mice.

An additional experiment to determine the dose response of DES revealed that DES also induces significant numbers of MOFs if the dose is high enough. As shown in Table 5.4, mice treated with DES-0.001 had 1/8 with MOF and that mouse had 2 MOFs. Mice treated with DES-0.01 had 2/7 mice with MOFs with 1 MOF each. However, mice treated with DES-0.1 and DES-1 all had MOFs (8/8 in each group) with 1.5 MOFs/mouse in the DES-0.1 group and 3.9 MOFs/mouse in the DES-1 group. If one assumed that only ERβ binding was necessary for this effect, it would take 0.877 mg/kg of DES (equal estrogenic dose of DES-0.001 multiplied by 877 = DES-0.877 mg/kg) to cause a similar incidence of MOFs as genistein-50 since there is a difference of 877 between the binding preference for ERβ between genistein and DES (Table 5.4). This appears to be a very good estimate since the incidence at the DES-1 dose (100%; 3.9 MOFs/mouse) correlates well with the number and frequency of MOFs at the genistein-50 dose (100%; 5.3 MOFs/mouse).

Western Blot Analysis of Selected Cell Adhesion Molecules Following Neonatal Exposure to Environmental Chemicals

Since several of the environmental chemicals showed the presence of MOFs similar to genistein, ovaries from mice treated with compounds from each category (high, medium and low) were further examined for similar alterations in α-catenin, VE-cadherin

and P-cadherin. Western blots showing expression levels of α-catenin, VE-cadherin, and P-cadherin in ovarian cytoplasmic fractions from control, genistein, daidzein, nonylphenol, coumestrol and DES can be seen in Figure 5.3. Image analysis quantification of the area of the immunoreactive bands can also be seen in Figure 5.3. The intensity was also determined using the image analysis system and similar results were seen as the area (data not shown). The expression levels of all three proteins were significantly elevated following genistein and daidzein treatment. Mice treated with nonylphenol showed a significant increase in VE-cadherin and P-cadherin while mice treated with coumestrol only showed a significant increase in P-cadherin. Mice treated neonatally with DES-1 appeared to have increased levels of all three proteins but these differences were not significant.

5.5 Discussion

Previous studies in our laboratory have shown that neonatal exposure to genistein causes alterations in ovarian differentiation as indicated by MOFs and that this effect is mediated by ERβ (27). We have also shown that genistein inhibits the breakdown of oocyte nests during the first week of life and that the oocytes themselves remain attached as shown by the presence of intercellular bridges in genistein-treated mice days after they are gone in control mice (36). Therefore, we hypothesized that cell adhesion molecules would be altered following neonatal genistein treatment.

Data from the current study has shown that this is indeed the case with 32 out of the 96 cell adhesion molecules represented on the array being altered with most of them

being increased compared to controls. Of the genes that were altered in the array, 11 were cadherins, catenins, or integrins. Previous studies have shown that these proteins are expressed during prenatal and neonatal ovarian differentiation and they are thought to be involved in the remodeling of the ovary during this time period (42-46, 51). Western blotting and immunohistochemistry further confirmed the array data with higher expression of α-catenin, VE-cadherin, and P-cadherin in the ovary following neonatal exposure to genistein. Immunohistochemical localization of α-catenin was simlar to previous reports with expression predominantly around the oocytes as well as in the granulosa cells with some staining in the interstitial/thecal cell compartment (48) and VE-cadherin was predominantly expressed in the vasculature as reported by other laboratories (52). P-cadherin was expressed predeominantly around the oocyte and in the granulosa cells with some staining in the interstitial/thecal compartment similar to other findings (45). Neonatal genistein treatment increased the expression of these proteins but did not alter the location of the proteins.

The cadherins are family of membrane bound proteins that mediate cell-cell adhesion; preferring to bind homophilically (53-55). The biological activity of cadherins is mediated by a group of connecting proteins called catenins propagating a signal from the extracellular portion of the cell to the interior (53, 56-58). The integrins have also been shown to be receptors for cadherins; integrin αE is a receptor for E-cadherin (59). The differential spatiotemporal expression of these proteins during development suggests that these proteins are very important normal ovarian differentiation (40, 41, 43-46, 51). For example, E-cadherin, is highly expressed on the surface of adjoining oocytes during

the prenatal period, however just prior to birth, E-cadherin expression is down-regulated in the oocytes and then reappears between the independent oocytes and granulosa cells (43). The importance of the expression of E-cadherin and normal differentiation was demonstrated in the same study using a disaggregated organ culture system. Ovaries that were dispersed just prior to birth were able to reform follicles after two to three days in culture; however the addition of an antibody to E-cadherin prevented the reaggregation of the follicles indicating the importance of this protein for normal development (43). The catenins have not been as well studied in the developing ovary, however both α-catenin and β-catenin have been shown to be present in most ovarian cell types and have been shown to be regulated by estrogens (42, 48) and in the current study several catenins were shown to be increased following genistein treatment. Integrins have also been shown to be present during the time of ovarian differentiation and are thought to be important in reorganization of the developing ovary (40, 46, 51). Integrin α6 was shown previously to be differentially expressed during ovarian differentiation (51) and this integrin was increased in the current study following genistein treatment. Another study showed increased integrin α6 expression in ovaries from transgenic mice lacking growth differentiation 9 (GDF-9); these mice have been shown previously to have disruptions in ovarian differentiation including MOFs (60). Integrin αE, has been shown to a receptor for E-cadherin (61) and in the current study integrin αE was increased on the array following neonatal genistein treatment while the message for E-cadherin was decreased. All of these studies suggest that these proteins are involved in normal ovarian differentiation and alterations in their expression correlates with disruption. Further

study into the expression of cadherins, catenins and integrins as well as other cell adhesion molecules during the time of neonatal genistein treatment should increase our basic understanding of ovarian differentiation.

