MECHANISMS OF ESTROGENIC ENDOCRINE DISRUPTION OF FEMALE PUBERTY AND REPRODUCTION AND FUNCTIONS OF LHFPL2 ON DISTAL REPRODUCTIVE TRACT DEVELOPMENT

by

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(Under the Direction of Xiaoqin Ye)

ABSTRACT

Reproduction is essential for the continued existence of species. Both environmental and genetic factors can affect reproduction. This dissertation has two main focuses: I) effects and mechanisms of estrogenic endocrine disruptors, zearalenone (ZEA) and diethylstilbestrol (DES), on puberty and female reproduction; II) effects of a point mutation in lipoma HMGIC fusion partner like-2 (Lhfpl2) gene on distal reproductive tract development in mice. Results from the 1st part of the research can be summarized as follows: A. Postweaning exposure to dietary ZEA advanced the timing of pubertal onset indicated by vaginal opening and disrupted embryo implantation in a dose-dependent way. Pre-mating or post-mating exposure to 40 ppm ZEA in diet affected fertilization or delayed embryo transport and embryo development, respectively, to impair embryo implantation (Chapter 2). B. Multigenerational exposure to 20 ppm dietary ZEA cumulatively impaired fertility, which could be partially alleviated upon exposure cessation (Chapter 3). C. Exposure timing is critical for endocrine disruption of puberty and early pregnancy. Peripubertal exposure to 50 ppb DES
significantly advanced the timing of vaginal opening. Peripubertal exposure to 50 ppb DES reduced the numbers of corpora lutea whereas post-mating exposure to 50 ppb DES affected postovulation events, both led to impaired embryo implantation (Chapter 4). The initial goal in part II of the dissertation was to investigate the mechanisms of vaginal opening using a Lhfpl2 mutant mouse model that had vaginal imperforation (Chapter 5). However, extensive studies showed that the vaginal imperforation was due to defective embryonic development of the distal vagina, resulting in female infertility. Interestingly, similar pathology was observed in Lhfpl2 mutant males that had abnormal structure of the distal reproductive tract, causing infertility in ~70% of males. In situ hybridization and immunohistochemistry localized Lhfpl2/LHFPL2 in the epithelium of female and male reproductive tracts. In vitro overexpression studies showed that wild-type and mutant LHFPL2 were both localized in the endoplasmic reticulum but only the wild-type LHFPL2 was expressed in the filopodia. This dissertation provides novel knowledge about mechanisms of estrogenic endocrine disruption of female puberty and reproduction, as well as a genetic basis of distal reproductive tract development.

INDEX WORDS: Diethylstilbestrol, embryo development, embryo implantation, exposure timing, embryo transport, endocrine disruptors, fertilization, lipoma HMGIC fusion partner like-2, ovulation, reproductive tract development, vaginal opening, zearalenone.
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LIST OF ABBREVIATIONS

**BPA**: bisphenol-A;  **cAMP**: cyclic adenosine monophosphate;  **CB1**: cannabinoid receptor 1;  **CFTR**: cystic fibrosis transmembrane conductance regulator;  **CND**: common nephric duct;  **D0.0-D19.5**: gestation day, mating night as D0.0, plug morning as D0.5;  **DES**: diethylstilbestrol;  **E2**: 17β-estradiol;  **EEDCs**: estrogenic endocrine disrupting chemicals;  **EGF**: epidermal growth factor;  **EMT**: epithelial-to-mesenchymal transition;  **FSH**: follicle-stimulating hormone;  **GAPDH**: glyceraldehyde-3-phosphate dehydrogenase;  **GPCRs**: G protein-coupled receptors;  **Hbegf**: Heparin-binding EGF-like growth factor;  **HET**: heterozygous;  **HPG**: hypothalamic-pituitary-gonadal;  **HPRT1**: hypoxanthine phosphoribosyltransferase 1;  **ICM**: inner cell mass;  **kg**: kilogram;  **LH**: luteinizing hormone;  **Lhfp**: lipoma HMGIC fusion partner;  **Lhfpl1-5**: lipoma HMGIC fusion partner like 1-5;  **MD**: Mullerian duct;  **mg**: milligram;  **mg/kg/day**: milligram per kilogram body weight per day;  **MUT**: mutant;  **OCC**: oocytes cumulus complex;  **PND**: postnatal day;  **s.c.**: subcutaneous;  **TBST**: Tris-buffered saline containing 0.1 % tween;  **TDI**: tolerable daily intake;  **TE**: trophoderm;  **Tmhs**: Tetraspan membrane protein of hair cell stereocilia;  **UGS**: urogenital sinus;  **WD**: Wolffian duct;  **WT**: wild-type;  **ZEA**: Zearalenone;  **µg**: microgram;  **µg/kg/day**: microgram per kilogram body weight per day.
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CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

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1.5 MAJOR HYPOTHESES AND SPECIFIC AIMS
The dissertation focuses on two major aspects: mechanisms of estrogenic endocrine disruption of female puberty and early pregnancy, and mechanisms of LHFPL2 on distal reproductive tract development in mouse models. The first chapter is to review the current literature about them to prepare you with background to understand Chapters 2-5 easier and better.

1.1 EARLY PREGNANCY EVENTS

1.1.1 Overview

Early pregnancy events are essential for successful pregnancy outcomes. They include ovulation, fertilization & embryo transport, embryo development, and embryo implantation. The female reproductive system is the major location where these events take place. The system mainly consists of ovary, oviduct (the fallopian tube in human), uterus and vagina. Events during early pregnancy happen in different compartments (ovary, oviducts and uterus). Specifically, ovulation occurs in the ovary; fertilization in the oviduct; embryo transport and development initiate in the oviduct and continue in the uterus; embryo implantation occurs in the uterus. These early pregnancy events as well as their endocrine regulations will be introduced sequentially.

1.1.2 Ovulation (Pre-pregnancy)

Ovulation is the release of the fertilizable female germ cell from ovarian follicles into the oviduct [1]. Normal development and maturation of ovarian follicles and oocytes are prerequisite for ovulation at adulthood. At the primordial follicle stage, all oocytes are kept in the prophase of first meiotic division before postnatal day (PND) 3 [2]. It is widely accepted that a pool of primordial follicles is the ovarian reserve that comprises
the source of female gametes for life. During the peripubertal period, some primordial follicles will be stimulated to initiate growth and develop into primary follicles [2].

The primary follicles will then start to grow in response to pituitary gonadotrophins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which work through their respective G protein-coupled receptors (GPCRs). A much smaller number of these growing primary follicles are selected for further development into preantral follicles, antral follicles and preovulatory follicles. In the preantral follicles, the pellucid zone between oocytes and follicle epithelium cell is visible and multiple antrums also begin to form. Formation of antrums indicates that follicles are at the antral stage [3]. Only a small number of antral follicles are selected to reach preovulatory stage by subtle increases in the basal levels of FSH and LH [4]. At the preovulatory stage, increased serum estrogen (E2) produced by mature follicles triggers the surges of FSH and LH through positive feedback at proestrus in mice. The increased level in FSH will stimulate granulosa cells LH receptor expression, thereby allowing only these follicles to respond to the LH surge. So FSH priming is critical for sufficient expression of granulose cells LH receptors. The high level of LH stops follicle growth and triggers a cascade of expression changes in a cohort of transcription factors (e.g. early growth response-1 and Sp1/Sp3) [5], which induce effector gene products (e.g. Egf-like ligands and cathepsin L) [5]. These gene expression changes prepare follicles for ovulation. After ovulation, follicles are remodeled to form corpora lutea. In summary, the surges of FSH and LH responding to the high-level E2 stimulate the preovulatory follicles to ovulate and luteinize (Fig. 1.1) [4, 6].
The corpora lutea formation from postovulatory ovarian follicles is called luteinization [7]. During the process, granulosa cells undergo structural and genomic changes that lead to differentiation into progesterone-producing luteal cells [7]. First, granulosa cells exit the cell cycle and express genes critical for luteinization rapidly and transiently, such as progesterone receptor and cyclooxygenase-2 [7]. During differentiation into a luteal phenotype, the granulosa cell undergoes expression changes in receptors for FSH, LH, estrogen, progesterone and prolactin, which subsequently alter its responsiveness to external signals. The granulosa cell also undergoes expression alteration in numerous intracellular signaling networks, such as cyclic adenosine monophosphate (cAMP) and calcium [7]. Along with these changes in granulosa cells, the follicular basal membrane breaks down; and endothelial cells, fibroblasts and theca cells evade into granulosa layers to establish neovascularization in

Figure 1.1. The hormonal profile during early pregnancy in mouse. P4, progesterone; E2, estrogen; LH, luteinizing hormone; Mating night as gestation day 0 (D0.0), morning plug was detected on D0.5, implantation occurs around D4.0. (Modified from [20])
the newly formed corpora lutea [7]. The corpora lutea will produce a substantial amount of progesterone by uptaking, synthesizing, transporting and converting cholesterol [7]. They also possess enzymes for converting progesterone to weak androgen (androstenedione) and estrogen [7].

Regression of the corpus luteum (also known as luteolysis and demise of corpus luteum) have two phases, the functional regression, which decreases the progesterone production, and the structural regression, which decreases the size and weight of corpus luteum [7]. Prostaglandin F2α and LH are involved in the waning progesterone production in the first phase [7]. Programmed cell death in luteal and vascular cell is involved in the structural alteration in the second phase. The regressed corpora lutea will become a scar within the stroma, also known as corpus albicans. Most corpora albicans will eventually reabsorbed and replaced by stroma cells [7].

Endocrine regulation of E2 production and ovulation: Both granulosa cells and theca cells are involved in E2 production (Fig. 1.2). The LH binds to its GPCRs in the theca cells, which in turn activates the adenylyl cyclase leading to increased cAMP level, which induces several enzymes for the production of androgen (testosterone and androstenedione) from cholesterol [8]. The androgen produced by theca cells diffuses into granulosa cells, which can increase the FSH receptor expression [9]. Granulosa cells are the only cell type in the ovary to express FSH receptors. FSH activates its GPCRs in granulose cells and increases the intracellular
cAMP level. The cAMP exerts a broad range of effects as a second messenger. It activates and induces aromatase, an enzyme responsible for converting the androgen into E2, and induces LH receptors in granulose cells. The E2 produced by granular cells will exert positive feedback to hypothalamic–pituitary–ovarian axis on gonadotropin secretion. More FSH and LH will be produced, which will further intensify proliferation of granulosa cells and theca cells and their coordinated E2 production. By this positive feedback, there will be FSH and LH surges to induce ovulation [3].

1.1.3 Egg transport & sperm transport

After ovulation, eggs will be transported into the oviduct. The oviduct is divided into three anatomical regions: infundibulum, ampulla and isthmus (Fig. 1.3). The infundibulum is surrounded by a fringe of tissue called fimbriae in the direction of ovary (Fig. 1.3)

Ovulated egg transport from ovary to ampulla: After ovulation, the oocyte cumulus complex (OCC) is picked up by the infundibulum and moves quickly to the ampulla where fertilization occurs. Infundibulum is the closest part of oviduct to the ovary but not physically connected to the ovary [10]. The process of fetching the OCC by infundibulum is suggested to be the result

Figure 1.3. The female reproductive system. The oviducts have three distinct segments: infundibulum, ampulla and isthmus. (Edited from [10])
of adhesion of the OCC to the ciliated epithelium of infundibulum and the ciliary beating. Muscle contraction might not be involved in the fetching process because its quiescence does not affect the process [10].

**Sperm transport to the site of the ovulated egg.** Upon successful mating, the sperm with semen is ejaculated into the female reproductive tract. Most of the semen is rapidly transported into the uterus, while some remains in the vagina where it coagulates to form a white plug. This plug has been demonstrated to be critical for sperm transport into uterus [11]. Then, the sperm passes the uterotubal junction to reach ovulated eggs in the oviduct [3]. The entrance to the oviduct forms a conical projection (called colliculus tubarius) which prevents sperm from entering [11]. Sperm reach the ampulla within 5 minutes after ejaculation in mice. The ciliary movement and fluid flow in the oviduct can aid sperm movement in the oviduct [3].

During transport in the female reproductive tract, the sperm must undergo capacitation before they are competent for fertilization. Capacitation is an irreversible process, through which motility is increased, surface proteins are removed and lipids are reduced [3, 12]. This reaction results in changes in both the head and the flagellum: the acrosome (a secretory vacuole-like structure) in the sperm head now is able to recognize the glycoprotein in zona pellucida of the ovulated egg; and the flagellum becomes hyperactivated enough to penetrate the cumulus mass and zona pellucida of the ovulated egg [12, 13]. Although the mechanism underlying capacitation is unknown, studies have shown that some critical factors and signaling pathways involve in this process, such as the epidermal growth factor receptor, ion channels, Ca$^{2+}$ and cAMP [13-15].
1.1.4 Fertilization & preimplantation embryo development

The fusion of the haploid sperm and the egg to produce diploid embryo is called 
fertilization. After ovulation, the egg is located in the ampulla of the oviduct. Sperm from 
males ejaculation are “swimming” and transported to the ampulla, where they become 
mature enough to penetrate the zona pellucida and the plasma membrane of the egg. 
Upon penetration, the egg is activated to resume its second meiotic division [3, 12]. Half 
of the chromatin (one haploid) is extruded in the second polar body. The left haploid 
transform into a female pronucleus. With the movement of cytoskeleton, the two 
pronuclei from the sperm and the egg move toward the center of the fertilized egg. 
During this process, chromatin of the two pronuclei loosen and their DNAs replicate, 
ready for the subsequent mitotic division. Then, the pronuclei membranes are broken 
down; chromosomes are condensed and assembled on the spindles, therefore 
reconstituting into a diploid organism. So the pronuclei from the sperm and the egg 
unite, forming the diploid zygote [16-18].

Preimplantation embryo development after fertilization in mice: the zygote sets 
off its first division, producing two equally-sized cells. By D1.5, the embryo reaches the 
two-cell stage (Fig. 1.4). Then, the embryo undergoes another three divisions to get to
four-cell, eight-cell and sixteen-cell stages on D1.5-D3.0 (Fig. 1.4). By the sixteen-cell stage, the compacted embryo is termed as a morula (~D3.0, Fig. 1.4). The morula is characterized by the first-time differentiation of an embryo, producing an outer rim of cells, the trophectoderm (TE) and the inner cell mass (ICM). The TE, also known as trophoblast, consists of a specialized, differentiated and polarized cells. The trophoblast cells are important for embryo attachment and implantation into the uterus and will eventually develop into the fetal part of placenta and yolk sac [19]. The ICM will differentiate into fetus and tissues supporting embryo development [12].

The trophoblast and the ICM regulate the development of each other to continue proliferation and compaction. The trophoblast excretes or transports watery fluid into basolateral lumen to form a cavity called blastocoel (Fig. 1.5). As a result of the cavitation, physical detachment and differentiation, trophoblasts gradually surround the ICM and the ICM gradually compact each other tightly (Fig. 1.5). Around D3.5 (Fig. 1.5), the embryo is at the stage of blastocyst, featuring the outer sphere of flatterned trophoblasts, the fluid-filled cavity (blastocoel) and the small round highly compacted ICM (Fig. 1.5) [12, 20].

Prior to implantation, the blastocyst continues expanding to become a fully developed blastocyst (late blastocyst) [21]. One feature of the late blastocyst is the

Figure 1.5. The structure of preimplantation blastocyst. The outside glycoprotein membrane is zona pellucida; the outer sphere of flatterned cells are trophoblast; the cavity is called blastocoel; the compacted inner cells are called the inner cell mass (ICM).
topological change. The early blastocyst has the ball-like structure, and late blastocyst is elongated along an axis due to the expansion of the blastocoel [22]. Another feature is the second round differentiation. Just before implantation on D4.0, a tissue called primitive endoderm differentiates on the surface of the ICM. The primitive endoderm is destined to generate only the extraembryonic parietal endoderm (part of visceral yolk sac) and visceral endoderm (part of parietal yolk sac). The remainder of the ICM will develop into a fetus [21].

On D4.0-D4.5, the blastocyst hatches from zona pellucida and gets ready for implantation. The mechanism of hatching is still not well-understood. Digestion enzymes synthesized by both the mural trophoblast cells (region opposite and farthest from the inner cell mass) and the uterus involve in the breakdown of zona pellucida. The rhythmic expansion and contraction of embryo may also play a role in hatching [12]. Afterwards, the blastocyst attaches the uterine epithelium, evades into the stroma, and establishes a placenta [23].

It has been noticed that preimplantation embryo development can be influenced by E2 and progesterone. E2 can reduce the number of embryos and the number of cells per embryo [23]. Ovariectomized mice show delayed embryo development. Treatment of E2 and progesterone can reverse the delays. Ovarian hormones may depend on growth factors or E2 metabolites from the uterus and the oviduct to regulate the early embryo development [23].

1.1.5 Preimplantation embryo transport

Another key event during early pregnancy is preimplantation embryo transport in the oviduct and in the uterus before implantation. It includes 1) transport of the
preimplantation embryo from the ampulla of the oviduct through the uterotubal junction into the uterus; 2) the blastocyst distribution in the uterus

**Embryo transport in the oviduct:** after fertilization, the embryo detaches from the ampulla epithelium and continues its migration toward the uterotubal junction [24]. Timing of embryo arrival in the uterus is critical since the “window of implantation” starts and ends in a very short period [10]. Passage of the embryo from the oviduct to the uterus requires the coordination of the ciliary beating and the smooth muscle contraction. The two processes can be regulated by ovarian hormones, embryonic factors and paracrine factors [25].

Ovarian hormones have been proven to play an important role. Administration of E2 after mating delays embryo transport in mouse [10, 26]. Administration of a progesterone antagonist after mating delays embryo transport in mice, suggesting the advancing effects of progesterone on embryo transport [27]. E2 stimulates the smooth muscles while progesterone relaxes them [10]. The opposite effects of E2 and progesterone on the smooth muscles may explain the opposite effects of the two ovarian hormones on embryo transport. There is no information about how ovarian hormones regulate the ciliary beating in the segment between the ampulla and uterotubal junction in mice.

Some factors released from embryos also play a role in embryo transport. The embryonic platelet-activating factor accelerates the transport possibly via increasing the ciliary beating by activating its receptor in the oviduct [10]. Embryos at the four-cell stage transport faster than the 1-cell stage when both are transferred to a recipient rat, indicating embryo signaling regulates the timing or rate of transport [28]. So the embryo
at different developing stages may produce unknown factors to regulate its transport to the uterus.

Several paracrine signals in the oviduct have been shown to regulate embryo transport. The prostaglandin E and prostaglandin F work the opposite way to contract or relax smooth muscle contraction, respectively [10]. Endothelin-1 and angiotensin II from the oviduct are also involved in the regulation of smooth muscle contraction [10]. Stimulation of α adrenergic receptor promotes the smooth muscle contraction whereas β activation inhibits the contraction [10]. Adrenomedullin, a peptide hormone produced by the oviduct, increases ciliary beating frequency and decreases smooth muscle contraction in rats [29]. Cannabinoid receptor 1 (CB1) is expressed in the oviductal smooth muscle and the developing embryo. Genetic deletion of CB1 causes retention of embryos in the oviduct, which can be reversed by administration of the β adrenergic receptor agonist [30, 31]. This indicates the importance of CB1 in embryo transport in the oviduct and its interaction with the adrenergic receptor signaling.

**Blastocyst distribution in the uterus:** When the blastocysts arrive in the uterus, they will be relatively evenly distributed along the uterine horns before hatching and embryo implantation take place [12]. The peristaltic movements of the uterine smooth muscles is thought to be responsible for the embryo alignment along the uterus in an equally distance between two adjacent embryos. Several factors are possibly involved in the embryo spacing in the uterus, such as lysophosphatidic acid receptor 3 [32] and β2-adrenoceptor [33], but the exact mechanism(s) is still unknown.
1.1.6 Embryo implantation

Implantation is a process by which the embryo comes into the intimate physical and physiological contact with the uterine epithelium, invading into the stroma and establishing the placenta. For successful implantation, the uterus must become receptive for an embryo to implant and the embryo is competent to implant into the uterine wall simultaneously [34].

