Perinatal Endocrine Disruption in the Female:
Multiple Latent Effects Throughout Murine Life

By

Benjamin James Moyer

B.S., Providence College, 2007

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This dissertation by Benjamin James Moyer is accepted in its present form by the Department of Biology and Medicine as satisfying the dissertation requirement for the degree of Doctor of Philosophy.

Date_____ _____
Mary Hixon, Ph.D., Advisor

Recommended to the Graduate Council

Date_____ _____
Kim Boekelheide, MD, Ph.D., Reader

Date_____ _____
Edward Filardo, PhD., Reader

Date_____ _____
Richard Freiman, PhD., Reader

Date_____ _____
Carmen Marsit, PhD., Reader

Approved by the Graduate Council

Date_____ _____
Peter M. Weber, Ph.D., Dean of the Graduate School

CURRICULUM VITAE

BENJAMIN JAMES MOYER
PhD Candidate
Pathobiology Graduate Program
Brown University Providence, RI 02912
Benajmin_Moyer@brown.edu

EDUCATION

2007 BS Biology, Providence College, Providence, RI

2012 (*anticipated)* PhD Medical Science, Brown University, Providence, RI
Thesis Title: *Perinatal Endocrine Disruption in the Female: Multiple Latent Effects Throughout Murine Life*

RESEARCH EXPERIENCE

2007-2012 **Doctoral Research***:* Department of Pathology, Brown University
Advisor: Dr. Mary Hixon

- Conducted long-term study of the effects of an *in utero* phthalate exposure on the adult female reproductive system, uncovering numerous previously unreported effects.

- Used a recently developed model of *ex vivo* ovary culture to assess the effects of bisphenol A on follicular recruitment in the perinatal mouse.

- Used human-derived ovarian-origin cell lines to assess the effects of MEHP on steroidogenic gene expression.

RESEARCH INTERESTS

-Effects of environmental toxicants on developing organisms.
-Novel models to assess endocrine disruption.
-Early ovarian development and function, including factors in germ cell cyst breakdown.
-Mechanisms of follicular recruitment in the ovary.

ACADEMIC HONORS AND AWARDS

2008 Levy Pre-Doctoral Travel Award to attend Affymetrix University
Northeastern Society of Toxicology Student Travel Award

2009 Brown University Sheridan Center for Teaching and Learning Certificate 1
Charles "Chick" Kuhn Graduate Award In Disease Pathogenesis
Northeastern Society of Toxicology Student Poster Award, 2nd place

2010 International Conference on Fetal Programming and Developmental Toxicity (PPTOX II) Student Travel Award. Miami, Florida.

2012 Brown University Sheridan Center for Teaching and Learning Certificate 2

PUBLICATIONS

Rogers, R, Ouellet, G, Brown CW, **Moyer BJ,** Rasoulpour T, and Hixon, ML. 2008. Crosstalk between the Akt and NFκβ signaling pathways inhibits MEHP-induced germ cell apoptosis. Tox. Sci. 2008 Dec;106(2):497-508. Epub 2008 Aug 28.

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MANUSCRIPTS

Moyer B, Conti J, Pietruska JR, Hixon ML. Bisphenol A alters ovarian follicular recruitment in an *ex vivo* exposure model. *(In final preparation)*

NON-REFEREED ARTICLES AND IMAGES

Moyer, BJ and Hixon, ML. 2009. Trimethyl H3K27 and SCP3 Staining of the mouse postnatal seminiferous epithelium. Molecular Reproduction and Development. 76(6): 601.

PLATFORM PRESENTATIONS AND INVITED TALKS

Guest Lecture: "The Bhopal Disaster: Toxicity, Poverty, and Consequences". Delivered for Brown University class *Environmental Health and Human Disease*, taught by Dr. Volkan Gürel. Spring 2009.

Moyer BJ, Hixon ML (2010). Effects of *in utero* exposure to Mono-2-ethylhexylphthalate (MEHP) on the adult female. Society of Toxicology. Salt Lake City, UT. March 7. Platform talk.

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Moyer BJ, Hixon ML (2009). Effects of *in utero* exposure to Mono-2-ethylhexylphthalate (MEHP) on the adult female. Northeastern Society of Toxicology. Cambridge, MA. October 16.

Moyer BJ, Hixon ML (2009). Effects of *in utero* exposure to Mono-2-ethylhexylphthalate (MEHP) on the adult female. PPTOX II. Miami Beach, FL. December 7-10.

Moyer BJ, Hixon ML (2010). A Dose-Response Study Following Late Gestational Exposure to the Phthalate Metabolite, Mono-2-ethylhexyl Phthalate (MEHP): Effects on Female Mouse Reproductive Development. Northeastern Society of Toxicology. University of Connecticut, Storrs, CT. October 15th.

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Moyer BJ, Hixon ML (2012). Reproductive Effects in F1 Adult Female Mice Exposed *In Utero* to Moderate to High Doses of Mono-2-ethylhexylphthalate (MEHP). Society of Toxicology. San Fransisco, CA, March 11-15th.

Preface

The sum of the work presented in this Ph.D. thesis was conducted in the laboratory of Dr. Mary Hixon. I have executed all of the experiments herein with the following exceptions:

Chapter **II**'s Affymetrix gene arrays were conducted by Dr. Christoph Schorl and analyzed with his assistance.

The experiments performed in chapter **III** were performed in collaboration with Jennifer Conti, in fulfillment of her senior honors thesis requirements. Ms. Conti performed some of the ovary culture experiments, performed follicle counts, and, with the help and supervision of Dr. Jodie Pietruska, conducted the western blot experiments.

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I would also like to thank my committee, Drs. Richard Freiman, Edward Filardo, Kim Boekelheide, and my outside reader Dr. Carmen Marsit, all of whom have provided keen insight and warm support over the years.

All the members of the Hixon lab, past and present, have been great colleagues and great friends. It's hard to imagine a better group of people to spend these past five years with.

Jennifer Conti's many hours of hard work and skill at the lab bench was instrumental for the success of chapter III, and she has my thanks. Dr. Jodie Pietruska's advice and guidance have been a great help to me in the preparation of this thesis.

I would also like to thank Paula Weston, Mindie Golde, Dr. Christoph Schorl, and a great many other people at 70 Ship Street who have all provided support- both technical and moral- throughout my years here.

Finally, I would like to thank all my family and friends for their love and support.

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ABSTRACT

Endocrine disruptors are a broad class of toxicants with the ability to alter and/or interfere with normal endocrine signaling. The perinatal period is particularly vulnerable to endocrine disruption, as a number of critical endocrine processes regulate aspects of gonadal development. In this thesis, two different endocrine disrupting chemicals-mono-2-ethylhexyl-phthalate, MEHP, and bisphenol A, BPA, were examined for their ability to alter perinatal ovarian development. The first aim of this thesis assessed the reproductive effects on the adult F1 female generation following exposure to 100, 500, and 1000 mg/kg MEHP during late gestation. We found that F1 females exposed to high doses of MEHP exhibit alterations in both fertility and endocrine function. The second of this thesis aim assessed the effects of BPA on neonatal mouse ovaries in an *ex vivo* ovarian culture model. We found that moderate to high doses of bisphenol A alter ovarian follicular survival and follicular recruitment signaling pathways. This thesis concludes that the neonatal period is a critical toxicological window in which exposure to endocrine disruptoring chemicals can result in lifelong alterations to the female reproductive tract.

Chapter 1: Introduction and Background

I. Ovarian Development

Early in mammalian *in utero* development , the developing gonads are tightly controlled both by endocrine and paracrine signaling. Later in development the gonads begin producing signaling of their own, informing development of secondary sexual characteristics *in utero*. For this reason, timing is critical in organogenesis, and for studies that focus on toxicant-induced disruption of the early ovary, the window of exposure may dramatically change the human health effects of a given toxicant exposure.

Organogenesis

In all mammals, sexual differentiation is determined chromosomally. Specifically by the gene *SRY,* (Sex Determining Region Y), which is located on the Y chromosome. Bipotential gonadal tissue exists prior to sexual determination. The presence of SRY will leads to the genesis of the testis. In the absence of SRY, ovary formation will occur. This bipotential gonadal primordia emerges from the surface of the mesonephros [1]. In the mouse, this typically occurs between gd10 and gd11. During this time, the germ cells of the animal will have migrated into the genital ridge. By gd11.5 the genital ridge will generally be structurally isolated from the mesonephros, which is presumed to halt any further migration into the ridge [2]. This process is mediated by a number of genes, but perhaps most significant are the genes WT1 (Wilm's tumor 1), without which neither kidneys nor gonad will develop [3]. The loss of SF1 (Steroidogenesis factor 1) will also result in no development of the adrenal gland or gonad [4].

At this point, regardless of sex, the germ cells remain clustered in groups, often connected via cytoplasmic bridges, and undergoing synchronous mitosis [5]. The fate of these germ cell cysts will be treated more in depth in the following section.

Following successful germ cell migration, from approximately gd11 to gd13 the genital ridge is largely made up of undifferentiated mysenchymal cells. In the male, greater vascularization is observed at this time. This is also the point at which the support cells of the gonad begin appearing: either Sertoli cells in the male or granulosa cells in the female. While the exact origin of these cells is a matter of dispute, it is believed that epithelial cells derived from the basement membrane underlying the genital ridge break off and enter the ridge [6]. It is during this time that the expression of SRY leads to the formation of these pre-Sertoli cells. In the absence of SRY, as in the case of females, these same cells will instead differentiate into granulosa cells, though often considered "pre-granulosa" cells at this point. The particular characteristics and function of these cells at this stage are poorly understood. By gd12.5, the pre-Sertoli cells, if present, have begun releasing anti-Müllerian hormone (AMH), suppressing the formation of the Müllerian duct, which otherwise provides the structural basis for the female reproductive tract.

It is then at approximately GD13 in which the gonad starts truly differentiating into either a testis or an ovary. The ovary is considered the "default" gonad. Based on this, the exact mechanism for ovary formation at the gene expression level is not as well defined as in the testis for which a number of critical genes have been identified. For the ovary, however, it is clear that the gene *Wnt-4* has a role in female development, *Wnt-4* mutant mice exhibit masculinization. These mice also lack a Müllerian duct while

developing a functional Wolffian duct. It is also clear that the germ cells in the female play an important role in the differentiation of the somatic cells; the pre-granulosa cells will fail to differentiate in the absence of germ cells and this can result in a "streak ovary" with undifferentiated mesenchymal cells composing the bulk of the ovary [7]. If germ cells are present but then removed or otherwise lost, the remaining somatic cells may even spontaneously redifferentiate into Sertoli cells, and even form structures not unlike empty seminiferous tubules [8].

Germ Cell Cyst (GCC) Formation.

As previously mentioned, shortly after genital ridge formation, germ cells that arrived at the ridge begin to undergo mitosis. During this time, they are considered to be oogonia. After several rounds of division, ending at approximately GD13.5 in the mouse, they will enter meiosis and become oocytes. During this time, cytokinesis is not completed. Cysts are connected cytoplasmically with microtubule bridges allowing organelle transport between sister oogonia. At this point in development, interactions of a poorly characterized nature with surrounding somatic (pre-granulosa) cells lead to the enclosing of these cysts in ovigerous or ovarian cords. These cords remain intact until the subsequent process of nest breakdown and primordial follicle formation [9]. At approximately GD13.5, after the repeated rounds of mitosis, these oogonia enter meiosis and are defined as oocytes. Eventually, these oocytes will then undergo arrest in the diplotene stage of meiotic prophase I [10]. The exact trigger by which the oogonia enter meiosis has not yet been determined. Early research suggested that the beginning was inherent and synchronized [11], but more recent research has indicated that oogonia enter meiosis in an anterior-to-posterior wave throughout the ovary [12].

The end result of this process is that the germ cells will exist in discrete clusters awaiting the next step in which these cysts undergo a systematic breaking apart and encapsulation into single units.

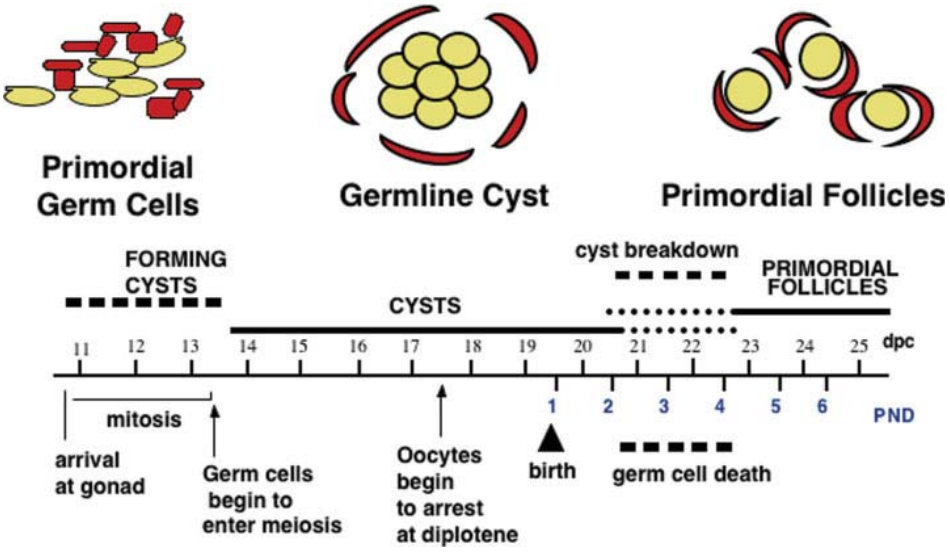

Fig. 1.1 Progression of germ cells in the mouse ovary. [10]

Germ Cell Cyst Breakdown

The oocytes remain at prophase I of meiosis until recruitment. However, during this period of meiotic arrest, they undergo a major structural change in which the cysts breakdown. The end result is each surviving oocyte forming a primordial follicle. This process typically happens shortly after birth in the mouse, but varies depending on species (See Fig. 1.1). In humans it begins at 4 months gestation and continues until 6 months after birth [13]. The exact nature of the breakdown is still poorly understood, though some mechanisms have been proposed [13,14]. During the time at which the nests begin breaking down, a significant number of oocytes begin undergoing apoptosis. In the mouse, over 60% of the germ cell population detected at GD13.5 will be lost by PND4, with a particular surge of programmed cell death among oocytes between PND1 and 3 [14]. Why some cells and not others undergo apoptosis, even when cytoplasmically

connected, has not been ascertained. However, it has been theorized that it may be a culling in which oocytes with genetic defects are lost [15]. Alternatively, the death of some cells may provide a support role for adjacent cells, which is believed to be the case in the similar GCC breakdown in drosophila [16].

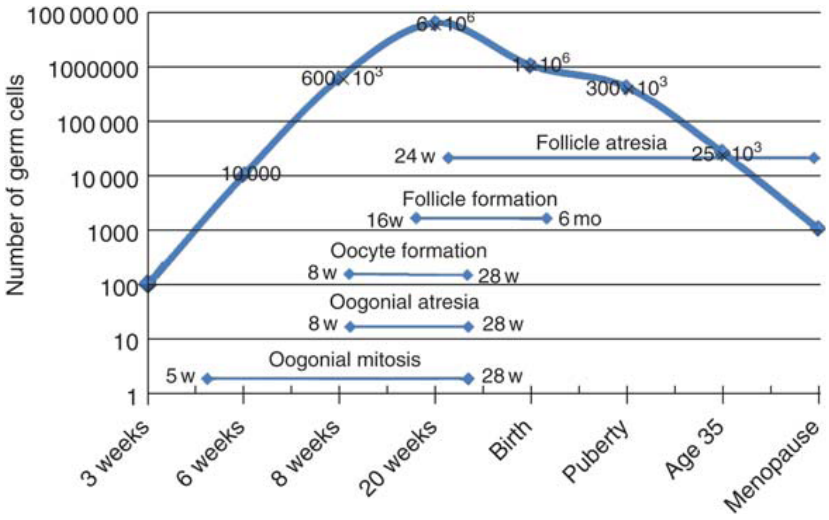

Fig. 1.2 Timeline of Human Folliculogenesis [13]

The genetic basis of GCC breakdown is still being elucidated, and it should be specified that because the majority of studies are conducted in mouse knockout models, it may be difficult to extrapolate to humans. A number of genes have been identified as playing a role in GCC breakdown and folliculogenesis. These genes have typically been identified via knockout or mutation resulting in abnormal or incomplete GCC breakdown. Such failure of GCC breakdown can result in multi-oocyte follicles (MOFs) [17] wherein multiple oocytes remain adjacent and encapsulated in the same sphere of granulosa cells. These structures persist in this state past normal GCC breakdown and into adulthood [14]. The exact physiological consequences of MOFs are unknown, but a study attempting *in vitro* fertilization of mouse oocytes from MOFs indicated they are typically 30% less likely to be successfully fertilized than their normal counterparts [18]. Two

TGFβ family members- bone morphogenic factor 15 (*bmp15)* and growth differentiation factor 9 (*gdf9)* are excreted by oocytes early in ovarian differentiation [19] and when either one is deleted the ovary exhibits significantly more MOFs [20]. Notch signaling also seems to play a role. Mice with a mutation in Notch-related gene lunatic fringe (*Lfng)* exhibit higher instances of MOFs and infertility [21]. *Nobox*, an oocyte-specific homeobox gene, is expressed throughout follicle formation and in primordial follicles; loss of this gene results in a delay in nest breakdown and higher attrition of oocytes during formation [22]. While these studies suggest a number of genes involved in the process of GCC breakdown, there is as yet no thorough study or proposed mechanism of how these genes interact to produce GCC breakdown and follicular formation.

A number of other factors in germ cell nest breakdown have also been studied, including soluble factors and paracrine elements. Growth differentiation factor 9 (gdf9), [23,24] stem cell factor (SCF) [25, 26, 27], and follicle stimulating hormone (FSH) when applied ectopically to ovaries in culture appear to induce primordial follicle formation. Conversely, the absence or blocking of these factors inhibit primordial follicle formation.

Finally, the effects of estradiol (and xenoestrogens) on GCC breakdown have been well-documented. Exposure to estrogenic substances consistently inhibits the process of GCC breakdown, resulting in higher instances of MOFs. Substances for which this has been reported includes 17-β-estradiol [28], the phytoestrogen genistein [29] and DES and BPA [30]. These reports clearly indicate that environmental exposures to endocrine disruptors, particularly xenoestrogens, are capable of altering the process of GCC breakdown and therefore capable of altering ovarian function throughout later life. Because of the role of estradiol in regulating germ cell nest breakdown discussed above,

the estrogen receptor family has become a target for understanding the mechanism of GCC breakdown. In a study using genistein, ERα-mutant and ERβ-mutant mice were both exposed, but only the ERα-mutant mice exhibited the characteristic MOF phenotype. This suggests that genistein's effects are partially mediated through ERβ. It is possible that this is true of the other estrogenic compounds that produce this effect [29].

In summary, the end result of the GCC breakdown process is that the germ cells of the ovary exist in discrete functional units. These functional units are the primordial follicles, consisting of the oocyte, surrounded by a layer of flattened granulosa cells. These follicles, initially quiescent, become the driving factor in female reproduction.

II. The Adult Ovary

Follicular Recruitment

The reserve of germ cells in the female is established in the process outlined above as primordial follicles. These follicles, consisting of both the oocyte and supporting granulosa cells, remain quiescent throughout the life of the individual until activation. The population established at the end of folliculogenesis is generally accepted as the total oocyte reserve of the individual. In humans, the population typically begins at approximately 1 million oocytes at birth and declines to about three to four thousand at puberty by atresia. Of those, only approximately a thousand will remain in the ovary at menopause [31]. Attrition due to atresia will claim all but approximately 300 of those follicles. That relatively tiny population must suffice for the duration of sexual maturity. Therefore, the organism must have a system by which the follicles are recruited gradually, a process referred to as follicular recruitment or activation. Once activated,

the primordial follicles proceed through a number of stages- primary, secondary, antral, and corpeal (following oocyte eruption). Each follicular stage has its own characteristics as to somatic cell behavior and endocrine activity.

The process by which a primordial follicle is recruited and progresses to the primary follicle stage is poorly understood and remains a question of intense interest. It has traditionally been held that some sort of intercellular communication between oocyte, granulosa cell, and thecal cell in the ovary control this initial follicular recruitment [13]. Knockout studies in mice have also demonstrated that a number of genes appear responsible for maintaining quiescence of the primordial follicle, and mutation or suppression of these genes leads to premature activation of the primordial follicle population. Some of these genes include *PTEN*, [32], *tsc-1* [33], and *Foxo3a* [34]. *Foxo3a* is a gene of particular interest because its phosphorylation and translocation to the nucleus is a useful marker for distinguishing activated follicles, and for this reason is prominently used in chapter 3's experiments.

There are also a number of possible paracrine regulators of primordial follicle activation. For example, AMH-null mice have increased follicular recruitment [35; 36]. AMH is produced by the granulosa cells of maturing follicles, so it may be that already-recruited follicles release signals suppressing recruitment of additional follicles until they proceed further in development. Kit ligand, also called stem cell factor, is expressed by granulosa cells. This receptor is found on oocytes and thecal cells [37]. Insulin has also been demonstrated in rat ovary culture models to stimulate the primordial-to-primary follicle transition [38]. Conversely, inhibin A (primarily produced by antral stage follicles) appears to impair activation of primordial follicles [39].

It can then be appreciated that while the primordial-to-primary transition is not yet understood, it is probably a complicated process in which the follicle is exposed to both intrinsic and extrinsic factors. Some of these factors are promoting recruitment and others suppress it. The challenge for researchers now is most likely in identifying the "tipping point" at which a particular follicle overcomes the inhibitory factors acting upon it in favor of the activating factors. Moreover, the degree to which exogenous factors, including toxicant exposure and/or endocrine disruption, remains a challenge.

Stages of Follicular Growth

Once a primordial follicle has been activated, it transitions to a primary follicle and a number of physical changes take place. The granulosa cells transition from flattened to cuboidal, the oocyte grows in size and the thecal layer begins to develop. The basal lamina and zona pellucida also begin to form [13]. At this point, the granulosa cells and newly developed thecal cells become hormonally active and appear to contribute to the process of further progression of the follicle [13]. Follicle-stimulating hormone receptor (FSHR) becomes expressed and from this point on FSH acts as a survival signal preventing follicular atresia [40], a process which will be discussed in-depth later. A number of other factors continue to determine the follicle's further growth and differentiation. In particular, production of growth differentiation factor 9 *(GDF-9)* in the oocyte has been shown in knockout experiments to be critical in at least two species for the progression of primary follicles to the later stages of maturation [41, 42]. Likewise, Bone morphogenic protein 15 *(BMP-15)*, produced by the oocyte, induces proliferation of the surrounding granulosa cells independent of FSH [43].

It is also during this time that the follicle becomes estrogenically active. The thecal cells and granulosa cells begin a complex interaction which results in the production of kit ligand, estradiol, and androgen precursors. Meanwhile, these cells also act as inhibitors of progesterone production by modulating FSH production via *BMP-4* and *BMP-7* [13]. Furthermore, the granulosa cells and thecal cells produce TGFβ family members that appear to mediate further follicle growth and maturation [44]. There appear to be inter-species differences as to the exact role of TGFβ in follicle maturation [45]. During this time, these smaller follicles will produce more activin A relative to inhibin A; activin A appears to have a role in both promoting follicular maturation and suppressing androgen production [45].

Eventually, the follicle will continue its maturation process, growing larger with additional layers of granulosa and thecal cells and develop the large fluid-filled space known as the antrum, and are now properly considered an antral follicle. Typically in humans, a number of follicles will reach this point at a similar time and a poorly understood process by which one of the follicles becomes "dominant" takes place; in other species, such as rodents, no such process occurs as numerous oocytes can be released simultaneously. During this time, the activin A-to-inhibin A ratio will begin to favor inhibin. As a result, androgen precursor production will increase, perhaps in preparation for the preovulatory surge in estrogens [13]. As previously mentioned, inhibin A also appears to inhibit maturation of other follicles. Inhibin A may act as a mechanism to prevent recruitment of additional follicles while large numbers of mature follicles prepare for eruption or thecalization. During this time, the follicle is increasingly steroidogenic under stimulation by luteinizing hormone (LH), released in a

pulsatile manner from the pituitary [46]. Following ovulation, LH will have an increasingly important role directing the follicular activities. A surge of LH will also trigger the eruption of the oocyte from the follicle and ovary as part of ovulation.