We also hypothesized that developmental exposure to other environmental chemicals with estrogenic activity would disrupt ovarian differentiation, as indicated by MOFs. The doses of compounds used in this study were chosen based on their level of estrogenic activity, previously assessed using an immature uterotropic bioassay (11, 12, 21, 62, 63). We have previously shown that genistein at a dose of 50 mg/kg is estrogenic in the mouse uterotropic bioassay at 19 days of age and causes MOFs at this dose given neonatally (11, 27). We compared other estrogenic chemicals at approximately equal estrogenic doses to genistein-50 to determine their ability to cause MOFs. The data from the current study clearly shows that genistein is not unique in its ability to cause altered ovarian differentiation. Several of the compounds tested in this study show an increased incidence of MOFs similar to genistein including daidzein, nonylphenol, coumestrol and HPTE.

Previously we have shown that the formation of MOFs is mediated through ERβ so we also compared the incidence of MOFs following a particular chemical to previously published binding affinities for either ERα or ERβ (9, 33-35). It appears from the current study that preferential binding to ERβ over ERα increases the severity of the disruption, i.e. more MOFs. In addition, DES, a compound that preferentially binds ERα, also caused MOFs if the dose is high enough. If the equal estrogenic dose of DES was increased by 1000 (the difference between ERβ preference of genistein and DES), the

incidence of MOFs was similar to genistein. This suggests that a chemical's estrogenic activity is important but an adjustment has to be made depending on the tissue and the end point. Since the ovary predominantly expresses ERβ, the compound's preference for ERβ is an important consideration for this tissue. This would suggest that knowing a compound's estrogenic activity alone would not be sufficient to predict the effect a compound would have on the ovary or perhaps other tissues that have higher levels of ERβ such as the prostate or the brain.

The presence of a high incidence of MOFs in the ovary is an indication that differentiation did not occur normally. In the ovaries of normal adult female mice, follicles consist of one oocyte surrounded by one or more layers of granulosa cells; follicles with more than one oocyte are rarely found (less than 1% in the Swiss strain;(64)). Humans have also been reported to have MOFs but they occur at a very low frequency of 0.6 to 2.44 % per ovary and up to 8 % of follicles collected for *in vitro* fertilization have multiple oocytes (65, 66). There is evidence in mice that oocytes derived from MOFs have a reduced fertilization rate compared to oocytes from single oocyte follicles (67), thus an increase in MOFs might lead to subfertility or possibly infertility. A recent study from our laboratory supports this, since mice treated with genistein (50mg/kg) are infertile and mice treated with lower doses of genistein (0.5 and 5 mg/kg) exhibit early reproductive senescence (7). It is unclear whether neonatal exposure to genistein or other environmental estrogens directly alters oocyte quality but studies in our laboratory are currently underway to determine this.

In addition to MOFs, neonatal exposure to environmental chemicals showed altered expression levels of several cell adhesion molecules similar to genistein treatment. In fact, protein expression levels correlated with the incidence of MOFs for a particular compound and its preferential binding to ERβ. While there is no direct evidence, the fact that increased preferential binding to ERβ exacerbates these effects and since ERβ is highly expressed in the ovary suggests that the normal control of this differential expression may be through ERβ. Further study into the molecular mechanisms involved in ERβ signaling in the developing ovary is currently underway in our laboratory.

Taken together, these data support the idea that genistein is not unique in its ability to disrupt ovarian differentiation as indicated by the presence of MOFs following neonatal exposure to several environmental estrogens. The increased incidence of MOFs is an indication that ovarian differentiation did not proceed properly and this could lead to problems with fertility. Interestingly, the severity of the disruption correlates with a chemical's preferential binding to ERβ over ERα further supporting our previous work showing MOF formation is mediated by ERβ. In addition, cell adhesion molecules including cadherins and catenins are altered following neonatal exposure to genistein as well as several environmental estrogens also correlating with ERβ binding preference. These data along with previous work cited earlier suggests that these proteins may be involved in normal ovarian differentiation and disruption of these proteins can lead to altered ovarian morphology and possibly ovarian function later in life.

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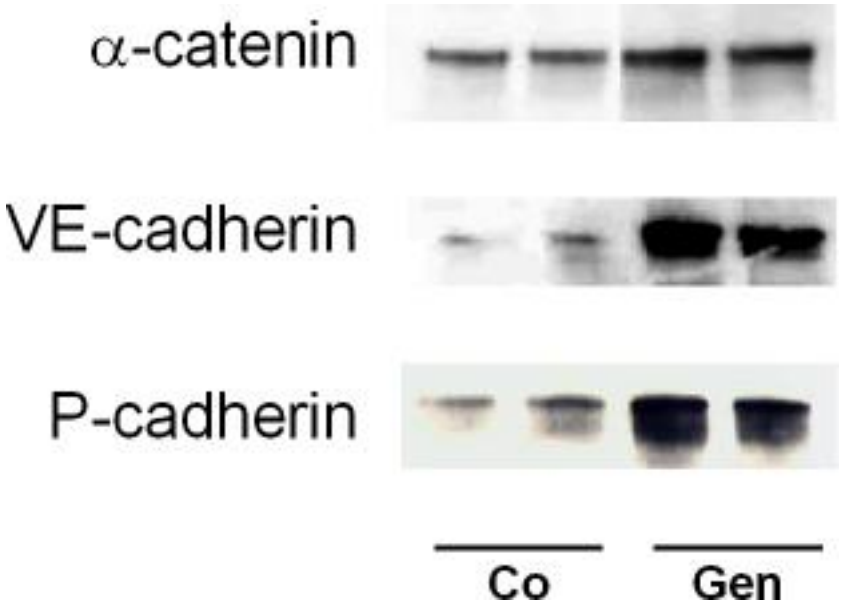

Figure 5.1: Western blot showing control and genistein treated ovarian expression of selected cell adhesion molecules. Panel A: α-catenin; Panel B: VE-cadherin; Panel C: P-cadherin.