**Endocrine regulation:** Implantation is mainly regulated by ovarian hormones, progesterone and E2, via their respective nuclear receptors. On D1 and D2, relative high level of E2 stimulates the proliferation of the uterine epithelium. On D3, progesterone produced by the newly-formed corpus luteum induces stroma proliferation. Around D4, progesterone reaches its peak. On the D3.5 morning, E2 at a surge level cooperates with progesterone to induce uterine receptivity. The epithelial cells stop proliferation and differentiate into a conducive tissue for embryos to implant. Around D4.0, the activated embryo implants into the receptive uterus (Fig. 1.1) [34].

**Establishment of uterine receptivity:** Uterine receptivity refers to a restricted and transient period when a uterus is receptive for blastocyst attachment. In mice, this period is limited to D4.0. So the uterus is non-receptive to embryos on D1.0-D3.0 and become refractory on D5.0 and afterwards [34]. In a receptive uterus, the uterine epithelial cells undergo morphogenesis and differentiation, becoming receptive for embryo implantation. The establishment of uterine receptivity requires the interactions among blastocyst trophoblasts, uterine luminal and glandular epitheliums, and stromal cells [34]. This process involves various signaling pathways, such as ovarian hormones (progesterone, E2 and their respective nuclear receptors), cytokines (leukemia inhibitory...
factor and interleukin-6), lipid signaling (cytoplasmic phospholipase A 2α, lysophosphatidic acid receptor 3, prostaglandin-endoperoxide synthase 2 and prostaglandins) and developmental genes (homeobox genes; bone morphogenetic proteins. Indian hedgehog proteins and their respective receptors) [34].

**Blastocyst activation**: The blastocyst must hatch from zona pellucida and acquire the implantation competency for successful implantation [23]. It should be noticed that the trophoblast is the embryonic tissue that establishes the interaction with uterus. The blastocyst activation involves in differentiation of the trophoblasts. During activation, trophoblasts express many adhesion-associated proteins, such as perlecan, for the embryo attachment to the uterus; and express some invasion-associated factors to aid the invasion [23]. Some signalings have been shown to play a role in the blastocyst activation. The gene *Hbegf* (Heparin-binding EGF-like growth factor) and its receptors are upregulated during the blastocyst activation. HBEGF may activate trophoblasts to differentiate and become adhesive during the embryo attachment and invasion [34].

Catecholestrogen, a catechol metabolite of endogenous E2, may be locally produced in the uterus and can activate dormant blastocysts in culture via a pathway different from classical nuclear estrogen receptor signaling [34]. CB1 is expressed in the trophoblast and its ligands, 2-arachidonoylglycerol and anandamide, are produced in the uterus [34]. The anandamide at the low level can bind CB1 to activate mitogen-activated protein kinase signaling and induce blastocyst competency while its higher level may affect this induction by inhibiting Ca²⁺ mobilization. This indicates the important role of the cannabinoid receptor signaling in the blastocyst activation.
1.2 TIME-DEPENDENT EFFECTS OF ESTROGENIC ENDOCRINE DISRUPTORS ON EARLY PREGNANCY

1.2.1 EEDCs

Estrogenic endocrine disrupting chemicals (EEDCs, also called estrogenic endocrine disruptors) are exogenous estrogenic chemicals in our environment that can interfere with normal functions of endocrine systems. They include persistent organohalogenes, some pesticides (such as methoxychlor), plasticizers (phthalates), naturally-occurring chemicals (such as, the phytoestrogen genistein and the mycotoxin zearaleone, ZEA), and drugs (such as diethylstilbestrol, DES) [35]. The dissertation uses ZEA (Chapter 2-3) and DES (Chapter 5) as testing compounds.

ZEA is a mycotoxin produced by several Fusarium species [36]. It is commonly found in livestock feed and human food, such as corn, rice, oats and wheat. Quantifiable ZEA was detected in 15% of 13,075 food samples and 9,877 unprocessed grain samples collected during 2005 and 2010 in Europe. Among them, detectable levels in processed food groups for human consumption were in the ppb range with the highest at 823 ppb, and those in unprocessed grains with the highest reaching 3 ppm (part per million) [37]. Worldwide, ZEA contamination levels in food are usually in the range of ppb and low ppm, with the highest reported reaching 600 ppm [36-39]. The tolerable daily intake (TDI) of ZEA established by the Panel on Contaminants in the Food Chain in Europe is 0.25 µg/kg body weight [37].

ZEA is mainly metabolized in the liver to the α- and β-zearalenol by the 3α- and 3β-hydroxysteroid dehydrogenases, respectively. The α-zearalenol is the dominant ZEA derivative in pigs [40], humans [41], rats and mice [42]. ZEA and its metabolites are
then glucuronidated by uridine diphospho-glucuronosyltransferase [36, 37, 43]. Cytochrome P450 may also be involved in ZEA metabolism [37]. ZEA and its metabolites have estrogenic effects due to their structural similarity with E2 [44]. The order of estrogenicity is E2> α-zearalenol>ZEA> β-zearalenol based on the gene induction assay [45], uterotrophic assay [46], and estrogen receptor binding assay [40].

ZEA has been associated with certain pathological conditions in females and shown to have adverse effects on embryo and/or female reproductive system in animal studies [37]. Also, human exposure to ZEA has been linked with precocious puberty in girls [47, 48] and neoplastic lesions in the reproductive tract [49]. So it has been a concern for female reproduction.

DES is a synthetic nonsteroidal E2 used previously as a drug for preventing miscarriage. DES exposure during pregnancy increased the risk of breast cancer in DES-exposed women, the risk of adenocarcinoma of the vagina and cervix and the risk of breast cancer in daughters of those women, and the risk of infertility, genitourinary anomalies and testicular cancer in the sons of DES-exposed women [50]. Based on different methods, DES has 1.1-2.5 times estrogenic potency compared to E2 [51]. Because DES has a potent estrogenic activity, it is often used as positive control [52, 53] and suggested as a model compound to reveal the reproductive toxicity with implication to other weak EEDCs [54].

E2 is crucial for successful female reproduction in mice. So EEDCs can interfere with E2-regulated physiology processes. EEDCs have been a risk factor for female reproductive health as exposure to EEDCs have been associated with precocious puberty in girls and decreased infertility in women [55]. The pre-pregnancy and early
pregnancy events prior to implantation include ovulation, fertilization, embryo transport and development, and establishment of uterine receptivity. All of them can be regulated by E2 and thus can be influenced by EEDCs. EEDCs have been associated with early pregnancy defects in human [56, 57]. So it is important to elucidate effects and mechanisms of EEDCs on early pregnancy.

Exposure timing plays an important role in effects and mechanisms of EEDCs on early pregnancy. Developmental exposure to EEDCs during developing stages, such as the in-utero, the neonatal and the peripubertal periods, can affect early pregnancy by disrupting the development and/or function of female reproductive organs [2, 58]. Different exposure timing to EEDCs on early pregnancy, especially embryo implantation, is reviewed in the following sections.

1.2.2 In-utero exposure

During organogenesis, primordial germ cells migrate into the indifferent gonad at D9.5 and rapidly proliferate until entering meiosis I at D13.5 (gestation day 13.5). At this point, female germ cells are referred to as oocytes [2]. The Mullerian duct develops and differentiates into three structurally distinct segments, the oviduct, the uterus and the upper vagina after D13.5 [59, 60]. The in-utero period is a well-established developmental window sensitive to EEDCs affecting reproductive organ development and subsequent early pregnancy at adulthood.

In utero exposure to DES (0.01 to 100 µg/kg/day) between D9-D16 in CD-1 mice decreased ovulation capability and caused structural abnormalities of the oviduct, uterus, cervix, and vagina in a dose-response way, which all contributed to infertility [61, 62].
1.2.3 Perinatal & neonatal exposure

During the perinatal and neonatal period, the ovary undergoes follicular assembly, i.e., the formation of primordial follicles starting from D13.5 to PND 2-3 [2]; and some of primordial follicles develop subsequently into primary follicles, secondary follicles and antral follicles around PND 3-21 [63]. The female reproductive tract is also undergoing morphogenesis and cellular differentiation, which is essential for its function at adulthood [64]. EEDCs can potentially affect the development of the ovary and the female reproductive tract to alter early pregnancy events as well as their collective event, embryo implantation.

Neonatal exposure to ZEA in ICR mice (PND1-10, 5-30 µg/animal) through subcutaneous (s.c.) injection caused delayed vaginal opening, persisted estrus and sterility. Further histology examination showed the vaginal epithelium thickening, indicating the induced proliferation in the reproductive tract [65]. C57BL/Crgrl mice were treated via s.c. injection of 1 µg/animal ZEA daily for 5 days after birth. At 8 months old, 74% the treated females had no corpora lutea; 56% had dense collagen deposition in uterine stroma and lacked uterine glands; 59% had squamous metaplasia in uterus; 32% had altered vaginal epithelium [66]. These adverse effects on reproductive tracts were not observed in ovariectomized ZEA treated mice, indicating that the alterations in the reproductive tract was depend on the ovary [66]. In CD-1 mice, exposure to 50 mg/kg/day genistein on neonatal days 1-5 via s.c. injection altered oviductal environment for preimplantation embryo development and uterine receptivity for embryo implantation. This developmental exposure may affect morphogenesis, immune
responses and adult gene expression patterns in the oviduct to cause preimplantation embryo loss [64, 67].

In the rat, a single s.c. injection of 0.1 mg E2 or 1.0 mg ZEA on PND 3 or 5 caused persistent vaginal estrus and no newly-formed corpora lutea in the ovary at adulthood. [68]. In rats, exposure to 20 mg/kg/day bisphenol-A (BPA) or 0.2 µg/kg/day DES on PND 1, 3, 5, and 7 significantly decreased the number of implantation sites possibly by altering uterine environment for implantation [52].

1.2.4 Peripubertal exposure

During the peripubertal period, the ovary continues to develop and the hypothalamic-pituitary-gonadal (HPG) axis is being established. Specifically, the granulosa cells continue to respond to gonadotropin to secrete E2 and the follicles continue to develop and grow; the brain begins to respond to the positive feedback of E2, resulting in the LH surge and the occurrence of the first estrous cycle and ovulation [69]. The first ovulation occurs about 5 days later after vaginal opening in mice. Due to the noninvasiveness, vaginal opening is usually used as an indicator of pubertal onset [70]. The uterus will increase its weight and size [71], and form more glands in this period [72].

In pigs, exposure to ZEA (3.61 ppm ZEA in 2 kg daily diet per gilt) from puberty to mating caused anestrous and edema uteri while the reproductive performance was not affected in gilt. In the same study, pregnancy exposure (4.33 ppm in 2 kg diet per sow) induced pseudopregnancy states in 45% nonpregnant gilts; decreased weight of uteri, placental membranes and fetus; and reduced heterogeneity of fetuses in the same litter compared with control [73].
There is inadequate knowledge about effects of peripubertal exposure to EEDCs on early pregnancy events, including embryo implantation, in animal models. This is also the significance of my dissertation as it fills the knowledge gap (Chapter 4).

1.2.5 Immediate exposure during early pregnancy

Immediate exposure is the period around the time of early pregnancy events, which is usually from D0-D4 and includes fertilization, embryo transport, preimplantation embryo development and embryo implantation. Early pregnancy is regulated by E2 in mice, therefore can be influenced by EEDCs.

S.c. injection to 100 mg/kg/day BPA on D0.5-D3.5 delayed embryo transport and preimplantation embryo development, and blocked embryo implantation [74]. Exposure to 60 or 90 ppm ZEA in 1.8 kg diet from pregnancy day 2-15 in gilts caused pregnancy loss when checked on pregnancy day 40-43 (implantation occurs around D13-14 in pigs) probably by inducing regression of corpora lutea and affecting ovarian hormone levels [75].

1.3 VAGINAL OPENING & LHFPL2

Cellular & molecular mechanisms of vaginal opening are the focus of the second part of this dissertation. Vaginal opening has been used as an indicator for pubertal onset in many toxicological studies to investigate effects on puberty. However, it is still unknown about the molecular mechanism of vaginal opening. I searched online and found that a spontaneous point mutation in Lhpfl2 caused imperforate vagina in mice, suggesting that Lhfpl2 may play a role in vaginal opening. Since no studies have been done to investigate Lhfpl2 and the phenotype of Lhfpl2 mutant mouse was relevant to
our EEDCs studies, I decided to use the Lhfpl2 mutant mouse as a model to investigate the mechanism of vaginal opening.

Lhfpl2 belongs to the family of lipoma HMGIC fusion partner (Lhfp), which was found to be the translocation partner of HMGIC in human lipoma tissue [76]. The Lhfp gene family includes six members, i.e. Lhfp, and Lhfpl1~ Lhfpl5 (lipoma HMGIC fusion partner like), all of which are predicted to be four transmembrane protein with 200-247 amino acids in both human and mice. As far as we know, there are only eight papers about this gene family since its discovery. Lhfpl1 is widely expressed in human tissue [77]; DNA hypermethylation in the promoter of Lhfpl4 is associated with cervical cancer in human [78]. Mutations in Lhfpl5 (also called Tmhs [tetraspan membrane protein of hair cell stereocilia]) could cause hearing disorder in human [79, 80] and mice [81, 82], and has been associated with hypospadias (a birth defect of abnormal placed opening of urethra), anal atresia with a recto-urethral fistula (a hole between urinary channel and the rectum) and hypoplastic kidney in a young boy [83]. Lhfpl5 may be link with PCDH15 (a cadherion) and the transduction channel to regulate mechanotransduction in cochlear hair cells [84]. Except these, we know nothing.

1.4 DISTAL REPRODUCTIVE TRACT DEVELOPMENT

My first intention was to use Lhfpl2 mutant mouse to investigate mechanism of vaginal opening. However the phenotype turned out to be defect in distal reproductive tract development. So I would like to review current literature about reproductive tract development in the following sections.
1.4.1 The reproductive tract development in mice

The female and male reproductive tracts are developed respectively from the Mullerian duct (MD) and the Wolffian duct (WD). Before sexual differentiation on D13.5, the mammalian embryos are sexually indifferent, with both the MD and the WD (Fig. 1.6).

In females, the WD degenerates and the MD will form morphologically and functionally different subsections, i.e. oviduct, uterus, cervix and 2/3 upper vagina in female. In males, the MD degenerates and the WD will differentiate into epididymis, seminal vesicle, and vas deferens (Fig. 1.6). Many developmental factors essential for the processes have been identified by investigating genetically modified mice [59, 60].

Figure 1.6. The embryonic development of reproductive tracts in mouse. (Edited from [59])

1.4.2 Distal reproductive tract development & genetic basis

The distal reproductive tract is the distal vagina and the distal vas deferens in female and male, respectively. The distal tracts need to fuse with the urogenital sinus (UGS, the embryonic origin of the bladder and the urethra) to develop into a complete
tract that form an open channel of vagina in female, and a complete tract that carries sperm from the testis to the urethra in male. Our knowledge about this fusion process is very limited.

In female mice, On D12.0, the MD is still on the way of extending posteriorly to reach the UGS. The WD is essential for guiding the MD elongation. By D13.5, MD somehow reaches the urogenital sinus [59, 85]. After D13.5, epithelium of the MD migrates posteriorly to form the entire vagina by PND7, which was different from previous hypothesis that upper two-thirds of vagina derives from the MD and the distal one-third develops from urogenital sinus [86]. While several genetically modified mice showed defects in vaginal opening (Table 1.1), it is still unknown about whether those vaginal imperforations are caused by distal reproductive tract developmental defects. So the genetic basis of distal MD development remains unknown.

In male mice, the distal WD on D12.0 connects with the common nephric duct (CND). On D13.5, the CND degenerates via apoptosis and the distal WD elongates and reaches the UGS [87], which may initiate the fusion process between them. The distal WD sequentially differentiates into ampulla, the wider duct of vas deferens at its connection with urethra, and seminal vesicle from lateral outgrowth [60, 88]. This fusion process may involve CFTR (CF SSR: Society for the Study of Reproduction knockout pigs showed distal vas deferens atresia [89]. However the mechanism of distal vas deferens remains a mystery and gene(s) potentially critical for distal WD development still await identification.
1.5 MAJOR HYPOTHESIS AND SPECIFIC AIMS

This dissertation focuses on effects and mechanisms of dietary exposure to two estrogenic endocrine disruptors, ZEA and DES, on female puberty and early pregnancy at different timepoints in mice; and effects and mechanism of a point mutation in Lhfpl2
on distal reproductive tract development in mice. These studies are based on a major hypothesis that are tested in 4 specific aims.

**The hypothesis of the dissertation**

Female pubertal onset and early pregnancy are regulated by estrogen and female reproductive tract development is controlled by genetic factors, it is hypothesized that estrogenic endocrine disruptors can affect female pubertal onset and early pregnancy and LHFPL2 is critical for distal reproductive tract development.

**Specific aims**

1. To determine effects and mechanisms of postweaning exposure to dietary zearalenone on pubertal onset and early pregnancy (Chapter 2);

2. To determine effects of multigenerational exposure to dietary zearalenone on puberty and embryo implantation (Chapter 3);

3. To determine exposure timing to dietary DES and potential recovery from exposure cessation on embryo implantation (Chapter 4);

4. To characterize the phenotypes of mice with a point mutation in *Lhfpl2* and to investigate mechanisms of LHFPL2 in distal reproductive tract development (Chapter 5).
CHAPTER 2
POSTWEANING EXPOSURE TO DIETARY ZEARALENONE (ZEA), A MYCOTOXIN, PROMOTES PREMATURE ONSET OF PUBERTY AND DISRUPTS EARLY PREGNANCY EVENTS IN FEMALE MICE

2.1 ABSTRACT

Zearalenone (ZEA) is a mycotoxin commonly found in contaminated livestock feed and human food with levels in the range of ppb and low ppm. It was hypothesized that ZEA, an endocrine disruptor, could affect puberty and early pregnancy. To test this hypothesis, newly weaned (3 weeks old) C57BL/6J female mice were exposed to 0, 0.002, 4, 10 and 40 ppm ZEA, and 0.05 ppm diethylstilbestrol (DES, positive control) in phytoestrogen-free AIN-93G diet. Females exposed to 10 and 40 ppm ZEA diets showed earlier onset of vaginal opening. Those treated with 40 ppm ZEA diet also had earlier first copulation plug and irregular estrous cyclicity. At 8 weeks old, all females were mated with untreated stud males on AIN-93G diet during mating. Treatment resumed upon identification of a vaginal plug on gestation day 0.5 (D0.5). Embryo implantation was assessed on D4.5. Exposure to 40 ppm ZEA diet resulted in reduced percentage of plugged mice with implantation sites, distended uterine appearance, and retained expression of progesterone receptor in D4.5 uterine epithelium. To determine the exposure timing and mechanisms of disrupted embryo implantation, four groups of females were fed with 0 or 40 ppm ZEA diets during premating (weaning~mating) and postmating (D0.5~D4.5), respectively. Premating exposure to 40 ppm ZEA diet reduced fertilization rate while postmating exposure to 40 ppm ZEA diet delayed embryo transport and preimplantation embryo development, which subsequently affected embryo implantation. These data demonstrate that postweaning exposure to dietary ZEA can promote premature onset of puberty and disrupt early pregnancy events.

Keywords: Zearalenone, vaginal opening, fertilization, embryo transport, embryo development, embryo implantation.
2.2 INTRODUCTION

Zearalenone (ZEA) is a mycotoxin produced by several *Fusarium* species [36]. It is commonly found in livestock feed and human food, such as corn, rice, oats and wheat. Quantifiable ZEA was detected in 15% of 13,075 food samples and 9,877 unprocessed grain samples collected during 2005 and 2010 in Europe. Among them, detectable levels in processed food groups for human consumption were in the ppb range with the highest at 823 ppb, and those in unprocessed grains with the highest reaching 3 ppm [37]. Worldwide, ZEA contamination levels in food are usually in the range of ppb and low ppm, with the highest reported reaching 600 ppm [36-39]. The tolerable daily intake (TDI) of ZEA established by the Panel on Contaminants in the Food Chain in Europe is 0.25 μg/kg body weight [37].