The final stage of the follicle occurs after ovulation, when the oocyte erupts from the follicle and out of the ovary into the uterine tubes. The follicle, now devoid of the oocyte, is referred to as a corpus luteum (CL). A number of changes take place. The granulosa and thecal cells undergo a process of luteunization characterized by increased expression of the LH receptor as well as StAR and the progesterone receptor (PR) [47]. The CL remains estrogenically active. Its major product is progesterone, which is necessary for successful implantation and gestation of any fertilized oocytes. It produces estradiol (which is also necessary for successful implantation) and inhibin A, which may inhibit additional follicular recruitment during pregnancy. If fertilization and implantation is successful, the developing blastocyst will release chorionic gonadotropin, which acts to maintain the CL. Without this signal, the CL will eventually degrade on its own.

From activation to degradation, the activated follicle undergoes continual transformation both structurally and functionally. At each stage, the three cell types- oocyte, granulosa, and thecal- play a different role, affecting each other within the follicle, as well as effecting neighboring follicles and indeed effecting the whole organism by the release of certain hormones. However, very few follicles will have an opportunity to progress through each of these distinct stages; at every step, a significant number will be lost through the process of atresia.

Follicular Atresia

At each stage of follicular development, there is a significant chance that a given follicle will undergo a process of apoptosis-mediated loss and regression known as atresia. As previously mentioned, the overwhelming majority of follicles will never release their oocyte in ovulation. While it is generally held to be a hormonally regulated process [40], it is still unclear why some follicles survive and others do not even in the same hormonal environment. In humans and other species in which typically only a single oocytes is released per ovulation, one follicle becomes dominant. This ensures the survival of the follicle, typically at the expense of all other antral follicles in the ovary at the time [13]. However, such a process does not appear to occur in animals such as the mouse, since numerous follicles will ovulate in a given cycle- typically 10 to 12 in the mouse.

The gonadotropins FSH and LH play a critical role. Studies indicate that absence of gonadotropins is sufficient to induce atresia in most follicles and early atretic follicles being rescued by exogenous gonadotropin exposure [40]. These signals appear to act on the granulosa cell via adenosine cyclase, increasing levels of cAMP and thereby activating protein kinase A (PKA) [48]. PKA is presumed to maintain granulosa cell viability which is key for inhibiting apoptosis and consequently atresia.

Evidence suggests that atresia in more mature follicles typically begins with the granulosa cells undergoing apoptosis and spreading to the thecal cells. With the loss of both granulosa and thecal cells, the oocyte then itself undergoes apoptosis [49]. However, in primordial and primary follicles, some studies have indicated it is the oocyte that initiates follicular atresia [50].

Follicles are structures containing numerous cells and three different cell types. Therefore it is not surprising that the major pathway in atresia is an extrinsic apoptosis pathway. Specifically, the Fas/Fas-ligand pathway has been implicated in a number of species as being a driving factor in granulosa cell apoptosis associated with follicular atresia [49]. Mice with a mutation in the Fas gene exhibit normal ovaries at birth; however by adulthood exhibit far more maturing follicles in the ovary than wild-type mice. These data suggest a failure of follicles to undergo apoptosis [51].

Caspase 3 also appears to be a key factor once apoptosis is initiated. Activated caspase 3 is strongly associated with granulosa cell apoptosis in follcular atresia. This is apparently untrue of oocytes, which can undergo atretic apoptosis without caspase 3 [52]. Interestingly, caspase 2 seems necessary for oocyte apoptosis and mice with caspase 2 mutations show abnormally high numbers of oocytes. These oocytes are surprisingly resilient to nutrient deprivation and chemotherapeutic compounds known to cause oocyte loss [53].

It is apparent that the majority of follicles in the ovary will never be ovulated to release their oocyte. The question of "why" remains; both in the sense of what causes the recruitment of "redundant" follicles, and what role, if any, these supporting follicles might play. The answers may lay in the hypothalamic-pituitary-gonadal (HPG) axis.

III. HPG Axis

The HPG, as the name implies, is a system consisting of the relationship between three hormone-producing bodies: the hypothalamus, the pituitary, and the gonad (for the purposes of this work, the ovary). Briefly, the hypothalamus releases gonadotropin-releasing hormone GnRH, which stimulates the pituitary to release FSH and LH. These

gonadotropins then reach the ovary [54]. The role of these gonadotropins has already been briefly discussed. FSH acts to maintain the population of active follicles and plays a role in progression and maturation of the follicles. LH plays both a role in the leutinization of follicles and in the LH surge. This surge plays a key role in triggering ovulation. The production of estradiol by the ovary acts to suppress production of both FSH and LH [55]. The luteal follicles of the ovary (along with the adrenal gland) also produce progesterone, which inhibits LH surges. This is presumably part of pregnancy maintenance [55]. Thus, the HPG acts dynamically to permit a "cyclic homeostasis", where levels of these different hormones change continually but predictably to maintain the normal reproductive cycle in healthy adult females. Exactly what this cycle entails varies somewhat depending on species.

In humans and other primates, the reproductive cycle is referred to as a menstrual cycle. In rodents and many other animals, it is referred to as an estrus cycle. There are some differences between the two, but the underlying hormone activity is generally conserved across mammalian species. One major difference is the corpus luteum. In humans, because only one oocyte is typically released during ovulation, the ovary will only have a single major corpus luteum. Rodents and other animals with multiple ova released may have several of these follicular remnants.

The general trend is the same, however (see fig. 1.3 for diagram of human cycle): in the time leading up to ovulation, estrogen levels increase, due in part to the follicles maturing and becoming more estrogenically active. Immediately prior to ovulation, the LH surge occurs, triggering the release of the ovum (or ova) and leutenizing the now oocyte-less antral follicles. Progesterone levels will continue to increase during this time;

if implantation occurs, the blastocysts will supplement the ovaries' progesterone production and the cycle will alter to support the pregnancy. In part due to the increase in progesterone, FSH and LH will be suppressed, as outlined above. Without successful implantation, the progesterone surge will pass, and FSH and LH levels will rise again to support the maturation of the next wave of follicles.

Because the HPG axis regulates estrogen production, it also has significant effects throughout the body, particularly in estrogen-sensitive tissue. For that reason, it is particularly crucial during puberty. At this point the reproductive cycle begins and secondary sexual characteristics, such as the mammary gland, start extensive development under the regulation of estrogen.

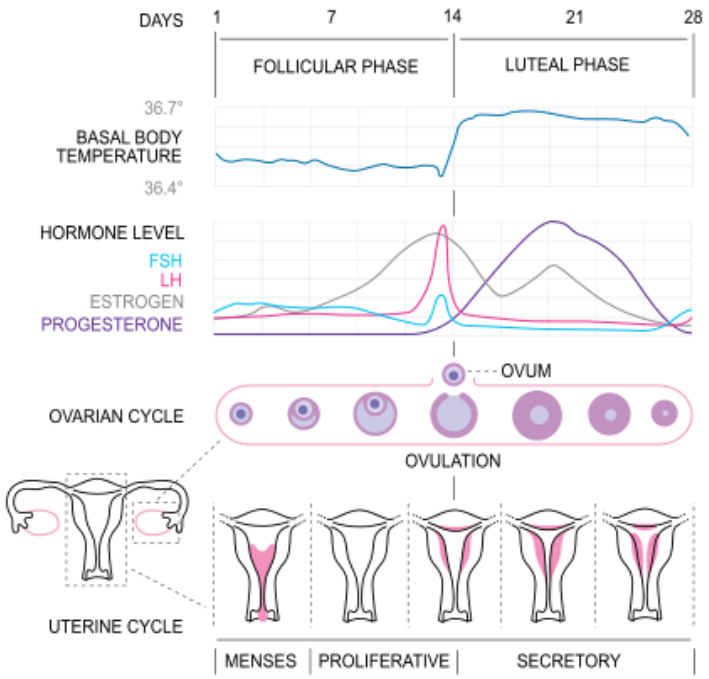

Fig. 1.3 Diagram of hormone cyclicity in human females [54b]

Effects of HPG Disruption

The HPG involves multiple organs and careful regulation of numerous signals which can inhibit or promote other elements in the axis. Therefore it is particularly susceptible to disruption. Dickerson and Gore identified three possible means of HPG disruption: "(1) direct stimulation or inhibition of the endocrine system; (2) mimicking or blocking the body's response to endogenous steroid hormones; or (3) altering biosynthesis or degradation of endogenous hormones." Compounds known or suspected of being able to disrupt the HPG include phytoestrogens such as genistein, polychlorinated biphenols (PCBs), [54], and a variety of other compounds including bisphenol-A and phthalates, both of which will receive extensive discussion in a later section. The particular effects of endocrine disruption are various; a few of interest will be briefly outlined below.

Mammary Gland Development and Hyperplasia

Alterations to the HPG axis can have profound effects throughout the body beyond hypothalamus, pituitary, and gonad. Any estrogen-sensitive tissue can be adversely affected by changes in normal HPG axis signaling. There has been much recent concern over trends in human populations for both the early onset of puberty and pre-pubertal breast development. The role of environmental factors is now receiving greater consideration [56, 57]. In recent years this early onset of breast development is both more widely reported and reported for earlier ages than premature menarche [58]. This indicates that the effect is not simply the result of earlier sexual maturity. Whether or not that prematurity is caused by environmental factors remains to be determined. A number of animal models using a variety of toxicants (including bisphenol A) suggest that early (gestational and lactational) environmental exposures may contribute to this

premature breast development [59,60]. By extension, it is clear that disruption of the HPG can cause effects that are organism-wide.

Concern is not limited to the early onset of breast development, though premature puberty has been linked to adverse health outcomes in humans [61]. There is also the ability of endocrine disruptors to cause abnormal development of the mammary gland and/or increased risk of breast cancer. A number of compounds have been shown to result in varying degrees of mammary gland hyperplasia in mouse and rat studies, including vinclozolin, the phytoestrogen genistein [62], and BPA [63]. DES, a strongly estrogenic compound, has been associated with increased risk of breast cancer in women exposed *in utero* [64].

This hyperplasia is typically characterized as having increased ductal branching of the mammary gland and an increased number of terminal end buds, or "beading" of hyperplastic ductal lining [60]. The exact mechanisms of this hyperplasia likely vary depending on the exposure, but estradiol and progesterone both play a role in inducing terminal end buds (TEBs) and branching respectively [65]. It has been proposed that even following mammary development, the adult retains a mammary stem cell population [66], which may be sensitive to estrogenic stimulation.

A correlation between hyperplasia and increased risk for breast cancer has been documented. Female rats with hyperplasia induced by genistein exposure had significantly higher rates of carcinogen-induced mammary tumors [67]. This same result was found using tamoxifen, a chemotherapeutic agent that acts as an estrogen antagonist in the adult breast but an agonist in the developing breast [68].

It appears that *in utero* or early exposure to endocrine disruptors can lead to malformations in the breast, and these malformations, while not necessarily tumorgenic on their own, pose an increased risk for cancer in the presence of other carcinogens. This constitutes another instance in which an endocrine disrupting chemical can have effects on secondary sexual characteristics with potential adverse health outcomes that might not otherwise be obvious given the earlier exposure.

Estrous Cyclicity

The effects of the cycling hormones are by no means limited to the HPG axis. Estradiol has been documented to affect a wide number of physiological systems. Of particular note for this thesis are the effects on the uterus. The estrus cycle in the mouse radically alters the tissue of the uterus and vagina allowing detection of stage of estrus cycle via vaginal lavage cytology [69]. Vaginal cytology has been a valuable tool for studying endocrine disruption in animal models, and is an important endpoint in a number of studies. A study with bisphenol A, for example, revealed that females exposed *in utero* exhibited abnormal cyclicity in adulthood [70]. Likewise, an acute exposure to DEHP in adult rats resulted in delayed estrous which correlated with the suppression of estradiol production observed in the study [71]. The continued correlation of cycle with hormone status makes estrus cyclicity in animal models an invaluable tool for detecting changes in hormone homeostasis.

Premature Ovarian Failure

Premature ovarian failure, also called premature ovarian insufficiency, is a condition in which the ovary has depleted its reserve of oocytes before the normal end of reproductive life in the female. In human pathology, its more specific diagnostic criteria

is the cessation of ovary function with high gonadotropin and low estrogen levels at the age of 40 or younger [72]. This condition is widespread, with a rate of 0.1% of all women at age 30, increasing to 1% of all women at age 40 [73]. A variety of factors have been identified, including autoimmune disorders, exposure to chemotherapeutic agents, certain infections, metabolic diseases, and idiopathic or genetic etiologies [74]. In the broadest sense, earlier onset of menopause could be considered a form of premature ovarian failure. In most circumstances, premature ovarian failure is associated with the loss of the follicle population of the ovary either by overrecruitment depleting the reserve or simply by direct destruction of the follicles.

Environmental factors are also considered as possible causes of premature ovarian failure. Cohort studies indicate that female infants of abnormal weight at birth (either heavier or smaller) are significantly more likely to have early onset of menopause [75]. Exposure to dioxin has also been linked with early onset of menopause [76]. A variety of animal models of premature ovarian failure have been developed. Perhaps the best documented is exposure to 4-vinylcyclohexene diepoxide (VCD), which appears to target primary and primordial follicles [77]. By depleting the ovarian reserve of both the quiescent and early active follicles, the ovary will soon run out of its entire population of follicles and the animal will enter a state that is hormonally similar to menopause.

The established ability for environmental factors- pathogens, toxicants, early perinatal weight and environment- to affect the ovarian follicle population with significant health outcomes suggests that identifying compounds that exhibit the ability to alter recruitment or viability of follicles is an important research goal.

IV. Endocrine Disruption.

Endocrine disruptors refer to any exogenous toxicant capable of inducing alterations to normal endocrine system homeostasis. Endocrine disruptors are now ubiquitous in the environment of industrialized countries. Pesticides, both those in current use and older chemicals still persist in the environment [78,79,80]. This thesis is focused on two particular chemicals, MEHP and Bisphenol A, which are used in the production of plastics.

DEHP and its metabolism into MEHP

Amongst the most widely used phthalates is di-2-ethylhexyl-phthalate (DEHP). DEHP is used for a variety of products, including cosmetics, pharmaceuticals [81], food packaging, and medical devices [82]. DEHP was formerly the most-widespread of phthalates, produced worldwide in volumes of 3 to 4 million tons [83,84]. While worldwide production has dropped in relation to other phthalates, such as di-butyl-phthalate and di-iso-nonylphthalate, DEHP production in the EU alone has stayed at levels of 200,000 tons [83]. DEHP itself can make up to 40% of the weight of PVC plastics [85].

DEHP and other phthalates are not covalently bound to the plastics to which they are added and therefore leach out readily, particularly in the presence of heat, acidic conditions, or lipophilic liquids. The fact that DEHP is used in many products directly ingested or applied (such as pharmaceuticals and cosmetics), results in a ubiquitous human exposure to phthalates in the industrialized world and urinary metabolites being detected in over 90% of urine samples from populations not occupationally exposed [86].

While exposure is ubiquitous in the population, a number of groups are considered particularly vulnerable either due to elevated exposure levels, increased risk for adverse effects, or a combination thereof.

Of particular concern, however, are prematurely born infants or newborns receiving intensive medical intervention in neonatal intensive care units (NICUs) [87]. These are considered a particularly susceptible population because of the high level of exposure due to the presence of phthalates in feeding tubes, blood bags, intravenous (IV) drips, and other medical devices.

DEHP is rapidly metabolized by esterases in the gut to a variety of phthalate ester metabolites. These metabolites include mono (5-carboxy-2-ethylpentyl) phthalate, 2-ethylhexanoic acid, and mono (2-ethylhexyl) phthalate (MEHP) [86]. MEHP is recognized as the major active metabolite of DEHP and constitutes approximately 70% of the metabolized DEHP.

Toxic Effects of MEHP.

MEHP has been observed to have toxic effects on a number of cell types, both *in vivo* and *in vitro.* Studies have indicated that MEHP alters cell differentiation in macrophage-monocyte cell lines [88] and bovine endometrial and ovarian cell lines [89], but is perhaps best characterized as a Sertoli cell toxicant. Acute exposure to MEHP causes germ cell sloughing in the testis. The mechanism of action is believed to be the effect of MEHP on structural proteins such as vimentin and claudin in the testis [90]. Other effects *in vivo* include atrophy of the testis and peroxisome proliferation in the liver of male rats [91].

Effects in the Male.

Epidemiological evidence has linked phthalate exposure to a number of adverse alterations in reproductive health. Acute exposures in the adult have been linked to reduced levels of free testosterone [92] and reductions in sperm quality [93]. *In utero* or perinatal exposure to phthalates have been linked to decreased AGD (a marker of feminization), increased instances of gynocomastia [94], and increased risk of hypospadias and other genital malformations [95]. Due to its established anti-androgenic properties, DEHP and phthalates in general have been linked to testicular dysgenesis syndrome (TDS) [95]. TDS is a proposed disorder of environmental origins associated with a trend observed over the past half century for increased incidence of male genital abnormalities, including cryptorchidism and hypospadias, and perhaps most alarmingly linked with increased rates of testicular cancer. In animal models, studies have indicated that *in utero* phthalate exposure suppresses testosterone production and induces gene expression changes [96], leydig cell hyperplasia, and multinucleated giant cells. Furthermore, *in utero* phthalate exposure appears to target the vimentin cytoskeleton and can disrupt the Sertoli cell-germ cell interface [97]. Because of the essential nature of the Sertoli cell to the survival of the germ cell, it has also been reported that high doses of MEHP will result in germ cell loss via apoptosis [98]. The specific mechanism of action is still being elucidated but it is clear that phthalates have effects both on the Sertoli cell (with resultant impact on the germ cells being supported by the Sertoli cell) and an effect on sex hormone production.

Effects in the Female.

While the majority of research on the toxic effects of MEHP has focused on its role as a testicular toxicant and anti-androgen, a number of effects have been observed in the female in *in vitro, in vivo,* and epidemiological studies.

Effects of MEHP on cultured cells of ovarian origin.

The effects of phthalate exposure on cultured cells have been well documented. One study [99] found a dose-dependent stimulation of gonadal steroidogenesis using both an immortalized mouse Leydig tumor cell lines (Line MLTC-1) and a mouse granulosa tumor cell line, KK-1. By assessing mRNA transcript levels and using a p450scc inhibitor, the researchers concluded that MEHP stimulates both of these steroidogenic cell types through a cAMP- and StAR- independent mechanism. This may be due to increased cholesterol trafficking. This finding was corroborated by another study which demonstrated that MEHP suppresses aromatase in cultured rat granulosa cells regardless of whether they were stimulated by FSHR or 8-bromo cyclic adenosine monophosphate, and therefore this suppression was not dependent upon cAMP. The authors concluded that this is a possible mechanism for known *in vivo* reductions in estradiol levels following acute MEHP exposure in the female. This will be discussed in more detail in the following section. A followup study [100] indicated that the effects of MEHP on aromatase may be unique amongst phthalate esters, but similar to PPAR agonist Wy-14,643. They conclude that MEHP may act through PPAR activation, summarized in fig 1.4 [101]. Briefly, the authors suggest that MEHP becomes bound to fatty acid binding protein (FABP) which acts to inhibit cAMP-mediated progesterone production.

Meanwhile, MEHP acts directly upon PPARα and PPARγ to suppress aromatase and therefore estradiol production.

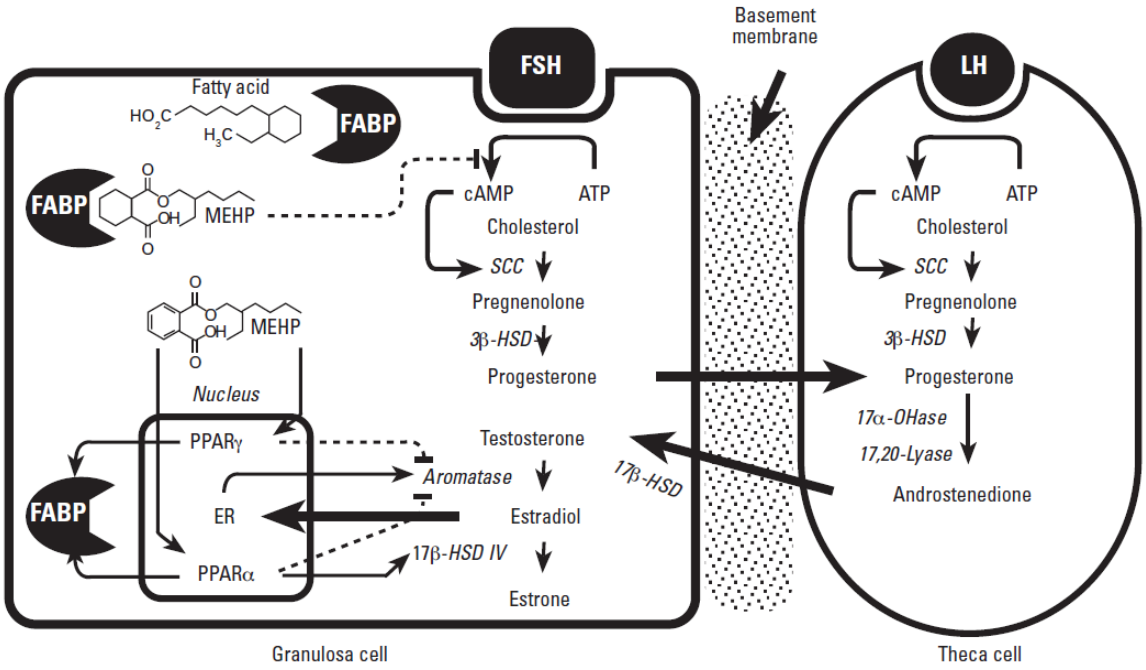

Fig. 1.4. Lovecamp-Swan and Davis' model of MEHP action via PPAR [101]

Another collaborating *in vitro* study used KGN cells, a granulosa-like human tumor cell line [102] under conditions of cAMP stimulation using forskolin. In this study, MEHP suppressed aromatase gene expression through the Cyp19 promoter II, the major aromatase promoter in humans.

***In Vivo* Female Studies**

A number of toxicity studies in female animal models have also been conducted. An adult exposure of 2 g/kg/day [103] caused a suppression of estradiol production, intriguingly not unlike the suppression of testosterone seen in similar *in utero* studies in the male. Furthermore, this exposure resulted in a suppression of ovulation and an

apparent shrinking of the granulosa cells of the maturing follicles suggesting a possible cellular target.

A number of studies have also assessed the effect of phthalates on the female *in utero.* One study by Gray et al 2006 [104] exposed pregnant rats to di-n-butyl phthalate, a phthalate structurally similar to DEHP. This study found that doses of 500 and 1000 mg/kg administered throughout pregnancy were sufficient to cause instances of mid-pregnancy abortions. However, live-born female offspring exhibited no significant reproductive abnormalities. *Ex vivo* examination of ovarian hormone production, however, indicated significant reductions.

Another study [105] was conducted using a wide range of doses of DEHP (from 0.015 to 405 mg/kg/day) given orally to pregnant Wistar rats from gestational day 6 through postnatal day 22, with a presumed lactational exposure. In the F1 generation, two markers of sexual maturity- day of vaginal opening and onset of estrous- were significantly delayed at doses as low as 15 mg/kg. Interestingly, secondary sexual characteristics, such as anogenital distance and nipple development, were not affected. This study strongly suggests that early developmental exposure to DEHP can alter at least some endocrine-mediated processes responsible for sexual development and maturation. However, a follow-up study using the same population [106] showed that these females exhibited higher levels of tertiary atretic follicles, but no other detectable alteration to their reproductive function once sexual maturity had been achieved.

Phthalate Studies in Human Populations

Occupational exposure to phthalates in factory workers has been linked to ammenhorea and infertility [107]. Phthalate exposure has also been reported as a risk

factor for a number of other conditions. Some of these may be related to its role as an endocrine disruptor. An association with diabetes [108], as well as some preliminary data suggesting that phthalates may be a risk factor in breast cancer [109], endometriosis, and uterine leiomyomata [110] have been published. Widespread detectable levels of metabolites in the urine of pregnant women have been observed [111]. This suggests that an *in utero* exposure to phthalates is extremely likely. However, a number of studies have also indicated that there is no association between phthalate exposure and precocious puberty [112] or endometriosis in infertile women [113].

Bisphenol-A

Bisphenol-A (BPA), like MEHP, is a chemical additive to plastics with known endocrine-disrupting properties. Phthalates are typically used to impart flexibility, while BPA is typically used to make plastics sturdier and more rigid, and is typically added to polycarbonate plastics. Unlike DEHP, BPA is widely used in food packaging, including polycarbonate water bottles, the linings of canned foods, and the inner bags of boxed wine. A number of other consumer products, such as thermal tape used in cash registers, are known to contain BPA [114]. BPA, like phthalates, also has the tendency to leach readily into liquids, particularly in the presence of heat or acidic or basic conditions. Due to this and its prominent role in many food packaging materials, ingestion is considered the major route of exposure for BPA although dermal absorbtion and inhalation have also been considered. Due to its ubiquity, exposure to BPA is considered universal in industrialized countries. The annual production may exceed 4 million tons, including an additional 100 tons released into the atmosphere [114].