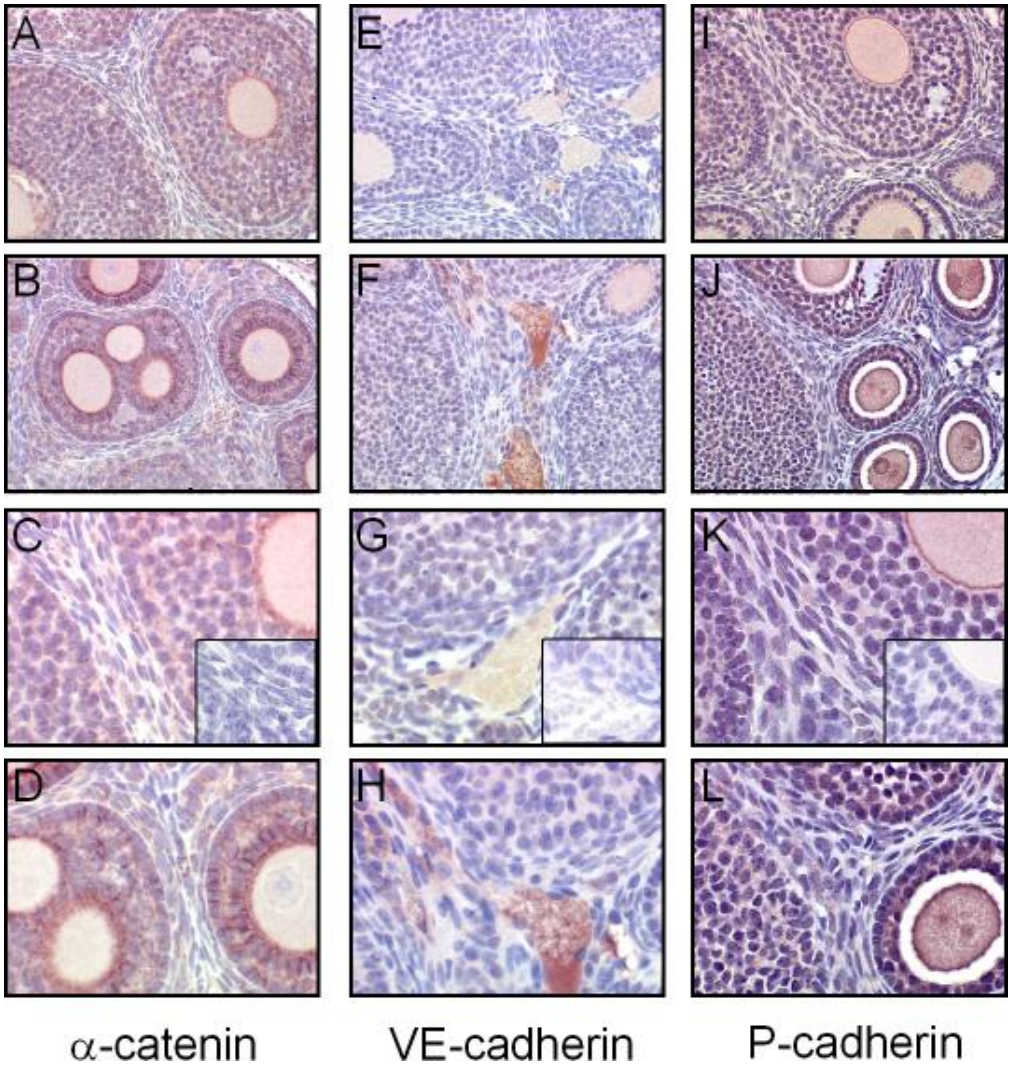

Figure 5.2: Immunohistochemical localization of selected cell adhesion molecules in the ovary following neonatal genistein treatment. Panels A, C, E, G, I, and K are controls and Panels B, D, F, H, J and L are genistein treated mice. The bottome 6 Panels (C,D, G, H, K and L) are higher power magnifications of panels (A,B, E, F, I and J, respectively). Panels A-D are α-catenin expression, note the localization around the oocyte and in the granulosa cells as well as in the interstitial/thecal cell compartment. The inset in Panel C is a negative control (no primary antibody). Panels E-H are VE-cadherin expression, note the localization predominantly in the vasculature; there is also some immunostaining around the oocyte. The inset in Panel G is a negative control (no primary antibody). Panels I-L are P-cadherin expression, note the localization around the oocyte, in the granulosa cells and in the interstitial/thecal cell compartment. The inset in Figure K is a negative control (no primary antibody). Also note the increased expression the genistein treated ovaries compared to controls for all three proteins.