ZEA is mainly metabolized in the liver to α- and β-zearalenol by 3α- and 3β-hydroxysteroid dehydrogenases, respectively. Alpha-zearalenol is the dominant ZEA derivative in pigs [40], humans [41], rats and mice [42]. ZEA and its metabolites are then glucuronidated by uridine diphospho-glucuronosyltransferase [36, 37, 43]. Cytochrome P450 may also be involved in ZEA metabolism [37]. ZEA and its metabolites have estrogenic effects due to their structural similarity with 17β-estradiol (E2) [44]. The order of estrogenicity is E2>α-zearalenol>ZEA>β-zearalenol based on gene induction assay [45], uterotrophic assay [46], and estrogen receptor binding assay [40]. Different metabolic pathways and varied estrogenicity of ZEA and its metabolites may contribute to the variable species susceptibility to ZEA exposure [102].

ZEA has been associated with certain pathological conditions in females and shown to have adverse effects on embryo and/or female reproductive function in
different species. In humans, dietary exposure to ZEA has been associated with precocious pubertal development [47, 48]. ZEA and α-zearalenol were also detected in the serum of patients with endometrial cancer [49]. In pigs, gestational exposure to ZEA caused blastocyst degeneration [103] and fetal loss [75]; exposure of sexually immature gilts to ZEA resulted in hyperestrogenism, including stimulated uterine cell proliferation [104], and affected the development and maturation of ovarian follicles [105]. Immature gilts seem to be more predisposed to ZEA insult than other age groups of swine [106]. In ewes, premating exposure to ZEA for 10 days reduced ovulation and fertilization, but the same doses and length of treatment starting 5 days postmating didn't affect pregnancy rate or cause embryonic loss [107]. In rats, neonatal exposure to ZEA caused persistent anovulatory estrous in adulthood [108]; and two-generation exposure (during mating and gestation only) to dietary ZEA (10 mg/kg/day) decreased pregnancy rate and increased embryo resorption in the uterus [109]. In mice, gestational (D15-18) or prepubertal (PND 15-18) exposure to ZEA (10 mg/kg/day, s.c.) for 4 days accelerated the onset of vaginal opening (VO), extended the estrous phase, and prolonged the period of anovulatory ovary [110, 111]; and neonatal exposure (PND1-10) to ZEA (5-30 μg/mouse) caused delayed VO, persistent estrous and sterility [112].

Despite extensive reports on the adverse effects of ZEA on female fertility [37], it has not been systemically studied with regard to potential effects of postweaning dietary ZEA exposure on early pregnancy events in vivo. Since only minimal amounts of ZEA and its metabolites could be transmitted to the milk [113], most mammals, including humans, start to be exposed to ZEA directly from food, which is the main route for ZEA exposure, once they are weaned from mother’s milk prior to puberty. The goal of this
study was to test the hypothesis that postweaning exposure to ZEA could affect female puberty and early pregnancy events in newly weaned C57BL/6J females fed with diets containing environmental relevant levels of ZEA. Various parameters, including the timing of VO and first copulation plug, estrous cycle, fertilization, embryo transport, embryo development, and embryo implantation were determined. The results could help risk assessment of ZEA and increase our understanding of the mechanisms of ZEA action on female fertility.

2.3 MATERIALS AND METHODS

**Animals.** C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME) to establish a colony in the Coverdell Rodent Vivarium at the University of Georgia. All mice were housed in polypropylene cages with free access to a casein-based phytoestrogen-free AIN-93G diet (Bio-Serv, Frenchtown, NJ) and water in polypropylene water bottles. The animal facility was maintained on a 12-hour light/dark cycle (0600 h to 1800 h) at 23 ± 1°C with 30–50% relative humidity. All methods used in this study were approved by the Animal Subjects Programs of the University of Georgia and conform to National Institutes of Health guidelines and public law.

**Diet preparation.** 0, 0.0005, 1, 2.5 and 10 mg of ZEA (Fermentek, Israel) and 0.0125 mg of diethylstilbestrol (DES, Sigma, USA) were first dissolved in 70 ml 100% ethanol (Alcohol 200 Proof, Decon Lab Inc) and then diluted with 30 ml ddH₂O to make 100 ml 70% ethanol solutions. Each solution was mixed with 250 g AIN-93G powder in a glass bowl to prepare 0, 0.002, 4, 10, and 40 ppm ZEA diets, and 0.05 ppm DES diet, respectively. Each diet was well mixed, squeezed by hand into pellets (about 2 cm in diameter and 6 cm in length), and dried for 48 h at room temperature in a dark hood.
The diets were prepared fresh every two weeks and kept at 4°C in a dark room until use. The rationale for the dose selection: 0.002 ppm ZEA diet was about the dose of 0.25 µg/kg/day, TDI for ZEA in Europe [37]; 4 ppm, 10 ppm and 40 ppm ZEA were based on the levels reported in contaminated foods [36]. Taking into account the average food consumption and body weight, these doses can be expressed approximately as 0.00025, 0.5, 1.25, and 5 mg/kg/day, respectively. The positive control 0.05 ppm dietary DES in diet was shown to affect pregnancy outcome [114].

**Treatments.** Postweaning exposure (Fig. 2.1A): Newly weaned female pups (3 weeks old) were randomly assigned into six groups (0, 0.002, 4, 10, and 40 ppm ZEA, 0.05 ppm DES) with littermates separated into different groups. At 8 weeks old, they were mated with young stud C57BL/6J untreated males (for all matings, mice were put together during daytime between 1100 and 1700). The mice were on control AIN-93G diet during mating. Once a vaginal plug was detected on gestation day 0.5 (D0.5), the original exposure regimen was resumed. Mice were assessed on D4.5 at 1100 h to determine embryo implantation using blue dye reaction [32]. If no implantation site was observed, one side of uterine horn and oviduct was flushed with 1xPBS to determine the presence, location, and morphology of embryos to indicate pregnancy status. Part of the other uterine horn was flash-frozen for immunohistochemistry and the rest was fixed in 10% formalin solution. During the treatment, body weight, food consumption and water consumption were measured weekly. Premating exposure (Fig. 2.1B): Newly weaned C57BL/6J females mice were exposed to 0 or 40 ppm ZEA diets. The treatment ended when they were mated with untreated stud males at 8 weeks old. One set of mice on the 40 ppm ZEA-treated group was assessed on D4.5 to determine embryo
implantation following the same procedure described in postweaning exposure. Another set of mice were analyzed on D1.5 to determine the presence and morphology of oocytes and embryos in the oviduct. To minimize the potential recovery from ZEA treatment, only the females plugged on the first night of mating were included in the study and analyzed on D1.5 in the 40 ppm ZEA-treated group. Both oviducts were flushed. The oocytes showing no pronuclei or polar body were considered to be unfertilized oocytes [115]. Postmating exposure (Fig. 2.1C): Untreated 8 weeks old C57BL/6J female mice were mated with untreated stud males. Once plugged, the
females were exposed to 0 or 40 ppm ZEA diets. One set of mice on the 40 ppm ZEA-treated group was evaluated on D4.5 to determine embryo implantation. Another set of females was analyzed on D3.5 at 1100 h. Both uterine horns and oviducts were flushed with 1xPBS to determine the presence, location, and morphology of embryos. Embryo development stage was categorized into morula, early blastocyst with small blastocoel [116], and blastocyst. Pregnancy rate was defined as the percentage of plugged mice with embryo(s) and/or implantation site(s) in the reproductive tract detected on D1.5~D4.5. The number of mice in each group was indicated in the “Results” and “Figure Legends”.

**Vaginal opening (VO), first copulation plug, and estrous stages.** VO was checked daily from PND 22 until PND 40. Once VO was detected, the female was mated with a stud male to determine the timing of the first copulation plug. A vaginal smear was collected daily between 1500 h and 1600 h for 21 days starting from 6 weeks of age to determine estrous stages. A complete cycle would include diestrus/ metestrus, proestrus, and estrous stages. A total of five mice were included in each of the 0, 10 and 40 ppm ZEA-treated groups [117].

**Histology.** Fixed D4.5 uterine horns were embedded in paraffin and cross-sectioned (5 μm). Sections were deparaffinized, rehydrated, and stained with hematoxylin and eosin, as previously described [118].

**Immunohistochemistry.** Frozen uterine cross sections (10 μm) were immunostained to detect progesterone receptor (PR) expression as previous described [74].

**Statistical analyses.** ANOVA type models were fitted using SigmaStat 3.5 for continuous outcomes. For the data that passed the normality and equal variance tests,
such as food consumption, water consumption, and duration days of estrous, one way ANOVA followed by Student-Neuman-Keuls (SNK) multiple comparison test was used. For the data that failed the normality and equal variance tests, such as age at VO, age at first copulation plug, interval between VO and first copulation plug, number of complete estrus cycles, number of implantation sites, and number of embryos and oocytes, ANOVA on ranks followed by Dunn’s method, which does not give precise P values but >0.05 or <0.05, was used. Postweaning body weights were analyzed with a two-way Repeated-Measures ANOVA (with time and dose as independent factors) followed by SNK multiple comparison test. Mean ± standard deviation (SD) was reported for the continuous outcomes. Chi-squared test and Fisher's exact test with P-value adjusted based on Holm's method for comparing multiple groups were used as appropriate for percentage of plugged mice with implantation sites, fertilization rate, embryo location, and embryo development. The significant level was set at p<0.05.

2.4 RESULTS

Postweaning exposure to ZEA diet accelerated the timing of vaginal opening (VO) and first copulation plug.

Postweaning dietary exposure to ZEA (Fig. 2.1A) at 0.002~40 ppm did not affect food consumption, water consumption, or body weight of the treated females. However, the females treated with 0.05 ppm DES diet had significantly increased water consumption starting from the third week of treatment and significantly increased body weight after 5 weeks of treatment (Supplementary figure S2.1 in Appendix A).
VO is a indication of puberty onset [70]. The mean age of VO in the control group (0 ppm ZEA) was 33.5 ± 2.1 days old (N=50). Those in 2 ppb ZEA (32.5 ± 1.5 days old, N=11) and 4 ppm ZEA (32.6 ± 2.1 days old, N=12) groups were comparable to the control. However, those in the 10 and 40 ppm ZEA-treated groups had significantly younger ages of VO compared to the control, at 27.1 ± 2.2 (N=30, P<0.05) and 25.6 ± 0.7 (N=30, P<0.05) days old, respectively. There was no significant difference in the age of VO between the 10 and 40 ppm ZEA-treated groups. The age of VO in the positive control 0.05 ppm DES-treated group (25.9 ± 0.8 days old, N=15) was comparable to that in both 10 ppm and 40 ppm ZEA-treated groups, but significantly younger than the remaining groups (Fig. 2.2A). The time of VO in both 40 ppm ZEA group and 0.05 ppm DES groups was only 3 days after the start of the treatments.

The first copulation plug is another indication of puberty onset [70]. It was detected at 40.6 ± 7.2 days old in the control group (N=9), 32.8 ± 4.8 (N=6, P>0.05 compared to the control) days old in the 10 ppm ZEA-treated group, and 28.7 ± 3.9
(N=19, P<0.05 compared to the control) days old in the 40 ppm ZEA-treated group. (Fig. 2.2B). Although the average age of the first copulation plug was reduced by ~8 days in the 10 ppm ZEA-treated group compared to the control, the difference was not statistically significant.

The average age intervals between VO and first copulation plug were 6.2 ± 6.3 (N=9), 6.2 ± 4.8 (N=6), and 2.7 ± 3.9 (N=19) days for 0, 10, and 40 ppm ZEA-treated groups, respectively. This interval was significantly decreased in the 40 ppm ZEA-treated group (P<0.05) compared to the control. In addition, a significantly higher percentage of mice had only one day gap in the 40 ppm ZEA-treated group (14/19=73.7%, P=0.0166) than in the control (2/9=22.2%) (Fig. 2.2C).

*Postweaning exposure to 40 ppm ZEA diet disrupted estrous cyclicity.*

Daily recording for 21 days revealed that the duration (days) in estrous stage per mouse was significantly increased in the 40 ppm ZEA-treated group (11.8 ± 4.0 days, P=0.008) but not that in the 10 ppm ZEA-treated group (6.4 ± 1.8 days), compared to the control group (5.4 ± 1.7 days) (Figs. 2.2D & 2.2E). In addition, the females in 40 ppm ZEA-treated group lacked a clear proestrus stage (Fig. 2.2D), thus no complete estrous cycle during those 21 days monitored (Fig. 2.2F). These data indicate that postweaning exposure to 40 ppm ZEA diet can disrupt estrous cyclicity.

*Postweaning exposure to 40 ppm ZEA disrupted embryo implantation.*

The postweaning exposure regimen included three segments: premating (3-8 weeks), mating (from cohabitation to identification of a vaginal plug), and postmating (D0.5-D4.5) (Fig. 2.1A). During the mating segment, both males and females were on the control AIN-93G diet to avoid any potential contribution from the males for any
adverse implantation outcome. The duration of mating segment was comparable among all the groups (Fig. S2.2 in Appendix A) and the average was about 2.86 days.

Figure 2.3. Effects of postweaning ZEA exposure on embryo implantation detected on D4.5. A. The percentage of plugged mice with implantation sites in each group. N=9-12. * P=0.0112; ** P=0.0015 compared to 0 ppm ZEA control group. B. Average number of implantation sites per pregnant mouse. N=6-9. Error bars: standard deviation. C. A representative D4.5 uterus in control group. Red arrows: implantation sites. D. A representative D4.5 uterus in 40 ppm ZEA-treated group. E. Histology of an implantation site in C. F. Histology of the uterus in D. G. Immunohistochemistry of progesterone receptor (PR) in a section from an implantation site in C. H. Immunohistochemistry of PR in a section from D. E & F. H & E staining of fixed uterine section (5 µm). G & H. Brown staining indicating PR expression in frozen uterine sections (10 µm). No specific staining in the negative control (data not shown). Black star: embryo; LE: uterine luminal epithelium; Lu: uterine lumen; Dec: decidual zone; Str: stroma; GE: glandular epithelium; Myo: myometrium. Scale bar: 100 µm.
Comparable percentages of plugged mice with implantation sites on D4.5 were observed among 0 ppm (9/12, the number of mice with implantation sites detected on D4.5 over the number of plugged mice detected on D0.5), 0.002 ppm (8/9), 4 ppm (6/10) and 10 ppm (9/11) ZEA-treated groups (Fig. 2.3A). A significantly decreased percentage of plugged mice with implantation sites was observed in 40 ppm ZEA-treated group (1/11, \( P=0.0112 \)), and the positive control 0.05 ppm DES-treated group (0/11, \( P=0.0015 \)) (Fig. 2.3A). There was no significant difference in the average number of implantation sites per pregnant mouse among 0 ppm (8.2 ± 0.8), 0.002 ppm (8.0 ± 1.0), 4 ppm (7.8 ± 1.0) and 10 ppm (7.2 ± 2.0) ZEA-treated groups (Fig. 2.3B), although there was a decreasing trend with the increase of ZEA doses. None of the females without implantation sites in these four groups had embryos in the reproductive tract, indicating that they were not pregnant, and all the pregnant ones had embryo implantation. All the uteri without implantation sites in the 40 ppm ZEA-treated group (10/11, 3 with embryos) and 0.05 ppm DES-treated group (11/11, one with embryos) had a distended appearance that was not present in the control (Figs. 2.3C & 2.3D, and data not shown).

In the postweaning 40 ppm ZEA-treated group, 5 of the 11 treated mice had plugs detected in the 1st morning after cohabitation, one of which had 7 implantation sites and the remaining 4 had neither implantation sites nor embryos in the reproductive tract detected on D4.5. The pregnancy rate was 1/5=20%, which was marginally different from that in the control group (5/6=83.3%, \( P=0.080 \)). The remaining 6 treated mice had plugs detected after the 3rd morning (between the 4th and 8th mornings). None had implantation sites, but three of them had embryos in the reproductive tract. The
pregnancy rate was 3/6=50%, which was comparable with that in the control group that had plugs detected after the 3rd morning (4/6=66.7%, P=1.000). Among these three females with embryos, one had two hatched blastocysts flushed from one uterine horn; one had a hatched blastocyst and a fragmented embryo with intact zona pellucida flushed from one oviduct; and the third one had 2 hatched blastocysts and one hatching blastocyst flushed from one oviduct (data not shown). These results indicate that postweaning exposure to 40 ppm ZEA diet affects embryo implantation and might also affect embryo development and embryo transport.

Postweaning exposure to 40 ppm ZEA altered uterine histology and PR expression in D4.5 uterus.

Uterine histology was examined in D4.5 fixed uterine tissues. In the control group, a section of an implantation site showed that implantation had occurred and decidualization was obvious (Fig. 2.3E). However, the uteri with distended appearance in both 40 ppm ZEA (Fig. 2.3F) and 0.05 ppm DES-treated groups (data not shown) had enlarged uterine lumen and tall uterine epithelium (Fig. 2.3F).

Progesterone receptor (PR) expression was detected in D4.5 uterus. In the D4.5 control uteri, PR had disappeared from the uterine luminal epithelium (LE) and was highly expressed in the stromal compartment, especially the primary decidual zone (Fig. 2.3G). In the D4.5 uteri without implantation sites from 40 ppm ZEA and 0.05 ppm DES-treated groups, PR remained expressed in the LE and highly expressed in the glandular epithelium, but it was relatively low in the stromal compartment (Fig. 2.3H and data not shown). Both uterine histology and PR expression confirm estrogenic effect and failed
implantation in the 40 ppm ZEA-treated and positive control 0.05 ppm DES-treated groups (Figs. 2.3E ~ 2.3H and data not shown).

DES (0.05 ppm) was included as a positive control in the postweaning study to determine any potential effects of ZEA on VO (Fig. 2.2A) and embryo implantation (Fig. 2.3A). Since postweaning study had established the positive effects of ZEA on promoting VO and blocking embryo implantation, which were similar as the effects of DES, DES was not included in the following premating study and postmating study.

Premating exposure to 40 ppm ZEA reduced fertilization rate and disrupted early embryo development.

Postweaning ZEA exposure (Fig. 2.1A) included premating and postmating exposure (D0.5-D4.5). Multiple early pregnancy events, such as ovulation, fertilization, embryo development, and embryo transport, can subsequently affect embryo implantation. Hence, further experiments were done to determine the critical exposure period(s) that could affect embryo implantation and the potential mechanisms that could account for the disrupted embryo implantation upon postweaning exposure to 40 ppm ZEA diet (Fig. 2.3). Newly weaned females were exposed to 40 ppm ZEA diet until mating day (8 weeks old) then placed on the control diet (premating) (Fig. 2.1B), or the mice were on control diet until a vaginal plug was identified then on 40 ppm ZEA diet (postmating) (Fig. 2.1C) and the early pregnancy events were analyzed. The control group (0 ppm ZEA) implantation data from D4.5 postweaning study (Figs. 2.1A & 2.3) were also used in these premating and postmating studies.