Due to concerns of its possible health effects, a number of regulatory actions have been taken against BPA in the past decade. The results of a two-year carcinogenesis study in 1988 concluded BPA had a LOAEL of 50 mg/kg/day. The Food and Drug Administration used this to extrapolate a reference dose of 50 μg/kg body weight per day. This reference dose has proven controversial with critics claiming that this does not take into account evidence suggesting BPA possesses a non-monotonic dose response. It also does not take into account that the initial study was conducted in adult animals, rather than animals at an age more vulnerable to endocrine disruption such as perinatal or peripubertal exposures. BPA has proven controversial most recently, with a number of countries taking legislative action to reduce its use particularly in products used by children.

BPA: Mechanisms of Action as Xenoestrogen

Bisphenol A drew attention for its effects as an endocrine disruptor and xenoestrogen because of its homology with diethylsilbesterol (DES) (See fig. 1.5). DES was widely proscribed throughout the 1950's and beyond for prevention of miscarriage, though studies never demonstrated its efficacy in this role. Later, DES would become notorious for its teratological effects on female fetuses exposed *in utero*. While the best-documented effect is a much greater risk for vaginal clear cell adenocarcenoma (otherwise a very rare tumor type), other effects included life-long alterations in reproductive function, including uterine and vaginal malformations [115].

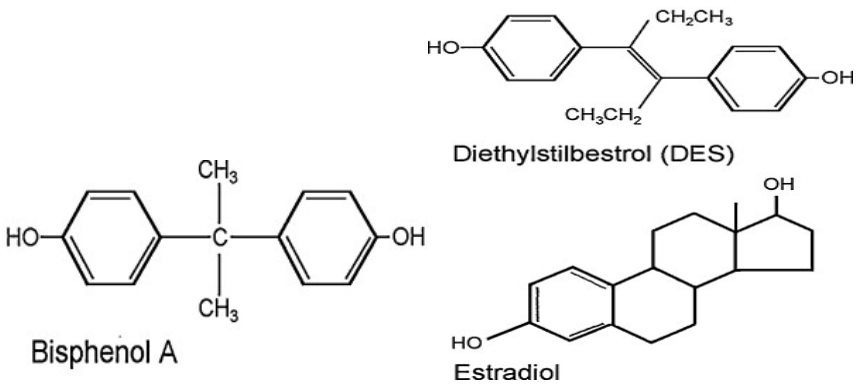

Fig. 1.5- structural similarities between BPA, DES, and Estradiol [114]

Like DES, BPA has been shown to bind to ERα and ERβ, though BPA has exhibited a significantly lower binding affinity. BPA is typically considered about 1000 to 10,000 times less potent than estradiol. However, a limited number of studies have suggested that BPA can, in certain cases, be equipotent with estradiol. These experiments, conducted in pancreatic [116,117] and adipose [118] cells, suggest that BPA may be acting through non-classic estrogen signaling pathways. Proposed alternate pathways include activation of G-coupled protein receptor 30 (GPR30) [119], and activation of membrane-specific and other non-classical ERs [120], as well as estrogen-related receptor γ, which does not bind estradiol and is expressed in the developing neonate and in the placenta [121]. Numerous other studies have emphasized the role of non-genomic responses to BPA exposure [122, 123], and a number of effects of BPA not observed with similar levels of exposure to other xenoestrogens suggesting that BPA may have a number of effects beyond its limited potency as an ER agonist [124]. Together, these studies suggest that while some aspects of BPA's biological activity are understood, much of its activity beyond its ability to act as a weak estrogen mimic is poorly understood.

BPA: Metabolism and Pharmacokinetics

Bisphenol A, once ingested, rapidly enters the circulation. However, it is rapidly glucuronidated in the liver, binding with glucoronic acid to form BPA-glucuronide, a metabolite which is rapidly eliminated in the urine. Unglucoronidated BPA is typically excreted in the feces [125]. This process is believed to be mediated by the liver enzyme uridine diphosphonate glucuronosyl transferase (UGT) [126]. BPA may also be sulfinated with similar results. Studies suggest that these metabolites do not appear to have any significant biological activity and do not bind the same receptors that BPA does [127].

One major consideration in BPA studies is the route of exposure. Because of the efficacy of glucuronidation, oral exposures typically show significantly higher LOAELs; following an oral exposure to rats, 60 to 100% of BPA found in circulating blood has been glucuronidated, as opposed to intraperitoneal or subcutaneous injections, where the majority of BPA is in its original form [125]. In similar human exposures to radiolabelled BPA, BPA-g was the only metabolite detected in serum and urine. Unchanged BPA was below the limit of detection in both serum and urine. These data strongly suggest that humans may be even less susceptible to exposure to BPA through oral exposure than suggested by metabolism in the rat [128]. Other studies corroborate this finding, showing that following oral exposures, unconjugated BPA does not exceed 2% of the circulating total BPA [129,130]. However, a more recent study [131] directly questions the findings from these studies, suggesting serious deficiencies in the experimental protocols. This study suggests that there is a detectable and non-negligible amount of unconjugated BPA in circulating serum within the ng/mL range.

Vulnerable Populations: BPA metabolism in non-adult populations

The studies discussed above were typically conducted in health adults, which is not necessarily the vulnerable population. Of concern are the potential effects of BPA *in utero*, on neonates, or on other individuals who may be at a stage of development vulnerable to endocrine disruption or unable to properly metabolize and excrete BPA. The National Toxicology Program, for instance, concluded that children under the age of 5 are likely to receive the highest BPA exposure by body weight [132]. For that reason, prenatal, neonatal, and young children are of particular concern for exposure and the ability of these populations to metabolize BPA deserves special mention.

In a study using healthy human placentas in an ex vivo model, BPA was able to cross the placental barrier with an efficiency of approximately 27%. The majority of the BPA remained in the unconjugated form by the time it crossed the placental barrier [133]. It should be mentioned that in this particular study, it was the unconjugated form of BPA that the placenta was exposed to. It is likely that *in vivo* a significant portion of BPA ingested by the mother would have gone through first pass metabolism before reaching the placenta and therefore the fetus. However, the study suggests that BPA can and will cross the placental barrier without any substantial metabolism in the placenta itself. The fetus' ability to metabolize unconjugated BPA is presumed to vary depending on its stage of development. Because of the known exposure and the variable ability of the fetus to metabolize or respond to this exposure, the developing fetus can be considered vulnerable to BPA.

The actual presence of BPA in various reproductive fluids has been assessed. Ikezuki et al [134] demonstrated the presence of BPA in both follicular fluid and

amniotic fluid, and discovered a significant elevation in detectable BPA in amniotic fluid at weeks 15-18 of gestation relative to other periods of pregnancy. BPA was found in amniotic fluid at concentrations 5-fold greater than the matching maternal serum levels. The authors suggest that fetuses swallow a significant amount of amniotic fluid during this period, though these increases in BPA concentration do not persist at term. This represents a period during which the fetus may be both exposed and be able to metabolize the BPA in the amniotic fluid.

Another study assessed BPA levels in urine samples from infants [135]. Fewer than half the samples had detectable levels of free BPA, and of those, BPA-g predominated relative to unconjugated BPA suggesting that infants metabolize BPA in a manner and degree similar to that of adults. However, because no serum samples were taken and unconjugated BPA is more typically excreted in the feces, the study did not definitively establish the efficacy of infant BPA metabolism but rather that glucuronidation did occur. The study also made the observation that infants fed by bottle exhibited BPA levels twice as high as those of infants fed by breast.

Premature births in neonatal intensive care units remain a population of concern (as with phthalates) because of their vulnerable status and the large quantities of plastics used in necessary medical interventions. A study by Calafat et al 2009 [136] suggest that these patients have ten-fold higher levels of circulating unconjugated BPA compared to the general population. While these patients also exhibit BPA-g, suggesting some capability of metabolism, the high levels of unconjugated BPA are still a source of concern.

These reports are just a limited survey of the information regarding perinatal BPA exposure. It is sufficient, however, to conclude that the perinatal population is of particular concern for a number of reasons: first, they may receive proportionally higher doses than adult populations; second, they may be unable to metabolize and clear BPA as effectively as adults; and finally, because depending on the stage of sexual development they may be acutely vulnerable to degrees of EDCs.

Effects of BPA on Ovarian Steroidogenesis

BPA has been used in *in vitro* experiments with cell lines as diverse as adipocytes, pancreatic cells, Sertoli cells, and a vast range of human-origin tumor lines. This section will limit itself to studies of particular relevance to this thesis; experiments specific to female reproduction. Both the female germ cell and ovarian support cells- granulosa and thecal cells- appear vulnerable to BPA exposure.

In Grasselli et al, 2010 [137], primary granulosa cells taken from pigs exhibited no alteration in proliferation, but a variety of alterations of endocrine effects, including reduced progesterone production and increased VEGF production. This latter effect, if found *in vivo,* might alter angiogenesis in the ovary. Perhaps the most intriguing finding, however, is a non-monotonic dose response for production of estradiol by the cultured granolusa cells. The lowest dose used, 0.1 μM, was sufficient to produce a small but significant increase in estradiol production, whereas the two higher doses (1 μM and 10 μM) suppressed estradiol production. The study does not speculate as to mechanism, and lacked a suitable positive control (e.g., ethnyl estradiol or genistein) to assess whether these results were strictly a result of BPA's ER agonist activity, but it provides additional support to the hypothesis that BPA exhibits a non-monotonic dose response.

These findings contradict another study [138] using primary granulosa and thecal-interstitial cells isolated from immature rats following prolonged estradiol stimulation. The authors found no non-monotonic dose response. Despite using identical doses as Grasselli et al, estradiol production was not significantly changed at the lowest dose in granulosa cells and remained suppressed at the higher two doses. Likewise, progesterone production was actually stimulated at all doses, in contrast with the Grasselli findings. These findings may not be directly comparable, due to the different species, but the difference in results suggests the difficulties inherent in comparing results between species.

A further study used isolated antral follicles to assess BPA's effects on steroidogenesis, arguing that the antral follicle is the most active functional unit of steroidogenesis in the ovary [139]. To that end, follicles isolated from mice were grown in culture and exposed to different concentrations of BPA. The authors conclude that high doses of 44 and 440 μM BPA are sufficient to cause significant inhibition of synthesis of a wide range of sex hormones, including progesterone, testosterone, and estradiol. This reduction was seen both in the levels of hormones released into the media and at the gene expression level with steroidogenic genes such as StAR and Aromatase down regulated in the presence of BPA. This study further demonstrates BPA's ability to alter sex hormone production *in vitro*, and using a model closer to *in vivo* effects by using the whole follicle as opposed to isolated cell populations.

Some epidemiological evidence exists that suggests similar effects in human populations. One study found a positive correlation between levels of BPA metabolites in the urine and gene expression of both ERα and ERβ in circulating leukocytes [140]. It

should be noted that circulating levels of sex hormones were not assessed and the role of ERs in leukocytes was not discussed.

A number of other possible endocrine-mediated pathologies have been associated with BPA exposure, including decreased sperm quality [141], sex-dependent differences in early childhood behavior [142], and diabetes mellitus [143]. While these epidemiological studies do not extend our understanding of BPA's mode of action, they do strongly suggest that BPA may pose a risk to human health through alterations of the endocrine systems.

Effects of BPA on Ovarian Function

Effects of BPA on the ovary are not limited to alterations in steroidogenesis, though those changes in steroid production and function would be sufficient to greatly alter ovarian function. Of particular interest for this thesis are alterations in follicular recruitment and maturation, as well as BPA's potential to interfere with germ cell cyst breakdown.

One study conducted in lambs demonstrated that an exposure of 50 μg/kg BPA (administered subcutaneously) daily between PND1 and 14 is sufficient to induce significant alterations in follicular dynamics [144]. Specifically, animals exposed to BPA or DES (as a positive control) had significantly fewer primordial follicles in the ovary, and a significantly elevated number of transitional and primary follicles. The small antral follicles in BPA-exposed lambs exhibited greater proliferation of both granulosa cells and thecal cells and higher instances of multi-oocyte follicles. At a molecular level, elevated levels of p27 were observed, which the authors used as a biomarker for follicular atresia. The authors conclude that BPA alters both the process of germ cell nest breakdown and

the activation of primordial follicles into maturing follicles. The significance of both the multioocytic follicles and the higher rate of follicular recruitment will be discussed in depth a later section.

The reduction in the primordial follicle pool has also been observed in a rodent model [145]. These animals received 4 IP injections over 8 days, with the highest dose being 20 μg/kg. The same tendency for follicular recruitment was detected at the high BPA dose. The same dose of DES exhibited a similar effect. This intriguingly suggests that while this phenotype may be due to ER agonist activity, BPA is acting at a greater potency than expected since DES is a much more powerful ER agonist. This study also had similar results with regards to p27 expression, but no significant changes were detected in the number of multi-oocyte follicles. This supports the findings of Rivera et al, and recapitulates the major findings in another species.

A somewhat contradictory finding was reported in a mouse model. In Zhang et al, 2011, [146] pregnant mice were exposed orally to doses of 20 to 80 μg/kg from gestational days 12 to 18. This study found an inhibition of germ cell nest breakdown at 80 μg/kg, but no other alteration of follicular recruitment. This difference may be due to difference in exposure (route of exposure, dose used, and duration of exposure all varied from previous experiments outlined) or species.

In summary, there is evidence to indicate that BPA may have effects on early folliculogenesis in the ovary, and that these effects may have profound effects throughout the organism's lifetime.

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Chapter II:

Reproductive Effects in F1 Adult Females Exposed *In Utero* to Moderate to High Doses of Mono-2-ethylhexylphthalate (MEHP)

This chapter has been published as "Reproductive Effects in F1 Adult Females Exposed *In Utero* to Moderate to High Doses of Mono-2-ethylhexylphthalate (MEHP)" by Moyer and Hixon, in Reproductive Toxicology. http://dx.doi.org/10.1016/j.reprotox.2012.02.006

ABSTRACT

Phthalates are widely used as plasticizers in everyday products. Yet, studies on the effects of phthalates on female reproductive health are limited. In this study, pregnant C57/Bl6 mice were exposed via oral gavage to corn oil, 100, 500, or 1000mg/kg MEHP from gestational days 17-19. Reproductive lifespan was decreased by one month in the highest F1 exposure group (9.8±0.4 versus 11.1±0.6 months in control F1 females). F1 females exhibited delayed estrous onset at the two higher exposures and prolonged estrus was observed in all MEHP-exposed females. Serum FSH and estradiol were significantly elevated at the highest exposure and altered mRNA expression was found for the steroidogenic genes LHCGR, aromatase, and StAR. At one year of age, mammary gland hyperplasia was observed in high dose MEHP-exposed females. In summary, late gestational exposure to MEHP leads to multiple latent reproductive effects throughout murine life resulting in premature ovarian senescence and mammary hyperplasia.

1. INTRODUCTION

Phthalate esters are used ubiquitously as plasticizers, with global production reaching over four million tons a year [1,2]. Di-ethyl-hexyl-phthalate (DEHP), the most widely used phthalate ester, is readily metabolized by esterases in the gut to mono-2-ethyl-hexyl-phthalate (MEHP). Exposure to DEHP can occur from a number of sources due to the widespread use of phthalates. Phthalate-containing plastics can leach phthalates readily into bodily fluids; consequently, food containers and medical devices such as blood bags and intravenous tubing can pose a significant route of human exposure to DEHP and other phthalates. Human exposure to phthalates has been established, as epidemiological studies show that an overwhelming majority of individuals have detectable levels of MEHP metabolites in their urine. High levels of the phthalate metabolite MEHP in the urine of pregnant women has been linked to cryptorchidism in their male offspring [2].

Phthalates have widely been considered to be male reproductive toxicants in rodents [3, 4] and epidemiological studies [5,6,7] and more recently xenotransplant models [8] suggest that exposure to plasticicizers in the human male results in reproductive abnormalities. Depending upon the phthalate ester (PE) and its route of exposure, *in utero* [3] or peripubertal [4], profound effects on the male reproductive system have been observed, including varying degrees of cryptorchidism, germ cell death, malformations of the epididymis, vas deferens, seminal vesicles, and prostate and decreased levels of testosterone [2,3,4]. In addition to the adverse reproductive effects observed in the male, studies have demonstrated that the ovary is also vulnerable to phthalate exposure [9]. High doses of DEHP have been shown to suppress serum

estradiol levels and ovulation in adult female rats [10]. Another study found that exposure from conception through lactation in the rat leads to increased tertiary atretic follicles by adulthood but no other reproductive abnormalities were observed [11, 12]. Chronic exposure to di-butyl-phthalate (DBP) from gestation through pregnancy reduces fertility in rats [13]. In humans, a report on female workers in a phthalate processing plant found that women exhibited anovulatory cycles in response to high occupational exposure to phthalates [14].

We conducted the present study to determine the effects of MEHP on the female murine reproductive system because no studies to date have evaluated the adult reproductive effects in mice following *in utero* exposure to this metabolite. Importantly, administration of 500 mg/kg MEHP is within the dose range previously shown to lead to altered reproductive function in the female rodent [11]. The doses utilized in the present study are not considered physiologically relevant. The Agency for Toxic Substances and Disease Registry in 1993 reported a maximum daily intake of 2 mg in the general population. However, subsets of the population may receive levels significantly higher than 2mg per day [15]. Medical interventions with DEHP-containing devices or pharmaceuticals have been demonstrated to contribute to elevated levels of DEHP metabolites in urine and/or blood. In rodent models, phthalates have been shown to cross the placental barrier, with concentrations in the fetus within two-fold compared to those found in the mother [16]. An exposure window from GD17 to GD19 was selected for *in utero* exposure to MEHP. This period of exposure was selected because although it is after the ovary has been established, it is a key period of folliculogenesis. This is the critical period of germ cell nest breakdown and primordial follicle formation [17]. This

process has been demonstrated to be sensitive to endocrine disruption in rodents [18] and was chosen as a narrow but relevant window of exposure. Based on prior studies [18,19], we hypothesized that late gestational exposure to MEHP effects the process of follicular recruitment in these animals resulting in adult life reproductive effects such as premature ovarian senescence and mammary gland hyperplasia.

2. Materials and Methods

2.1 Mice Timed-pregnant C57/Bl6 mice were purchased from Charles River Laboratories (Shrewsbury, MA). Animals were kept in climate-controlled colonies (23.3±2 degrees Celsius; humidity 30-70%) with a 12 hour light/dark cycle. All procedures involving animals were performed in accordance with the guidelines of the Institutional Animal Care and Use committee of Brown University in compliance with the guidelines established by the National Institute of Health.

2.2 Exposure Paradigm At gestational days 17, 18, and 19 (with day of plug detection considered GD1), pregnant dams were exposed via oral gavage to 100, 500, or 1000 mg/kg MEHP (TCI America) dissolved in corn oil, or an equivalent volume of corn oil as a vehicle control. Typical dosage volumes ranged from 40 µl to 60 µl. Seven dams were exposed per treatment group. Dams were monitored for changes in weight throughout the exposure period. Pups were counted at birth and weighed every other day from PND1 to 21 and weaned at PND 25. Whenever mice were necropsied, they were sacrificed on the first observed day of the estrous cycle to maintain consistency within and between treatment groups.

2.3 Reproductive Endpoints Reproductive endpoints assessed included age at vaginal opening, age of first estrous cycle, and estrous cycle length and phase. Estrous cycles were determined via vaginal lavage cytology followed by staining with toluidine blue and recorded daily for 17-day spans from PND40 to PND56, and for mice not sacrificed at PND56, for at least 7 sequential days per month. All cycles were measured at approximately 10 A.M. daily. Assessments were completed following guidelines set by Pedersen [20].

2.4 Continuous-Breeding Assay. In order to assess the reproductive fitness of female mice, pregnant C57/Bl6 dams were exposed via oral gavage to 100, 500, or 1000 mg/kg MEHP dissolved in corn oil or an equivalent volume of corn oil as vehicle control from gestational days 17-19 as described above. The F1 female pups were then paired with healthy C57/Bl6 male mice for breeding starting at an age of 8 weeks. Mating pairs were placed in separate cages and inspected each morning. After a litter was weaned, the female was paired with a new male to avoid low reproducibility due to male reproductive dysfunction. If no litter was produced, the female was paired one more time with a healthy male to confirm female infertility. Litter size and cumulative number of progeny per female were recorded for each group and used to evaluate the reproductive fitness of female mice. The ages of the control and F1 exposed females at their final delivered live litters were recorded.

2.5 Mammary Gland Whole Mount Analysis F1 females were sacrificed at PND365, following either continuous breeding or non-breeding isolation. At sacrifice, inguinal mammary glands from the fourth and ninth positions were removed, and mounted via the whole mount protocol described in Fenton et al, 2002 [21]. Briefly, mammary glands

were placed on microscope slides and fixed in Carnoy's fixative. Following rehydration they were stained using carmine solution, dehydrated via ethanol and xylene washes, and photographed via a dissecting microscope (Zeiss Stereo Discovery V.8, Carl Zeiss International).

2.6 Hormone Assessment To measure serum FSH, estradiol, and LH levels, blood samples were collected by cardiac puncture from female mice at the first detected day of estrus. Mice were euthanized via carbon dioxide asphyxiation and all necropsies were performed within an hour of establishing estrus stage. The serum was stored at −80°C until assayed. FSH and LH levels were assayed by the University of Virginia Ligand Core Facility as previously described for radioimmunoassay of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (http://www.healthsystem.virginia.edu/internet/crr/ligand.cfm; Specialized Cooperative Center Program in Reproductive Research, National Institute of Child Health and Human Development/National Institutes of Health U54 HD28934). Estradiol levels were measured utilizing the Estradiol ELISA kit (11-ESTHU-E01) obtained from Alpco Immunoassays (Salem, NH) according to the manufacturer's specifications.

2.7 RNA Isolation Mice were necropsied at PND56, ovaries were then flash-frozen, and stored at -80°C until further processing. RNA was extracted from whole ovaries by homogenization with Tri-Reagent (Sigma-Aldrich, St. Louis, MO), followed by isolation using an RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA quality and concentration was determined using a NanoDrop Spectrophotometer (Thermo Scientific, Wilmington, DE). RNA Samples with concentrations less than 100

ng/μl or with a 260/280nm absorption ratio less than 1.95 were not included in further studies.

2.8 Gene Array At PND56, RNA was isolated from the ovaries of different litters of F1 females exposed *in utero* to 1000 mg/kg MEHP (N=4 ovaries) and from the ovaries of different litters of F1 females exposed *in utero* to corn oil (N=3 ovaries). The ovarian RNA was then hybridized for gene array experiments. Microarray analysis was conducted using Affymetrix GeneChip Mouse Gene 1.0 ST Arrays (Affymetrix, Santa Clara, CA) following manufacturer's protocols. Statistical analysis of the raw CEL files was performed with ParTek Genomics Suite Software (Partek, St. Louis, MO) using Robust Multiple Array (RMA) analysis with log2 transformation and gating to include only genes differentially expressed with a p-value of less than 0.01 and at least a 2-fold difference relative to controls.

2.9 Quantitative RT-PCR Total RNA was treated with DNase-I (Invitrogen, Carlsbad, CA) and then reverse-transcribed with iScript cDNA Synthesis Kit (Bio-Rad) following the manufacturer's protocols, and the cDNA templates were amplified with QuantiTect primers (Qiagen) using iQ SYBR Green Supermix (Bio-Rad) on an iCycler iQ Multicolor Real-time PCR Detection System (Bio-Rad). Each sample was run in triplicate, and mRNA levels were analyzed relative to hypoxanthine phosphoribosyltransferase (HPRT), a housekeeping gene unaltered by MEHP exposure [22, 23]. Log2-transformed relative expression ratios were calculated as described using the equation set forth by Pfaffl [24], in which efficiencies for both the gene of interest and the calibrator hypoxanthine phosphoribosyltransferase (HPRT) were used. To avoid litter effects, only one female per litter was used for RNA, with 7 litters per treatment group used for RT-PCR.

2.10 Follicle Counts Ovaries were removed at sacrifice from PND56 or PND365 mice and fixed in Bouin's Fixative (Sigma-Aldrich) for 24h, then processed and embedded into paraffin for sectioning. Selected ovaries were sectioned completely into 7μm sections. Every other section was counted and follicles classified as either primordial, primary, secondary, or antral. Primordial follicles were defined as those containing an oocyte with a single layer of flat granulosa cells. Follicles were classified as primary if they contained an oocyte surrounded by 7 or more cuboidal granulosa cells. Secondary follicles were classified as having two or more complete layers of granulosa cells. Antral follicles were classified as having a visible antrum with an area greater than that of the oocyte. Only follicles with visible nuclei were counted to prevent counting the same oocyte multiple times. Total ovary area per section was measured using Aperio Imagescope Software (Aperio Technologies, Vista, CA) and the number of follicles were divided by ovary area to compensate for possible differences in ovary size and section location. As with the RT-PCR, only one ovary per litter was selected for counting to avoid litter effects. Follicle counts were normalized to area counted.