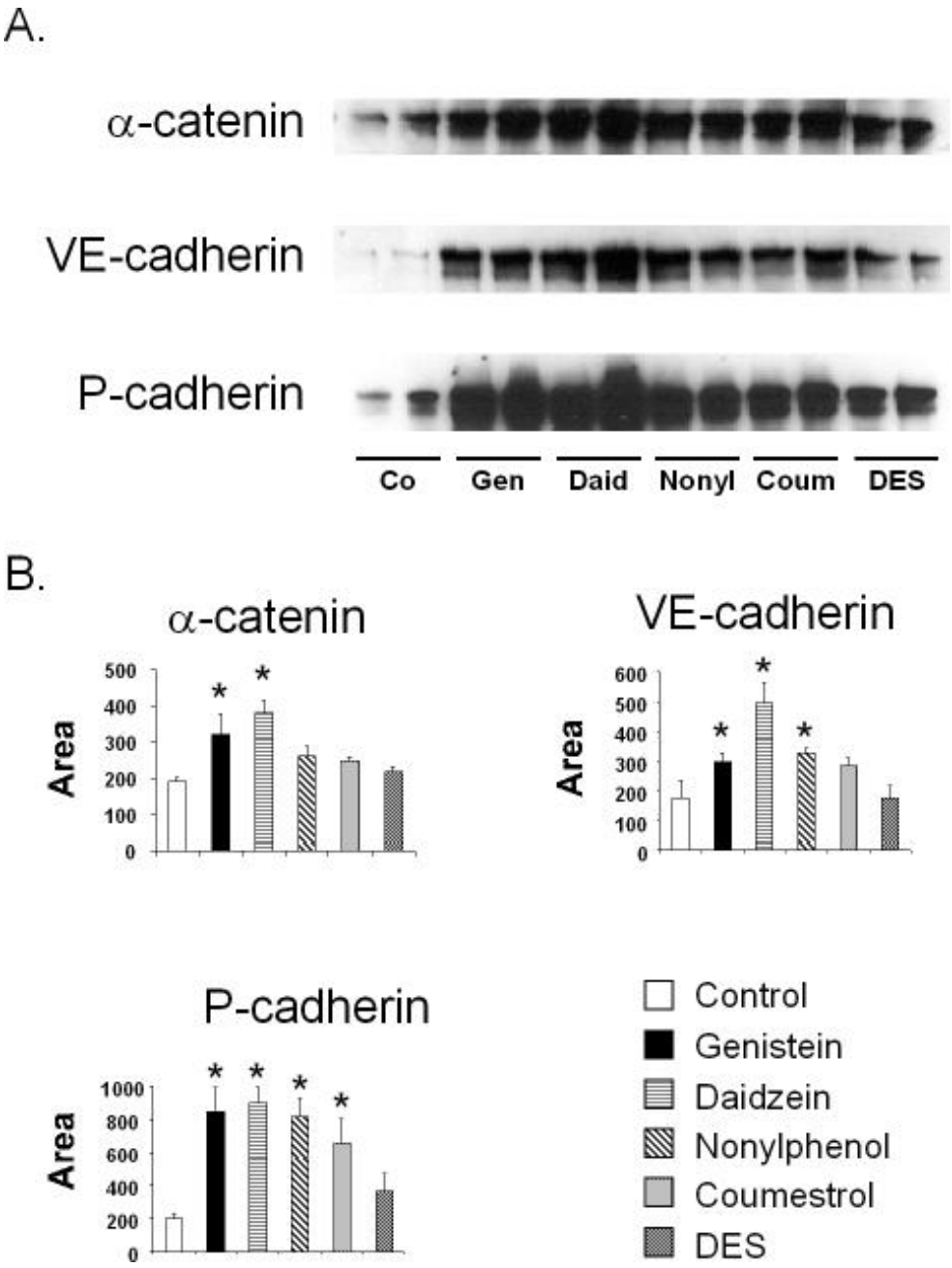

Figure 5.3: Western blot showing ovarian expression of selected cell adhesion molecules following neonatal treatment with environmental estrogens. Panel A: Western blots of α-catenin, VE-cadherin, and P-cadherin from control (Co), genistein (Gen), daidzein (Daid), nonylphenol (Nonyl), and DES treated ovaries (2 mice each). Panel B: Image analysis quantification of the area ± sem of the immunoreactive bands (4 mice each) from all three proteins. * indicates statistical significance using Student t-test at $p<0.05$.

Table 5.1: Compounds and neonatal doses used in this study.

Compound	Dose (mg/kg) [a]	Company
Genistein	50	Sigma Chemical Co., St. Louis, MO
Daidzein	250	Alfa Aesar, Ward Hill, MA
Coumestrol	2	Sigma Chemical Co., St. Louis, MO
Nonylphenol	200	Schenectady International, Schenectady, NY
HPTE, metabolite of methoxychlor	200	Cedra Corp., Austin, TX
Bisphenol A	250	Aldrich Chemical Co. Inc., Milwaukee, WI
DES	0.001	Sigma Chemical Co., St. Louis, MO
DDT	2	Lancaster Synthesis Inc., Windham, NH
ICI	10 [b]	Gift from Alan Wakeling
DHT	1 [b]	Sigma Chemical Co., St. Louis, MO
Progesterone	10 [b]	Sigma Chemical Co., St. Louis, MO

[a] Equal estrogenic dose of this compound was determined using uterotropic bioassay data from the following references and from unpublished laboratory data (10, 11, 20, 60, 61).
[b] These compounds do not exert estrogenic activity.

Table 5.2: Antibodies used in this study.

	Company	Western blot	IHC
Primary Antibody			
Mouse α-catenin	Zymed	1:1000	1:50
Mouse VE-cadherin	Santa Cruz	1:1000	1:50
Mouse P-cadherin	R & D	1:1000	1:50
Secondary Antibody			
HRP anti-mouse	Amersham	1:50,000	
Biotinylated anti-mouse	Vector		1:500

Table 5.3: Neonatal genistein exposure alters gene expression in the ovary at 19 days of age.

Low Expression[b]		Medium Expression[b]		High Expression[b]	
Gene	**Fold Control[a]**	**Gene**	**Fold Control[a]**	**Gene**	**Fold Control[a]**
Down-regulated <1.5 fold		*Down-regulated <1.5 fold*		*Down-regulated <1.5 fold*	
Mmp2	0.11	Cadherin 1 (E)	0.19		
Cathepsin E	0.18				
Caspase 9	0.37				
Thrombospondin4	0.45				
Up-regulated >1.5 fold		*Up-regulated >1.5 fold*		*Up-regulated >1.5 fold*	
Cadherin 2 (N)	6.25	**Cadherin 3 (P)**	**2.96**	Col4a2	2.42
Pecam	3.19	Integrin αE	2.87	Serpinb5	2.36
Caveolin	2.29	Cathepsin H	1.90	**Catenin α1**	**2.20**
Cathepsin D	2.21	Integrin α6	1.87	Catenin α2	2.10
Ncam2	1.97	Sparc	1.85	Catenin δ2	1.96
Selectin	1.89	Fibronectin rec. 1	1.71	Ecm1	1.93
Jcam1	1.77	Catenin α-like 1	1.65	Plasminogen act.	1.88
				Basignin	1.86
				Cadherin 5 (VE)	**1.74**
				Col1a1	1.64
				Fibronectin 1	1.57
				Integrin α X	1.55
				Laminin B1	1.53

[a] Numbers are counts from phosphoimager of genistein treated ovaries divided by counts from control ovaries using the cell adhesion molecule array (MM010, SuperArray) following a one day exposure. The cut-off for genes that showed an increase or a decrease was 1.5 fold over or under control.

[b] The categories are based on the actual counts from the array with low expression = <500, medium expression = 500-3000 and high expression = >3000

Bold = genes selected for further study.

Table 5.4: Incidence of multi-oocyte follicles following neonatal exposure to environmental compounds.