Premating exposure to 40 ppm ZEA did not seem to have a significant effect on the percentage of plugged mice with implantation sites detected on D4.5 (8/19=42% vs.
9/12 = 75% in the control, \( P=0.1378 \)). However, an interesting observation was made between the time a vaginal plug was detected and the percentage of plugged mice with implantation sites detected on D4.5. Out of the 19 plugged females in the 40 ppm ZEA-treated group, 8 were detected in the 1\(^{st}\) morning after cohabitation with 2 of them having implantation sites on D4.5 (2/8 = 25.0%); 4 were detected in the 2\(^{nd}\) and 3\(^{rd}\) mornings with 1 having implantation sites (1/4 = 25.0%); and the remaining 7 were detected after the 3\(^{rd}\) morning (between the 5\(^{th}\) and 12\(^{th}\) mornings) with 4 of them having implantation sites (4/7 = 57.1%). If the data from the mice with plugs detected between 1\(^{st}\) and 3\(^{rd}\) mornings were combined, 3 out of 12 plugged mice (25.0%) had implantation
Figure 2.4. Effects of premating exposure to 40 ppm ZEA diet on embryo implantation detected on D4.5 and oocytes/embryos detected on D1.5. A. The percentage of plugged mice with implantation sites detected on D4.5. * P=0.043. B. The average number of implantation sites per pregnant mouse detected on D4.5. Error bars: standard deviation. A & B: All, combined data for each group; 1st – 3rd morning / >3rd morning, the time when a vaginal plug was detected after cohabitation. C. Average number of oocytes and embryos per mouse. N=6 and N=13 for 0 and 40 ppm ZEA-treated groups, respectively. Error bars: standard deviation. D. Percentage of mice with embryos on D1.5. * P=0.047. N=7 and N=14 for 0 and 40 ppm ZEA-treated groups, respectively. E. Relative percentage of unfertilized oocytes, two-cell stage embryos and 4~8-cell stage embryos from mice with embryos (0 ppm ZEA, N=49; 40 ppm ZEA, N=87). * P<0.0001, percentage of unfertilized oocytes compared to 0 ppm ZEA-treated group. F, G and H. Representative images of an unfertilized oocyte (F), a two-cell stage embryo (G), and a 4~8-cell stage embryo (H), respectively.

sites on D4.5, which was significantly lower than that in the control group (5/6=83.3%, P=0.043). However, no significant difference was observed in the mice with plugs detected after the 3rd morning (40 ppm ZEA-treated group: 4/7=57.1%; control group: 4/6=66.7%, P=1.000) (Fig. 2.4A). Although no significant difference was observed between 0 ppm and 40 ppm ZEA-treated groups in the average number of implantation sites per pregnant mouse on D4.5, a trend of increased number of implantation sites was observed in the 40 ppm ZEA-treated group if the plugs were detected after the 3rd morning (Fig. 2.4B). These data (Figs. 2.4A & 2.4B) suggest that premating ZEA treatment affects embryo implantation, but the effect diminishes after the treatment ends.

To determine the potential cause for the reduced percentage of mice with implantation sites in the 40 ppm ZEA-treated group (Fig. 2.4A), another set of newly weaned females underwent premating exposure to 0 and 40 ppm ZEA diets. Only the mice with a plug detected in the 1st morning after cohabitation with untreated stud males
were included in the study to minimize the potential recovery from premating ZEA treatment. On D1.5, the percentage of mice having ovulated, indicated by the presence of unfertilized oocytes and/or embryos detected in the oviduct, was similar between 0 ppm ZEA-treated group (7/8=87.5%) and 40 ppm ZEA-treated group (14/16=87.5%, P=1.0). The total number of both unfertilized oocytes and embryos was comparable between 0 ppm ZEA-treated group (8.2 ± 1.6, N=6, excluding the first mouse, which had four 2-cell embryos but the number of unfertilized oocytes was not recorded) and 40 ppm ZEA-treated group (9.7 ± 4.6, N=13, excluding the first mouse, which had unfertilized oocytes only but the number was not recorded; P=0.478) (Fig. 2.4C). These data demonstrate that premating exposure to 40 ppm ZEA diet did not affect the number of oocytes that were ovulated. However, the percentage of ovulated mice with embryos was significantly reduced in the 40 ppm ZEA-treated group (7/14=50% vs. 7/7=100% in the control, P=0.047) (Fig. 2.4D). Among the mice with embryos, the percentage of unfertilized oocytes was significantly higher in the 40 ppm ZEA-treated group (53/87=60.9% vs. 7/49=14.3% in the control; P<0.0001) (Figs. 2.4E & 2.4F). Interestingly, all embryos in the control group (42/42) were at 2-cell stage (Fig. 2.4G), but 67.5% (23/34, P<0.0001) of the embryos in the 40 ppm ZEA-treated group were at 4~8-cell stages (Fig. 2.4H). Collectively, these data demonstrated that premating exposure to 40 ppm ZEA diet did not affect the percentage of mice that ovulated or the number of oocytes ovulated (Fig. 2.4C). However, it adversely affected fertilization (Figs. 2.4D & 2.4E), and possibly early embryo development (Figs. 2.4E ~ 2.4H), which could subsequently affect embryo implantation (Fig. 2.4A).

*Postmating exposure to 40 ppm ZEA delayed embryo transport and development.*
Postmating exposure (D0.5-D4.5) to 40 ppm ZEA diet did not affect the pregnancy rate (9/10 vs. 9/12 in the control, P=0.59) detected on D4.5. This was expected because postmating exposure did not affect ovulation and fertilization, both of which normally occur during the dark cycle before 0500 h of the mating night in mice [119], before ZEA exposure. All 9 pregnant mice in the control group had more than 6 implantation sites (Figs. 2.3C & 2.5A). Among the 9 pregnant mice in the 40 ppm ZEA-treated group, 3 had more than 6 implantation sites; 3 had less than 4 implantation sites (Fig. 2.5B), with some showing faint blue bands, an indication of delayed implantation [120]; and 3 had no implantation sites, indicating that postmating exposure to 40 ppm ZEA diet also adversely affected embryo implantation. In the three mice without implantation sites, 11 embryos were flushed from the oviducts of two mice and he reproductive tract of one mouse was not flushed at the time of dissection. Among these 16 embryos recovered from the oviducts, 7 appeared normal D4.5 embryos without zona pellucida (Fig. 2.5C), but the other 9 embryos were underdeveloped and still surrounded by the zona pellucida (Fig. 2.5D), suggesting that embryo transport and embryo development were also affected by postmating exposure to 40 ppm ZEA diet.

To further demonstrate the effects of postmating exposure to ZEA on embryo transport and preimplantation embryo development, the location and morphology of embryos were examined in the D3.5 reproductive tract. There was no significant difference in the pregnancy rate (based on the presence of embryos) between the control group (7/8) and 40 ppm ZEA-treated group (7/11, P=0.34) and the number of embryos per pregnant mouse (41/7=5.9 vs 42/7=6.0). Among the 7 pregnant mice in the
the 40 ppm ZEA-treated group, 3 had embryos in the oviduct only, 2 had embryos in both oviduct and uterus; and the remaining 2 had embryos in the uterus only. The percentage of pregnant mice with embryos in the oviduct was significantly increased in the 40 ppm ZEA-treated group (P=0.021) (Fig. 2.5E). Among all flushed embryos in this group, 54.8% were from the oviduct (P<0.0001) (Fig. 2.5F), indicating delayed embryo transport upon postmating 40 ppm ZEA treatment.

The stage of development was also examined for embryos flushed from uteri and oviducts on D3.5 (Fig. 2.5G). Among the 41 embryos recovered in the control group, 3 were at the morula stage (7.3%) (Fig. 2.5H), 1 was an early blastocyst (2.4%) (Fig. 2.5I), and 37 were expanded blastocysts (90.2%) (Fig. 2.5J). Among the 42 embryos recovered from the 40 ppm ZEA-treated group, 11 were at the morula stage (26.2%) (Fig. 5H), 9 were early blastocysts (30.9%) (Fig. 2.5I), and 18 were expanded blastocysts (42.9%) (Fig. 2.5J). These ratios were significantly different from those in
the control (P<0.0001, 2x3 Fisher's exact test). The percentage of early blastocyst and morula was significantly higher in the 40 ppm ZEA-treated group than that in the control (57.1% vs 9.7%, P<0.0001), indicating delayed embryo development. These data demonstrated that postmating exposure to 40 ppm ZEA diet delayed embryo transport and embryo development, subsequently affecting embryo implantation (Figs. 2.5A & 2.5B).

2.5 DISCUSSION

Postweaning exposure to ZEA accelerates vaginal opening (VO) (Fig. 2.2A). VO is an early sign of puberty that can be affected by endocrine disruptors in rodents [121-125]. ZEA is an endocrine disruptor with estrogenic effects [45, 126-128]. Late gestational exposure (GD15-18) to ZEA (0.5 or 10 mg/kg/day, s.c. injection) accelerated VO in CD-1 mouse offspring for more than 1 day [111]. Our study indicates that postweaning exposure to 40 ppm ZEA diet for an average of 3-4 days can accelerate VO age by an average of about 8 days (Fig. 2.2A). Different exposure periods, doses, exposure routes, and mouse strains could all potentially contribute to the varied effects seen in the above two studies. VO is under neuroendocrine control [122, 129]. However, the molecular mechanisms for VO and the effects of endocrine disruptors, e.g., ZEA, on VO, are still largely unknown.

Postweaning exposure to ZEA also disrupts estrous cyclicity (Figs. 2.2D ~ 2.2F). Many endocrine disruptors can affect estrous cyclicity, such as bisphenol A [130], chlorotriazine simazine [131], estradiol valerate [132], and genistein [133]. Although it has not been investigated about the molecular mechanism of how the ZEA treatment regimen in this study affects estrous cyclicity, based on the literature, it is possible that
ZEA and its derivatives may affect estrous cyclicity via hypothalamus-pituitary-ovarian axis. ZEA could alter the concentration of neuronal progestin receptors, which are neuroendocrine integrators [134], in ventromedial hypothalamus [135]. Neonatal exposure to ZEA early in development alters postpubertal pituitary response to gonadotropin-releasing hormone in rats [136]. Zeranol (α-zearalanol), a synthetic derivative of ZEA and a growth promoter in livestock production [137], can increase pituitary volume in Suffolk wethers [138]. ZEA could also alter levels of gonadotropins and sex steroids [139].

Postweaning exposure to ZEA could also disrupt embryo implantation. Postweaning ZEA exposure seemed to yield two sets of data on embryo implantation: those with plugs detected in the 1st morning after cohabitation appeared to mimic the premating data from overnight mating, and those with plugs detected after the 3rd morning had phenotypes more similar to those from postmating exposure. The late set of data suggests recovery from premating exposure during extended mating interval. Premating and postmating exposure regimens helped dissect the contributing factors, including reduced fertilization, delayed embryo transport, and delayed preimplantation embryo development, for the disrupted embryo implantation observed upon postweaning exposure to 40 ppm ZEA diet.

Premating exposure to 40 ppm ZEA diet significantly reduces the percentage of plugged mice with implantation sites if the mice are mated within 3 days of cohabitation (Fig. 2.4A). A reduced fertilization rate (Fig. 2.4D) could be a main mechanism. Fertilization involves both oocytes and sperms. Our pilot experiment indicated that untreated females mated with males treated with 40 ppm ZEA diet for 3 weeks
produced comparable litter size as those untreated females mated with untreated males (Fig. S2.3 in Appendix A). This result suggests that the sperms, which were only exposed to residual ZEA and its metabolites in the ZEA-treated female reproductive tract between mating and fertilization, is not a significant contributing factor for the reduced fertilization rate. This observation led us to speculate that the oocyte quality and/or the oviductal environment might be affected by 40 ppm dietary ZEA treatment. Studies have shown that over half of primordial follicles formed by postnatal day 6 are eliminated by the time of puberty [140]. The accelerated onset of puberty by ~8 days in 40 ppm ZEA-treated group (Fig. 2.2A) may disrupt this normal follicle elimination process and lead to the development and ovulation of immature or poor quality oocytes that were not competent to be fertilized (Figs. 2.4D & 2.4E). Alternatively, ZEA may directly affect oocyte quality. Reports indicate that ZEA and its derivatives can inhibit oocyte maturation and induce chromatin abnormalities in cultured oocytes [141, 142]. Since fertilization occurs in the oviduct, it is also possible that the estrogenic ZEA may alter the oviductal environment that is less conducive for fertilization.

Postmating exposure to 40 ppm ZEA diet delays embryo transport (Figs. 2.5E & 2.5F). This adverse effect of ZEA on embryo transport in the oviduct is most likely attributed to its estrogenicity. It has been documented that estrogen and estrogenic chemicals can delay oviductal oocyte or embryo transport in cows [143], rabbits [144], mice, guinea pigs and hamsters [26, 74], but accelerate oviductal oocyte or embryo transport in rats [26, 145, 146]. The molecular mechanism of estrogen and estrogenic compounds on oviductal transport is largely unknown.
Postmating exposure to 40 ppm ZEA diet also delays preimplantation embryo development (Fig. 2.5G). Delayed embryo development seemed to be associated with delayed embryo transport. Correlation analysis showed that the embryos retained in the oviduct were less developed than those in the uterus of 40 ppm ZEA-treated mice (P=0.0003). This seeming correlation may reflect the varied sensitivity of individual mice to ZEA treatment, e.g., both embryo transport and embryo development are more affected in mice that are potentially more sensitive to ZEA exposure. Since estrogen can influence the expression of oviductal glycoprotein(s) that could interact with the gametes and early embryo and could affect litter size [147, 148], it is possible that the estrogenic ZEA may affect preimplantation development via altered oviductal environment.

Our data appeared to show that premating ZEA exposure accelerated early embryo development (Fig. 2.4E) while postmating ZEA exposure delayed preimplantation embryo development (Fig. 2.5G). Here is one possible explanation: premating ZEA exposure to 40 ppm ZEA diet increased duration of estrous and disrupted estrous cyclicity (Figs. 2.2D ~ 2.2F), thus influenced the mating time. Since the females in the premating study (Fig. 2.4E) were put with stud males between 1100 h and 1700h and removed the next morning, it was possible that the ZEA-treated females were plugged much earlier than the control females, which normally mated during the night. We did one quick pilot experiment to support this hypothesis. We treated 24 adult female mice (2~4 months old) with 40 ppm ZEA diet for ~2 weeks. The females were cohabitated with fertile males ~1100 and a vaginal plug was detected in 9 females within 2.5 hours. Seven of these 9 plugged females were dissected on D1.5 (real D2.0),
3 females had 4~8-cell stage embryos and the other 4 females didn’t have embryos in the oviduct. This pilot experiment supports that dys-synchronized estrous cycle caused by ZEA treatment could lead to early mating time. Subsequently, ovulation, fertilization, and early embryonic cell division occurred earlier and led to seemingly accelerated embryo development (Fig. 2.4E).

In summary, peripubertal and early pregnancy are two sensitive periods that can be influenced by ZEA exposure, which affects not only puberty and estrous cyclicity but also early pregnancy events, including fertilization, embryo development, embryo transport, and embryo implantation.

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CHAPTER 3
MULTIGENERATIONAL EXPOSURE TO DIETARY ZEARALENONE (ZEA), AN ESTROGENIC MYCOTOXIN, AFFECTS PUBERTY AND REPRODUCTION IN FEMALE MICE

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3.1 ABSTRACT

This study investigated potential cumulative effects of multiple pregnancy and multigenerational exposure to dietary ZEA (0, 0.8, 4, or 20 ppm) on female puberty and reproduction in C57BL/6J mice. Multiple pregnancies did not significantly affect litter size or offspring puberty. Significant effects were observed in 20 ppm ZEA-treated females: advanced puberty onset in F0, F1, and F2 generations; decreased implantation rate, pregnancy rate, and litter size, and increased pregnancy gap and gestation period in F1 and F2 generations; and reduced fertility index in F2 generation. F3 females from 0 and 20 ppm groups were split into 0 or 20 ppm ZEA diets at weaning, with advanced puberty onset seen in 0-20 and 20-20 groups and decreased implantation rate observed in 20-20 group. In summary, 20 ppm dietary ZEA advanced puberty onset without obvious cumulative effect and impaired fertility with multigenerational cumulative effect, which could be partially alleviated upon exposure cessation.

Keywords: Zearalenone, multipregnancy, multigeneration, vaginal opening, embryo implantation, litter size, female reproduction.
3.2 INTRODUCTION

Mycotoxin zearalenone (ZEA) is commonly found in livestock feed and in human food. Its contamination levels are in the range of ppb to low ppm with the highest reported at 600 ppm [36, 37]. Plant foods (e.g., corn and wheat) can contain ZEA through fungal contamination. Animal foods (e.g., meat and dairy products) can be contaminated with ZEA via intake of fungus contaminated feedstuff by livestock or can contain zeranol (α-zearalanol), a derivative of ZEA used as a growth promoter in livestock [48, 149]. Contaminated food is the main source of human exposure to ZEA [36, 37, 149]. The median and 95th percentile daily dietary ZEA exposure in European population were estimated to be <0.1 μg/kg body weight and <0.3 μg/kg body weight, respectively [37]. The tolerable daily intake (TDI) for ZEA established by the Panel on Contaminants in the Food Chain in Europe is 0.25 μg/kg body weight [37].

A study on 76 girls/2 boys with idiopathic precocious puberty (IPP) and 99 girls/1 boy in the control indicated positive correlation between ZEA and IPP, with an odds ratio of 8.833 (95% confidence interval: 2.281-34.208) [47]. Epidemiological studies support ZEA and its derivative mycotoxins as a triggering factor for precocious pubertal development in prepubertal exposed girls (reviewed in [48]). Although human data on definitive causative effects of ZEA and its metabolites on puberty and female reproductive system are unavailable, the estrogenicity of ZEA and its metabolites renders them the potential to influence puberty and functions of the female reproductive system [36].

Female puberty and reproduction are regulated by estrogen [150-152], therefore, these processes could be affected by ZEA. Our previous study showed that
postweaning exposure to 10 ppm or 40 ppm dietary ZEA promoted premature onset of puberty and 40 ppm dietary ZEA also disrupted early pregnancy events in female mice [153]. ZEA is quickly absorbed and the unconjugated ZEA has an elimination half-life of 16.8 hours after oral administration in male rats [37]. Placental transfer of ZEA and its metabolites in rats and pigs, as well as lactational transfer of ZEA and its metabolites in the bovine milk, have been demonstrated (reviewed in [37]). Given that ZEA is a common contaminant in human diet [37], which is consumed daily across generations, it is natural to ask if there are any cumulative effects of ZEA on female puberty and reproduction during multigenerational exposure.

Multigenerational studies on reproduction usually cover the F0 (parental), F1 and F2 generations, with exposure commencing from the F0 generation prior to mating and continuing through the F2 generation until the F2 offspring are weaned [154], allowing the investigation of reproduction upon exposure for one or two generations. A survey of multigenerational studies of 316 chemicals in rats indicates that more chemicals have reproductive effects on the F1 generation than on the F0 generation and more adult reproductive effects are seen in the F1 generation than in the F0 generation [155]. Therefore, it is necessary to assess the effects of ZEA on female puberty and reproduction in a multigenerational setting. It was hypothesized that ZEA in the diet could have cumulative adverse effects on female puberty and reproduction. This hypothesis was tested in C57BL/6J mice. Vaginal opening, pregnancy rate, and litter size were among the parameters used to determine effects of ZEA treatments on female puberty and reproduction.
3.3 MATERIALS AND METHODS

Animal. The C57BL/6J mice were initially derived from animals at Jackson Laboratories (Bar Harbor, ME) [153]. All mice were housed in polypropylene cages with free access to a casein-based phytoestrogen-free AIN-93G diet (Bio-Serv, Frenchtown, NJ) or a ZEA in AIN-93G diet and to water in polypropylene water bottles. Generally, ZEA levels in rodent diets were undetectable or very low (<30 ppb) [156, 157]. The animal facility was maintained on a 12-h light/dark cycle (0600 h to 1800 h) at 23 ± 1°C with 30–50% relative humidity. All methods used were approved by the Animal Subjects Programs of the University of Georgia and conform to National Institutes of Health guidelines and public law.

ZEA treatment, mating, and data collection. Homemade diets containing 0, 0.8, 4, and 20 ppm ZEA (Fermentek, Israel) were prepared as previously described [153]. These ZEA levels were within the range of levels in the highly contaminated food [36, 37]. The treatment regimen is outlined in Figure 1. F0 females were treated from weaning (3 weeks old) to dissection; F1 and F2 females were exposed to ZEA diets during their entire lives, from gestation to dissection (Fig. 3.1A).

Newly weaned F0 generation females were randomly assigned into 0, 0.8, 4 and 20 ppm ZEA-treated groups with littermates assigned into different groups. At 8 weeks old, the F0 females in each group were mated with fresh stud males, which were exposed to the same ZEA diets as the females only during mating, to produce three consecutive litters, F1a, F1b and F1c (Fig. 3.1B). Another set of F0 females were mated and dissected on gestation day 4.5 (D4.5) to determine embryo implantation in the F0 generation. At 8 weeks old, F1a and F1b females were mated with fresh stud males.
The F1a females would eventually produce three consecutive F2 litters, F2a, F2b, and F2c. The F1b females were dissected on D4.5 to determine embryo implantation in the F1 generation (Fig. 3.1B).