2.11 KGN Cell Culture The human granulosa-like KGN cell line [25], grown in DMEM/F-12 medium supplemented with penicillin (50 U/ml), streptomycin (50 μg/ml) and FCS (10% v/v) from cells kindly provided by Richard Freiman (Brown University). Cells were maintained as monolayer cultures in 100 mm dishes at 37°C in 5% CO_2. Cells were serum-starved for 24h, followed by 24h exposure to MEHP at 10 or 100 μM or EtOH vehicle control, in combination with either forskolin (25 μM) or additional EtOH, following the protocol established by Ohno et al [26]. Ethanol did not exceed 0.05% by

volume in any treatment group. Following exposure, cells were harvested for RNA isolation and real-time RT-PCR as described above.

2.12 Statistical Analysis All animal data are presented as the mean ± SEM. Data were analyzed using two-way ANOVA analysis (SigmaPlot software version 8). Each data point for animal experiments represents a single randomly selected individual from an *in utero* exposed litter. A $p < 0.05$ was considered statistically significant.

3. RESULTS

3.1 Maternal body and pup weights. Administration of MEHP at 100, 500, and 1000mg/kg from GD17-19 did not affect maternal body weight or pup growth. Maternal body and pup weights are shown in **Table 1**. Maternal body weight was recorded immediately prior to administering the first dose of MEHP (GD17) and at the time of final dosing of MEHP (GD19). Seven pregnant dams were examined per exposure group. A minimum of two pups were randomly selected from control and exposed litters and weighed at postnatal day 7. Again, we found no significant differences in pup weight between corn oil exposed and MEHP exposed pups (**Table 1**). This trend continued throughout the lifetime of the animal, including at PND28 (within 2-3 days of the mean day of vaginal opening for all treatment groups) and at PND56, the young adult sacrifice timepoint. There were no significant differences in animal weight between exposure groups when matched for age.

Table 1.

Treatment	Weight (g) at PND7	Changes in maternal weight during dosing (g)
Control	3.1 ± 0.7 (n=14)	2.0 ± 0.6 (n=7)
100 mg/kg	3.3 ± 0.9 (n=14)	2.3 ± 1.0 (n=7)
500 mg/kg	3.6 ± 0.8 (n=14)	1.7 ± 1.1 (n=7)
1000 mg/kg	3.2 ± 1.0 (n=14)	1.6 ± 1.5 (n=7)

Table 1. *In utero* exposure to MEHP does not induce overt maternal toxicity or changes in pup weight.

3.2 Vaginal Opening and Estrous Cyclicity. Beginning at 25 days of age, randomly selected F1 females from both control and MEHP-exposed litters were monitored daily for vaginal opening. A trend was found for delayed vaginal opening in both the 500mg/kg and 1000mg/kg MEHP exposure groups (**Table 2**). Following vaginal opening, F1 females were monitored for the onset of estrous (**Table 2**). At both the 500mg/kg and 1000mg/kg MEHP exposure groups, there was a four-day delay in estrous onset ($p < 0.01$). Body weights for these animals remained consistent across treatment groups (**Table 1)**, suggesting that alterations in maturity are not related to general growth differences. In both the 500mg/kg and 1000mg/kg exposure groups, F1 females exhibited a prolonged estrous phase of approximately twice the length of control F1 females ($p < 0.01$) (**Fig. 1**). The duration of metestrous was also significantly increased relative to controls in the 500mg/kg and 1000mg/kg F1 adult females (**Fig. 1**). Adult F1 females in the 100mg/kg MEHP exposure group exhibited a similar pattern to the higher exposure groups; however, only the length of metestrous was significantly increased relative to control females ($p< 0.05$). A strong trend for an increase in the time spent in the estrous stage of the cycle was observed in F1 females in the 100mg/kg MEHP exposure group ($p = 0.06$) (**Fig. 1**). A total of 14 females from 7 random litters per group were examined.

Moreover, altered estrous cyclicity was observed in exposed F1 females for up to one year.

Table 2.

Treatment	Vaginal Opening (PND)	Onset of Estrous (PND)
Control	30.0 ± 1.3 (n=14)	35.1 ± 1.9 (n=14)
100 mg/kg	30.0 ± 1.4 (n=14)	35.6 ± 1.0 (n=14)
500 mg/kg	30.7 ± 2.7 (n=14)	38.9 ± 1.8** (n=14)
1000 mg/kg	30.8 ± 2.5 (n=14)	39.3 ± 1.9** (n=14)

Table 2. Markers of sexual maturity in *in utero* exposed F1 females. * indicates significant difference from controls ** p< 0.01.

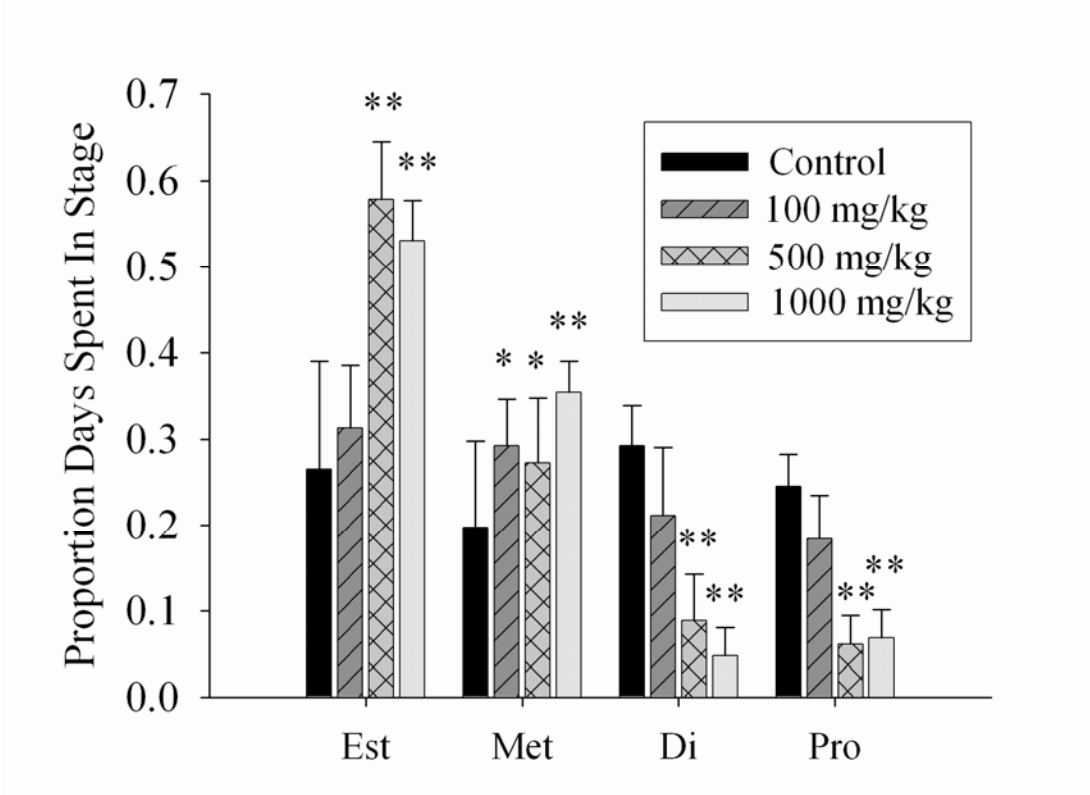

Figure 1. Effects on the estrous cycle of F1 females exposed *in utero* to vehicle control, 100mg/kg, 500mg/kg, or 1000mg/kg MEHP. Graph depicts the fraction of time spent in each stage by exposure group. Numerical values represent the percent of time spent per day in a particular stage of estrus; means + standard deviation are shown. * indicates significant difference from controls as determined by (1 or 2-way?) ANOVA * $p < 0.05$; ** $p < 0.01$.

3.3 Litter size and Reproductive Lifespan. Adult F1 females exposed *in utero* to MEHP were housed with unexposed male mice of proven fertility. All breeding pairs successfully had 3 or more litters and litters were weaned at PND25. Initially, at 1000mg/kg MEHP, adult F1 females exhibited a significant increase in average litter size (7.6 ± 1.6) relative to control females (5.9 ± 1.0) ($p < 0.05$) (**Fig. 2a**). Since C57/Bl6 mice typically have litter sizes of 5-6 pups, the observed increase in litter size (7-9 pups per litter) prompted us to determine the reproductive lifespan of the *in utero* exposed F1 female generation. We found that over a ten month period, the total number of pups delivered per group did not differ significantly among the exposure groups (23.6$\pm$3.1 pups [corn oil]; 21.5$\pm$1.2 pups [100mg/kg MEHP]; 21.3+ 4.8 pups [500mg/kg MEHP]; and 23.0$\pm$2.6 pups [1000mg/kg MEHP]). However, continual breeding of exposed F1 females with males of proven fertility revealed a one month decrease in the overall reproductive lifespan of the F1 female generation in the highest exposure group relative to controls. Adult F1 females exposed *in utero* to 1000mg/kg MEHP delivered their final litter at 275.5 ± 11.4 days of age, compared to 310.5 ± 16.8 days of age in the controls ($p < 0.05$) (**Fig. 2b**).

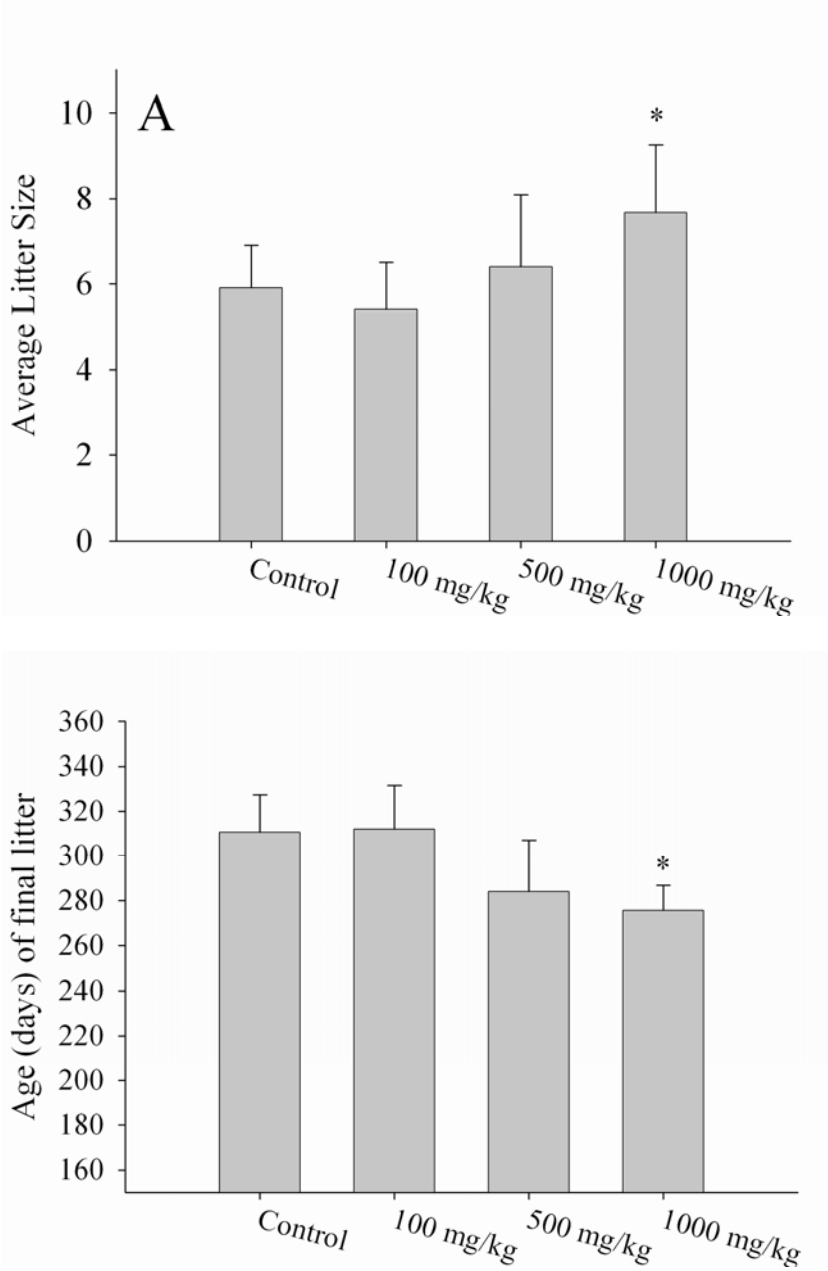

Figure 2. Effects on fertility of F1 females exposed *in utero* to 0mg/kg, 100mg/kg, 500mg/kg, or 1000mg/kg MEHP. F1 females were bred with unrelated male mice of proven fertility. (a) Total number of pups per litter were counted and averaged for all the exposure groups. N = 12 litters/4 breeding pairs (control); N= 8 litters/3 breeding pairs (100 mg/kg); N= 10 litters/3 breeding pairs (500 mg/kg); and 9 litters/4 breeding pairs (1000 mg/kg); means and standard deviation are shown. (b) Effect of *in utero* exposure to MEHP on F1 female reproductive lifespan. The graph depicts the age of F1 females at a given exposure at delivery of last live litter. All F1 female mice were sacrificed at day 365. Values on the figure are average ages and SD. indicates the mean differed from control as determined by ANOVA; * $p < 0.05$.

3.4 Follicle counts Figure 3 depicts the follicle counts of corn oil exposed and MEHP exposed ovaries from adult F1 females sacrificed on the first day of estrous. Follicle counts were determined in one ovary per mouse from one random female per litter (N=7 litters). To determine the number of follicles present, we counted the number of follicles per type per section, and normalized the number of follicles counted per section to the total ovary area of that section to control for possible differences in ovary size, orientation, or section depth. We found no significant differences in the number of primordial follicles between either the control or exposure groups at any of the doses of MEHP administered. Scoring of primary follicles revealed that in 500mg/kg/day MEHP (1.05 ± 0.14) and 1000 mg/kg/day MEHP (1.04 ± 0.16 mm^2) exposure groups, there was a trend for higher numbers of primary follicles relative to the corn oil exposed group (0.90 ± 0.12 mm^2) (**Fig 3**). We also observed a significant increase in the number of secondary follicles at 500 and 1000mg/kg/day MEHP. In the 500 mg/kg/day exposure group, there were 0.79 ± 0.13 secondary follicles per mm^2 ($p < 0.05$) and at 1000mg/kg/day there were 0.91 ± 0.25 follicles per mm^2 compared to 0.55 ± 0.12 follicles per mm^2 in control ovaries ($p < 0.05$). The number of antral follicles was significantly increased at the higher exposure groups relative to control ovaries with 0.20 ± 0.07 antral follicles per mm^2 in controls compared to 0.29 ± 0.06 at 500 mg/kg/day and 0.30 ± 0.07 follicles per mm^2 at 1000 mg/kg/day ($p < 0.05$**).**

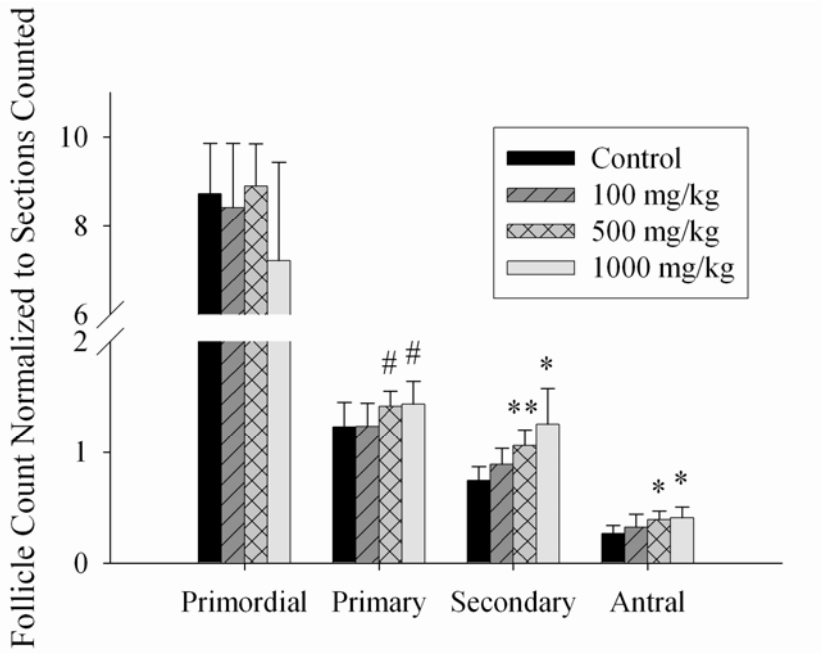

Figure 3. Effects on ovarian follicle counts in F1 females exposed *in utero* to 0mg/kg, 100mg/kg, 500mg/kg, or 1000mg/kg MEHP. Representative H&E stained sections of corn oil control (A) and 1000 mg/kg MEHP exposed (B) ovaries at PND56. Bar indicates 100 μM. (C) Ovarian follicle counts in females exposed *in utero* to MEHP, normalized to section area. Bars represent the mean and SD of ovaries from at least three separate litters for both F1 control and F1 exposed litters. indicates the mean differed from control as determined by ANOVA; * $p < 0.05$.

3.5 *Mammary Gland Whole Mounts.* Inguinal mammary glands (positions 4 and 9) removed from F1 females at 365 days of age were mounted, fixed, and stained for gross assessment. **Figure 4** depicts the whole mount images of mammary glands from F1 virgin (**Fig 4. c,d**) and F1 breeding females (**Fig. 4a,b**) exposed to corn oil (**Fig 4. a,c**) or 1000mg/kg MEHP *in utero* (**Fig 4. b,d**). Regardless of breeding status, F1 females exposed to 1000 mg/kg MEHP *in utero* (**Fig. 4.b,d**) exhibited significant hyperplasia relative to corn oil exposed (**Fig 4a,c**) with more extensive branching and the presence of hyperplastic nodules (**Fig 4c,d**).

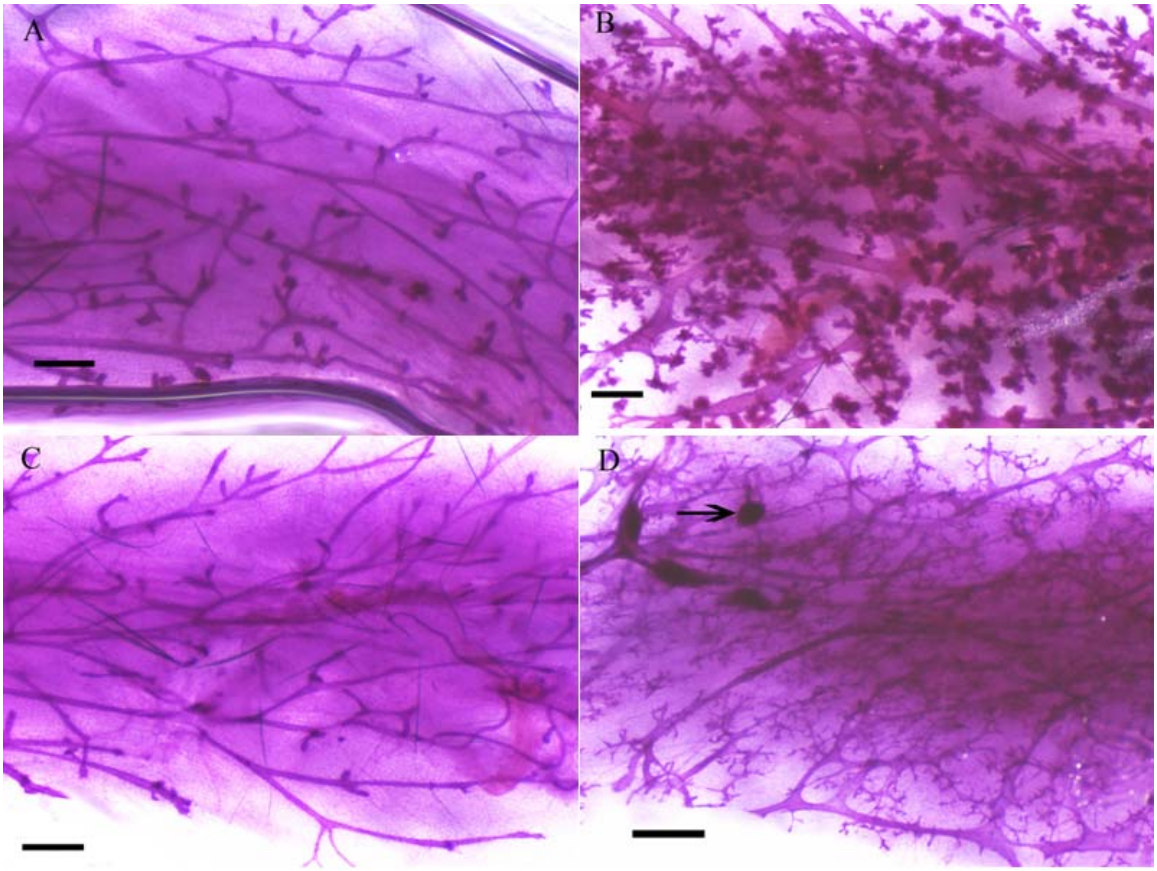

Figure 4. Effects of *in utero* exposure to 1000 mg/kg MEHP on mammary gland hyperplasia at PND365. Mammary glands were removed and whole mounted and stained with carmine solution. A. Mammary gland of virgin control female. B. 1000 mg/kg *in utero* exposed virgin female. C. Mammary gland of breeding control dam. D. 1000 mg/kg *in utero* exposed breeding dam. Bar indicates 1 mm. Arrows indicate hyperplastic alveolar nodules.

3.6 Serum Hormone Levels Levels of estradiol, FSH, and LH in serum isolated from F1 females at PND56 were measured by ELISA. All serum samples were obtained during the first day of estrous stage of the estrous cycle. Mean estradiol levels were significantly higher ($p < 0.05$) in the 1000mg/kg/day exposure group (153 ± 17 pg/mL) compared to controls (92 ± 37 pg/mL) (**Fig. 5a**). A strong trend for increased serum estradiol levels was detected at 500mg/kg (135 ± 31 pg/mL, $p = 0.075$). No significant difference was detected in the 100mg/kg/day group compared to control. For the estradiol

assay, the numbers of mice utilized per group were the following: N=6 for control group and 1000 mg/kg, n=5 for 100 and 500 mg/kg. Serum FSH levels (**Fig. 5b**) were significantly elevated at 1000mg/kg (7.6 ± 3.1 ng/μL, $p < 0.05$) relative to control (3.7 ± 2.0 ng/μL). Serum LH levels (**Fig. 5c)** showed no significant differences at the 100 or 500mg/kg/day exposure groups; however, at 1000mg/kg/day there were lower levels of LH (0.15 ± 0.10 ng/μL) relative to controls (0.25 ± 0.08 ng/μL, $p = 0.05$). For the FSH and LH assays, the numbers of mice utilized per group were the following: N=6 controls, N=6 1000 mg/kg, N=5 500 mg/kg, N=4 100 mg/kg.

Additionally, 4 control mice and 4 1000 mg/kg exposed females were sacrificed at PND365 and the serum also analyzed for estradiol concentrations **(Fig. 5d)**. In these animals, control mice exhibited significantly higher serum estradiol than the exposed mice (73.3 ± 14.0 pg/mL vs. 51.2 ± 4.1 pg/mL, respectively).