Treatment	Level of Effect	#Mice with MOF [a]	#MOF / Mouse with MOF [b]	ERβ/ERα [c] (mRBA)	ERβ/ERα [d] (hRBA)
Control		1/16 (6)	1.0	NA	NA
Genistein	High	6/6 (100)	5.3	87.7	17.5, 21.8, 50.2
Daidzein		5/7 (71)	5.6	29.9	5.0, >12.2
Nonylphenol	Medium	7/8 (88)	2.6	ND	1.8
Coumestrol		4/8 (50)	2.0	17.6	1, 7, 7.4
HPTE		3/8 (38)	2.3	ND	5.0
Bisphenol A	Low	2/8 (25)	1.0	ND	0.6, 1.0
DES		1/8 (13)	2.0	0.1	0.04, 0.9, 1.3
DDT		0/8 (0)	0.0	ND	2.0

[a] The numbers are the number of mice that had at least one MOF out of the number of mice with acceptable sections.
[b] The numbers are the average number of MOFs (only mice with at least 1 MOF were included in this number).
[c] The numbers are the relative binding affinity (RBA) for each compound for ERβ divided by the RBA for each compound for ERα using mouse receptors.
[d] The numbers are the relative binding affinity (RBA) for each compound for ERβ divided by the RBA for each compound for ERα using human receptors.
ND = not determined
Data was taken from previously published articles (8, 32-34).

6. CONCLUSION

The possibility that naturally occurring chemicals with estrogenic activity could cause alterations in the developing reproductive system is a concern since many humans are exposed to these chemicals in the diet. The studies presented herein describe the effects of neonatal exposure of CD-1 mice to the phytoestrogen, genistein on the developing female reproductive system with particular emphasis on the ovary. The doses of genistein used in these studies were chosen to span the range of human exposure levels including higher doses producing serum-circulating level of genistein similar to the levels found in infants on soy-based infant formulas and lower doses similar to levels found in human breast milk of vegetarian mothers.

The studies in Chapter 2 were designed to determine if developmental exposure to genistein could cause effects on the ovary. The most notable effect on the ovary was the appearance of multi-oocyte follicles (MOFs). Since genistein has properties other than estrogenic activity such as tyrosine kinase inhibitory activity, several experiments were carried out to determine the mechanism of action responsible for the formation of MOFs. The tyrosine kinase inhibitor, lavendustin was used to rule out genistein's tyrosine kinase inhibitory activity as a cause of MOFs. Two transgenic mouse models lacking either ERα or ERβ were used to determine which receptor subtype was involved if genistein's estrogenic activity was responsible. Since mice treated with lavendustin did not develop MOFs, this property of genistein was determined not to be responsible. Mice lacking ERα still developed MOFs when treated neonatally with genistein while mice lacking ERβ did not, suggesting that genistein interacts with ERβ to cause MOFs.

Although these results suggest that genistein directly affects the ovary to cause these effects, this question is not answered definitively herein. There are several other studies that more specifically address the direct effects of estrogens on the developing ovary. One study using neonatal ovaries grown *in vitro* showed that the follicles remained unassembled when exposed to estradiol leaving oocytes in clusters or nests; this suggests that estrogens exhibit a direct effect on the differentiation of the ovary [1]. Further evidence for estrogen's direct effects on the developing ovary comes from an *in vitro* study showing neonatal ovaries grown in culture and then transplanted into a host (under the kidney capsule) develop MOFs in the presence of DES [2]. While these studies do not directly answer the question about genistein, it is highly suggestive that the effects on ovarian differentiation, namely inhibition of oocyte nest breakdown leading to MOFs, are direct and that genistein's estrogenic activity is responsible.

Since genistein was shown to cause effects on the ovary, the functional effects on the ovary as well as reproduction were studied in Chapter 3. The most striking finding in this study was the infertility seen in mice treated neonatally with genistein at a dose of 50 mg/kg. Two lower doses of genistein, 0.5 and 5 mg/kg also caused early reproductive senescence. These studies also showed the complete lack of corpora lutea or anovulation in the high dose by 4 months of age suggesting ovarian function was disrupted. The lack of estrous cyclicity in mice treated with the high dose of genistein also suggests that ovulation was not occurring. Whether this is due to a direct effect on the ovary or an indirect effect on the hypothalamic-gonadal axis is not known but there have been several studies by other laboratories that support an indirect effect. One study showed that neonatal exposure of rats to genistein altered pituitary responsiveness to gonadotropin releasing hormone (GnRH) [3]. Higher

neonatal doses of genistein were associated with decreased pituitary responsiveness by producing less leutinizing hormone(LH) in response to GnRH stimulation [3]. The LH surge is necessary for ovulation so lower levels of LH may explain the lack of ovulation as evidenced by no corpora lutea at 4 months of age observed herein. Interestingly, in that same study mice treated with lower doses of Gen (0.01 mg/kg) were hyper-responsive to GnRH stimulation leading to enhanced ovulation rates similar to what we have shown previously in younger mice treated neonatally with genistein and again in older mice in this study [3, 4]. Altered pituitary responsiveness later in life could also account for the early reproductive senescence observed in mice treated with lower doses used in our study. Another indication that the effects on ovulation are secondary is the fact that mice treated neonatally with genistein were able to ovulate when given exogenous gonadotropins. Even though these mice are capable of ovulation, the quality of the oocytes is not known.

The other significant finding in Chapter 3 was the characterization of the infertility seen at the high dose of neonatal genistein treatment. Some of the mice in this treatment group showed signs of pregnancy, but the implantation sites were very small, reabsorptions were present and many fewer implantation sites were seen compared to controls. While this is not direct evidence for poor oocyte quality, this could be one explanation. Additional evidence that neonatal genistein may lead to poor oocyte quality is the study done by Iguchi, et al. showing that oocytes collected from MOFs were much less fertilizable than oocytes from single oocyte follicles [2]. Since there is a much higher incidence of MOFs in neonatal genistein treated mice, fewer oocytes may be competent for fertilization or if fertilized, not capable of normal development. Future plans include fertilized oocyte culture to determine if the fertilization rate or the oocyte quality is affected in these mice. The other possibility is

that the environment of the uterus or the hormonal milieu is not suitable for implantation. Serum hormones measured during pregnancy did not reveal any deficiencies in hormones thought to be needed to maintain pregnancy such as progesterone and estradiol suggesting that this is a less likely cause of the implantation defects. However, previous studies from our laboratory have shown that mice treated neonatally with higher doses of DES lack the ability to respond to estrogen stimulation [5]. This suggests that the uterus may not be able to respond to pregnancy hormones. Decidualization experiments as well as embryo transplantation in mice treated with genistein should help determine if the uterus is capable of responding to implantation.