**Figure 3.1.** Treatment and breeding regimens. A. Treatment protocols. F0 generation was treated with 0, 0.8, 4, or 20 ppm ZEA diets during postweaning period and pregnancy; F1 and F2 generations were exposed to 0, 0.8, 4, or 20 ppm ZEA diets during gestation and lactation via maternal exposure and treated with the same ZEA diets during postweaning and pregnancy via direct dietary exposure. Females in the 0.8 and 4 ppm ZEA-treated F2 generation were dissected on D4.5 without producing F3 generation. In the F3 generation, 0 ppm and 20 ppm ZEA-treated groups were split into two groups each at weaning, and then treated with 0 ppm or 20 ppm ZEA during postweaning period and pregnancy. B. Breeding regimen. F0 dams produced three litters: F1a, F1b, and F1c; F1a dams produced three litters: F2a, F2b, and F2c; F2a and F2b dams produced two litters: F3a and F3b.
At 8 weeks old, F2a and F2b females were mated with fresh stud males. Since no obviously adverse effects on puberty and fertility were observed in the 0.8 and 4 ppm ZEA-treated F0 and F1 females, F2a and F2b females in the 0.8 and 4 ppm ZEA-treated groups were dissected on D4.5 to determine implantation without producing an F3 generation for these two dose groups. F1c and F2c pups were sacrificed before or at weaning. In the 0 ppm ZEA group, 11 F2a and 2 F2b females were mated to produce F3a and F3b litters; in the 20 ppm ZEA groups, 3 F2a and 8 F2b females were mated to produce the F3a and F3b litters (Fig. 3.1B). The rest of the F2b females in the 0 and 20 ppm ZEA were dissected on D4.5 to determine embryo implantation in the F2 generation.

At weaning, F3 female littermates from each dam in the 0 and 20 ppm ZEA groups were randomly split into 0 or 20 ppm ZEA-treated groups, resulting in a total of four groups: 0-0, 0-20, 20-0, and 20-20 (Fig. 3.1A). At 8 weeks old, all F3 females were mated with fresh stud males and dissected on D4.5 to determine embryo implantation in the F3 generation.

During mating in each generation, the females were checked every morning for the presence of a vaginal plug to determine mating in the previous night. They were separated from the mating males and housed individually when an enlarging belly (to indicate pregnancy) was observed. Some females had demonstrated more than one mating plug before a pregnancy was evident. Dams that lost all pups before weaning or had pups weaned at three weeks old were back to mating after one week of rest if they were scheduled to produce more litters.
Water consumption and food consumption were recorded weekly during postweaning (F0 and F1) and lactation (F0, F1, and F2). Postnatal body weights were monitored at 7, 14, 21, 22, 29, 36, 43, 50, and 57 days of age. Body weights during pregnancy were recorded every 5 days from D2.5 to D17.5 for F0, F1, and F2 dams and their body weights during lactation were measured weekly. Litter size was recorded at birth. Gender ratio of the pups was determined at weaning (3 weeks old).

Female pups were monitored daily after weaning for signs of vaginal opening; the age at vaginal opening was recorded as an indication of puberty onset [153, 158, 159]. As previously described [32, 153], embryo implantation and pregnancy status on D4.5 were determined by using the blue dye reaction, or for those without implantation sites, by uterine flushing to detect the presence of healthy-looking embryos. All male pups were sacrificed at weaning without further study.

The number of animals in each group was indicated in the figure legends. All groups began with at least 6 mice. However, since not all mated mice were pregnant, not all F0 and F1 pregnant mice produced three litters as planned, and there was further impaired fertility in the F1, F2, and F3 20 ppm ZEA-treated groups, the numbers of pregnant females per group analyzed for food and water consumption during lactation, body weight during gestation and lactation, length of the gap between mating and delivery (pregnancy gap), age of dams at delivery, and number of implantation sites may be less than 6.

Statistical analysis. ANOVA analyses were done using SigmaPlot 12.0. ANOVA on ranks followed by Dunnett’s test was used for analyzing the age at vaginal opening and the number of implantation sites. One way ANOVA followed by Dunnett’s test was used
for analyzing food and water consumption, body weight during gestation and lactation, mating duration, and pregnancy gap. Two-way repeated measures ANOVA followed by Dunnett’s methods was used for analyzing gestational period and litter size from the females that gave birth two (F2) or three (F0 & F1) times and postweaning body weight for each mouse that was repeatedly measured weekly. Two-way ANOVA followed by Holm-Sidak test was used for analyzing preweaning body weight. Fisher’s exact test was used to analyze pregnancy rate, implantation rate, mating index, and fertility index. The significance level was set at P< 0.05, two-tailed test.

3.4 RESULTS

Food consumption, water consumption, and body weight

Although the numbers fluctuated, no consistent differences in either food consumption or water consumption were observed among 0, 0.8, 4 and 20 ppm ZEA-treated groups at the same ages in the same generation during postweaning or lactation (Supplementary Tables S3.1 & S3.2). The only consistent difference observed in body weight was a significantly higher body weight in the F1 and F2 20 ppm ZEA-treated groups during weeks 6-8 (Supplementary figure S3.1). Based on food consumption and body weight, the estimated ZEA doses from postweaning growth (lowest) to the end of lactation (highest) were: 0.1~0.24 mg/kg body weight per day in the 0.8 ppm ZEA group; 0.5~1.2 mg/kg body weight per day in the 4 ppm ZEA group; and 2.5 to 6 mg/kg body weight per day in the 20 ppm ZEA group. The gender ratios at weaning were comparable among all treatment groups in different generations (Appendix B).
Vaginal opening in F0, F1, and F2 females

No significant difference in the age at vaginal opening was observed among all F0, F1, and F2 females in the 0, 0.8, and 4 ppm ZEA treated groups (Fig. 3.2). However, the females in the 20 ppm ZEA-treated groups in each generation had significantly younger ages at vaginal opening compared with the control in each generation, indicating accelerated puberty onset upon 20 ppm ZEA dietary treatment. Among the F0, F1, and F2 generations treated with 20 ppm ZEA, no significant difference in the ages at vaginal opening was observed (Fig. 3.2). The females in different litters from the same dams (litters F1a, F1b from the F0 dams; and litters F2a, F2b from the F1a dams (Fig. 3.1B)) in the same treatment group had comparable ages at vaginal opening (Appendix C).

![Figure 3.2. Effect of ZEA on the ages at vaginal opening in F0, F1, and F2 female mice. ANOVA on ranks followed by Dunnett’s test was used for analyzing the differences among groups. The numbers above bars, the total numbers of females in the indicated groups; * P<0.05, compared with the 0 ppm group in the same generation; error bar, standard deviation; N=13-34.](image-url)
Mating behavior, 1st pregnancy rate, fertility index, litter size, gestation period, and pregnancy gap in F0, F1, and F2 females

ZEA treatment did not affect the mating behavior of female mice among different treatment groups or different generations as indicated by a comparable mating index (number of vaginal plug-positive females / number of mating females x 100%) and comparable mating duration (time from cohabitation to detection of a vaginal plug) (Appendix D).

The 1st pregnancy rate was determined as the percentage of the first mating, indicated by a vaginal plug, leading to a full term pregnancy (number of females producing litters / number of females with 1st vaginal plugs x 100%) in the same group. The F0 and F1 dams potentially had three 1st vaginal plugs and the F2 dams potentially had two 1st vaginal plugs during the production of three and two litters, respectively. Results revealed that 20 ppm ZEA-treated F1 and F2 females, but not the F0 females or the 0.8 and 4 ppm ZEA-treated F0, F1, and F2 females, had significantly reduced 1st pregnancy rates compared with 0 ppm ZEA-treated females in the same generation (Fig. 3.3A). No significant difference was observed in the 1st pregnancy rates between F1 and F2 20 ppm ZEA-treated groups, both of which were significantly lower than that in the F0 20 ppm ZEA-treated group (Fig. 3.3A). Interestingly, there was a significant increase in the 1st pregnancy rate of the F2 control compared with the F0 control (Fig. 3.3A).
Figure 3.3. Effect of ZEA on fertility in F0, F1, and F2 female mice. A. Pregnancy rate following the first mating (number of females producing litters / number of females with 1st vaginal plugs x 100%). N=15-29 first vaginal plugs from 6-13 dams. B. Fertility index (number of females ever producing litters / number of females ever mated x 100%) from all matings. N=16-29 from 6-13 dams. C. Litter size. N=10-26 litters from 6-13 dams. D. Gestation period. N=7-24 from 6-13 dams. E. Pregnancy gap from cohabitation to delivery. The numbers above bars, the total numbers of dams in the indicated groups; N=3-13. A & B, Fisher’s exact test; C & D, Two-way repeated measures ANOVA followed by Dunnett’s test; E, One-way ANOVA followed by Dunnett’s test. A-E: * P<0.05, compared with respective 0 ppm ZEA-treated group; # P<0.05, compared with the F2 0 ppm ZEA group (A); ## P<0.05, compared with F0 20 ppm ZEA group (A); error bar (C~E), standard deviation. A-D: X-axis, dams’ generation.

The fertility index is the percentage of females in which vaginal plugs were detected (some plugged multiple times) that then produced litters. The only difference was seen in the F2 20 ppm ZEA-treated group, in which 5 out of 11 females with vaginal plugs never became pregnant. The other 6 females had full term 1st pregnancies (F3a). However, one of them had to be sacrificed due to a delivery problem. The remaining 5 females continued to give birth to a second litter each (F3b). The fertility index in the F2 20 ppm ZEA-treated group was significantly lower than that in the F2 0 ppm ZEA-treated group (P=0.0232) but was not significantly different from the F0 (P=0.0788) or the F1 (P=0.2565) 20 ppm ZEA-treated groups (Fig. 3.3B).

No obvious effect of birth order (F1a, F1b, and F1c from the F0 dams; F2a, F2b, and F2c from the F1a dams; F3a and F3b from the F2a and F2b dams) on litter sizes and gestation periods were observed in each treatment group (Appendix E), indicating that increased exposure time of the dams did not significantly alter any effect of ZEA on their fertility. When all the litters from the same dams were counted, no significant
difference in litter sizes was observed among all treatment groups at different generations except the 20 ppm ZEA-treated F1 and F2 dams, which produced significantly smaller litter sizes compared with the 0 ppm ZEA-treated F1 or F2 dams. There was no significant difference in litter sizes produced by 20 ppm ZEA-treated F1 or F2 dams. However, the litter size from the F1 dams was significantly smaller than that from the F0 dams in the 20 ppm ZEA-treated group (Fig. 3.3C). In addition, F1 and F2 dams in the 20 ppm ZEA-treated groups also had prolonged gestation periods compared with their respective control groups in the same generation (Fig. 3.3D). The pregnancy gaps (duration between cohabitation and delivery) in the F1a dams producing F2a, F2b, and F2c litters and the F2a/b dams producing F3a and F3b litters in the 20 ppm ZEA-treated groups (Fig. 3.1) were significantly longer than those in their respective 0 ppm ZEA-treated control groups (Fig. 3.3E). The ages of F1a dams at delivering F2a and F2b litters and F2a/b dams at delivering F3a litters in the 20 ppm ZEA-treated groups were significantly older than those in their respective 0 ppm ZEA-treated groups (Supplementary figure S3.2).

*Embryo implantation in F0, F1 and F2 females*

Embryo implantation was examined on D4.5 to investigate the mechanism of impaired fertility upon 20 ppm ZEA diet treatment. Among all groups in the F0, F1, and F2 generations, only the F1 and F2 females in the 20 ppm ZEA-treated groups had significantly lower implantation rates (number of females with implantation sites / number of females with vaginal plugs in the same group x 100%) compared with their respective controls (Fig. 3.4A). However, the dams with implantation sites had comparable numbers of implantation sites among all four different treatment groups in
all three generations (F0, F1, and F2) (Fig. 3.4B). It was noticed that all the mice in the 0, 0.8, and 4 ppm ZEA-treated groups had distinct blue bands (Fig. 3.4C and data not shown), but about 1/3 of the pregnant mice with implantation sites in the F0 (4/12) and the F1 (3/9) 20 ppm ZEA-treated groups had faint blue bands (Fig. 3.4D), an indication of delayed implantation [120]. In the F2 generation, only 3 out of 10 females had implantation sites on D4.5 and all of them had distinct implantation sites (data not shown) as seen in the control (Fig. 3.4C).
Figure 3.4. Effect of ZEA on embryo implantation detected on gestation day 4.5 (D4.5) in F0, F1, and F2 generations. A. Implantation rate. Fisher’s exact test. * P<0.05, compared with the 0 ppm ZEA treated group in the same generation; # P<0.05, compared with the F0 20 ppm ZEA-treated group; N=10-22. B. Average number of implantation sites per mouse with implantation sites. ANOVA on ranks. Error bar, standard deviation; the numbers above bars, the numbers of females with implantation sites; N=3-14. C. A representative D4.5 uterine image from control (0 ppm ZEA). Red arrow, on-time implantation site. D. A D4.5 uterine image from the F1 20 ppm ZEA-treated group showing faint blue bands. Red arrowhead, delayed implantation site.

Vaginal opening and embryo implantation in F3 females

The treatment regimen in the F3 females consisted of four groups designated 0-0, 0-20, 20-0, and 20-20 (Fig. 3.1A). This treatment regimen could potentially provide clues to the following questions: Would generational cumulative effects continue upon 20 ppm ZEA treatment (20-20 group)? Would any adverse effects lessen if the treatment was discontinued (20-0 group)? Would some adverse effects be associated with only immediate exposure (0-20 group)?

Significantly advanced ages at vaginal opening were observed in the 0-20 and the 20-20 groups compared with the 0-0 control group, and there was no significant difference between the 0-20 and the 20-20 groups (Fig. 3.5A). Interestingly, although the average age at vaginal opening in the 20-0 group was comparable with that of the 0-0 group, the ages at vaginal opening in the 20-0 group were shown in two distinct clusters, a phenomenon not observed in their littermates in the 20-20 group (Fig. 3.5A).
Figure 3.5. Effects of switched ZEA exposure on age at vaginal opening and embryo implantation at gestation day 4.5 in the F3 generation. A. Ages at vaginal opening. ANOVA on ranks followed by Dunnett’s test. Black diamond, individual data point; red line, average age in each group; * P<0.05, compared with the 0-0 group; N=7-24. B. Implantation rate. Fisher’s exact test. The ratio above each bar, the number of females with implantation sites over the total number of plugged females; N=7-13; * P<0.05, compared with the 0-0 group. C. Number of implantation sites. ANOVA on ranks. Black diamond, individual data point; red line, average number of implantation sites in each group; only mice with implantation sites included. D. Progressively decreasing implantation rates in the F0, F1, F2, and F3 20 ppm ZEA-treated groups. Fisher’s exact test. * P<0.05, compared with F0; red curve, exponential trend of the four data points.
The implantation rate in the 0-20 group was comparable with those in the 0-0 group (Fig. 3.5B) and the 20 ppm ZEA-treated group in F0 generation (Fig. 3.4A), which also had the treatment only after weaning (Fig. 3.1A). A significantly decreased implantation rate was observed in the 20-20 group (F3 20 ppm ZEA-treated group) compared with the 0-0 group. The implantation rate in the 20-0 group (5/10=50%) was in between the 0-0 (8/11=72.7%) and the 20-20 (1/7=14.3%) groups, although there was no significant difference between the 20-0 and the 0-0 groups or the 20-0 and the 20-20 groups due to the small sample sizes. Females without detectable implantation sites had no embryos detected in the reproductive tracts except for one animal in the 0-20 treated group, which had three embryos flushed from the oviduct but none from the uterus, indicating delayed embryo transport [153].

The average numbers of implantation sites in the mice with implantation sites were 7.5±0.8 (N=8) in the 0-0 group, 4.4±2.7 (N=9) in the 0-20 group, 5.2±3.4 (N=5) in the 20-0 group, and 3 (N=1) in the 20-20 group (Fig. 3.5C). Although there was no significant difference among the 0-0, 0-20 and 20-0 groups (P=0.066, ANOVA on ranks), large variations among animals in the 0-20 and the 20-0 groups were noticed. In addition, females with implantation sites in the 0-20 group had a significantly higher percentage of mice (8/9, compared to 0/8 in the 0-0 group, P<0.05) with only faint blue bands in the uterus, indicating delayed implantation as seen in Fig. 3.4D, while all mice in the 0-0 and the 20-0 groups had distinct blue bands as seen in Fig. 3.4C, indicating on-time implantation. The only pregnant mouse in the 20-20 group had distinct implantation sites.
3.5 DISCUSSION

This multiple pregnancy and multigenerational study revealed a promoting effect of 20 ppm ZEA diet on female puberty. However, it did not show any obvious cumulative effect either via multiple pregnancies or through multiple generations under the experimental setting in this study. It also showed an adverse effect of 20 ppm ZEA diet on female reproduction, which was cumulative over multiple generations but not over multiple pregnancies in the same generation. Cessation of exposure to the 20 ppm ZEA diet in the F3 generation after three generations (F0, F1, and F2) of exposure could partially reverse these adverse effects.

Vaginal opening is an early indication of puberty onset in rodents [153]. The lack of a difference among different litters from the same dams (Appendix C) or among different generations under the same ZEA treatment (Fig. 3.2) suggested the absence of a cumulative effect of ZEA on female puberty onset. However, this study design was not capable of revealing any cumulative shortening of the time to pubertal onset if an average of 25~26 days old was the earliest possible age for vaginal opening in C57BL/6J mice upon postweaning ZEA treatment. This possibility was supported by our previous published observation [153] and unpublished observations that stronger estrogenic treatments of newly-weaned C57BL/6J females mice with a 40 ppm ZEA diet, a 0.05 ppm diethylstilbestrol (DES) diet, or with daily i.p. injection of 12.5 µg 17β-estradiol (E2, a dose equivalent to ~10 ppm E2 in the diet) all led to a similar age (25~26 days old) at vaginal opening. Decreased age at vaginal opening was mainly seen in the mice with direct exposure to 20 ppm ZEA during postweaning period (Figs. 3.2 & 3.5A), a phenomenon also seen in rats treated with 500 ppm dietary genistein for
multiple generations [160]. These observations suggested that the period immediately prior to puberty onset was a vulnerable window for ZEA to influence puberty, something also observed in CD-1 mice subcutaneously injected with 10 mg/kg/day ZEA [161]. However, based on the switched exposure regimen in the F3 generation, F3 females in the 20-0 group showed two distinct clusters of ages at vaginal opening (Fig. 3.5A). The observation suggested that gestational and lactational exposure to 20 ppm ZEA might have segregated effects on puberty onset of female offspring due to possible genetic segregation of capacity to clear ZEA. It also suggested that dam-mediated gestational and lactational exposure could contribute to the effects of estrogenic endocrine disruptors on offspring puberty onset [37, 159].

A generational cumulative effect on implantation rate was seen as a dramatic exponential decrease from F0 (comparable to control) to F1 with a further gradual decline from F1 to F3 upon 20 ppm ZEA treatment (Fig. 3.5D). However, a more subtle effect, such as delayed implantation, was already evident in the F0 20 ppm ZEA-treated group. Since reduced pregnancy rates at term correlated with reduced implantation rates at D4.5 in the F1 and F2 20 ppm ZEA-treated groups (Figs. 3.3A & 3.4A), it suggested that one or more early pregnancy events, e.g., fertilization, embryo transport, preimplantation embryo development, embryo implantation [153], or oocyte quality [141], were adversely affected by the continuous exposure to 20 ppm ZEA from gestation to pregnancy. One study in rats revealed reduced numbers of corpora lutea in the F1B female offspring from parents exposed to 10 mg/kg ZEA [109], suggesting that follicle development and/or ovulation might also be affected by a higher dose of ZEA exposure. Since no embryos were detected in the reproductive tracts of 6 out of 7 F3
20-20 females (P<0.05 compared with the 0-0 group, 3/11) on D4.5 in this study, it implied that ovulation and/or fertilization were adversely affected in the F3 20-20 females.