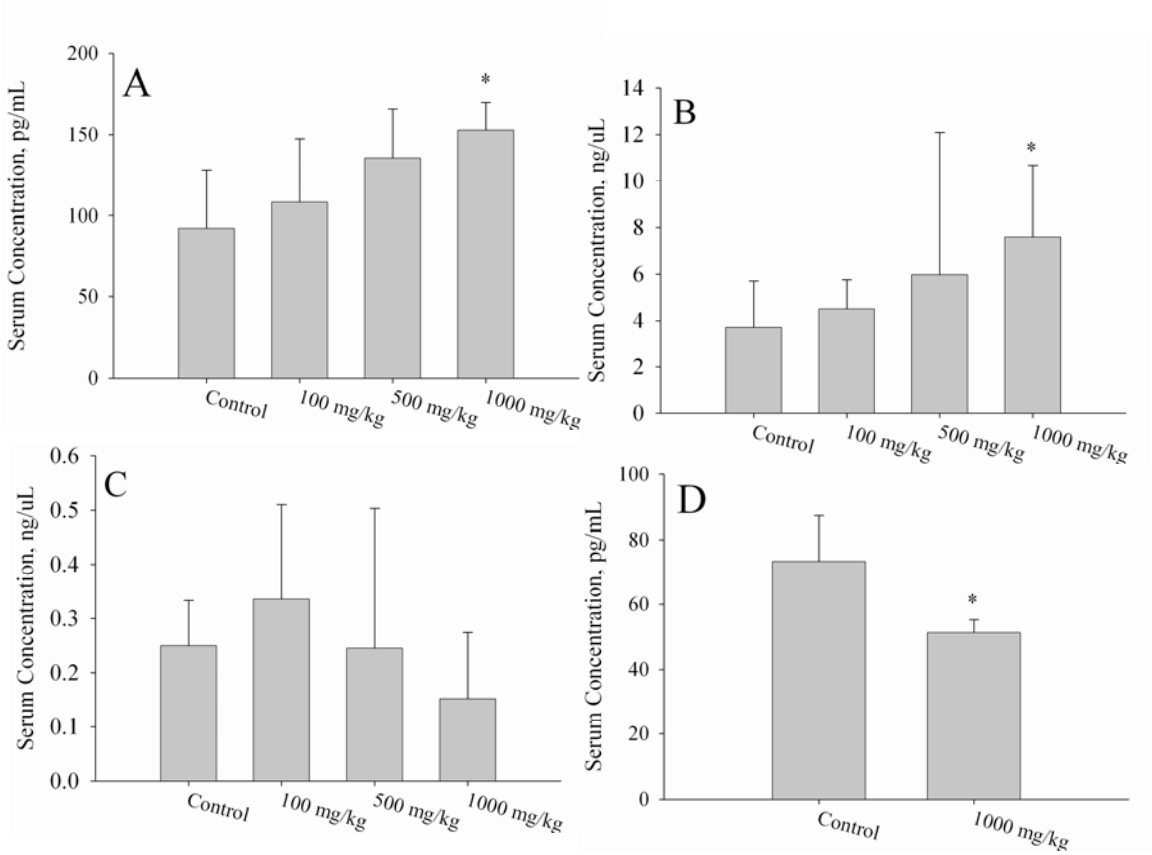

Figure 5. Effect of *in utero* to 0mg/kg, 100mg/kg, 500mg/kg, or 1000mg/kg MEHP on A. Estradiol at PND56; B. FSH; C. LH hormone production, and D. Estradiol at PND365. Serum was collected via cardiac puncture at approximately two months of age during estrus and various hormone measurements were made by ELISA. A minimum of 3 litters each per control and per exposure group were examined. Each bar represents the mean and SD of serum from mice of at least 3-5 separate litters for both F1 control and F1 exposed litters. * indicates $p < 0.05$ relative to controls.

3.7 ***Affymetrix Gene Array and Quantitative RT-PCR*** Table 3 depicts selected Affymetrix Gene array results performed on mRNA isolated from ovaries of different litters of corn oil exposed (N=3 litters) and 1000mg/kg/day MEHP exposed (N=4 litters) F1 adult females (PND56). PND56, as a young adult age in the mouse, was selected to see whether the *in utero* exposure had caused gene expression changes detectable at sexual maturity. Our results indicated 85 differentially expressed genes which exhibited both a 2-fold difference in expression and a p value of < 0.05 (**Table S1**). Genes of

interest were selected because of their role(s) in hormone signaling and/or reproductive development and validated by quantitative RT-PCR. We found a strong correlation between our array results and the quantitative RT-PCR analyses (**Fig 6**). Aromatase mRNA expression was reduced by 50% relative to corn oil exposed controls at both the 500mg/kg/day ($p < 0.01$) and 1000mg/kg/day MEHP ($p < 0.05$) exposure groups (**Fig. 6a**). The most striking changes were observed for the steroidogenic acute regulatory protein (StAR) with decreased expression of 30% at 100mg/kg/day ($p < 0.05$), 50% at 500mg/kg/day ($p < 0.01$) and 60% at 1000mg/kg ($p < 0.01$) (**Fig. 6b**). Luteinizing hormone/choriogonadotropin receptor (LHCGR) mRNA expression was also reduced by approximately 50% in both 500 and 1000mg/kg/day ($p < 0.01$) exposure groups relative to control litters (**Fig. 6c**).

Table 3.

ID	Gene Symbol	Gene Name	Fold Change	P value
NM_020278	**LGI1**	leucine-rich, glioma inactivated 1	4.840	1.94E−02
NM_019779	**CYP11A1**	cytochrome P450, family 11, subfamily A, polypeptide 1	−2.096	6.35E−03
NM_181754	**GPR141**	G protein-coupled receptor 141	−2.620	4.17E−02
NM_013582	**LHCGR**	luteinizing hormone/choriogonadotropin receptor	−2.776	9.81E−03
NM_207683	**PIK3C2G**	phosphoinositide-3-kinase, class 2, gamma polypeptide	−3.077	1.68E−04
NM_011485	**STAR**	steroidogenic acute regulatory protein	−3.405	5.71E−04
NM_016668	**BHMT**	betaine--homocysteine S-methyltransferase	−9.009	5.98E−04

Table 3. Affymetrix gene array indicates a number of significantly altered genes of interest.

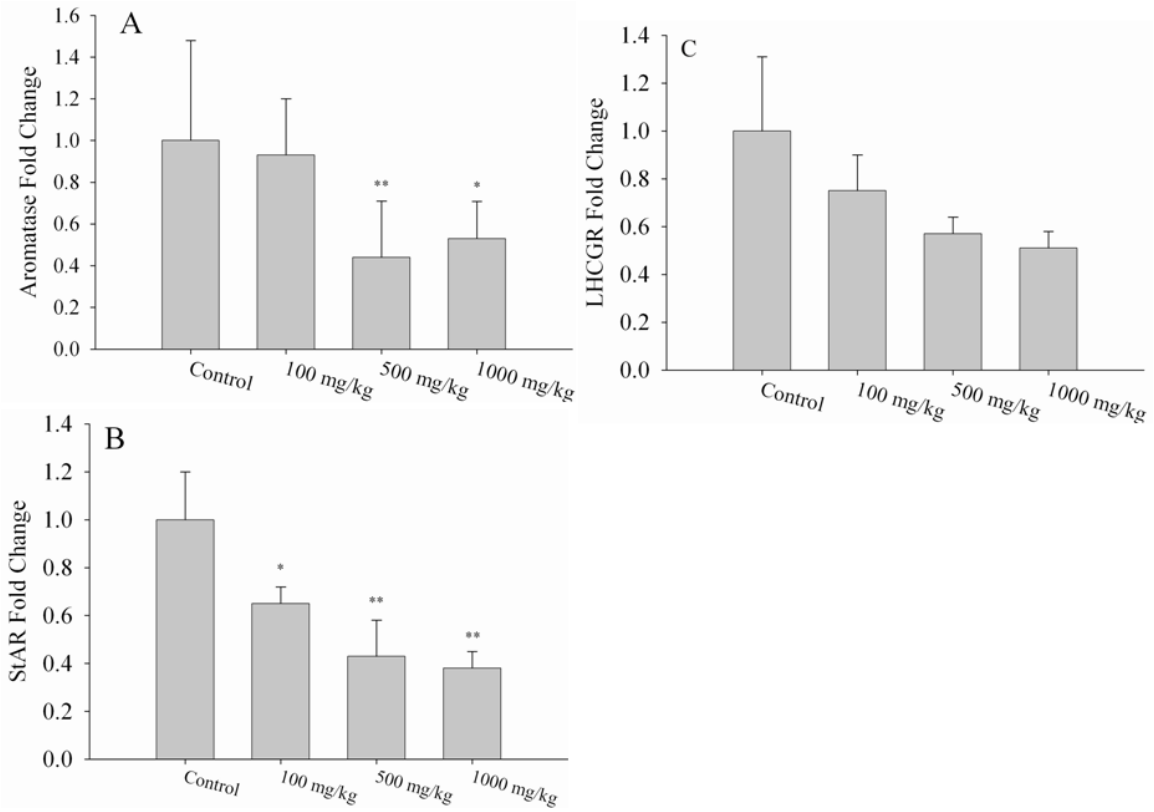

Figure 6. Effect of MEHP on **A.** Aromatase; **B**. StAR,; and **C.** LHCGR mRNA expression from *in utero* exposed and control F1 female ovaries. All expression was normalized to the house keeping gene, HPRT. Each bar represents the mean and standard deviation of ovaries from at least three separate litters for both F1 control and F1 exposed litters.* indicates significant difference from controls: * indicates $p < 0.05$; ** indicates $p < 0.01$.

3.8 KGN in vitro cell culture To determine if *in utero* exposure to MEHP targets the granulosa cell population, we examined the expression of StAR, Aromatase, LHCGR, and FSHR in the KGN cell line [25,26], a human granulosa-like cell line. We chose doses of both forskolin (FSK) and MEHP that did not significantly alter KGN cell viability (**Fig. S2**). RNA was then isolated from the KGN cell line for real time RT-PCR. Consistent with our *in vivo* observations, aromatase (**Fig. 7a**), StAR (**Fig. 7b**), LHCGR (**Fig. 7c**) and FSHR (**Fig. 7d**) expression were all significantly decreased at 100 μM MEHP relative to forskolin controls ($p < 0.01$ for Aromatase, LHCGR, and FSHR; $p <$

0.05 for StAR). At 10 μM MEHP both aromatase and LHCGR were significantly decreased ($p < 0.05$). No significant differences were detected between non-FSK controls and non-FSK MEHP-exposed KGN cells.

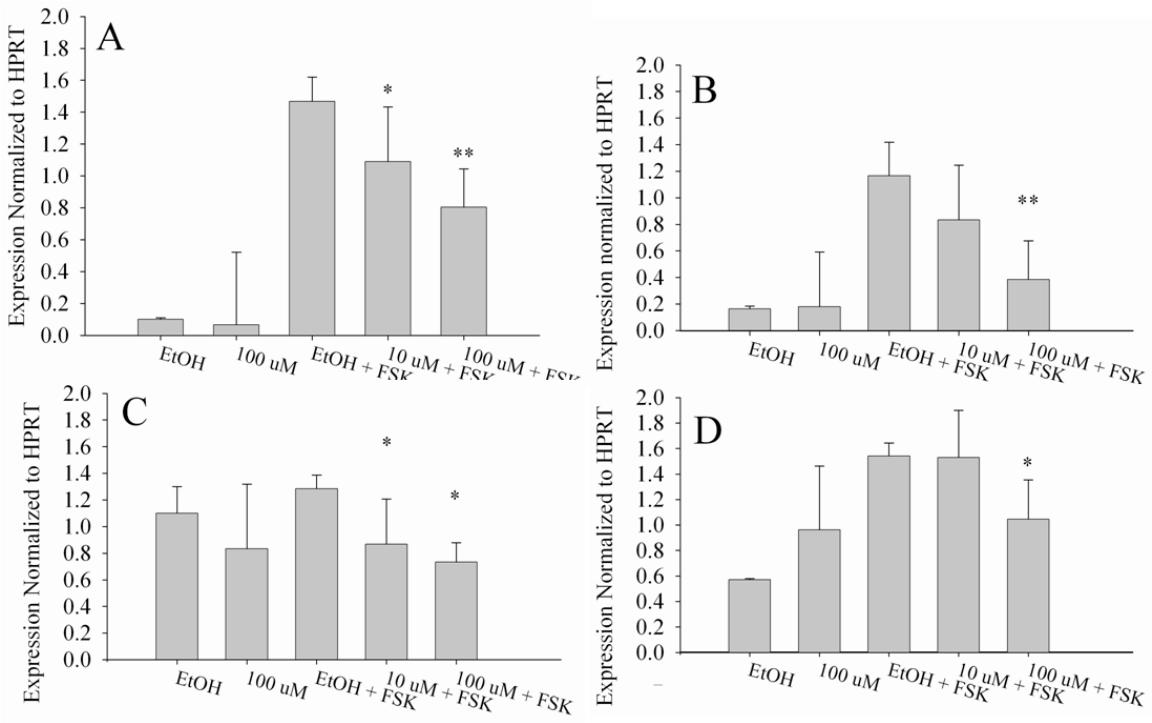

Figure 7. Effect of MEHP on **A.** Aromatase; **B**. StAR; **C.** LHCGR; and **D.** FSHR mRNA in a granulosa-like cell line, KGN. The KGN Granulosa-like cell line was cultured for 24 h with 100μm MEHP, 25 μM Forskolin (FSK), 10 μm MEHP, or 100 μm MEHP plus FSK or vehicle control (ethanol). **A.** Aromatase; **B**. StAR; **C.** LHCGR; and **D.** FSHR mRNA was measured using real time RT-PCR and normalized to HPRT. Each bar represents the mean and/SD of at least three separate experiments. * indicates $p < 0.05$ relative to forskolin control. ** indicates $p < 0.01$.

4. DISCUSSION

Our present study adds to the growing body of evidence that *in utero* or early life exposures to endocrine disrupting chemicals accelerates female reproductive senescence in the rodent [27,28]. The consequences of such exposures in humans remain to be determined. Epidemiological studies have demonstrated an association between EDC exposure and either anovulatory cycles or early menopause. For example, a group of

female workers in a phthalate processing plant exhibited anovulatory cycles following high occupational exposure to phthalates [14]. Another study indicated women exposed *in utero* to diethylstilbesterol have increased risk of earlier onset of menopause [29]. Moreover, exposure to the endocrine disrupting chemicals dioxin and DDE (dichlorodiphenyltrichloro- ethane), a metabolite of DDT (dichlorodiphenyltrichloroethane) has been associated with early menopause [30, 31].

In the present study, we observed no overt maternal toxicity following *in utero* exposure to 100, 500, or 1000mg/kg/day MEHP. *In utero* exposure to MEHP did not significantly affect maternal body weight or pup growth at any of the doses examined (Table 1); however, we observed changes in the estrous cycle of exposed females at all doses examined (Table 2). We postulate that these changes may be due to alterations in normal hormonal signaling which extend to the uterine histology, as measured by vaginal smear cytology [32]. Altered estrous cyclicity has been reported in adult female rats following acute exposure to extremely high doses of MEHP [33]. Likewise, prolonged estrous has been observed in rats following *in utero* exposure to bisphenol A, another endocrine disrupting chemical [34].

We also observed an increase in the number of pups per litter in young adult F1 females exposed to the highest dose of MEHP (1000mg/kg/day), an intriguing finding since C57/Bl6 mice generally have small litters. Endocrine disrupting compounds such as phthalates are postulated to activate the primordial follicle pool [19] and our observations of increased numbers of mature follicles in adult females in the highest exposure group and the subsequent decrease in overall reproductive lifespan support this hypothesis.

In the present study, we found evidence for a reduced estrogenic effect, indicated by the late onset of sexual maturity and decreased aromatase expression. However, we also found evidence for estrogenic effects, as estradiol levels were significantly elevated and LH levels were suppressed by 1000mg/kg/day MEHP. We postulate that at this dose, larger estradiol- and testosterone-producing follicles and ovarian stroma remain functional, which may account for the higher estradiol levels. Our findings are consistent with a study of marmoset monkeys receiving DEHP at 1000 to 2000mg/kg, which showed increases in both blood estradiol levels and ovarian and uterine weight suggesting that DEHP led to accelerated development in the non-human primate [35]. The observation of elevated estradiol levels is reversed by PND365, at which point the controls exhibit higher levels than the 1000 mg/kg exposed animals. This is consistent with the understanding that these high-exposed animals have entered a menopausal state before their control counterparts.

Another intriguing finding of our study was the hyperplasia observed in the mammary glands of F1 females exposed to 1000 mg/kg MEHP regardless of breeding status. Mammary gland hyperplasia is associated with exposure to high serum estradiol levels following xenoestrogen exposure [36, 37] and may be the result of the elevated estradiol levels seen in the F1 females in our study. Based on our findings of higher estradiol levels at 1000mg/kg/day, we examined the expression of aromatase *in vivo* and *in vitro*. Reduced aromatase activity at the transcript level inhibits estradiol production, and previous studies have shown that *in vitro* exposure of either isolated rat antral follicles or rat granulosa cells to DEHP or MEHP at moderate to high doses suppresses aromatase mRNA expression [38]. Our experiments in the current study using the human

granulosa-derived cell line KGN suggest that this is also the case in humans, as well as the suppression of FSHR, StAR, and LHCGR. Though the KGN cell line is granulosa-based and not thecal, it still expresses StAR [39]. However, while these data suggest the granulosa cell population is susceptible, this is not meant to indicate the sole target of MEHP because the time of exposure in our animal studies occurs before any large population of mature granulosa cells in the ovary. Furthermore we cannot speculate on the effects of MEHP on thecal cells based on the current findings, though the perturbation of estrogen signaling is apparent.

Similar to the findings of others, we found significant decreases in ovarian aromatase expression in both the 500 and 1000mg/kg/day MEHP exposure groups relative to controls. One possible explanation for high estradiol/low aromatase expression is that aromatase protein levels are altered in our system. A more intriguing possibility and one that deserves further investigation is that of reprogramming of the HGP axis. In previous studies involving MEHP or DEHP, the toxicant is administered directly and biological samples are examined immediately. In our study the exposure is *in utero* and the adult effects are examined months later, suggesting the potential for perinatal reprogramming. A recent study by Gore et al [27] demonstrates that early life exposure to methoxychlor leads to lifelong molecular reprogramming of the hypothalamus and premature reproductive aging similar to the premature reproductive aging observed in our study with MEHP.

It must be stated that the overwhelming majority of results seen in this study are found at doses of 500 and 1000 mg/kg, doses that are well beyond the scope of physiologically relevant doses. Interestingly, the only statistically significant effect seen

at 100 mg/kg, a dose one or two orders of magnitude above that which might be considered physiologically relevant [14,15,40], is a decrease in StAR mRNA expression. In the absence of any other observed toxicological or physiological effects at this dose, and indeed without assessing StAR at the protein or transcript level, 100 mg/kg appears to be a no observable adverse effect level (NOAEL).

Based on our study, we propose that exposure to MEHP activates primordial follicle recruitment; whether the mechanism involves reprogramming of the HPG axis remains to be determined. However, it is possible that the increased rate of follicle maturation results in higher estradiol levels due to the presence of active granulosa cells. From early adulthood, this suspected rapid recruitment of follicles continues, ultimately resulting in increased litter size. The large-litter phenotype, along with the prolonged estrus phenotype, continues until approximately 9 to 10 months of age, at which point the high dose-exposed females cease having litters, and by 1 year of age exhibit significantly lower serum estradiol levels relative to the control animals. This is consistent with the hypothesis that these animals are recruiting follicles at an accelerated rate. In agreement with these findings, MEHP-exposed F1 females sacrificed at approximately one year of age exhibit extensive mammary gland hyperplasia, an observation consistent with elevated estradiol levels throughout much of their lifetime.

In conclusion, our study demonstrates that an acute exposure to MEHP *in utero* from gestational days 17-19 leads to an initial increase in litter size, an increase in the number of mature follicles and increased estradiol levels in young adult F1 females (PND56), the consequence of which is decreased overall fertility later in life. Therefore, considering the differences between our study and previous studies, our data suggests that

the timing, duration, route of exposure, and species used are all important variables that determine the effect of MEHP on steroid biosynthesis, ovarian and mammary gland biology, and reproductive lifespan.

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Chapter III:

Bisphenol A Alters Follicular Survival and Akt Signaling In the Neonatal Mouse Ovary

Abstract: Bisphenol A, a chemical in which industrialized populations have near-ubiquitous exposures, has been implicated as an endocrine disruptor. The ovary is an endocrine-sensitive organ particularly at perinatal timepoints during which primordial follicle reserve is being established. To assess whether the mouse neonatal ovary is susceptible to BPA toxicity, ovaries were removed from mice at postnatal day 4 (PND4) and grown in culture. Following two days of acclimatization, the ovaries were exposed to doses of 25 μM, 25 nM, or 2.5 nM BPA, 10 nM ethinylestradiol, or vehicle (EtOH) via the culture media. Following 4 days of exposure, ovaries and media were collected for analysis. At 25 μM, ovaries exhibited significantly fewer primordial follicles and higher levels of primary follicle apoptosis relative to controls. Ovaries exposed to 25 nM BPA exhibited higher levels of primary follicle apoptosis but no significant change in primordial follicle population number. Protein phosphorylation of Akt and Foxo3a, members of the same pathway implicated in regulating both apoptosis and follicular recruitment were assessed. Intriguingly, 25 nM and 25 μM BPA, exhibited inverse trends in Akt and Foxo3a activity. We conclude that cultured mouse ovaries exposed to high doses of BPA exhibit increased follicular atresia, but that this toxicity is mediated by different signaling mechanisms dependent upon the dose of toxicant exposure.

1. Introduction

Bisphenol A (BPA) is a ubiquitous additive used in polycarbonate plastics with an annual global usage in excess of 4 million tons [1]. BPA is a weak estrogen receptor agonist, with a binding affinity estimated to be approximately 1,000 to 10,000 times lower than estradiol [2,3]. However, numerous studies have indicated that BPA may act through mechanisms outside the canonical ER pathway [4,5,6,7]. The effects of BPA on the female reproductive system, whether mediated through canonical ER signaling pathways, or by other mechanisms, have been documented. A study using primary granulosa cells taken from pigs revealed that BPA reduced progesterone production [8]. Isolation and culture of mature antral follicles demonstrated that exposure to doses of 44 μM and 440 μM are sufficient to down-regulate *StAR* and *Aromatase*, key steroidogenic genes, at the mRNA level [9]. CD-1 mice exposed perinatally to BPA exhibit decreased fertility and fecundity [10] and hyperplasia of the mammary gland including increased estrogen sensitivity [11,12]. Another study indicated that mice exposed *in utero* to BPA showed a higher volume of the ovary filled by antral follicles relative to controls, suggesting a possibility that BPA may have altered follicular recruitment [13]. Neonatal exposure to BPA has also been shown to alter follicular dynamics. Lambs injected subcutaneously with BPA or DES demonstrated altered follicular dynamics with a reduction of primordial follicles and increased numbers of more mature follicles [14]. BPA has also been shown to decrease the primordial follicle pool in rat ovaries when delivered subcutaneously subsequently increasing the rate of follicular recruitment [15].

It is generally accepted that in mammals, the total reserve of germ cells in the female is established very early. Once this pool of primordial follicles is established,

they remain quiescent until recruited, at which point they mature in stages. During this time, they are both steroidogenic and particularly steroid-responsive, and these maturing follicles will ultimately undergo atresia or erupt from the ovary for possible fertilization.

The process regulating the activation of primordial follicles is poorly understood. A number of factors, both intrinsic and extrinsic to the follicle have been identified [16]. A number of studies have also demonstrated the ability of environmental exposures to either inhibit or accelerate the activation of primordial follicles. Paracrine and endocrine factors shown to induce follicular recruitment include kit ligand [17], leukemia-inhibiting factor [18], and insulin [19]. Environmental exposures have also been shown to alter follicular recruitment. Benzo(a)pyrene, a constituent of cigarette smoke, has been shown to increase primordial follicle recruitment and it has been linked with premature ovarian failure [20]. Conversely, diethylstilbesterol has been shown to inhibit follicular maturation in the neonatal mouse [21].

The EPA has identified 50μg/kg/day as the safety reference dose for bisphenol A [1]. This dose has proven controversial [22]; and, there has been much scientific debate over relevant human exposure levels and the effects of route of administration of BPA in animal studies. In the current study, three doses were selected: 25 μM, 25 nM, and 2.5 nM. These doses were chosen due to the fact that estimates of normal human serum levels of BPA vary widely. One study found that following a high dietary exposure to BPA, serum levels of total BPA were generally below the detection limit of 1.3 nM and therefore concentrations of unconjugated BPA would not exceed nanomolar levels [23]. However, another study found unconjugated BPA serum levels of approximately 0.5 to 1.5 nM [24]. Therefore, the doses selected in the current study range from well outside

expected human exposure levels down to levels that may be within an order of magnitude of normal circulating levels. It should be specified that this study is not intended for risk assessment purposes, as the model used is a single-organ culture model using an ectopic media exposure. This study is intended to explore mechanism(s) of action.

In the current study, a recently developed model of *ex vivo* ovary culture was utilized to assess the effects of BPA on the follicular dynamics of the perinatal ovary. Previous studies have identified the potential effects of BPA on the ovary, particularly follicular dynamics; therefore, we hypothesized that perinatal mouse ovaries exposed *ex vivo* to BPA would have altered follicular recruitment. To test this hypothesis, we exposed ovaries from PND4 CD1 mouse pups to 25 μM, 25 nM, and 2.5 nM BPA for four days with a 10 nM exposure of ethinyl estradiol as a positive control for ER-mediated effects. At the end of the exposure, the ovaries were harvested for histology and protein assessment.