Since we had previously shown that genistein causes MOFs, we wanted to study the developmental process that led to this abnormal phenotype. The studies in Chapter 4 followed changes in the ovary during the first week of life during the time of neonatal treatment with genistein. It became clear in this study that genistein altered ovarian differentiation. At birth, mice have large oocyte clusters or nests; these break down over the first week of life. Treatment with genistein inhibits this process leaving the oocytes still attached by intercellular bridges, leading to the presence of MOFs later in life. Therefore, a high incidence of MOFs is an indication that ovarian differentiation did not occur normally. The fact that the oocytes remained attached suggested that genistein may alter the expression of cell adhesion molecules, thus leading to the study presented in the next chapter.

Another important finding from the studies in Chapter 4 was the effect of genistein treatment on the total numbers of oocytes as well as its effect on normal oocyte cell death that occurs during the first week of life. During normal oocyte nest breakdown, approximately two thirds of the oocytes die by apoptosis [6]. There was an apparent

attenuation of oocyte cell death leaving higher numbers of oocytes in genistein treated ovaries than in their control counterparts. This suggests that cell survival and/or cell death pathways are altered following neonatal genistein treatment. In addition, estrogens have been shown to alter cell survival usually inhibiting cell death and enhancing cell survival [7, 8]. In the developing ovary, evidence from two transgenic mouse models suggests that the B-cell lymphoma/leukemia-2 (Bcl-2) family of proteins may be involved in regulating apoptosis during oocyte nest breakdown. Mice over-expressing Bcl-2 (a cell survival gene) and mice lacking Bax (a cell death gene) both have increased numbers of oocytes during neonatal life [9, 10]. In addition, adult females with a targeted disruption of *bcl-2* have fewer oocytes and follicles with either a degenerating oocyte or no oocyte at all [11]. Since neonatal genistein affects oocyte cell death during development, further study into the cell death / cell survival pathways may prove useful in determining the process by which normal oocyte cell death occurs giving us a better understanding of what types of exposures may affect this process.

The studies in Chapter 5 were carried out to study further the mechanism involved in the formation of MOFs and to determine if other environmental estrogens could cause similar alterations. Using a small array specific for cell adhesion molecules, alterations between control and genistein treated ovaries were determined. These results revealed that several cell adhesion molecules including cadherins, catenins and integrins were altered following neonatal genistein treatment. These proteins have been shown by other laboratories to be involved in ovarian differentiation, specifically the restructuring of the ovary during prenatal and neonatal development [12-17]. Previous studies have shown that disruption of some of these proteins leads to altered differentiation [15]. Since genistein alters the expression of

some of these cell adhesion molecules, this may play a part in the disruption of ovarian differentiation. A careful study of the expression of these proteins during the time of exposure may further delineate the mechanism by which genistein causes these effects.

In addition to cell adhesion molecules, several other genes have been shown to lead to the presence of MOFs and may be involved in normal ovarian differentiation. For example, mice lacking bone morphogenetic factor 15 (BMP-15) or growth differentiation factor 9 (GDF-9) have an increased number of MOFs as well as other defects of ovarian differentiation [18]; both proteins are oocyte-secreted factors expressed early in ovarian differentiation [19]. Transgenic mice over-expressing another member of the TGFβ family, inhibin-α show disrupted ovarian development and ovaries that exhibit MOFs [20]. Therefore, it appears that there are many factors that contribute to proper differentiation of the ovary during the neonatal time period and disruption of any of these appears to cause permanent alterations in ovarian morphology and possibly function later in life. Studying the effects of neonatal genistein treatment on the ovary using more global microarray analysis, including thousands of genes, should prove useful in determining the pathways that are involved in ovarian differentiation and the disruption of this process; these studies are currently underway.

Since genistein altered ovarian differentiation, other environmental estrogens were tested in Chapter 5 to determine the potential of these compounds to have similar effects. Several environmental estrogens at equal estrogenic doses caused MOFs similar to genistein. A comparison of these compounds binding preference for ERβ compared to ERα revealed that a compound that preferentially binds to ERβ causes more MOFs. Several phytoestrogens, including genistein, daidzein and coumestrol, have been shown to

preferentially bind to ERβ [21]. Thus, this class of chemicals might be particularly harmful to the developing ovary and perhaps other ERβ rich tissues such as the prostate and the brain. The results from the studies presented herein suggest that this is indeed the case with more severe effects on the ovary than compounds that preferentially bind to ERα, such as DES. It remains to be seen if adverse effects on other ERβ rich tissues are exacerbated by compounds that preferentially bind to ERβ. Two additional chemicals tested in this study with preferential binding to ERβ also showed increased MOFs, nonylphenol and the methoxychlor metabolite, HPTE. This suggests that other chemical classes also have the potential to cause more severe effects on ERβ rich tissues than one might predict from their estrogenic activity alone. This is a very important concept as assays are designed to detect estrogenic activity currently do not include this aspect of a compound's activity. There has already been much debate over the design and standardization of estrogenic activity assays and although the immature rodent model does a very good job detecting estrogenic activity of a compound, particularly an ERα agonist, a compound that is a specific ERβ agonist might be missed. In fact, this has been shown to be the case, since the ERβ specific agonist, diarylpropionitrile, did not increase uterine wet weight [22].