Litter sizes from F1 and F2 dams were also significantly reduced but not those from F0 dams exposed to 20 ppm ZEA (Fig. 3.3C). Reduced litter size from F1 but not F0 dams was also reported in rats exposed to another estrogenic endocrine disruptor, genistein [162]. However, the litter sizes from both F0 and F1 dams were reduced in rats treated with 10 mg/kg ZEA (~80 ppm in the diet for adult mice) [109]. This discrepancy was most likely caused by the use of higher ZEA doses because a higher ZEA dose at 40 ppm could also adversely affect fertility of the F0 females [153]. Despite reduced litter sizes (Fig. 3.3C) and reduced implantation rates (Fig. 3.4A) in the F1 and F2 20 ppm ZEA-treated groups, the numbers of implantation sites from the remaining pregnant F1 and F2 females were not significantly changed (Fig. 3.4B), indicating postimplantational lethality in these pregnant females. Increased postimplantational lethality upon treatment with 10 mg/kg ZEA was demonstrated in rats [109]. The generational cumulative effect of 20 ppm ZEA diet on female fertility was also manifested in a reduced fertility index in the F2 but neither the F0 nor the F1 generations (Fig. 3.3B) and increased pregnancy gap in the F1 and F2 generations (Fig. 3.3E).

The switched exposure regimen in the F3 females provided further information on those developmental windows sensitive to ZEA exposure. Since implantation rate, pregnancy rate, and litter size were not adversely affected in the F0 20 ppm ZEA-treated group (Figs. 3.3A, 3.3C, 3.4A) and implantation rate was unaffected in the F3 0-
20 group (Fig. 3.5B), which all started ZEA treatment after weaning, the results imply that maternal exposure during mating, gestation, and lactation played an important role for the adverse effects of 20 ppm ZEA on F1 and F2 fertility. Although no significant difference in implantation rate or number of implantation sites was observed in the F3 20-0 group due to small sample sizes, both parameters fell in between those of the F3 0-0 control group and the F3 20-20 group (Figs. 3.5B & 3.5C), indicating that exposure cessation (20-0 group) could partially alleviate the adverse effects while extending exposure for one more generation continued the trend of diminished fertility (Fig. 3.5D). The worsened fertility might be caused by dam-mediated in-utero and/or lactational exposure [37], a phenomenon that could be influenced by epigenetic mechanisms [163, 164].

The contribution of the mating males to the adverse effects of 20 ppm ZEA is insignificant based on the following observations. First, male rats exposed to 10 mg/kg body weight of ZEA via diet for at least 10 weeks did not have any reported obvious pathological changes in the testis [109]. Second, male mice intraperitoneally injected with 15 mg/kg ZEA for 7 consecutive days did not have visible effects on sperm quality and testis histology [165]. Third, young adult C57BL/6J males treated with 40 ppm ZEA diets for three weeks plus one week with gavage during mating did not affect mating activity, testis weight, sperm counts, or the fertility of the mated females (Supplementary figure S2.3 in Appendix A; Appendices F & G). Since the effect of ZEA on female fertility was dose-dependent [153] and the mating males in this study were exposed to ZEA at lower doses (0.8-20 ppm, or <2.5 mg/kg body weight per day [153]) for shorter periods, the adverse effects on fertility observed in the 20 ppm ZEA treated females in this study
were assumed to be mainly attributed by the effects of ZEA on female reproductive system.

In summary, our study demonstrated that exposure to a 20 ppm ZEA diet promoted female puberty onset without obvious cumulative effect and diminished female fertility over generations under the current experimental design. While direct exposure to ZEA immediately prior to puberty onset was a sensitive window for ZEA to affect female puberty, different exposure periods from gestation, lactation, to premating could all contribute to the adverse effects of ZEA on female fertility. Cessation of exposure to ZEA seemed to partially alleviate these adverse effects.

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**Supplementary Table S3.1. Water and food consumption during postweaning**

**Table S3.1A. Water consumption (g/week per mouse) during postweaning *.$

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* Rough estimation of the average water consumption per mouse (N=16-36) in cages with multiple mice thus no standard deviation was available in each group.

**Table S3.1B. Food consumption (g/week per mouse) during postweaning *.$

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* Rough estimation of the average food consumption per mouse (N=16-36) in cages with multiple mice thus no standard deviation was available in each group.
Supplementary Table S3.2. Water and food consumption during lactation

Table S3.2A. Water consumption (g/week per dam) during lactation.

<table>
<thead>
<tr>
<th>Generation</th>
<th>ZEA</th>
<th>1\textsuperscript{st} week</th>
<th>2\textsuperscript{nd} week</th>
<th>3\textsuperscript{rd} week</th>
</tr>
</thead>
<tbody>
<tr>
<td>F0</td>
<td>0 ppm</td>
<td>73.5± 24.7</td>
<td>104.2± 31</td>
<td>120.4± 21.4</td>
</tr>
<tr>
<td></td>
<td>0.8 ppm</td>
<td>81.3± 25.7</td>
<td>110.7± 35.8</td>
<td>110.4± 42.2</td>
</tr>
<tr>
<td></td>
<td>4 ppm</td>
<td>90.8± 38.6</td>
<td>122.9± 54.6</td>
<td>132.9± 37.4</td>
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<tr>
<td></td>
<td>20 ppm</td>
<td>79.0± 16.3</td>
<td>111.9± 26.7</td>
<td>124.4± 30.8</td>
</tr>
<tr>
<td>F1</td>
<td>0 ppm</td>
<td>79.8± 16.5</td>
<td>119.2± 35.7</td>
<td>121.9± 31.6</td>
</tr>
<tr>
<td></td>
<td>0.8 ppm</td>
<td>87± 41.8</td>
<td>105.6± 29.4</td>
<td>126.1± 36.6</td>
</tr>
<tr>
<td></td>
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<td>90.6± 19.4</td>
<td>122.4± 29</td>
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<td>20 ppm</td>
<td>53.8± 5.2</td>
<td>88.3± 2.8</td>
<td>123.3± 9.2</td>
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<tr>
<td>F2</td>
<td>0 ppm</td>
<td>71.6± 25</td>
<td>114± 33</td>
<td>128.1± 25.1</td>
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<tr>
<td></td>
<td>20 ppm</td>
<td>57± 7.1</td>
<td>89± 15.6</td>
<td>113.5± 24.7</td>
</tr>
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</table>

N=4-13

Table S3.2B. Food consumption (g/week per dam) during lactation.

<table>
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<tr>
<th>Generation</th>
<th>ZEA</th>
<th>1\textsuperscript{st} week</th>
<th>2\textsuperscript{nd} week</th>
<th>3\textsuperscript{rd} week</th>
</tr>
</thead>
<tbody>
<tr>
<td>F0</td>
<td>0 ppm</td>
<td>41.6± 9.1</td>
<td>60.9± 10.6</td>
<td>58.9± 11</td>
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<tr>
<td></td>
<td>0.8 ppm</td>
<td>40.5± 12.2</td>
<td>54.2± 19.8</td>
<td>60.3± 18.3</td>
</tr>
<tr>
<td></td>
<td>4 ppm</td>
<td>41.5± 7.7</td>
<td>56.7± 17.3</td>
<td>61.3± 12</td>
</tr>
<tr>
<td></td>
<td>20 ppm</td>
<td>44.7± 3.7</td>
<td>61.1± 15.9</td>
<td>57.7± 17.3</td>
</tr>
<tr>
<td>F1</td>
<td>0 ppm</td>
<td>39.8± 5.3</td>
<td>54.5± 8.4</td>
<td>58.3± 14.2</td>
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<td>0.8 ppm</td>
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<td>63.4± 14.1</td>
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<tr>
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<td>4 ppm</td>
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<td>60.6± 4.7</td>
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</tr>
<tr>
<td></td>
<td>20 ppm</td>
<td>35.1± 3.5</td>
<td>48.1± 4.9</td>
<td>59.3± 15.3</td>
</tr>
<tr>
<td>F2</td>
<td>0 ppm</td>
<td>35.7± 6.6</td>
<td>51.7± 10</td>
<td>62.1± 16.9</td>
</tr>
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<td>20 ppm</td>
<td>38.6± 17.8</td>
<td>47± 21.8</td>
<td>46.2± 14.3</td>
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</table>

N=4-13
Supplementary Figure S3.1. Postnatal body weight
Supplementary Figure S3.1. Female body weight during postnatal development. All litters from the same dams were included. Litter order did not affect body weight. A. F0 females. B. F1 females. C. F2 females. D. Preweaning F3 females. E. Postweaning F3 females. Error bar, standard deviation; * P<0.05, 20 ppm ZEA group compared with the 0 ppm ZEA group at the same time point; #, P<0.05, 4 ppm ZEA group compared with the 0 ppm ZEA group at the same time point; F0, N=16-20; F1, N=19-36; F2, N=14-30; F3, N=7-26.
Supplementary Figure S3.2. Age of the dams at litter delivery

Supplementary Figure S3.2. Aages of the F0, F1, and F2 dams at delivery of different litters. Error bar, standard deviation; * P<0.05, 20 ppm ZEA group compared with the 0 ppm ZEA group at the same birth order in the same generation; N=3-13.
CHAPTER 4

TIMING AND RECOVERY OF POSTWEANING EXPOSURE TO DIETHYLSILBESTROL ON EARLY PREGNANCY IN CD-1 MICE

Fei Zhao, Jun Zhou, Ahmed E. El Zowalaty, Rong Li, Elizabeth Ann Dudley, Xiaoqin Ye.

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4.1 ABSTRACT

Exposure timing could play an important role in the effects of estrogentic endocrine disrupting chemicals (EEDCs) on early pregnancy. This study examined the sensitivity of different exposure periods from weaning to gestation day 4.5 (D4.5) to 50 ppb diethylstilbestrol (DES, as a test EEDC) diet on embryo implantation and potential recovery upon temporary cession of DES exposure in CD-1 mice. Peripubertal (3-5 weeks old) DES exposure reduced the numbers of corpora lutea and implantation sites. Postpubertal (5-7 weeks old) DES exposure did not have significant effects on early pregnancy. Postmating (D0.5-D4.5) DES exposure affected postovulation events leading to impaired embryo implantation. A 5-day premating rest from 5-week DES exposure (3-8 weeks old) resulted in recovery of early pregnancy rate. These data demonstrate that peripubertal and postmating are sensitive periods to endocrine disruption of early pregnancy and temporary cession of exposure could partially alleviate adverse effects of DES on early pregnancy.

**Keywords:** Estrogenic endocrine disrupting chemical (EEDC), diethylstilbestrol (DES), peripubertal exposure, postmating exposure, corpus luteum, embryo transport, embryo implantation, early pregnancy.
4.2 INTRODUCTION

Estrogenic endocrine disrupting chemicals (EEDCs) are exogenous estrogenic chemicals that include man-made chemicals, such as plasticizers (e.g., bisphenol A (BPA)), pesticides (e.g., methoxychlor), and drugs (e.g., diethylstilbestrol (DES)), as well as naturally-occurring compounds, such as phytoestrogens (e.g., genistein) and mycotoxins (e.g., zearalenone (ZEA)) [35, 36]. EEDCs could interfere with the endocrine system to potentially disrupt estrogen-regulated physiological processes, including puberty and pregnancy. Indeed, EEDCs have been associated with precocious puberty in girls and decreased fertility in women [55, 56, 166].

Toxicological studies in rodents have demonstrated that exposure to EEDCs could affect early pregnancy events, which include ovulation, fertilization, embryo transport, preimplantation embryo development, establishment of uterine receptivity, and embryo implantation. Adverse effects on any of the above events could lead to impaired embryo implantation. In utero exposure to DES decreased ovulation capability and caused structural abnormalities in female reproductive tract, which all contributed to DES-induced infertility in CD-1 mice [62]. Neonatal exposure to genistein (via subcutaneous (s.c.) injection) increased preimplantation embryo loss and decreased uterine receptivity leading to impaired embryo implantation in CD-1 mice [64, 67, 167]. Neonatal exposure to BPA or DES decreased the number of implantation sites in rats [52]. Postmating exposure to BPA (via s.c. injection) or ZEA (via diet) could also interfere with early pregnancy events leading to impaired embryo implantation in C57BL6 mice [74, 153]. These studies demonstrate that besides dose, route, and
duration, exposure timing is another important factor for potential effects of EEDCs on early pregnancy.

A common route of exposure to EEDCs in mammals is via diet, especially direct dietary exposure starting from weaning, but limited studies have been focused on postweaning dietary exposure to EEDCs on early pregnancy and no studies have dissected the sensitivity of different postweaning periods to EEDC exposure on early pregnancy. In addition, it is unknown about any recovery upon cession of EEDC exposure. It was hypothesized that exposure timing during postweaning could modulate effects of EEDCs on early pregnancy and some effects could be transient upon cession of exposure. This study was designed to achieve two goals using DES as a test EEDC [168] in CD-1 mouse model: 1) to determine the sensitivity of different postweaning periods to DES on embryo implantation; and 2) to determine potential recovery from postweaning exposure to DES on embryo implantation. Embryo implantation was chosen as the end point because it is a collective point for all successful early pregnancy events.

4.3 MATERIALS AND METHODS

Animals. CD-1 mice (8 weeks old females and males) were purchased from Charles Rivers. They were mated 10 days after arrival at Coverdell animal facility to produce offspring for this study. They were housed in polypropylene cages with free access to water (in polypropylene bottles) and regular rodent diet. The Coverdell animal facility at the University of Georgia was maintained on a light/dark cycle 12h/12h (0600 h~1800 h) at 23 ± 1°C with 30–50% relative humidity. All methods used in this study were
approved by the Animal Subjects Programs of the University of Georgia and conform to National Institutes of Health guidelines and public law.

**DES treatment.** Diets containing 0, 5, 20, and 50 ppb DES were homemade using AIN-93G powder (Bio-Serv, Frenchtown, NJ) and DES (Sigma-Aldrich, USA) as previously described [153]. Newly-weaned littermate females (3 weeks old) were randomly assigned into A1/B1, A2, A3, A4/C1, B2, B3, B4, C2, C3, and C4 groups (Fig. 4.1). Set A was to determine DES dose-response on embryo implantation: 0 (A1/B1), 5 (A2), 20

Figure 4.1. Treatment regimen. Red dotted period, mating with untreated stud males on control vehicle diet; black shaded period, 5 days on control vehicle diet prior to mating; A1/B1, vehicle control group for set A (dose-response) and set B (timing); A4/C1, the highest dose group in set A and the group to be compared in set C (recovery). At least 10 mice were included in each group.
(A3) and 50 (A4/C1) ppb DES diets (Fig. 4.1). After 5 weeks of exposure, females in all groups were set up for mating at 8 weeks old with untreated CD-1 young stud males. There was no DES exposure during mating in all groups in this study to exclude any potential effect of DES on male fertility. Females were checked for a vaginal plug each morning. The morning with plug identification was designated as gestation day 0.5 (D0.5) and the plugged females resumed their premating DES diets (Fig. 4.1). Body weight was measured weekly, and food and water consumption were recorded per week per cage from 3 to 8 weeks old. Set B was to determine exposure timing to 50 ppb DES diet on embryo implantation: B1 (vehicle control, same as A1), B2 (3-5 weeks old), B3 (5-7 weeks old), and B4 (D0.5 to D4.5) (Fig. 4.1). Set C was to determine recovery from 50 ppb DES exposure: C1 (same as A4, DES exposure: 3-8 weeks old + D0.5-D4.5), C2 (DES exposure: 3-8 weeks old + D0.5-D4.5, with a 5-day rest prior to mating), C3 (DES exposure: 3-8 weeks old), and C4 (DES exposure: 3-8 weeks old, with a 5-day rest prior to mating) (Fig. 4.1). Vaginal opening was monitored daily from weaning till it was detected except for several females at the beginning of the study in A1/B1 and A4/C1 groups. All mice were dissected on D4.5 to determine implantation sites as previously described [32, 153, 169]. If no implantation site was observed, the uterine horns and oviducts were flushed with 1xPBS to determine the presence of embryo(s) thus the pregnancy status. At least 10 females were included in each group.

Immunohistochemistry. Cross sections (10 µm) of representative D4.5 uteri from B1 and B4 groups were immunostained to detect progesterone receptor (PR) expression as previously described [120, 153, 170].
**Ovary histology.** One ovary per animal in the B1, B2, B3, B4, C1, and C2 groups was fixed in formalin, dehydrated, embedded longitudinally in paraffin as previously described [170]. Sections were cut at 5 μm. Every 10th section was collected (about 20-40 sections per ovary) and stained with H & E. The numbers of follicles and corpora lutea were counted from 5 random sections at the center 1/3 ovary (those in the middle one third of all sections for that ovary, e.g., sections No. 10~20 for an ovary with 30 10th sections). Three categories of follicles were counted: primordial (types 1-3b), growing (types 4-5b), and antral (types 6-8) [171]. The average numbers of follicles at these three stages and corpora lutea from 5 sections were recorded for each ovary.

Statistical analyses. All the data were analyzed using SigmaPlot 12.0. Two-Way repeatedly measured ANOVA followed by Dunnett’s method was used for postweaning body weight. One-Way ANOVA followed by SNK method or One-Way ANOVA on ranks (if data failed normality test or equal variance test) was used for the numbers of implantation sites, follicles, and corpora lutea. Fisher’s exact test was used to analyze implantation rate and pregnancy rate. The significant level was set at p < 0.05.

### 4.4 RESULTS

**Dose-response**

Newly-weaned 3 weeks old CD-1 females treated with 0, 5, 20 and 50 ppb DES diets had comparable water and food consumption during the 5 weeks of treatment prior to mating (data not shown). However, significant lower body weights were observed in 20 ppb DES-treated group from 6 to 8 weeks old and 50 DES-treated group at 8 weeks old. No significant difference in body weight was observed in 5 ppb DES-treated group (Fig. 4.2A).
Vaginal opening was used to indicate pubertal onset and it showed a DES dose-dependent decrease of age. Since vaginal opening occurred before 5 weeks old, groups with the same treatment between weaning (3 weeks old) and 5 weeks old were combined: A1/B1, B3 and B4 on 0 ppb DES diet; A4/C1, B2, C2, C3, and C4 on 50 ppb DES diet (Fig. 4.1). All mice in 50 and 20 ppb DES groups had vaginal opening within 4 days of treatment, while those in 5 and 0 ppb DES groups were within 6 and 10 days of

Figure 4.2. Postweaning DES exposure on body weight and vaginal opening. A. Body weight during treatment from 3-week to 8-week old. N=12-27. * P<0.05, 20 ppb group compared with 0 ppb control group; # P<0.05, 50 ppb group compared with 0 ppb control group. B. Cumulative percentages of mice with vaginal opening during DES treatment. N=12-44. C. Average age at vaginal opening upon different doses of DES treatment. N=12-44; error bar, standard deviation; * P<0.05 compared with 0 ppb DES control group.
treatment, respectively (Fig. 4.2B). The average ages at vaginal opening were: 25.6±2.8 days old (N=24) for 0 ppb; 24.0±1.5 days old (N=12) for 5 ppb, 23.3±0.8 days old (N=12) for 20 ppb, and 22.6±0.9 days old (N=44) for 50 ppb DES-treated females, indicating a dose-dependent decrease of age at vaginal opening, although significant difference was only observed in 50 ppb DES-treated females (Fig. 4.2C).

Figure 4.3. Dose-response effects of DES exposure on early pregnancy. A. Pregnancy rate and implantation rate. N=10-15; * P<0.05 compared with A1 control group. B. Average number of implantation sites on D4.5. Only mice with implantation sites were included. N=5-15 in A1-A3 groups; N=0 in A4 group due to 0% implantation rate (A); error bar, standard deviation. C. Representative uterine images. Red arrows, implantation sites.