2. Materials and Methods

2.1 Mice Timed-pregnant CD1 mice were purchased from Charles River Laboratories (Shrewsbury, MA). Animals were kept in climate-controlled colonies (23.3±2 degrees Celsius; humidity 30-70%) with a 12 hour light/dark cycle. All procedures involving animals were performed in accordance with the guidelines of the Institutional Animal Care and Use committee of Brown University in compliance with the guidelines established by the National Institute of Health.

2.2 Ovary Culture Model At PND4 (PND1 = day of birth), pups were sacrificed via CO2 asphyxiation. Following protocol outlined in Devine et al [25], ovaries were

removed under a dissecting microscope and extraneous tissue was carefully removed. Ovaries from the same animal were separated randomly such that no treatment group had both ovaries from the same animal to reduce any effect of abnormal or underdeveloped individuals. Ovaries were then placed on pieces of membrane suspended on the surface of phenol-free DMEM-F12 (Gibco) media supplemented with 1 mg/ml bovine serum albumin (Fisher Scientific), 0.5 mg/mL Albumax (Gibco), 50 μg/mL asborbic acid (Sigma), 27.5 μg/mL transferrin (Sigma), and penicillin/streptomycin (Invitrogen). Each ovary had its own membrane cut from sterile CM 0.4μM cell culture inserts (Millipore) in a single well of a 24-well cell culture plate (Corning) with 400 μL of media. A single droplet of media was placed on top of the ovaries, which were then placed in a humidified 37°C incubator.

2.3 Exposure Paradigm. Ovaries were kept in normal media as described above for 48 hours to adjust to culturing conditions. Following this period, media was removed and replaced with media containing either 25 μM, 25 nM, or 2.5 nM BPA (dissolved in ethanol), 10 nM ethnyl estradiol, or ethanol control. All media contained equivalent concentrations of ethanol not exceeding 0.05% by volume. At time of exposure, a fresh droplet of media was placed on the ovaries. Following 48 hours of exposure, the media was again replaced. Following 4 total days of exposure, the ovaries were harvested.

2.4 Follicle Counts Ovaries selected for follicle counts were placed in Bouin's fixative for 24 hours, and then placed in successive washes of ethanol, xylene, and paraffin. Following paraffin fixation, the ovaries were sectioned at 5 μM thickness and stained with hemotoxylin and eosin following standard protocols. Every other section of every other slide was counted with approximately 6 sections counted per ovary. Each treatment

Primordial follicles were characterized by a small single layer of flattened granulosa cells and little to no extranuclear space visible in the oocyte. Primary follicles were characterized by larger size, a prominent layer of cuboidal granulosa cells, and an oocyte containing a substantial extranuclear compartment. These primary follicles tended to localize towards the middle of the ovary. Apoptotic follicles were identified by the oocyte being highly eosiniphilic and pyknotic. The status of these follicles as apoptotic was confirmed via TUNEL staining. N= at least 7 different ovaries per group, taken from at least 4 different litters. Total number of sections counted: 25 μM, 35 sections; 25 nM, 34 sections; 2.5 nM, 37 sections; control 62 sections; EE 13 sections.

2.5 Western Blot Ovaries designated for protein were flash-frozen in liquid nitrogen prior to protein isolation and determination. Due to the small size of the cultured ovary, each sample used for western blot consisted of 20 pooled ovaries, each of which from a different individual and at least 4 different dams. The pooled ovaries were lysed in cold RIPA buffer (20 mM Tris 8.0, 150 mM NaCl, 0.5% sodium deoxyocholate, 0.1% SDS, 1% NP-40, 10 mM sodium pyrophosphate, 10 mM sodium fluoride with 60 μl/ml HALT protease/phosphatase inhibitors (Thermo Scientific, Waltham, MA) and 1 mM PMSF (Roche, Indianapolis, IN). Lysates were incubated on ice for 20 minutes with frequent vortexing and cleared twice by centrifugation (13,200 rpm, 10 minutes, 4°C). The DC Protein Assay (Bio-Rad) was used to assess protein concentration. Fifty micrograms of total protein was subjected to SDS-PAGE and transferred onto Immobilon-P PVDF (Millipore, Billerica, MA). Membranes were blocked for 60 minutes in 5% non-fat milk/Tris-buffered saline/0.1% Tween (TBST) at room temperature. Membranes were incubated for 2 hours at room temperature then overnight at 4°C with primary antibody.

Membranes were washed 3 times (5 minutes per wash) in TBST, and incubated for 60 minutes at room temperature in horseradish peroxidase conjugated goat anti-rabbit IgG (1:2000, Cell Signaling) or goat anti-mouse IgG (1:10,000 Millipore) diluted in 5% milk/TBST. Membranes were washed 3 times (5 minutes per wash) in TBST and once in TBS prior to visualization using enhanced chemiluminescence (Thermo Scientific).

2.6 Estradiol determination Media was removed from each well prior to changing media or immediately following harvesting of the ovaries. Estradiol levels were measured utilizing the Estradiol ELISA kit (11-ESTHU-E01) obtained from Alpco Immunoassays (Salem, NH) according to the manufacturer's specifications, along with a panel of control media samples to control for any differences due to media content.

2.7 Statistical Analysis All animal data are presented as the mean ± SEM. Follicle count data were analyzed using two-way ANOVA analysis (SigmaPlot software version 8). ELISA data was analyzed using a paired T-Test (SigmaPlot).

Results

3.1 Effects of BPA on follicular recruitment. Sections of H&E stained ovaries from each treatment group were assessed for number of follicles at primordial and primary stages, as well as number of primary follicles with visibly apoptotic oocyte. The 25 μM exposure group had significantly ($p = 0.002$) fewer primordial follicles relative to control, with an average of 112 ± 72 per section at 25 μM compared to 164 ± 82 per section in controls. No significant difference between controls and any other treatment group was observed. No significant difference was observed in number of healthy primary follicles, though there was a trend ($p = 0.13$) for a higher number of healthy primary follicles at 25

nM (6.44 ± 7.27) relative to control (4.69 ± 4.05). The number of apoptotic primary follicles per section was significantly elevated for both 25 nM ($p = 0.04$; 5.0 ± 4.56) and 25μM ($p=0.02$; 5.22±4.88) relative to controls (3.2 ± 3.6). Neither 2.5 nM nor EE demonstrated altered follicular dynamics.

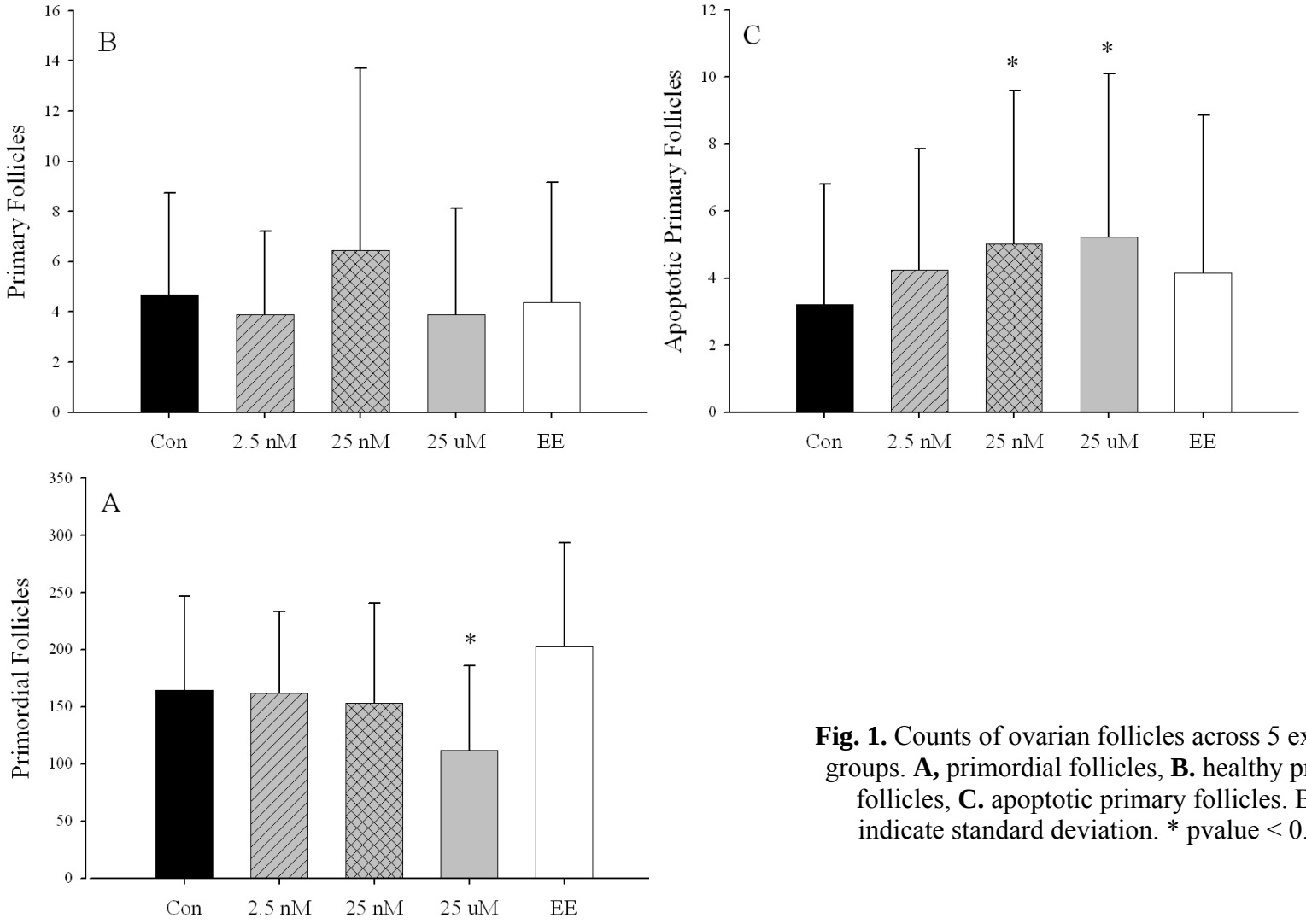
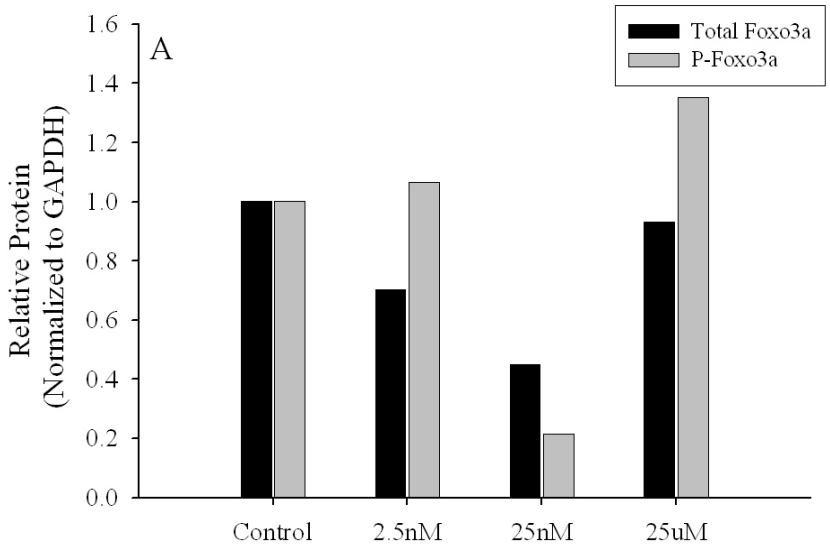
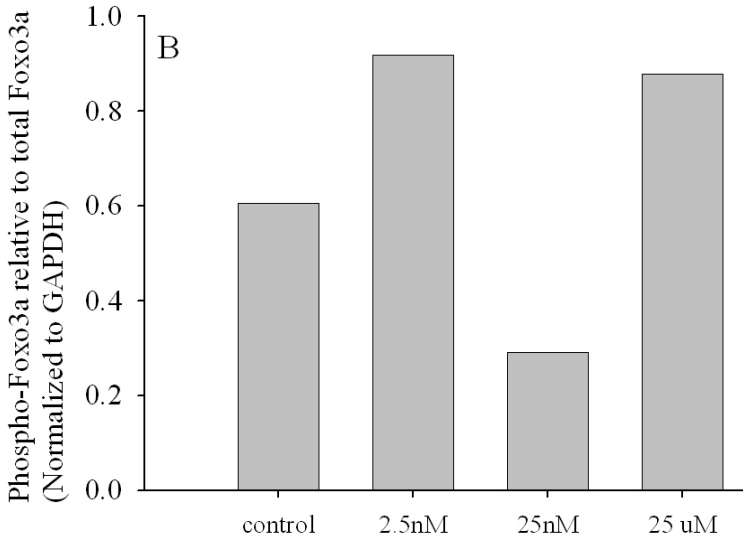


Fig. 1. Counts of ovarian follicles across 5 exposure groups. **A,** primordial follicles, **B.** healthy primary follicles, **C.** apoptotic primary follicles. Bars indicate standard deviation. * pvalue < 0.05.

3.2 Foxo3a Protein expression and phosphorylation. Protein was isolated from pooled ovaries (20 ovaries per treatment group from at least 4 different litters) and used for Western Blot analysis. Total and phospho-Foxo3a was assessed. Total levels were similar between control and 25μM, but both 2.5 nM and particularly 25 nM exhibited major reductions in total Foxo3a levels (Fig.2a). The degree of phosphorylation also varied greatly and in a non-monotonic manner: 2.5 nM and 25 μM exhibited substantially higher degrees of phosphorylation relative to controls, with nearly all Foxo3a being phosphorylated (Fig. 2b). Intriguingly, 25 nM had drastically lower levels of Foxo3a phosphorylation.





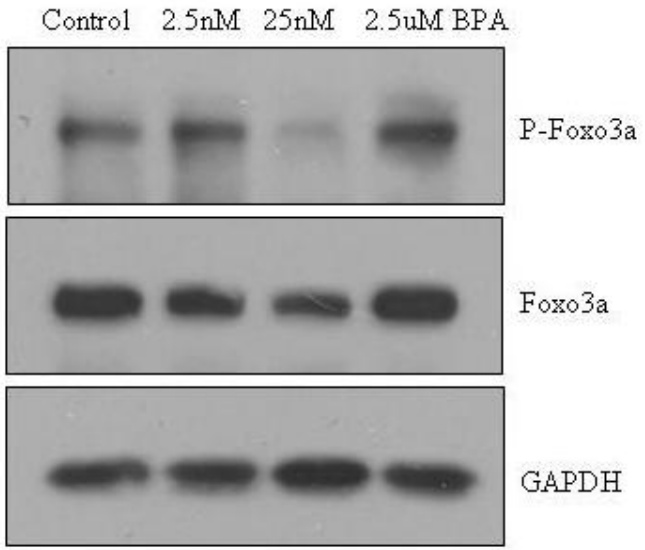
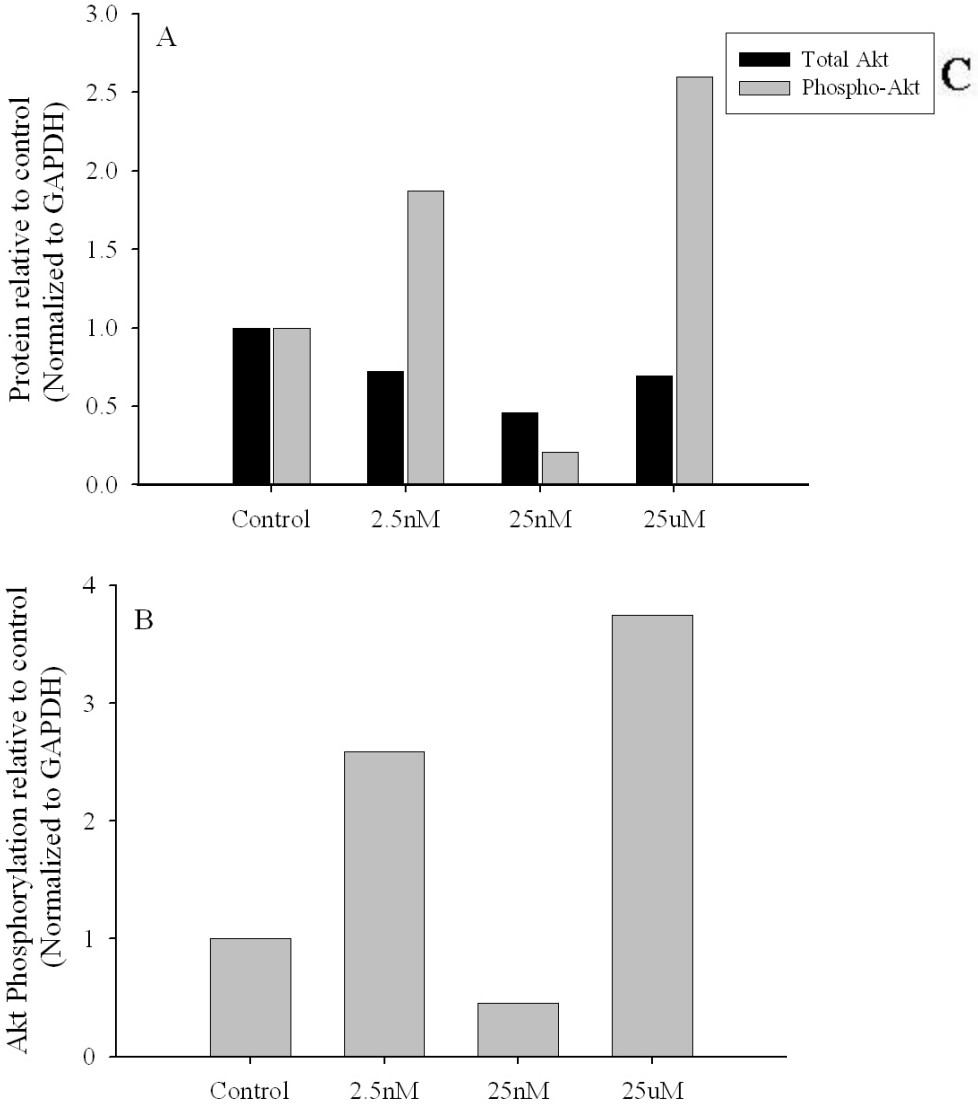


Fig. 2 Foxo3a Protein Expression and Phosphorylation. Densitometry of protein relative to control, normalized to GAPDH, of Foxo3a and Phospho-Foxo3a (a) and ratio of phosphorylated to non-phosphorylated Foxo3a (b).

3.3 Akt protein and phosphorylation. The same samples were then re-probed for pan-Akt and phospho-Akt. All BPA exposure groups showed some reduction in total Akt protein levels, particularly the 25nM group (Fig 3a). Most striking, however, was the substantial increase in Akt phosphorylation. The ratio of phosphorylated Akt to total Akt (Fig 3b) was approximately 2.5 times higher at 2.5 nM and 3.5 times higher at 25 μM. However, 25 nM showed a decrease in relative Akt phosphorylation, with less than half of the relative phosphorylation seen in control samples.



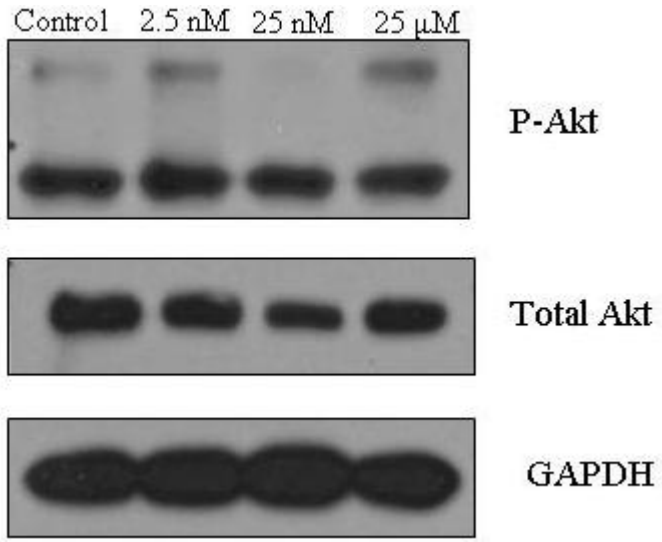


Fig. 3. Akt Phosphorylation. Densitometry (a) of western blot (c) probing for total Akt and phospho-Akt relative to GAPDH, normalized to controls. (b), ratio of total Akt to phospho-Akt.

3.4 p27 and IRS1 protein expression. Insulin Receptor Substrate 1 (IRS-1) and p27 were also assessed via Western Blot (Fig. 4). Densitometry was conducted, followed by normalization to GAPDH loading control. Densitometry graphs are shown normalized to controls. All treatment groups had elevated levels of p27 relative to controls (Fig 4a). The highest increase was seen at the lowest dose, with an approximate 2-fold increase relative to controls. 25 nM protein expression was approximately 120% of controls, and 25 μM expression was 160% of control samples. IRS-1 protein expression (Fig 4b) is slightly elevated (115% of control by densitometry, normalized to GAPDH) at 25 μM and substantially decreased at 25 nM (~50% of controls), with no changes to IRS-1 expression at 2.5 nM.

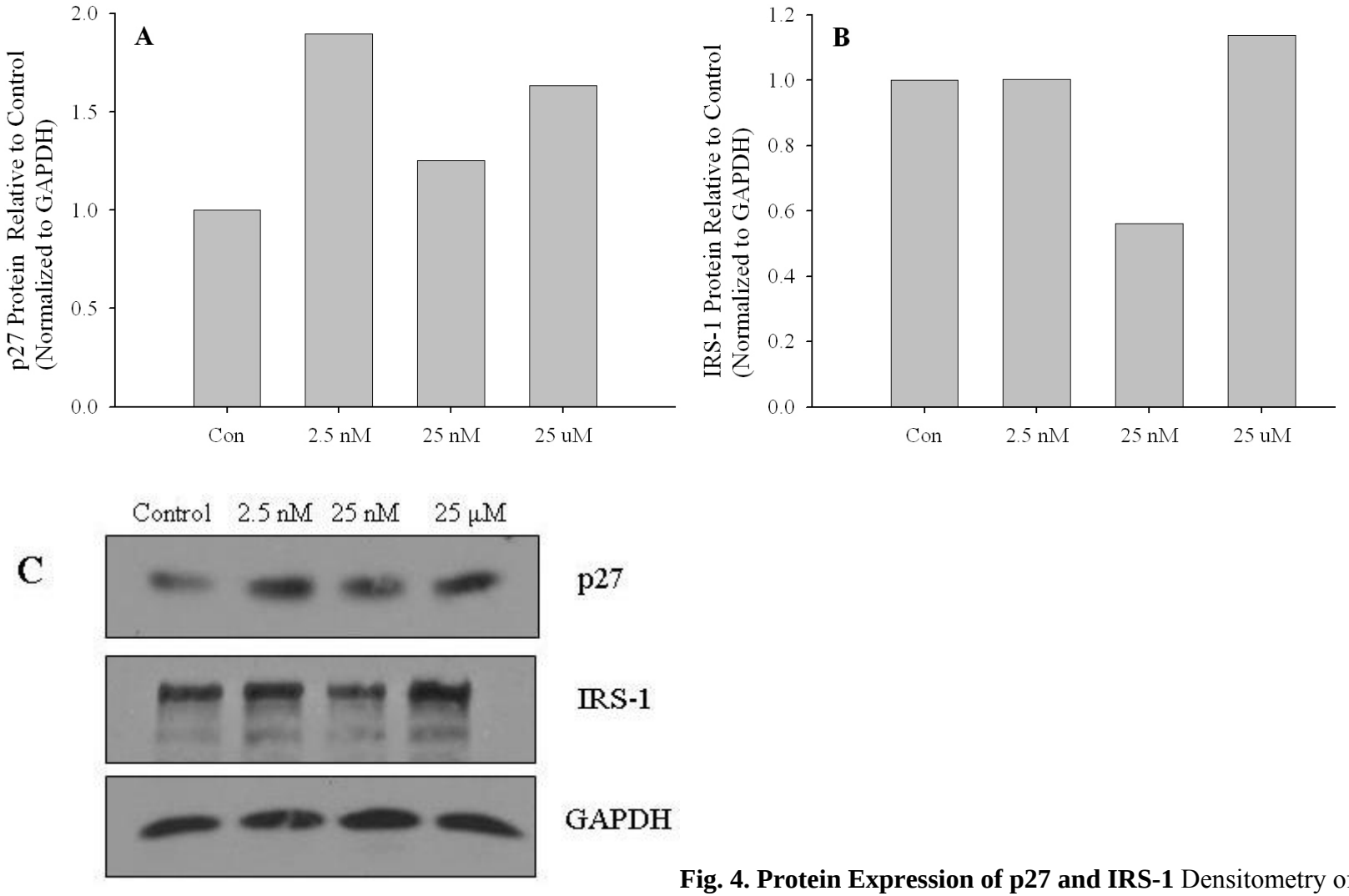


Fig. 4. Protein Expression of p27 and IRS-1 Densitometry of blot (c) for p27 expression (a) and IRS-1 (b).