Another interesting finding from the studies in Chapter 5 is the very low incidence of MOFs following DES at an equal estrogenic dose to genistein. This is most likely explained by the relatively low binding preference for ERβ compared to ERα; preferring to bind to ERα in a mouse by a factor of 10 [23]. The fact that the equal estrogenic doses of these compounds were determined by an immature uterotropic bioassay also contributes to this dose differential as mice express predominantly ERα in the uterus. Therefore, equal estrogenic doses are mostly determined by a compound's activity through ERα. This being

said, a mathematical correction of the dose for the difference between the binding preferences for ERβ between the two compounds produces similar results. The difference between the two is approximately 1000 (0.1 to 87.7) and DES treatment of a dose 1000 times its equal estrogenic dose results in a similar incidence of MOFs as from genistein treatment. The reason this works for the ovary and for this endpoint is that the ovary predominantly expresses ERβ and the formation of MOFs are mediated through ERβ making the correction a simple one. Other tissues that express both ERα and ERβ or an endpoint that depends on both receptors would be more complicated to deal with but perhaps a mathematical model could be developed to predict the dose at which an estrogenic chemical would cause a particular effect in a specific tissue. This provides further evidence that knowledge of a chemical's estrogenic activity alone is not sufficient to determine what effect it might have on a particular tissue; information is also needed about the binding preference of that chemical as well as the receptor content of the target tissue and the receptor responsible for the effect. Until such information is available, *in vivo* tests of these compounds are necessary to determine their potential adverse effects.

Recently, concern over possible transmission of adverse effects to subsequent generations has emerged. Several studies from our laboratory showed that prenatal or neonatal treatment with DES leads to cancer of the female and male reproductive tract and that these lesions were transmitted to their descendents [24, 25]. The mechanism for these events is not known but recent studies have offered some possibilities. One of those is altered methylation patterns and their transmission through the germ line. Altered methylation patterns have been identified in several genes that are permanently dysregulated following developmental DES treatment. For example, the estrogen-responsive proteins

lactoferrin and c-fos are permanently up regulated in the uterus following developmental exposure to DES and the promoter region of these genes has been shown to be hypomethylated [26, 27]. Although the consequences of these types of alterations are not known, studies suggest that methylation patterns can be passed to subsequent generations [28]. In fact, a recent study supports this theory as prenatal exposure to vinclozolin or methoxychlor causes adverse effects on testis morphology and male fertility and this effect is transmitted to subsequent generations [29]. In addition, this study showed that these two chemicals caused epigenetic alterations on the DNA, specifically hyper- and hypo-methylation and that these alterations are also apparent in subsequent generations [29]. Since MOFs are formed during development, this phenotype may be transmitted to subsequent generations. Since the MOF phenotype is rare in control mice of this strain and since genistein treatment causes such a high incidence of MOFs, the transmission of this effect would be possible. In addition, since MOFs can be observed at 19 days of age, this endpoint would be much easier to monitor for subsequent generation effects compared to uterine cancer which requires 18 months to develop [24, 25]. Another unique opportunity to study trans-generational effects is that we have an indication of which genes might be altered; these genes could be studied for possible alterations in methylation patterns as seen with DES exposed mice [26, 27] as well as the recent study using methoxychlor and vinclozolin [29]. Future studies into the possible trans-generational effects of genistein and other environmental chemicals and the mechanisms that govern these effects will be an exciting field of research for many years to come.

All of this data taken together suggests a possible mechanism by which genistein causes adverse effects on the developing ovary and a schematic representation of this

proposed mechanism is shown in Figure 6.1. Prior to birth, estrogen levels are high in the developing fetus and the influence of estrogen on the ovary may be a signal for the oocytes to remain together in oocyte nests until shortly after birth when estrogen levels fall. Genistein treatment during neonatal life prolongs the estrogenic stimulus. The studies presented herein show that genistein acts as an estrogen and that it's interaction with ERβ (most likely in the granulosa cells) is responsible for the formation of MOFs. I propose that genistein continues the estrogenic signal to the ovary, specifically the granulosa cells rich in ERβ. This estrogenic interaction with ERβ produces a subsequent signal sent the oocytes to remain together in oocyte nests. This continued signal results in maintenance of the oocyte bridges and aberrant expression of cell adhesion molecules facilitating maintenance of oocyte nests. The mechanism by which the granulosa cells surround the oocytes appears to be intact since follicles are formed; however, since many of the oocytes are still together when the granulosa cells surround the oocytes, multi-oocyte follicles are formed. These abnormal follicles most likely do not develop proper communication between the oocyte and granulosa cells.

In conclusion, the work presented herein demonstrates that the naturally occurring plant estrogen, genistein causes adverse effects on the developing reproductive system and in particular on the ovary at environmentally relevant doses. These studies suggest that more epidemiological studies on humans exposed to phytoestrogens during development are warranted.

6.1 References

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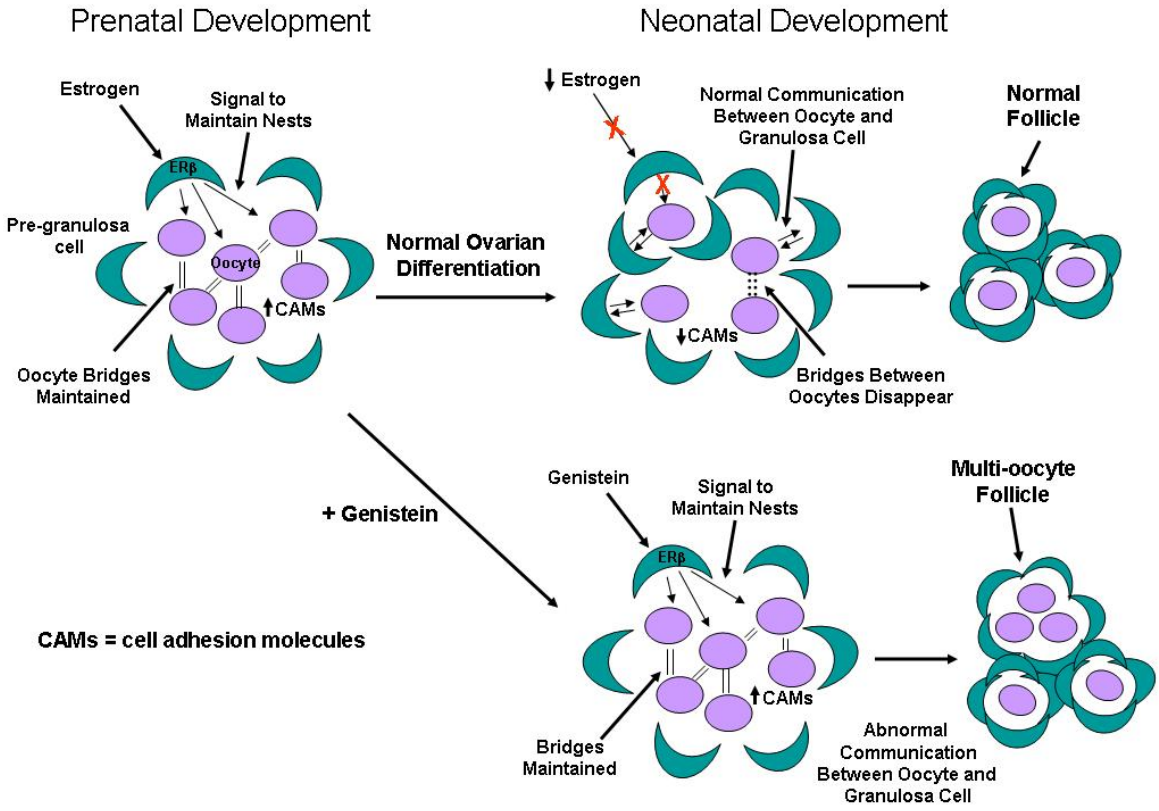