DES dose-dependent effect was also seen in early pregnancy rate (% of plugged females with embryos and/or implantation sites) and embryo implantation rate (% of
plugged females with implantation sites) detected on D4.5 (Fig. 4.3A). Significantly decreased pregnancy rate and implantation rate were observed in both A3 (20 ppb) and A4 (50 ppb) groups compared with A1 (vehicle control) group (Fig. 4.3A). In A4 group, the pregnancy rate (20%) was higher than the implantation rate (0%), although no significant difference was detected due to small sample size; while in A1~A3 groups, the pregnancy rate and implantation rate were the same (Fig. 4.3A). There was a DES dose-dependent trend of reduced number of implantation sites (P=0.067) (Fig. 4.3B) and no implantation site was detected in any of females in the A4 group (Fig. 4.3B). All uteri in A1 and A2 groups didn’t show distended appearance (Fig. 4.3C); 9 out of 12 females in A3 group had similar appearance as those in A1 and A2 groups (Fig. 4.3C), and the rest 3 females showed distension (data not shown); all uteri in A4 group had distended appearance (Fig. 4.3C).

*Exposure timing on embryo implantation*

To determine exposure timing on embryo implantation, three different periods were examined: 3-5 weeks old (B2, peripubertal), 5-7 weeks old (B3, postpubertal), and D0.5 to D4.5 (B4, postmating) (Fig. 4.1). While exposure to 50 ppb DES during none of these periods affected pregnancy rate, peripubertal two weeks of exposure (B2 group) significantly reduced the number of implantation sites compared with B1 vehicle control group, and exposure from D0.5 to D4.5 (B4 group) significantly reduced both implantation rate and the number of implantation sites compared with B1-B3 groups (Figs. 4.4A & 4.4B). B3 group had the number of implantation sites in between those in B1 and B2 groups but didn’t differ significantly from them (Fig. 4.4B).
Ovary histology from D4.5 females revealed comparable numbers of primordial, growing, and antral follicles among the B1-B4 groups. However, significantly reduced number of corpora lutea was observed in B2 group compared with B1 and B4 groups (Figs. 4.4C & 4.4D), although both B2 and B4 groups had reduced numbers of implantation sites on D4.5 (Fig. 4.4B). No significant difference was observed for B3 group compared with the rest three groups (Fig. 4.4B). The relative numbers of corpora lutea correlated with the relative numbers of implantation sites among B1, B2, and B3 groups ($R^2=0.9882$) but not that in B4 group (Figs. 4.4B, 4.4C, 4.4D).

The pregnant females with or without implantation sites in B4 group showed distended uterine horns (Fig. 4.4E). Retained expression of progesterone receptor in the uterine luminal epithelium of pregnant B4 uterus without implantation sites also indicated that embryo implantation had not occurred yet (Fig. 4.4F) [120]. Among the 7 females without implantation sites in B4 group, 3 females did not have embryos flushed.
from the reproductive tract, indicating they were not pregnant; the rest 4 females had 2, 4, 4 and 7 embryos flushed from reproductive tracts, respectively. The average number of embryos (4.3±2.1, P=0.012) flushed from the entire reproductive tract of these 4 females in B4 group and the average number of implantation sites (2.5±2.1, P=0.0003) in both uterine horns of the 6 females with implantation site(s) in B4 group were significantly lower than the average number of corpora lutea per section in one ovary in B4 group (8.8±3.4 corpora lutea/ovary), whereas in B1-B3 groups, the numbers of implantation sites in both uterine horns were higher than the numbers of corpora lutea per section in one ovary (Figs. 4.4B & 4.4D). These observations indicated impaired postovulation process(es) in the B4 group. Among the 4 pregnant females without implantation sites in B4 group, 2 females had 2 and 4 blastocysts, respectively, flushed from the uterus (Fig. 4.4G) but not oviduct; one female had 3 blastocysts in the uterus and 1 morula in the oviduct (data not shown); and the fourth one had 2 blastocysts in the uterus and 5 blastocysts in the oviduct (Fig. 4.4H), indicating delayed embryo transport and potentially delayed embryo development.

Partial recovery from a 5-day premating break

Our previous study on ZEA-treated C57BL/6J females implied potential recovery in females with delayed mating activity: females plugged within 3 days of cohabitation with stud males without treatment had an implantation rate significantly lower than that in the control, while those plugged after 5 days of cohabitation had a comparable implantation rate with the control [153]. To determine potential recovery from DES treatment, a 5-day cessation of DES exposure was introduced prior to mating in C2 and C4 groups, while C1 and C3 groups were mated immediately after DES exposure from
week 3 to week 8 (Fig. 4.1). Therefore, the only difference in the exposure regimens between C1 and C2 groups or C3 and C4 groups was the 5-day break from DES exposure prior to mating (Fig. 4.1). Although no significant difference in the implantation rate between C1 (0/10=0%) and C2 (2/11=18.2%) groups was detected, the pregnancy rate was significantly increased in C2 group (10/11=90.9%, P=0.002) compared with C1 group (2/10=20%) (Fig. 4.5A). The two pregnant females in C1 group had 2 and 3 blastocysts in the uterus (data not shown), respectively. The two pregnant females with implantation sites in C2 group had 2 and 11 implantation sites, respectively (Fig. 4.5B); among the rest 8 pregnant females without implantation sites in C2 group, 3 had embryos recovered in the uterus only, 4 had embryos recovered in the oviduct only, and the 8th one had embryos flushed from both uterus and oviduct (data not shown). Among the 45 embryos flushed from the reproductive tracts of these pregnant females, 40 were hatched blastocyst, 3 were unhatched blastocyst, 1 was hatched morula, and 1 was unhatched morula (data not shown). C1 and C2 groups had comparable numbers of follicles and corpora lutea (Figs. 4.5C & 4.5D). The numbers of corpora lutea in both C1 and C2 groups were significantly lower than that in the B1 vehicle control group but comparable with that in the B2 group (Fig. 4.4D).
C3 and C4 groups did not have DES treatment after 8 weeks old (Fig. 4.1). Although C4 group, which had a 5-day break between DES treatment and mating (Fig. 4.1), appeared to have higher pregnancy rate and implantation rate (Fig. 4.5A) and...
more implantation sites (Fig. 4.5B) than those in C3 group, there was no significant difference in all three parameters between these two groups. However, when compared with B1 vehicle control group, C3 group had significantly decreased pregnancy rate (8/13 vs. 15/15, P=0.013), implantation rate (8/13 vs. 15/15, P=0.013), and number of implantation sites (10.1±2.9 vs. 13.3±1.5, P=0.009) at D4.5, whereas C4 group only had significantly reduced number of implantation sites (11.9±2.1 vs. 13.3±1.5, P=0.04) at D4.5 (Figs. 4.1, 4.4A, 4.4B, 4.5A, 4.5B). These data suggest less severe adverse effects in C4 group that had a 5-day premating rest from DES exposure.

The difference in the exposure regimens between C1 and C3 groups or C2 and C4 groups was the 4-day postmating exposure to DES (Fig. 4.1). The pregnancy rate was statistically marginally higher in C3 group (61.5%, P=0.09) compared with C1 group (20%). The pregnancy rates between C2 and C4 groups were comparable (Fig. 4.5A). However, the implantation rates were significantly higher in both C3 and C4 groups compared with C1 and C2 groups, respectively (Fig. 4.5A), consistently demonstrating the sensitivity of D0.5-D4.5 postmating period to endocrine disruption.

4.5 DISCUSSION

Estrogen signaling plays important roles in pubertal development [172] and early pregnancy [20]. It is not surprising that EEDCs could interfere with these processes. Mammals begin direct dietary exposure to EEDCs from weaning, yet very limited studies have been focused on the sensitivity of mammals during postweaning period, which covers peripubertal development, to EEDC exposure on early pregnancy. This study focuses on postweaning dietary exposure to DES (as a test EEDC) to identify
sensitive periods to EEDC exposure and potential recovery from EEDC exposure on early pregnancy with embryo implantation as the endpoint.

The adverse effects from 50 ppb DES exposure on embryo implantation was most severe from postmating (D0.5-D4.5) exposure, less severe from peripubertal (3-5 weeks old) exposure, and least severe (no significant adverse effects) during postpubertal (5-7 weeks old) exposure. Considering the fact that peripubertal exposure was prior to postpubertal exposure thus having more time to eliminate DES from the body, yet peripubertal exposure but not postpubertal exposure significantly reduced the number of implantation sites, the data clearly indicated that peripubertal was a more sensitive period than postpubertal period upon endocrine disruption. Peripubertal DES exposure also significantly reduced the age at vaginal opening, supporting peripubertal as a sensitive period to endocrine disruption.

Although postmating exposure had more severe adverse effects than peripubertal exposure on embryo implantation, it was hard to directly compare the sensitivity to endocrine disruption between these two exposure periods for the following two reasons: 1) there was over three weeks of break from DES exposure between peripubertal exposure and embryo implantation, while postmating exposure was immediate to embryo implantation; and 2) only the treated females were at risk during peripubertal exposure, while both the treated females and the embryos were at risk during postmating exposure. Indeed, the mechanisms for the adverse effects of peripubertal DES exposure and postmating exposure on embryo implantation were different.
Peripubertal DES exposure significantly reduced the number of corpora lutea without significantly reduced the number of follicles, indicating inhibition of ovulation, an effect that has also been reported in rats that were prepubertally treated with EEDCs polychlorinated dibenzo-p-dioxins [173]. The reduction of corpora lutea correlated with the reduction of implantation sites, suggesting that peripubertal DES exposure affected embryo implantation via its effect on ovulation. The formation of corpora lutea depends on preovulatory surge of luteinizing hormone (LH) [7] and it has been reported that neonatal exposure to DES could affect LH producing cells in mice [174].

However, postmating DES exposure did not affect ovarian function, including follicle development and ovulation, indicated by comparable numbers of follicles and corpora lutea between DES-treated group (B4) and the vehicle control (B1). Instead, postmating DES exposure interfered with fertilization process, indicated by the reduced number of embryos either as hatched but un-implanted blastocysts or implanted embryos in the reproductive tract; embryo transport process, indicated by the retention of blastocysts in the oviduct on D4.5; potentially preimplantation embryo development, indicated by the occasional presence of morula on D4.5; and embryo implantation process, indicated by the un-implanted hatched blastocyst. Regardless whether or not there was DES exposure during premating, or whether or not there was a 5-day premating break from DES exposure, postmating exposure aggravated the adverse effects of DES on early pregnancy (B4 vs. B1; C3 vs. C1; C4 vs. C2) (Figs. 4.4 & 4.5; Table 4.1).
The significant reduction of numbers of corpora lutea and implantation sites indicated persistent adverse effects of peripubertal DES exposure, while our previous study on EEDC ZEA suggested that there was potential recovery when the untreated mating period was prolonged in mice previously treated with ZEA [153]. Indeed, when a 5-day premating break from DES treatment was introduced prior to mating in C2 group (weeks 3-8 + postmating DES exposure) or C4 group (weeks 3-8 DES exposure) in this study, there was a significant increase in the pregnancy rate in C2 group although the rest parameters showed recovery but didn’t reach significant difference (C2 vs. C1), or the adverse effects from weeks 3-8 DES treatment was general less severe in C4 group (significantly reduced number of implantation sites) than in C3 group (significantly

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<tbody>
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<td>B1</td>
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<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
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<td>C1</td>
<td>+</td>
<td>+ **</td>
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<tr>
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<td>C2</td>
<td>-</td>
<td>+ **</td>
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<td></td>
<td>C3</td>
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<td></td>
<td>C4</td>
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<td>C1</td>
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<td>C3</td>
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* P=0.09; +, P<0.05; -, P>0.05; ** C1 had 100% implantation failure (N=0) and C2 had only two females (N=2) with implantation sites thus preventing statistical analysis on the number of implantation sites; B1, vehicle control; B2, peripubertal (3-5 weeks old) DES exposure; B3, postpubertal (5-7 weeks old) DES exposure; B4, postmating (D0.5-D4.5) DES exposure; C1, weeks 3-8 + postmating DES exposure; C2, C1 + 5-day premating break from DES exposure; C3, weeks 3-8 DES exposure; C4, C3 + 5-day premating break from DES exposure (Fig. 4.1).
reduced pregnancy rate, implantation rate, and number of implantation sites) when they were compared with B1 vehicle group (Figs. 4.4 & 4.5; Table 4.1). About 90% of a single dose DES can be quickly metabolized and excreted within 24 hours in mice [175], thus a 5-day premating break from DES exposure in C2 and C4 groups could provide sufficient time to eliminate the majority of DES from the body. Therefore, factors leading to embryo implantation, such as oocyte quality for fertilization as well as reproductive tract environment for fertilization, preimplantation embryo development, embryo transport, and uterine receptivity, could have potentials to recover from premating DES insult.

This study provides three important messages: 1) peripuberty is a sensitive period for endocrine disruption and there could be persistent adverse effects; and 2) early pregnancy is an extremely sensitive period for endocrine disruption, which could potentially interfere with multiple early pregnancy events; and 3) avoiding significant exposure to EEDCs before getting pregnant could be beneficial to the outcome of pregnancy.

**Funding Information**

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**Acknowledgements**

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Interdisciplinary Toxicology Program, and Department of Physiology and Pharmacology at University of Georgia, and National Institutes of Health.
CHAPTER 5

LHFPL2 IS ESSENTIAL FOR DISTAL REPRODUCTIVE TRACT DEVELOPMENT IN MICE

Fei Zhao, Jun Zhou, Shuang Huang, Xiaoqin Ye. To be submitted to Biology of Reproduction.
5.1 ABSTRACT

Distal reproductive tracts include the lower part of the vagina in females and the prostatic end of the vas deferens in males. These structures are essential for reproductive success and health. However, the development of distal reproductive tracts in both genders remains a mystery. A spontaneous point mutation (G to A in the 2nd nucleotide of the codon for 102 amino acid residue) in lipoma HMGIC fusion partner like 2 (Lhfpl2) caused atresia of the distal vagina and the distal vas deferens in mice, resulting in 100% female and ~70% male infertility. Further studies indicated defective development of distal reproductive tracts in both females and males during embryonic development. Gene expression studies indicated Lhfpl2 was expressed in the epithelium of postnatal vagina and vas deferens. In vitro over-expression studies showed both wild-type (WT) and mutant (MUT) LHFPL2 was expressed in endoplasmic reticulum but only WT LHFPL2 was expressed in the filopodia. It is possible that LHFPL2 in the filopodia of the epithelium helps extension and development of the distal reproductive tract.

Keywords: reproductive tract development; distal atresia; Lhfpl2; vagina; vas deferens

5.2 INTRODUCTION

In the chapters 2-4, estrogenic endocrine disruptors advanced timing of pubertal onset in mice, indicated by vaginal opening. However, the mechanisms of vaginal opening are largely unknown. The initial goal of this chapter was to investigate the mechanisms of vaginal opening using the Lhfpl2 mutant mouse model that had vagina permanently closed. However, our extensive studies revealed that Lhfpl2 did not involve in estrogenic endocrine disruptor induced vaginal opening. The vaginal imperforation in
the mutant females was the result of defective embryonic development of the distal female reproductive tract.

The reproductive tract is essential for successful production of offspring in mammals. The female reproductive tract, which includes oviduct, uterus, cervix and vagina, is the site for fertilization, embryo transport, embryo implantation, embryo development and fetus delivery; the male reproductive tract, which consists of the epididymis, seminal vesicle and vas deferens, provides a ductal channel for the maturation, transport and ejaculation of sperm. Every reproductive event after the release of gametes (oocytes / sperm) from gonads (ovaries / testes), such as fertilization, embryo transport and development, occurs in the reproductive tracts. The prevalence of congenital female reproductive tract abnormality is about 1% in the population of women with normal fertility and with fertility [176]. Congenital absence of vas deferens is responsible for 1-2% of male infertility (11 out of 749 men presenting with infertility between April 1975 and December 1981) and the unilateral absence of vas deferens occurs in about 0.06-1% of men having elective vasectomy [177].

Before sexual differentiation, the mammalian embryo is sexually indifferent, with both the mullerian duct (MD) and the wolffian duct (WD). In females, the WD degenerates and the MD will form morphologically and functionally different subsections of the female reproductive tract, including oviduct, uterus, cervix and 2/3 upper vagina in female; in males, the MD regresses and the WD differentiates into epididymis, seminal vesicle, and vas deferens. Many developmental factors in this process have been revealed in genetically modified mice [59, 60]. However, the least known, as well as more controversial, is how the distal part of reproductive tracts develops. At the stage of
sexually indifferent embryo on gestation day (D) 12.0, distal WD connects with the common nephric duct, which connects with urogenital sinus (UGS, the embryonic origin of the bladder and urethra); but the MD is still extending posteriorly and both WD and MD reach the urogenital sinus by D13.5 [59, 85]. After D13.5, the epithelium of MD in female mice was recently thought to migrate posteriorly to form the entire vagina by PND7, which was different from the previous hypothesis that upper two-thirds of vagina derived from the MD and the lower one-third from the UGS [86]. While several genetically modified mice showed abnormal development of the vagina as indicated by imperforate vagina (Table 1.1), its molecular mechanisms of these genes in the distal MD development is still unknown. In males, after reaching the UGS, the distal WD somehow differentiates into ampulla, the wider duct of vas deferens at its connection with the urethra, and the seminal vesicle from lateral outgrowth [60, 88]. This developmental process may involve CFTR (CF transmembrane conductance regulator), of which knockout pigs showed partial or total vas deferens atresia [89]; however, other genes potentially critical for distal WD development still await identification. Hence, the molecular network in regulating distal reproductive tract development is largely unknown.

Lhfpl2 belongs to the family of lipoma HMGIC fusion partner (Lhfp), which was found to be the translocation partner of HMGIC in the human lipoma tissue [76]. The Lhfp gene family includes six members, i.e. Lhfp, and Lhfpl1~Lhfpl5, all of which are predicted to be four transmembrane protein with 200-247 amino acids in both humans and mice. In total, only eight papers have been published describing these genes [77-84]. Lhfpl1 is widely expressed in human tissues [77]; DNA hypermethylation in the
promoter of Lhfpl4 is associated with cervical cancer in human [78]. Mutations in Lhfpl5 (also called Tmhs [tetraspan membrane protein of hair cell stereocilia]) could cause hearing disorders in human, [79, 80] and mice [81, 82], and has been associated with hypospadias (a birth defect of abnormal placed opening of urethra), anal atresia with a recto-urethral fistula (a hole between urinary channel and the rectum) and hypoplastic kidney in a young boy [83]; Lhfpl5 may be linked with PCDH15 (a cadherion) and the transduction channel to regulate mechanotransduction in cochlear hair cells [84].

Here, we report that a spontaneous point mutation in Lhfpl2 causes congenitally absence and blocked reproductive tracts at the distal ends in male and female mice. The study reveals the function of a novel gene Lhfpl2 and its critical role in the normal development of the reproductive tract. It will be a good model to investigate the formation of the distal reproductive tract and the consequence of its defects.

5.3 MATERIALS AND METHODS

**Animals.** Lhfpl2 MUT mice were purchased from Jackson Laboratories (Bar Harbor, ME) to establish a colony in Coverdell Rodent Vivarium at University of Georgia. All mice were housed in polypropylene cages with free access to food and water in polypropylene water bottles. The animal facility was maintained on a 12-hour light/dark cycle (0600 h to 1800 h) at 23 ± 1°C with 30–50% relative humidity. All methods used in this study were approved by the Animal Subjects Programs of the University of Georgia and conform to National Institutes of Health guidelines and public law.
Table 5.1. Primers used in RT-PCR, point mutation confirmation, *in situ* hybridization and FLAG expression plasmid construction.

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<th>Primers</th>
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<td>NM_029990.1</td>
</tr>
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<td>point mutation</td>
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<td>Expression plasmid</td>
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<td>ACC</td>
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<td>into CT pFLAG</td>
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<td>into CT pFLAG</td>
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<tr>
<td>mLhfpl2 e4R1</td>
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**Confirmation of the point mutation and genotyping.** Genomic DNAs were extracted using DirectPCR (Viagen, USA) according to manufacturer’s protocol. The primer pair (conF/conR) (Table 5.1) was used to amplify 400 basepair PCR products covering the point mutation around middle. The PCR products were subsequently digested using BgIII at 37°C overnight. Digested products were run in the 10% gel. Custom Taqman SNP genotyping assay (Life technologies, USA) was used for genotyping. Taqman SNP genotyping primers were designed according to the manufacturer’s instructions.