3.5 Estradiol production Media was removed from the wells of the ovary culture dishes following the two days of acclimation in normal media and then following the first two days of exposure to BPA or ethanol vehicle control. This media was used in an ELISA to assess any changes in estradiol production by the ovary over the course of treatment (Fig. 5). Media of each treatment group not used for ovary culture was used to assess background detection for estradiol. None of the BPA or ethanol-containing media exhibited significantly higher estradiol concentrations than media alone, suggesting that neither BPA nor ethanol was providing false signal to the ELISA. Following exposure, 25 μM samples exhibited significantly higher levels of estradiol (277 ± 55 pg/mL) ($p < 0.05$) relative both to pre-exposure levels (183 ± 14 pg/mL) and relative to control post-exposure levels (192 ± 29 pg/mL). 25 nM samples had a significant decrease in estradiol concentration in paired testing between pre-exposure (209 ± 88 pg/mL) and post-exposure (160 ± 65 pg/mL) ($p < 0.05$), but did not differ significantly in an unpaired analysis with post-exposure controls. N = 5 per group, from a total of 4 litters.

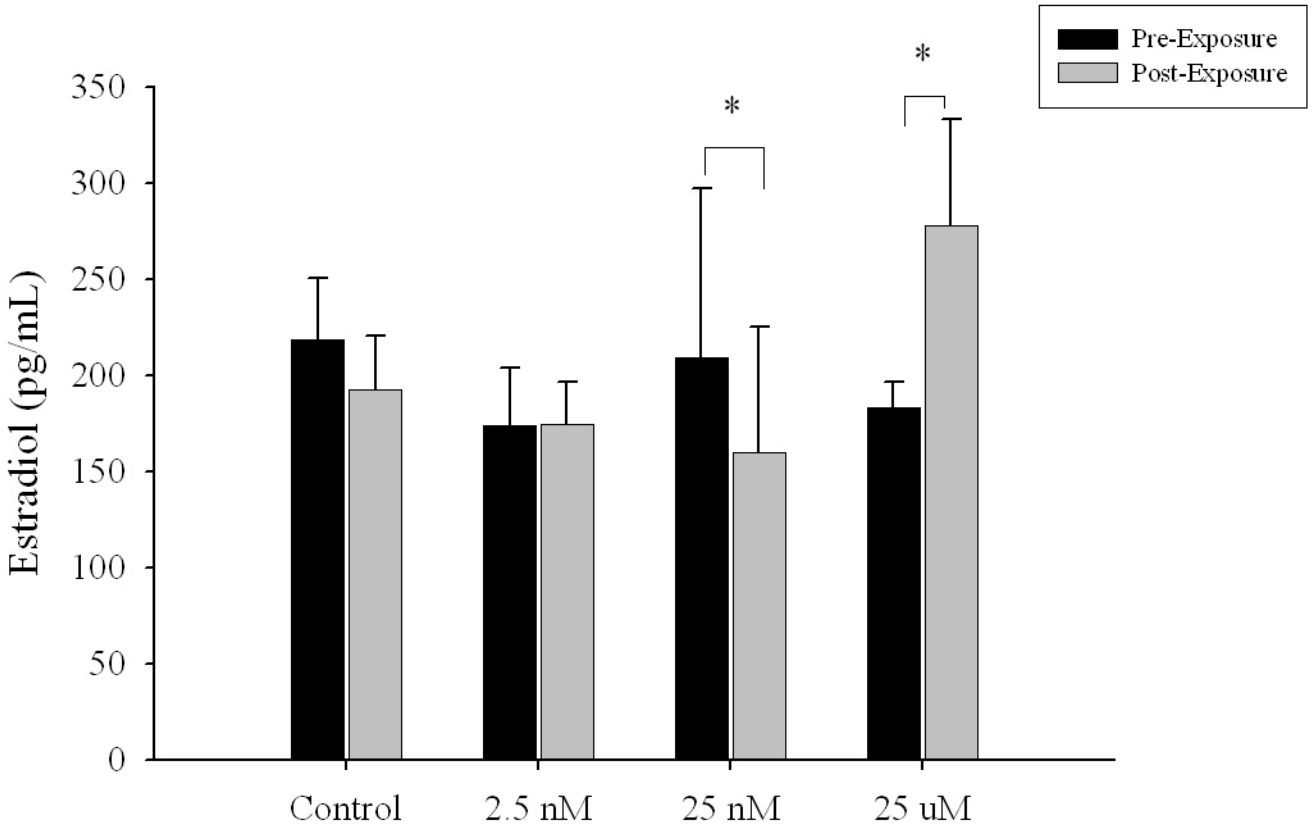


Fig. 5. Estradiol Production ELISA analysis of media used in culturing ovaries, with pre-exposure media taken from culture wells following 2 days of incubation with untreated media and post-exposure media following 2 days of incubation with media containing either BPA or ethanol control. * p value < 0.05.

4. Discussion: In the current study, we have found that cultured neonatal ovaries exhibit altered follicular dynamics following exposure to BPA; as well as, protein-level changes in key signaling pathways. Ovaries exposed to a dose of 25 μM BPA exhibited significantly higher numbers of apoptotic primary follicles and decreases in the number of primordial follicles relative to control ovaries. 25 nM BPA exposure was sufficient to increase the number of apoptotic primary follicles, but did not show significantly fewer primordial follicles. Ethinyl estradiol at 10 nM, a dose projected to be roughly equipotent to 25 uM BPA in terms of ER agonist activity, did not induce any significant changes in

follicle numbers, suggesting that the effects of BPA are not being mediated primarily through ER pathways.

To elucidate the mechanism of this increased follicular activation, the levels of forkhead transcription factor Foxo3a and protein kinase Akt were assessed. Foxo3a is known as both a pro-apoptotic factor for oocytes [26] and an inhibitor of follicular recruitment [27]. Foxo3a is regulated in part by phosphorylation by the PI3K/Akt signaling pathway, which phosphorylates Foxo3a resulting in its shuttling from the nucleus and eventually its degradation. Because 25 μM exhibited both elevated apoptosis and possibly elevated follicular recruitment (because of the depletion of primordial follicles without any substantial levels of primordial follicle apoptosis detected), Foxo3a-mediated activity was suspected. The protein analysis strongly supports this hypothesis for 25 μM. At that dose, p-Foxo3a relative to total Foxo3a was approximately 20-30% times higher than in controls. Furthermore, Akt also had approximately 3.5-fold higher relative phosphorylation at 25 μM relative to controls. Because one of Akt's many targets is the phosphorylation of Foxo3a, this suggests that BPA at this high dose is stimulating Akt, which is generally implicated in cell survival and growth pathways. As a result of this increase in Akt phosphorylation, Foxo3a is phosphorylated and no longer functions as an inhibitor of follicular maturation. The exact mechanism of BPA inducing Akt activation is unclear, though it may be a result of general stress from such a high dose to BPA. However, it is likely that the increased recruitment and follicular loss is not just a function of Foxo3a phosphorylation via Akt, because the 2.5 nM group exhibits similar protein phosphorylation trends without the same phenotype of decreased primordial follicle number and increased primary follicle apoptosis. One interpretation would be

that BPA induces an Akt response at both 25 μM and 2.5 nM, but at 2.5 nM the ovary can maintain a compensatory mechanism to prevent increases in recruitment or apoptosis. Higher levels of p27, a cell cycle regulator, suggest that at 2.5 nM the ovary is acting to inhibit cell cycle progression and perhaps slow increased follicular recruitment. Alternatively, 25 μM may be inducing other pathways beyond Akt and Foxo3a that play a role in follicular recruitment. The slight increase in IRS-1 protein at this exposure but no other may suggest that insulin-responsive pathways are involved, and follicle recruitment has been shown to be insulin sensitive [19], though . Furthermore, BPA has been shown to induce oxidative stress in the testis [28], which may be true in the ovary as well. If so, then this stress may act synergistically with other pathways to induce follicle loss.

At the 25 nM dose the pattern of protein phosphorylation and expression is quite different. While the general trend of increased primary follicle apoptosis is found at this lower dose as at 25 μM, the protein pattern is completely reversed: protein levels of Foxo3a and Akt are both lower than controls, and proportionally less phosphorylated than controls. Because there is no significant decrease in number of primordial follicles but an increase in the number of apoptotic primary follicles, it may be that 25 nM BPA is increasing pro-apoptotic signaling via decreased Foxo3a phosphorylation, and this leads to greater loss of primary follicles via atresia.

Among other differences observed between 25 μM and 25 nM-exposed ovaries is the relative production of estradiol following expressed. ELISA data suggest that 25 μM-exposed ovaries are more estrogenic than controls. This would be consistent with the hypothesis that this dose is inducing accelerated follicular recruitment, as maturing

follicles produce estradiol while primordial follicles are held to be non-steroidogenic. Ovaries exposed to 25 nM, contrarily, appear to produce less estradiol than controls.

The dose response curve found in this study is unusual: the follicular loss phenotype is shared at two doses at 1000x difference with a completely different protein profile in a pathway that plays a role in follicular recruitment and apoptosis. Meanwhile, the protein profile is shared between 25 μM and 2.5 nM, two doses that share no other phenotypic response. The current finding of a non-monotonic dose response to BPA is not unique. Previous studies have found that low doses of BPA can cause effects that are qualitatively different than high doses [22,6], though this too is controversial with other studies finding strictly monotonic dose responses [29,30].

The ability of BPA to alter follicular recruitment in the neonatal ovary may indicate an adverse health outcome of BPA exposure in neonates. Loss of these follicles, taken from what is normally held to be a limited ovarian reserve, may result in a premature failure of the ovary. Although the in vivo (i.e. whole animal) consequences of the altered steroidal environment can not be assesses in this study, the steroidogenic alterations caused by BPA have a potential to cause alterations in reproductive health. In addition, our study does suggest a non-monotonic dose response to BPA. Future studies to examine the pathways responsible for the differential effects are warranted.

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Chapter IV: Discussion and Future Directions

1. Endocrine Disruption of the Ovary.

Current dogma in reproductive biology dictates that female mammals are born with the entire complement of oocytes that they will have for their entire lifespan. Therefore, the process by which these germ cells are rationed must be a carefully regulated process. Additionally, the ovary plays a critical role in the regulation of sex hormones, a process tightly linked to the process of oocyte recruitment and ovulation. It is of great concern, then, that these processes can be disrupted by environmental exposures. Toxicant exposures that alter the follicular dynamics can alter fertility. Exposures that effect normal endocrine homeostasis can also affect fertility. Furthermore, they may potentially disrupt other endocrine-mediated processes and may also act as risk factors for certain cancers, particularly breast, ovarian, and uterine.

Because there are a number of systems involved and each of those systems contain numerous factors that can be perturbed, there are a wide range of mechanisms by which the ovary can be disrupted. Below are the current working hypotheses for the mechanism of disruption in each of the studies presented here.

Aim 1: MEHP induces lifetime changes in follicular recruitment and HPG axis

In Aim 1, it was demonstrated that an exposure *in utero* to MEHP leads to lifelong alterations in the female reproductive tract. The phenotypic changes are extensive and suggest that both the ovary and systemic HPG function have been targeted by MEHP exposure. The key characteristics of the MEHP exposed phenotype are:

1. Alteration of the estrous cycle; wherein the mice spend a disproportionate amount of time in the estrous (ovulatory) stage of the cycle.
2. Increased recruitment of ovarian follicles from young adulthood. This is also

presumed responsible for the increase in litter sizes found in the higher exposed treatment groups. These animals also enter reproductive senescence prematurely.

3. Altered HPG axis functionality, including altered serum hormone levels. This includes elevated estradiol levels, mammary gland hyperplasia (presumed due to additional serum estradiol), and altered gene expression in the ovary of steroidogenic proteins (StAR, Aromatase) and gonadotropin receptors (LHCGR, FSHR) These three characteristics all indicate a profound alteration in the normal functioning in the female reproductive system, consistent with an altered HPG axis. The extent of this perturbation has not been completely elucidated in the current study, nor has the precise mechanism of action. Future experiments to further characterize this phenotype and investigate the mechanism of MEHP-induced ovarian dysgenesis are outlined in a later section. However, a mechanism can be hypothesized.

It is clear that the HPG axis has been disrupted. This axis contains a substantial amount of feedback mechanisms which produce a cyclic homeostasis. Of particular importance is the negative feedback loop; wherein the maturing ovarian follicles produce estradiol, which acts on the pituitary to inhibit the release of the gonadotropins FSH and LH. FSH and LH in turn regulate the maturation of follicles. The MEHP-exposed phenotype is characterized by increased follicle activation, higher serum estradiol levels, and reduced gonadotropin receptor gene expression. Therefore, it is extremely likely that the phenotype is a result of a global alteration of the estradiol/gonadotropin feedback mechanism in favor of higher levels of estradiol. Because this process is cyclical, it is currently unclear at which point in the cycle the fundamental disruption occurs. Constitutively higher levels of estradiol originating from increased follicular recruitment

would suggest an ovarian origin of the phenotype. Alternatively, constitutively decreased gonadotropin production in the pituitary (or ovarian insensitivity to gonadotropin signaling) might result in accelerated follicular recruitment and increased estradiol production. In either case, the phenotype could be expected to become self-sustaining if basal estradiol levels remain high. This also explains the secondary pathologies observed, particularly the mammary gland hyperplasia (found in other animal models of persistent elevated estrogen levels). See Fig. 4.1 for a graphic characterization of this hypothesis.

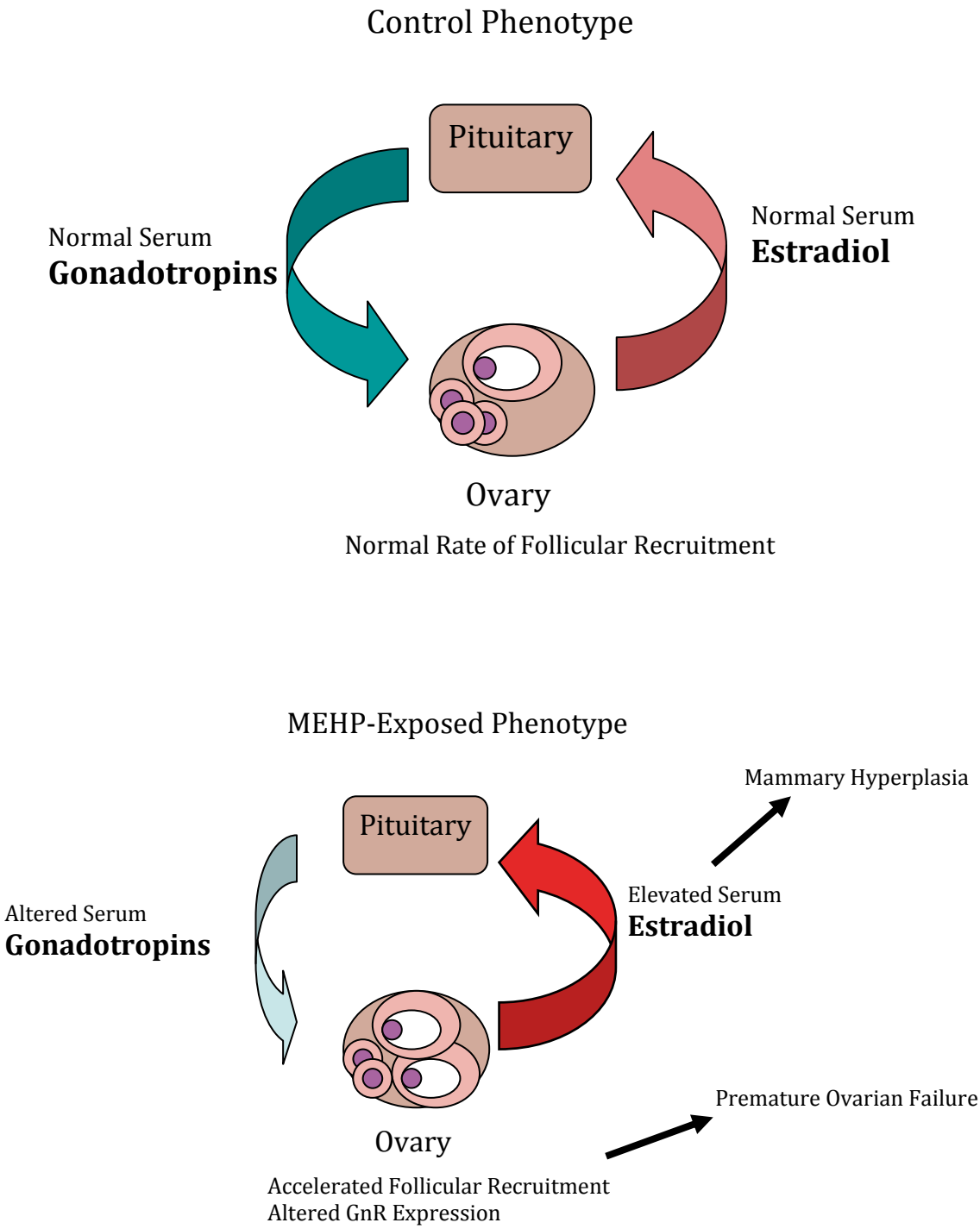

Fig. 4.1 Illustration of normal HPG/ovarian function and MEHP-exposed HPG/ovarian function

Aim 2: BPA alters follicular recruitment in the perinatal ovary.

Aim 2 demonstrated that in an ex vivo perinatal ovary model, the recruitment of follicles was altered by exposure to bisphenol A. At doses of 25 uM, follicular recruitment was enhanced, with a higher proportion of apoptotic primary follicles relative to primordial follicles compared to controls. This finding was supported by western blot, where the highest dose had substantially elevated levels of Foxo3a phosphorylation, a marker of follicular activation. Interestingly, these experiments suggested a non-monotonic dose response curve. At 25 nM, a dose a thousand-fold lower than the highest dose used, an opposite effect was observed, with significantly lower levels of Foxo3a phosphorylation. A non-monotonic dose response has been reported in a number of studies using bisphenol A. A lower dose, 2.5 nM, displayed marked similarities to the highest dose, despite no matching changes to follicular recruitment as determined by follicle count, suggesting that Foxo3a is not the sole determinant of follicular recruitment following BPA exposure.

Signaling kinase Akt was also extensively phosphorylated at the highest and lowest BPA doses with reduced phosphorylation (and total protein) at 25 nM. Because Akt phosphorylates Foxo3a, this strongly suggests that BPA's ability to induce Foxo3a phosphorylation is mediated through the PI3K/Akt signaling pathway.

For these experiments, a positive control was used: ethnyl estradiol. The dose of 10 nM was selected because this provides an equivalent dose to BPA regarding these compounds' actions as ER agonists. The follicle counts of the EE-exposed ovaries revealed no differences from controls. This suggests that BPA is not acting strictly through canonical ER pathways. This is consistent with other studies, which found effects

induced by BPA exposure but not by equivalent estrogen exposure.

The current study has not identified the mechanism of action of BPA on the perinatal ovary. Given the non-monotonic dose response, it is likely it is a complicated process that may vary depending on both the dose and the exposure window. Further experiments to expand our understanding of this effect and its mechanism are outlined below. A mechanism can be hypothesized based on the current findings and previous studies. First, it is likely that BPA is acting on some level as an ER agonist, as its receptor affinity has been well established. This, in turn, has known effects on the perinatal ovary: xenoestrogens suppress the process of GCN breakdown and alter follicular recruitment dynamics typically by stimulating primordial follicle activation. Graselli et al.'s [1] study of the effects of BPA on mature granulosa cells also indicates an alteration in estradiol production in a non-monotonic manner, with low exposures causing an upregulation and higher exposures suppressing production. Other studies have also noted the changes in follicular dynamics seen in the current study, such as Rivera et al's [2] 2011 study wherein lambs exposed perinatally to BPA or DES exhibited increased follicular recruitment. As discussed in the background, other studies using both BPA and DES have indicated similar effects on follicular recruitment when used at identical doses. Because BPA has a much lower ER binding affinity to DES, this suggests that BPA is acting in a manner in addition to its role as ER agonist, though there is as yet little reliable data to suggest a mechanism.

The protein analysis of BPA-exposed ovaries in the current study indicate that 2.5 nM and 25 μM have upregulated Akt phosphorylation, which can be assumed to be responsible for the increased Foxo3a phosphorylation also observed. However, while the

degree of phosphorylation of these two proteins is similar, the effects on follicle populations are not. This suggests that while BPA may be acting via Akt signaling, increased Foxo3a phosphorylation is not sufficient in and of itself to induce increased follicular maturation, but at 25 μM BPA is inducing some additional pro-recruitment effect not seen at the lower doses.

It should be emphasized that these ovaries are isolated from the normal HPG axis. As a result, the cultured ovary will not receive gonadotropins produced outside the ovary. Conversely, the hypothetical mouse the ovary "belongs to" is not receiving any hormones that are produced in the ovary. In the current study, the high dose of BPA induces increased estradiol production by the ovary. Given that these are preadolescent animals, the effects of this increased estradiol production (in addition to the xenoestrogenic activity of the BPA) might be profound on the developing animal. BPA has been shown to promote premature mammary gland development [3]. This pathology would be compounded by additional estradiol production. A study of recovery following the removal of BPA is beyond the scope of the current study. This is due to the in vitro culture system, which does not contain factors extrinsic to the ovary affecting follicular recruitment or steroidogenesis. However, at the very least it is certain that prematurely recruited follicles will be exhausted before sexual maturity. This, in turn, reduces the follicular reserve of the animal and reduces its window of fertility, resulting in premature ovarian failure.

A more severe later-life phenotype would be a "runaway recruitment" of the sort hypothesized in Aim 1. In this case, the recruited follicles' production of estradiol suppresses gonadotropin production in the pituitary, establishing a lifelong higher rate of

recruitment, compounding the rate at which the animal will suffer premature ovarian failure.

2. Future Directions

While the current studies have revealed compelling information about the effects of both MEHP and BPA on the developing ovary, there is much yet to be discovered particularly regarding mechanism of action. A number of experiments can be conducted to help elucidate both the mechanisms of action and other potential effects of perinatal endocrine disruption.

Aim 1: MEHP and Its Effects on Ovarian Dynamics

The study in aim 1 revealed a distinctive phenotype in females exposed *in utero* to high doses of MEHP. This phenotype consists of changes in serum hormone levels, ovarian gene expression, estrous cyclicity, follicular recruitment, and fertility. Such an extensive list of phenotypic effects suggests that a number of systems may be targeted by the exposure, but the exact mechanism is unclear. A number of experiments could be undertaken to expand our understanding of the mechanism of MEHP-induced ovarian dysgenesis. *In vivo* experiments can be conducted to develop a better understanding of targets of MEHP on a tissue level, while *in vitro* experiments can eludicate cellular and molecular mechanisms of MEHP-induced toxicity.

***In Vivo* experiments: Ovarian Transplant Experiments**

The MEHP *in utero* study indicates that the exposure results in a lifelong alteration to the reproductive system. Gene-level changes are detected in the ovary, but the current study does not fully reconcile the ovary findings with all of the observed effects, particularly alterations in serum hormone levels. Because the HPG axis is a

complex system involving multiple organs, it would be very useful to identify whether the key changes induced by MEHP are largely focused on the ovary or if the changes also affect the pituitary or other steroidogenic tissues. An essential question is basically one of "the chicken and the egg"- whether effects observed in the ovary are informing systemic hormone level changes which alter other systems, or whether the changes observed in the ovary are the result of a more general HPG axis disruption. Of course, because of various feedback mechanisms, it is likely that it is not an absolute, but it would be valuable to assess each organ's role in the exposed phenotype.

One possible method of assessing this would be to repeat the basic exposure paradigm, but perform ovarian transplants between control and exposed animals (along with necessary sham surgeries of "control-to-control" and "exposed-to-exposed"). These transplants could be conducted at different timepoints- ideally, one set performed prepubertally (approximately PND20) and another performed during adulthood (PND112).

Following transplant and recovery, the lifetime phenotype could be reassessed, particularly the estrous cycles and litter sizes. This would elucidate whether the phenotype is mediated by changes to the ovary, the pituitary, or some combination thereof. For instance, if an animal exposed *in utero* were to receive a transplant of unexposed ovaries and resumed normal estrous cyclicity and breeding characteristics, this would strongly suggest that the ovary itself has been fundamentally altered by the *in utero* exposure. This finding would of course be strengthened considerably if the unexposed recipient of the exposed ovary developed the phenotype.