Figure 6.1 Schematic representation of the mechanism by which genistein causes its effects on the developing ovary. The left half of the figure is prenatal life and the right half of the figure is neonatal life. The diagram represents a developing follicle with pre-granulosa cells surrounding an oocyte nest on the left and oocyte nest breakdown with subsequent single oocyte follicle formation on the right. Treatment with genistein neonatally (bottom) results in maintenance of oocyte bridges, increased cell adhesion molecule expression (CAMs) and abnormal communication between the oocytes and granulosa cells. The visible sign of this abnormal differentiation is the presence of MOFs.

APPENDICES

APPENDIX A. Publications authored or co-authored by Wendy N. Jefferson

Newbold, R. R., Teng, C. T., Beckman, W. C., Jr., **Jefferson, W. N.**, Hanson, R. B., Miller, J. V., and McLachlan, J. A. Fluctuations of lactoferrin protein and messenger ribonucleic acid in the reproductive tract of the mouse during the estrous cycle. Biology of Reproduction. 47: 903-915, 1992.

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Jefferson, W.N., Padilla-Banks, E. and Newbold, R.R. Neonatal Exposure to Environmental Estrogens Alters Ovarian Differentiation and Expression of Cell Adhesion Molecules: Preferential Binding to Estrogen Receptor β Determines Severity. (in preparation).

APPENDIX B. Selected abstracts by Wendy N. Jefferson

Jefferson, W.N., Padilla Banks, E., Phillips, A., and Newbold, R.R. Low Doses of Diethylstilbestrol (DES) During Development Result in Functional, Morphological and Biochemical Changes in the Reproductive Tract. The Triangle Consortium for Reproductive Biology, January, 2001.

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Jefferson, W.N., Couse, J.F., Padilla Banks, E., Korach, K.S. and Newbold, R.R Neonatal Exposure to Genistein Induces Estrogen Receptor α Expression and Multi-oocyte Follicles in the Maturing Mouse Ovary: Evidence for ERβ-mediated and Non-estrogenic Actions. NC Society of Toxicology, March, 2002.

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Jefferson, W.N., Couse, J.F., Padilla Banks, E., Korach, K.S. and Newbold, R.R Neonatal Exposure to Genistein Induces Estrogen Receptor α Expression and Multi-oocyte Follicles in the Maturing Mouse Ovary: Evidence for ERβ-mediated and Non-estrogenic Actions. Society for the Study of Reproduction, July, 2002.

Jefferson, W.N., Padilla Banks, E., and Newbold, R.R. Altered Gene Expression in the Murine Uterus Following Developmental Treatment with Genistein, a Soy Phytoestrogen. The Triangle Consortium for Reproductive Biology, February, 2003.

Jefferson, W.N., Padilla-Banks, E. and Newbold, R.R. Altered Gene Expression in the mouse uterus following developmental treatment with genistein, a soy phytoestrogen. North Carolina Society of Toxicology, March, 2003.

Jefferson, W.N., Padilla-Banks, E. and Newbold, R.R. Altered Gene Expression in the mouse uterus following developmental treatment with genistein, a soy phytoestrogen. Society of Toxicology, March, 2003.

Newbold, R.R. Diethylstilbestrol (DES) exposure during development alters uterine gene expression: influence on cancer later in life, Society of Toxicology, March 2003. Platform presentation by **Wendy N. Jefferson**.

Jefferson, W.N., Padilla-Banks, E., and Newbold, R.R. ERα Mediated Uterine Effects Following Neonatal Exposure to the Soy Phytoestrogen, Genistein: Differential Effects of Low Versus High Doses. Endocrine Society, June, 2003.

Jefferson, W.N., Pepling, M., Padilla-Banks, E. and Newbold, R.R. Ovarian Differentiation is Altered by Neonatal Exposure to Genistein, Triangle Consortium for Reproductive Biology, February, 2004

Jefferson, W.N., Pepling, M. Padilla-Banks, E. and Newbold, R.R. Neonatal Exposure to Genistein Alters Ovarian Differentiation Resulting in the Formation of Multi-oocyte Follicles, Society of Toxicology, Platform Presentation, March, 2004.

Jefferson, W.N., Pepling, M. Padilla-Banks, E. and Newbold, R.R. The Phytoestrogen Genistein Alters Ovarian Differentiation and Subsequent Fertility, Society for the Study of Reproduction, August, 2004.

Jefferson, W.N., Pepling, M. Padilla-Banks, E. and Newbold, R.R. Ovarian Differentiation is Altered by Neonatal Exposure to the Phytoestrogen Genistein, NIEHS Science Day, Oral presentation, November, 2004

Jefferson, W.N., Padilla-Banks, E. and Newbold, R.R. Neonatal Exposure to Environmental Estrogens Alters Ovarian Differentiation and Gene Expression: Preferential Binding to Estrogen Receptor β Determines Severity, Triangle Consortium for Reproductive Biology, February, 2005.

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