At sacrifice, the other endpoints of interest of the ovary could also be assessed. Follicle counts might reveal whether the accelerated recruitment of follicles is intrinsic to the ovary or mediated by hormone signaling outside of the ovary. Of particular interest would be an assessment of gene expression to match with previous results. For example, if the ovary maintains the phenotypic suppression of hormone-related genes, this may suggest an epigenetic effect resulting in reduced expression of those genes.

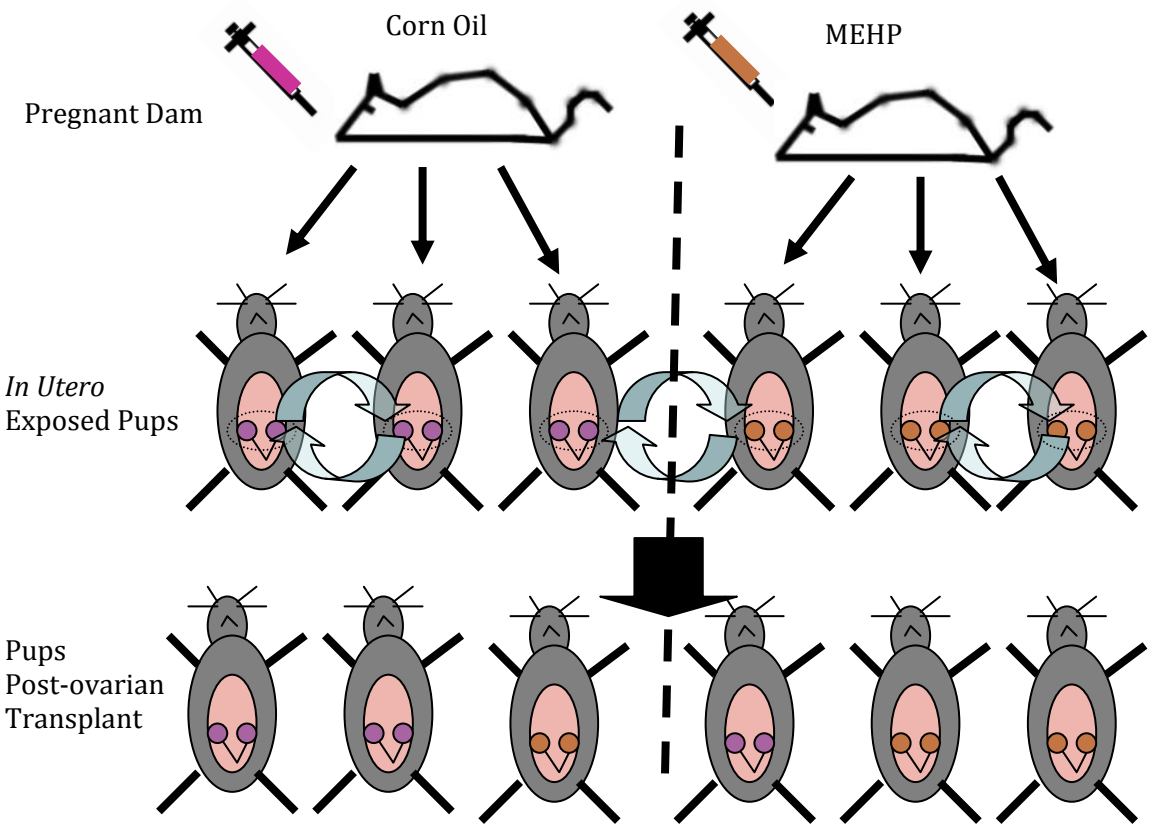

Fig. 4.2 Proposed Model of MEHP Exposed Ovarian Transplant

This approach has potential pitfalls. The stress of even a successful ovary transplant might significantly alter the hormonal characteristics of the animal, in which case the effects of the MEHP exposure may be difficult to differentiate from effects of the surgery even with appropriate controls. Furthermore, if the exposed phenotype previously documented is multifactorial, it is possible that this phenotype might disappear

completely in both recipient groups. While this recovery would itself be an interesting finding, it would complicate further efforts at understanding the mechanism underlying the MEHP-exposed phenotype.

Other *in vivo* experiments can be conducted using the existing paradigm used in Aim 1, particularly if the above experiments suggest that the ovary itself is not the driving force of the phenotype. The data currently suggest that the ovary has altered gonadotropin receptor expression, and the animals themselves appear to have altered serum levels of LH and FSH. Further perturbation of these signaling pathways might yield important information. For example, reduced LH receptor expression may play a role in the accelerated recruitment of follicles. One experiment might be treatment of MEHP-exposed females with exogenous LH to see if this alters either the estrous cycle or rate of follicular recruitment. Treatment with an LHCGR antagonist in control animals might act as a companion experiment to see if follicular recruitment increases with the reduction of LH signaling. In addition to co-exposure experiments of animals to both MEHP and a known pituitary toxicant such as methoxychlor or chlorpyrifos, which are known to alter expression of gonadotropin releasing hormone (GnRH) [4].

The goal of these alternate *in vivo* models would be to further perturb the HPG axis to establish whether these co-exposures would be additive, synergistic, or antagonistic. If MEHP is acting by a mechanism similar to these known pituitary toxicants, the result might be additive toxicology. Alternatively, because of MEHP's apparent effects in reducing gonadotropin receptor levels in the ovary, it is entirely possible that a reduction in gonadotropin levels induced by targeting the pituitary may not have as powerful an effect as they would in a healthy animal. By using toxicants with

better-known mechanisms that may act on the same target organs as MEHP, it would provide useful insight into MEHP's actions on the HPG axis.

In vitro Models: The Pregranulosa Cell as Target

The current study and other previous research suggest that the human granulosa cell may be a target of MEHP, altering its steroidogenic properties. Other studies have used whole follicles removed from the ovary and exposed these to phthalates in culture. The current study in Aim 1 does not suggest that MEHP's effects are mediated by the granulosa cell (particularly because the exact character of the granulosa cell or pre-granulosa cell population is poorly understood). However, the granulosa cell remains one of the few points in this exposure paradigm that we know MEHP has a direct effect upon, so it seems reasonable to focus *in vitro* studies on the granulosa cell to assess potential mechanisms.

As previously mentioned, the pre-granulosa cells at the window of exposure used in the Aim 1 study are currently poorly understood, both in general characteristics of behavior and differentiation and more specifically their role in hormone synthesis. One promising approach would be to attempt to isolate and culture this cell population. Techniques exist to isolate the different component cell populations of an adult follicle. While nothing in the literature suggests this has been attempted with germ cell cysts in the perinatal ovary, it may be possible. Ideally, a perinatal ovary would be isolated and ruptured under a dissecting scope, the pregranulosa cells removed and grown in culture. The actual isolation may be straightforward: the ovary's volume is largely taken up by germ cell cysts and pregranulosa cells at this time; if the germ cells have completed their mitotic expansion, the majority reproductively competent cells remaining will be the pre-

granulosa cells. Developing an adequate culture system to maintain the pregranulosa cells may pose its own challenges; it is unclear what triggers the progression from pregranulosa to granulosa, what actually characterizes this progression, or how these cells will act in the absence of oocytes. However, each of these challenges will also yield critical information about this currently poorly understood cell population.

Once the cell population has been isolated and grown (and potentially immortalized), a number of experiments could be conducted. There would be a number of useful experiments to address a number of questions regarding the nature of the pregranulosa cell itself such as estradiol sensitivity and growth characteristics. However, the breadth of such experiments would potentially be beyond what could be considered a "future direction" for the current study, and therefore further experiments described will focus on these cells' response to MEHP.

The first step would be to repeat the experiments conducted on the KGN cells in Aim 1, to see if these cells respond similarly to exposure to MEHP. This is critical, because the suppression in KGN cell aromatase expression may be an important element in the HPG axis disruption found in the previous study. In addition to assessment of mRNA levels of genes of interest, media can be collected for ELISA analysis of estradiol levels. Other endpoints may include viability and growth, and there may be methods of assessing morphologically whether these cells undergo differentiation.

Once a basic understanding of the effects of MEHP on this cell population has been established, a more thorough dissection of the mechanism of action can begin. While speculative, the current data and previous studies suggest that PPARα, PPARγ, and GPR30, amongst others, might be receptors of interest. These can be tested *in vitro* by

use of known receptor agonists or antagonists. Other genes of interest, such as StAR and aromatase, can be altered via siRNA. The end goal would be to assess which genes are critical in the cellular response to MEHP exposure. Their role may be detected either by inducing the MEHP phenotype without MEHP via receptor activation, or by ameloriating the MEHP-exposed phenotype by blocking pathways that may be critical for inducing the phenotype.

These experiments are contingent on these cells being isolated and cultured successfully without losing their defining characteristics. If the cells fail to thrive in culture or lose their essential characteristics, a number of alternate approaches might be taken. Rather than culturing the pregranulosa cells and then exposing them to MEHP, animals could be exposed as in Aim 1, but sacrificed immediately at birth. Ovaries removed from these animals could be flash-frozen and used for laser capture microdissection (LCM). LCM would allow the targeted removal of pregranulosa cells for mRNA extraction and therefore RT-PCR or gene array studies. This would also allow the simultaneous removal of the oocyte, which while no current evidence suggests is a direct target of MEHP exposure, would also warrant investigation.

The ultimate goal of these experiments would be to provide a molecular understanding of MEHP-induced female reproductive toxicity. These experiments would provide a companion to the *in vivo* experiments outlined above. The general strategy would be that the whole organism approach would help investigate the tissue or systemic origins of the exposed phenotype, while the *in vitro* experiments would help explore the molecular mechanisms of the toxicity. The *in vivo* experiments may also inform what potential mechanisms deserve the most focus for the more molecular questions. For

instance, if the ovarian cross-transplant experiments suggest that the exposed ovary is the origin of the wider phenotype, then the focus would likely be on the granulosa cells and thecal cells, particularly the steroidogenic properties of the ovary. If instead the experiments suggest that the phenotype is not "carried" with the ovary, then experiments may focus on either the pituitary or on the ovary's responsiveness to gonadotropin signals.

Future Directions: Aim 2. Bisphenol A and accelerated follicle recruitment.

The findings of aim 2 strongly suggest that at sufficiently high doses, BPA will accelerate the recruitment of follicles in the perinatal ovary. Intriguingly, evidence also suggests that lower doses have an opposite effect, inhibiting activation of primordial follicles. Given the ubiquity of BPA exposure and the potential health consequences of BPA, a number of follow-up experiments will illuminate the mechanisms and degree of risk associated with a perinatal exposure to BPA. As with aim 1, there are both experiments that can be conducted within the existing model (in this case, the perinatal *ex vivo* ovary culture) and experiments based on expanding current findings into novel model systems.

BPA Exposure in an *ex vivo* ovary model: additional experiments

The ability to remove ovaries from perinatal mice, grow them in culture, and expose them to toxicants via the media has proven to be a convenient and fruitful model. A number of additional experiments can be conducted based on the existing model to expand our current findings and provide insight into lingering questions.

The current studies focused on a particular window of exposure with ovaries removed at PND4 and cultured over 6 days. While PND4 is an important time period in

ovarian development at the very end of germ cell nest breakdown, other windows of exposure might constitute a more vulnerable stage of development. For that reason, other periods of development would be an excellent starting point for future experimentation.

Of particular interest would be repeating the experiments at PND1 rather than PND4. PND1 is a time point early in germ cell cyst breakdown in the mouse [5], and this process has been shown to be disrupted by xenoestrogen exposure [6]. Even though specific Aim 2 experiments described in this thesis are within the window of GCN breakdown, this period is towards the end of this window. PND 1 pinpoints the early stages of GCN breakdown and therefore is more likely to be disrupted by BPA's effects as a xenoestrogen. The current use of ethinyl estradiol as a positive control suggests that the effects of BPA on the ovary are not simply a result of its role as an ER agonist. The possibility of multiple effects due to this non-ER-mediated toxicity and its role as an estrogen mimic suggest that a period known to be estrogen-sensitive would make this a particularly interesting window to investigate. The end-points used in the current study could also be applied to these experiments: Foxo3a phosphorylation as marker of follicle recruitment, estrogenic activity of the ovary, and histology for follicle counts. A greater emphasis on intact GCNs would be necessary as this process is anticipated to be disrupted. Because of the three-dimensional nature of these structures, conventional histology and microscopy would likely not provide a complete representation of germ cell cyst populations. For that reason, confocal microscopy using fluorescent antibodies against known markers of oocytes (such as STAT3) could be used for assessing the presence of GCNs. Particularly because of the small size of the ovary at these early stages, it may be possible to conduct a "whole mount" of the ovary and use digital

tomography to produce a 3d composite image of the ovary in order to assess the germ cell nests. It would be predicted that, as a xenoestrogen, BPA would inhibit GCN breakdown and therefore the ovary would contain more GCNs and these nests would also be substantially larger than their counterparts following the course of exposure of several days. As mentioned above, however, there are effects of BPA that are not mediated by its action as a xenoestrogen. Higher levels of BPA exposure induce primordial follicle activation and increased estradiol levels. It is possible that the observed increase in estradiol may be caused at least in part due to the follicular activation, as the primary follicles are estrogenic whereas primordial follicles are not. Because the GCN breakdown is inhibited by estradiol, then BPA exposure may be a double-edged sword because it appears to both induce follicular activation and act as xenoestrogen. For example, if the ovary at PND1 consists of both primordial follicles and GCNs, each of these will be targeted in a distinct way. The primordial follicles will be activated by BPA, becoming estrogenic before undergoing atresia. Simultaneously, the GCNs are exposed to abnormally high levels of estrogens, both from direct action of the BPA and estradiol produced by the prematurely activated follicles. From this, it could be predicted that there would be a "splitting" of the population, wherein a BPA exposed ovary would have both abnormally high levels of intact GCNs as well as activated follicles [see fig. 4.2].

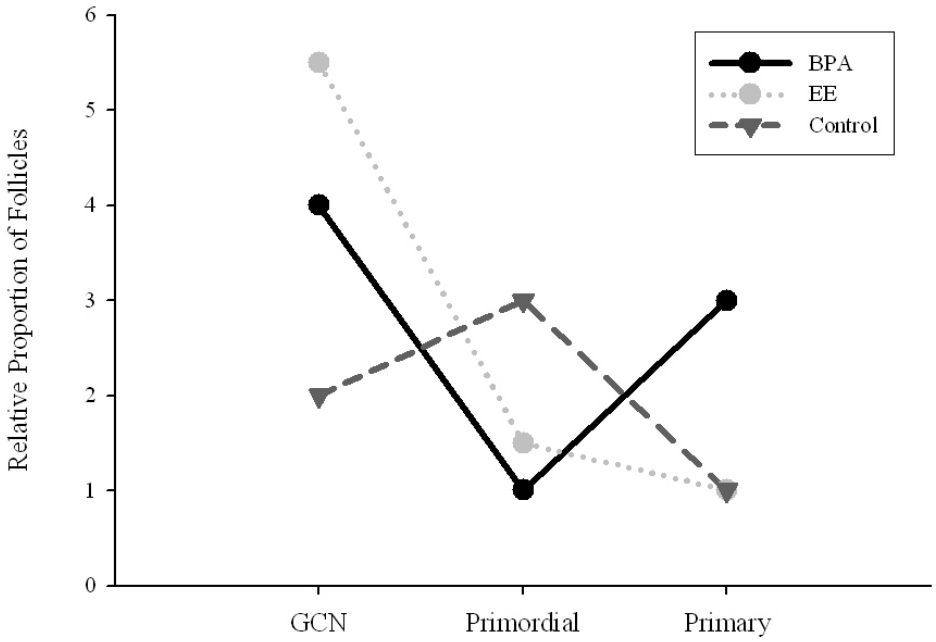

Fig. 4.3: Hypothesized changes to ovarian follicle characteristics following BPA or EE Exposure, PND1 to PND5. By PND5, under normal conditions the ovary will have few GCNs remaining, with a high proportion of primordial follicles. EE is known to alter GCN breakdown, resulting in fewer primordial follicles. BPA exposure is suspected to act via two mechanisms, resulting in an inverse of the control curve: higher levels of both intact GCNs and activated follicles, with few intact primordial follicles.

Ovarian Follicular Recruitment

Additional experiments might focus on the mechanisms of action regarding ovarian follicle recruitment. Previous research, as well as the current study, indicate that Foxo3a is a marker for follicular recruitment and its phosphorylation and trafficking out of the nucleus is a key step in recruitment. Experiments could be conducted to see whether BPA is acting directly on this pathway at high doses to induce follicular recruitment or whether it is acting through other mechanisms to induce follicular activation. The question is essentially whether Foxo3a phosphorylation is acting as a target of BPA or whether increased phosphorylation of Foxo3a following BPA exposure is simply indicating that BPA is inducing follicular activation by other means. **Because**

the mechanism of how the whole ovary (or whole animal) regulates when, how many, and which follicles are activated is still poorly understood, this is a challenging question for assessing BPA's role in follicular activation. One way of testing whether BPA is acting strictly through canonical Foxo3a signaling pathways would be to expose BPA in the presence of inhibitors of upstream activators of Foxo3a phosphorylation. Akt, shown to undergo increased phosphorylation at 2.5 nM and 25 μM in the current study, is one such target. If Akt's upstream activator, PI3k, is targeted with an inhibitor such as wortmannin during BPA exposure, this might identify a pathway either necessary or dispensable for BPA-induced follicular activation. Because of the unusual similarities and differences seen at the lowest and highest dose (but not at an intermediate dose), it is clear that multiple pathways are involved, and therefore numerous targets for these kinds of studies will be necessary to identify alternate mechanisms at each dose.

Experience with the cultured ovary model suggests that most small molecules can permeate throughout the whole ovary. While it may not offer the versatility of conventional cell culture, a wide number of experiments are possible to dissect out the relevant signaling pathways. Because follicular recruitment is a process involving multiple cell types, the ovarian *ex vivo* culture model has a great deal of potential as a complementary technique.

Significance of Research: Human health relevance and consequences

The toxicants used in this study are found ubiquitously in the industrialized world. While the exact levels of exposure to MEHP or BPA amongst the general population are contested, it is clear that virtually everyone has some level of exposure and this level will

vary greatly based on a number of factors. Occupational exposures are possible either through primary exposure (i.e., working in a plastics plant producing either DEHP or BPA or products containing these compounds) or through professional products (thermal tape, cosmetics, etc.) Personal exposure will vary depending on the products used. Phthalates are widely used as stabilizers in pills and other pharmaceuticals, while BPA is typically used in the lining of canned foods.

It should be remembered that individuals exposed to these EDCs are not being exposed in a vacuum as BPA and MEHP are just two prominent members of a wide range of EDCs to which the population is routinely exposed. While co-exposure to multiple EDCs is beyond the scope of the current study, co-exposure is the rule and not the exception within the human population.

It should be noted that the MEHP exposure used in Aim 1 was well beyond what would be considered physiologically relevant. The 1,000 mg/kg exposure group in which most of the discussed data were found is several orders of magnitude beyond what is reported in the general population. However, while a linear dose response cannot and should not be assumed, the current study does identify a distinct MEHP-exposed phenotype which will inform both further mechanistic studies and draw attention to certain pathologies that might otherwise not be associated with phthalate exposure, such as premature ovarian failure.

Similarly, a wide range of doses were used for the BPA study. The lowest dose used, 2.5nM, corresponds with, or approaches, frequently reported levels of BPA found in human serum. 25nM, the middle dose, is one order of magnitude higher but still falls within the EPA safe exposure level, and at this level significant change relative to control

were observed at the follicle level. The highest dose, 25uM, was three orders of magnitude higher than the next lowest dose used and would not generally be considered physiologically relevant. Despite this, this exposure is useful because it again identifies targets of BPA action that might otherwise go unnoticed and provides further evidence for a non-monotonic dose response to BPA. This unusual dose response should be taken into consideration for later studies and interpretation of results.

Premature Ovarian Failure

Premature ovarian failure (POF) is the loss of ovarian function before the normal end of an animal's reproductive lifespan. The exact characteristics of this disease state have already been discussed in the background chapter. It bears repeating, however, that this disease has become increasingly prevalent within the past century, and the role of environmental exposures as a risk factor remains a question of great scientific interest. In the current studies, both MEHP and BPA can reasonably be considered potential risk factors for POF. In the case of MEHP, it is quite clear that the high dose of MEHP has induced premature ovarian failure: the animals exposed at this dose show loss of fertility significantly earlier than control counterparts. Furthermore, these exposed animals at one year of age have significantly lower levels of serum estradiol, indicating loss of ovarian endocrine function in a classic indication of menopause. While the dose that achieved this phenotype was far beyond regular human exposure, this study clearly indicates that MEHP can induce POF in mice, and therefore it is not unreasonable to expect that it has the potential to play a role in human POF.

Because the current BPA study only assessed the effect of the exposure over the course of 4 days, it would be overstating the case to say BPA also definitively causes

POF. However, it is clear that BPA at higher doses causes significant increases in follicular recruitment, as the highest dose demonstrated both reduced numbers of primordial follicles and increased numbers of apoptotic primary follicles. These follicles recruited by BPA will be lost well before sexual maturity and therefore subtracted from the follicular reserve of the animal. Assuming the current dogma regarding the ovary's inability to produce additional oocytes is correct, one can reasonably expect that any follicles ultimately lost to premature activation will shorten the animal's reproductive lifespan by the appropriate amount.

Again, the current study was limited to only the ovary and during a particularly short span of time. However, during that span of time, there is both an increased recruitment of follicles and increased secretion of estradiol by the exposed ovary. While it is quite possible that this accelerated recruitment would immediately end following exposure, it is may be possible that the increase in estradiol production and primary follicles might ultimately alter the HPG-axis and cause "runaway recruitment" as proposed in the case of the MEHP studies. While there is no direct observation of this phenomenon at this time, it appears plausible enough to warrant further investigation.

Mammary Gland Hyperplasia

was observed as a result of *in utero* MEHP exposure at a year of age. This effect is presumably due to constitutively higher levels of estradiol. Numerous previous studies have indicated hyperplasia as a consequence of prolonged xenoestrogen exposure or elevated endogenous estradiol [3,7,8]. In the MEHP study, this hyperplasia was accompanied by hyperplastic alveolar nodules suspected of being precancerous lesions. While these results are difficult to extrapolate to humans, it is not unreasonable to suspect

that if women were to have the elevated estradiol phenotype associated in this study with MEHP exposure they would be at additional risk for breast cancer. The connection between estrogen (both endogenous and exogenous) and breast cancer has been well-established. Therefore, while it cannot be concluded that MEHP exposure is directly a risk factor for mammary gland hyperplasia and possible tumorigenesis, the secondary effects of altered HPG-axis behavior may be a risk.

Other Potential Health Consequences

Endocrine disruption *in utero* has been shown to cause a wide range of reproductive abnormalities. The best example would be diethylstilbesterol (DES), a potent estrogen mimic widely prescribed to pregnant women for several decades. The first indications of adverse effects of this compound were numerous young women presenting with a particular vaginal cancer otherwise considered both rare and restricted to older women. Eventually, a wide range of other abnormalities have been linked to DES exposure, including uterine malformation and impaired fertility.

While the current studies of MEHP and BPA clearly show that these compounds are not nearly as potent as DES and do not necessarily act by the same mechanism, the case of DES still illustrates that the effects of early endocrine disruption can be both profound and not easily predicted. For that reason, there may be other effects of these exposures not discovered or assessed in the current study or effects that may only be relevant to humans.

One area of intense interest is an increasing trend for premature puberty among human girls. While the average age of menarche has slowly decreased over the past decades, particularly in industrialized countries, the age of thelarche has been dropping

considerably faster. Environmental factors have been indicated as a contributing factor, including phthalate exposure [9], though findings have been varied [10,11]. While the current MEHP study actually suggested a delay in puberty (with delays in the onset of estrous in high treatment groups) and the BPA study occurs strictly in a perinatal system, the possibility that these exposures may play a role in human premature puberty must be considered. Because both exposures can apparently cause elevated estradiol levels (persistently in the case of MEHP and of unknown duration in the case of BPA), it is not unreasonable to suspect a role. Again, there is no evidence in the current studies that these exposures cause premature puberty, though MEHP does cause changes in the timing of sexual maturity (albeit by delay rather than prematurity).

However, it is clear that these exposures can cause a major alteration to the ovary and by extension the entire HPG axis. Because this alteration happens early in life and before many estrogen-mediated changes and growth, the full lifetime effects of this perturbation are difficult to predict but are likely to be profound, lifelong, and adverse. For this reason, *in utero* endocrine disruption remains a topic of serious concern requiring extensive investigation to ensure the health of all who are, during critical stages of development, ubiquitously exposed to numerous compounds of uncertain action and safety.

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