**Animal treatment and tissue collection.** WT and MUT mice were sacrificed on postnatal day 3, 7, 14, 21, 28 and on the day of vaginal opening, or after estrogen treatments; and collected tissues were either fixed or flash frozen on dry ice. For the estrogen treatment, 12.5 µg E2 with the sesame oil as vehicle was injected intraperitoneally into newly-weaned females for 3 consecutive days. Vaginal opening was monitored. One day after the third treatment, mice were sacrificed to measure weights of body, reproductive tracts and uterine fluids.

**Bioinformatics analysis.** The Lhfpl2 was predicted to have 222 amino acids with the weight of 23.88 KD. Transmembrane prediction was done in the website (http://www.cbs.dtu.dk/services/TMHMM/) [178] and secondary topology was analyzed in Protter (http://wlab.ethz.ch/protter/start/).

**Histology.** Fixed tissues were embedded in paraffin and sectioned (5 µm). Sections were deparaffinized, rehydrated, and stained with hematoxylin and eosin, and then dehydrated and mounted as previously described [179]
RT-PCR. Specific primers were used in the (Table 5.1). Quantitative Realtime PCR was done as previously described [180]. The mRNA expression levels were normalized by the expression of GAPDH (glyceraldehyde-3-phosphate dehydrogenase). The PCR products were run in 10% agarose gel and visualized in the UV light. Pictures were taken with 1 second exposure. HPRT1 (hypoxanthine phosphoribosyltransferase 1) served as the loading control.

In situ hybridization. Probe preparation and In situ hybridization were performed as previously described [181]. Sense and antisense probes for Lhfpl2 were synthesized from cDNA fragments amplified with the specific primer pair in the Table 5.1. The T-easy vector with Lfhpl2 cDNA was sequenced by GENEWIZ. INC.

Immunohistochemistry. Immunohistochemistry was done according to [120]. Briefly, frozen sections (10 µm) were mounted, fixed, and subjected to antigen retrieval. Non-specific staining was blocked by 10% goat serum in 1XPBS. Sections were then incubated with rabbit LHFPL2 antibody (1: 250, Thermo Fisher Scientific) in blocking reagent at 4°C for overnight. After washing in 1xPBS, sections were incubated with biotinylated goat anti-rabbit secondary antibody (1: 200 Santa Cruz Biotechnology, USA) for 30 minutes at room temperature. Sections were then incubated with ABComplex/HRP (Santa Cruz Biotechnology, Inc) for 30 minutes at the room temperature, washed, incubated with 3, 3'-diaminobenzidine tetrahydrochloride, counterstained with Hematoxylin, and mounted for imaging. Negative control was processed exactly the same except that the primary antibody was replaced with normal rabbit IgG.
**Western blotting.** Uterine samples were homogenized in RIPA buffer with protease inhibitor (1:100). Protein concentrations were measured by Bradford assay with Nanodrop. Samples (30 µg) were loaded into polyacrylamide gel. Separated proteins were transferred onto PVDF membrane and blocked with 5% non-fat milk for 1 hour at room temperature on an orbital shaker. After washed with the Tris-buffered saline containing 0.1 % tween (TBST), the membrane was incubated in the primary antibody (1:200) over 2 nights at 4°C in TBST containing 1% BSA (bovine serum albumin) on an orbital shaker. The membrane was then washed and incubated with the appropriate peroxidase-labeled secondary antibody (1:3000) diluted in 5% non-fat milk. The image was developed on the film after incubation with Pierce ECL western blotting substrate.

**Plasmid construction.** Full length WT Lhfpl2 and MUT Lhfpl2 cDNA were derived from WT and MUT uteri, respectively by PCR with the specific primer pair (orfF/orfR) with BamHI and HindIII recognition sites at the 5’ end. The PCR products were cloned into T-easy vector and sequenced. Double digested by BamHI and HindIII from the T-easy vectors, WT and MUT full open reading sequences with the sticky ends were subcloned into the p3xFLAG-CMV-14 vector [182].

**Cell culture and transfections.** Hela cells were kindly provided by Dr. Biao He at the University of Georgia. They were cultured in DMEM media with 4.5 g/L glucose, L-glutamine, sodium pyruvate, 100 I.U/mL penicillin-streptomycin, and 10% fetal bovine serum. Transient transfections were conducted in a 24-well plate with Lipofectamine LTX reagent (Invitrogen, USA) according to the manufactory instruction.

**Immunocytochemistry.** Hela cells were seeded on the coverslips and transfected after 24h proliferation. Transfected Hela cells were fixed with 4% paraformaldehyde for 20
min; then washed 3 times with 1xPBS. Pore creation was achieved using 0.5% Triton X-100 in PBS with 3% BSA for 20 min. Then cells were washed 3 times with 1xPBS and non-specific staining was blocked by preincubation of 10% goat serum at 37°C for 1 hour. The coverslips were incubated with rabbit anti-DYKDDDDK tag primary antibody (1:800 Cell signaling technology, USA) and mouse anti-KDEL (1:100, ab12223 Abcam, USA) in blocking reagent for overnight at 4°C. After washed 3 times with 1xPBS for 5 min each and removed excess buffer, coverslips were incubated with Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 568 goat anti-mouse (Invitrogen, USA) for 1 hour at the room temperature. After washed with 1xPBS for 3 times for 5 min each, cells were mounted with VECTASHIELD mounting medium with DAPI and imaged using the confocal microscope.

5.4 RESULTS

Expression of Lhfp family members
To determine the expression of Lhfp gene members, RT-PCR were used to detect their mRNAs in different organs (Fig. 5.1). Lhfp, Lhfpl1 and Lhfpl2 had ubiquitous expressions; Lhfpl3, Lhfpl4 and Lhfpl5 were mainly expressed in brain (Fig. 5.1A). In mature vagina, the most expressed ones were Lhfp, Lhfpl1 and Lhfpl2 (Fig. 5.1B).

Confirmation of point mutation

The point mutation (G-A at the 305th nucleotide in Lhfpl2 cDNA) in MUT mice caused a missense mutation, resulting in glutamic acid at 102th amino acid in MUT mice instead of glycine in WT mice (Fig. 5.2A). The point mutation created the restriction site that could be recognized by BgIII (Fig. 5.2B). BgIII digested total, partial or none PCR products covering point mutation into two parts in MUT, HET (heterozygous) and WT mice, respectively (Fig. 5.2C). This confirmed the point mutation in HET and MUT mice. The point mutation was also confirmed by Taqman SNP genotyping assay. Allelic discrimination was achieved by relative FAM and VIC fluorescent intensity after thermal cycles, with more FAM intensity in MUT mice, and more VIC intensity in WT mice (Fig. 5.2D). This method was used for genotyping.
Figure 5.2. Confirmation and genotyping of Lhfpl2 mice. A. Sequences of cDNA and their translation covering the point mutation. B. Recognition sites by BglII in mutant mice. C. Gel result of PCR. U, undigested; D, digested; M, molecular-weight size marker. D. A representative result of Taqman SNP genotyping.
Predicted secondary topology of WT and MUT Lhfpl2

Both WT (Fig. 5.3) and MUT Lhfpl2 (Appendix C) were predicted to have four transmembrane alpha-helices with the N terminal inside the cell and the point mutation in the secondary transmembrane alpha-helix.
Figure 5.3. Predicted secondary topology of mouse LHFPL2. The secondary topology analyzed in Protter (http://wlab.ethz.ch/protter/start/). The red circle, the location of the point mutation.

**Vaginal imperforation in Lhfpl2 MUT females**

At 8 weeks, all WT and HET females had vagina opened (Figs. 5.4A & 5.4C) with no difference in ages at vaginal opening (Fig. 5.4D). However, none of MUT females had vagina opened (Figs. 5.4B & 5.4C). Since imperforate vagina was present as early as PND3 (Fig. 5.7) and before vaginal opening, it caused severe dilation of reproductive tracts (Figs. 5.4F) at adulthood compared to that of WT at estrus stage (Figs. 5.4E), which was also confirmed by their significantly heavier weights and longer lengths compared to those in WT females (Figs. 5.4G & 5.4H). Compared to WT female (Figs. 5.4I & 5.4K), the vaginal imperforation kept uterine fluid in the lumen (Fig. 5.4J), making the stroma denser and inducing small white dots in the epithelium (Fig. 5.4L).
Figure 5.4. Imperforate vagina in Lhfpl2 mutant mice. A & B. Representative pictures of WT (A) and MUT (B) mice at 8-week old. C. Percentage of mice with vaginal opening. * P<0.05. N=18, 26 and 35 for WT, HET and MUT, respectively. D. Ages at vaginal opening. N=18 and 26 for WT and HET. NP, not applicable since vagina was closed in all mutant females. E & F. Representative reproductive tracts of WT and MUT females at 8-week old. I-L. Uterine histology in WT (I & K) and MUT (J & L) females. Dotted box, the enlarged area for showing small white dots in MUT epithelium. G. Reproductive tract length at ~10 weeks old in WT and Mut females. * P<0.05. N=4-6. H. Reproductive tract weight at ~10 weeks old in WT and Mut females. * P<0.05. N=4-6.
**Vagina imperforation is not caused by estrogen signaling deficiency**

Vaginal opening depends on estrogen [101]. To investigate whether vagina imperforation was due to estrogen deficiency, ovarian histology was examined in WT and MUT females at 4-weeks and 8-weeks old (Figs. 5.5A ~ 5.5D). Comparable ovarian histology was observed between WT (Figs. 5.5A & 5.5C) and MUT (Figs. 5.5B & 5.5D) females with growing follicles and corpora lutea, suggesting normal ovarian histology in MUT females. Besides, MUT females did not have vagina opened upon 12.5 µg exogenous estrogen 3-day treatment while all WT females had vaginal opening (Fig. 5.5E).

Estrogen-treated MUT females and WT females had comparable increases of epithelial height in the uterus and the vagina (Figs. 5.5F ~ 5.5I). In the E2 treated females, both MUT and WT had significant increases in the relative weights of intact reproductive tracts, lumen fluid, uterine horns and cervix plus vagina compared with oil-treated females (Fig. 5.5J). However, weights were comparable between estrogen-treated WT and MUT females (Fig. 5.5J). All these data suggested the comparable responsiveness to estrogen in the MUT females, and estrogen signaling did not play a role in vaginal imperforation in MUT females.
WT  MUT

4 week

8 week

E

Percentage of mice with open vagina after 3-day treatment (%)

WT

MUT

WT E2

MUT E2

Uterus

Vagina

J

Relative weight (weight/body weight x 10^3)

- WT Oil
- WT E2
- Mut Oil
- Mut E2

NS

*
Figure 5.5. Imperforate vagina was not caused by deficiency in the estrogen signaling. A-D. Ovarian histology of WT and MUT females at 4 and 8 weeks old. A. WT at 4 weeks old; B. MUT at 4 weeks old; C. WT at 8 weeks old; D. MUT at 8 weeks old. E. Percentage of mice with vaginal opening after three-day treatment. Number above column is No.mice with vaginal opening/No. all treated mice. F & G. Histology of uterus after 3-day E2 treatment in WT (F) and MUT (G) females. Scale bar, 20 µm. H & I. Histology of vagina after 3-day E2 treatment in WT (H) and MUT (I) females. Scale bar, 20 µm. J. relative weight of reproductive tracts after 3-day E2 treatment. NS, no significant difference. * P<0.05. N=5-9.

The distal vagina atresia with persistent medial walls in the upper vagina

A close look at the distal part of vagina revealed the presence of distal vaginal atresia (absence of distal vagina) in MUT females (Figs. 5.6B & 5.6C) was not in the WT females (Fig. 5.6A). Estrogen treatment did not rescue the distal vagina atresia in MUT females (Fig. 5.6E) even though they became swelled and filled with fluid as the WT females (Fig. 5.6D). The distal vagina atresia was observed as early as PND3, the earliest age examined (Fig. 5.7), suggesting that it might be caused by defective embryonic development.

Figure 5.6. The distal vagina atresia. A. A representative picture of paraformaldehyde-perfused WT female reproductive tract at 8 weeks old. B-C. Representative pictures of paraformaldehyde-perfused MUT female reproductive tracts at 8 weeks old. ~30% MUT females were like B; ~70% MUT females were like C. D & E. Representative pictures of female reproductive tract upon 3-day estrogen treatment in WT (D) and MUT (E) females. The red arrows, the distal vagina atresia.
To further characterize the distal vagina atresia, vaginal histology was examined by H & E staining (Fig. 5.8). Unlike the connection of distal vagina with skin in WT females (Figs. 5.8A & 5.8C), the closed upper vagina and the absence of distal vagina were observed in MUT females on PND7 (Fig. 5.8B) and PND28 (Fig. 5.8D). Interestingly, there was a persistent medial wall in upper vagina of MUT females (Figs. 5.8F & 5.8H).

In summary, the distal vagina atresia caused vaginal imperforation in Lhfpl2 MUT females and the upper vagina in the MUT females were closed with medial walls.
Figure 5.8. The distal atresia with persistent medial wall in postnatal vagina in mutant females. A & B. Representative pictures of coronal sections of vagina at PND 28 (postnatal day) in WT (A) and in MUT female (B). C & D. Representative pictures of coronal sections of vagina at PND 7 in WT (C) and in MUT (D) females. E & F. Representative pictures of transverse sections of vagina at adult age in WT (E) and MUT (F) females. G & H. Representative pictures of transverse sections of vagina on PND 7 in WT (G) and MUT (H) females. A-D. Scale bars, 1 mm. E & F. Scale bar, 0.5 mm. G & H. Scale bar, 200 µm.
Male infertility due to posttesticular azoospermia

MUT males had infertility due to posttesticular azoospermia. Within 2-month cohabitation with 3 HET females, only 30% (5/17) MUT males yielded pups, which was significantly lower than 93.3% (14/15) WT males (Fig. 5.9A). The 30% MUT male yielding pups produced comparable number of pups at weaning (Fig. 5.9B), suggesting comparable fertility with WT males.

To investigate the infertility in MUT males, we first examined their plugging capability. The days between cohabitation and plugging 1st female (Fig. 5.9C), as well as days between plugging 1st and 2nd female (Fig. 5.9D), was comparable between WT and infertile MUT males, suggesting normal mating activity in the infertile MUT males. We then investigate testicular function. The comparable absolute and relative testis weights (Figs. 5.9E & 5.9F), comparable numbers of sperm at cauda epididymis both at 10 weeks and 6 months of age (Fig. 5.9G), and comparable testicular histology between WT and Infertile MUT males (Fig. 5.9I), suggested normal testicular function in the infertile MUT males. However, females plugged by infertile MUT males did not have any sperm in their reproductive tracts (Figs. 5.9J & 5.9K). These data suggested the blockage of sperm delivery into the female reproductive tract.
A. Percentage of males yielding pups (%)

B. No. pups at weaning

C. Days between cohabitation and 1st plug

D. Days between plugging 1st and 2nd females

E. Absolute testis weight (g)

F. Relative testis weight (g/kg body weight)

G. Sperm count (x10^6)

H. WT

I. MUT

J. Percentage of plugged females with sperm in reproductive tract (%)

K. No. sperm in flushing from uterus and vagina (x10^6)
Figure 5.9. Infertility due to posttesticular azoospermia in mutant males. A. 8-weeks old males with 3 heterozygous females for 2 months. Male producing pups as fertile; those did not as infertile. * P<0.05. B. No. pups at weaning. Pups were from HET females mated with either HET or fertile MUT males. N=10-16 litters. C. Days between cohabitation and 1st plug. D. Days between plugging 1st and 2nd plug. E. Absolute testis weight. F. Relative testis weight. G. No. sperm per cauda (x10^6). H & I. Histology of WT (H) and MUT (I) testis. J. Percentage of plugged females with sperm in reproductive tracts, including plugs, flushing of uterine horns and flushing of vagina on the morning with plugs. Ratio above column was number of females with sperm in reproductive tracts over number of females plugged by 4 WT or 6 infertile mutant males.* P<0.05. K. No. sperm in flushing of uterine horns and vagina on the morning with plugs. The numbers above each column was number of plugged females by 4 WT or 6 infertile mutant males.

Distal atresia led to obstructive azoospermia

Examination of WT/HET and MUT male reproductive tracts revealed a “knot” structure at the distal vas deferens (Figs. 5.10B ~ 5.10D). It seemed that the “knot” was the result of absence of distal vas deferens, which should insert into urethra as in WT males (Fig. 5.10A).

Figure 5.10. Different extents of distal vas deferens atresia in MUT males. A-D. Representative pictures of WT/HET (A) and MUT (B-D) male reproductive tract at the site of distal vas deferens. Red arrows, the obstructed “knots”. Black arrows, the abnormal seminal vesicles. MUT I, MUT II and MUT II were different extents of distal vas deferens atresia. MUT I, obstructed “knot” observable with background light; MUT II, bigger obstructed “knot” observable with naked eyes; MUT III, biggest obstructed “knot” with defect in structures of seminal vesicles. N, number of males.
To investigate the consequence of distal vas deferens atresia, blue dye was injected into one horn of vas deferens to visualize the sperm transport routes in WT and MUT males (Fig. 5.11). The dye traveled into urethra and bladder in WT males (Fig. 5.11A). However, in infertile MUT males, the dye was blocked at the distal vas deferens (1/3) (Fig. 5.11B), or traveled to seminal vesicle not urethra (2/3) (Fig. 5.11C). In the MUT fertile males, the dye could travel to urethra but via seminal vesicle (Fig. 5.11D). These data indicated the distal vas deferens atresia caused the obstructive azoospermia in infertile MUT males and altered sperm transport in fertile MUT males.

Figure 5.11. Visualization of sperm transport obstruction in MUT males by injection of blue dye via one horn of vas deferens. A. Representative picture of WT male after dye injection via one horn of vas deferens. Te, testis; Ep, epididymis; Bl, bladder; uVD, uninjected vas deferens; SV, seminal vesicle; iVD, Injected vas deferens; Ur, Urethra. The dye traveled into urethra and bladder. B-C. Representative pictures of MUT infertile males after dye injection via one horn of vas deferens. The dye could not get through vas deferens (B); the dye were kept in seminal vesicle and could not get into urethra (C). D. Representative pictures of MUT infertile males after dye injection via one horn of vas deferens. The dye traveled to seminal vesicle then into urethra.
Lhfpl2 expression in vagina and vas deferens

*In situ* hybridization detected *Lhfpl2* expression at vaginal epithelium on PND7 (Fig. 5.12A), PND21 (Fig. 5.12B) and cornified vagina (~PND30) (Fig. 12C) using antisense probe. A sense probe didn't detect any specific signal (Fig. 5.12D).

![Figure 5.12](image-url)

Figure 5.12. Lhfpl2 expression in the epithelium of vagina and vas deferens. A-D. *In situ* hybridization detection of Lhfpl2 in the PND vagina. A, PND7 vagina; B, PND21; C, Cornified vagina. As, anti-sense probe; Se, sense probe. Scale bar, 20 µm. E. Western blotting to detect Lhfpl2 in the uterus. kDa, kilodalton. F-H. Immunohistochemistry detection of Lhfpl2 in PND7 vagina and PND7 vas deferens. F, Lhfpl2 in vagina; G, Lhfpl2 in vas deferens; H, IgG, the negative control; Scale bar, 100 µm.
Western blotting detected a specific and strong band between 20-26 kDa (Fig. 5.12E) using a commercially available LHFPL2 antibody. Immunohistochemistry using this antibody detected LHFPL2 in epithelium of vagina (Fig. 5.12F) and vas deferens (Fig. 5.12G) on PND7 without strong signaling with the negative control (Fig. 5.12H). These data suggested the expression of LHFPL2 in the epithelium of postnatal vagina and vas deferens.

**Subcellular localization of Lhfpl2 in vitro**

To determine the subcellular localization of LHFPL2, FLAG expression plasmids were constructed to express WT Lhfpl2 or MUT LHFPL2 fused with FLAG at the C-terminal (WT+FLAG or MUT+FLAG). The transiently transfected Hela expressed WT (Fig. 5.13A) and MUT LHFPL2 (Fig. 5.13B) in the endoplasmic reticulum as suggested by their colocalization pictures (Figs. 5.13C & 5.13F) with an endoplasmic reticulum marker (KDEL. K, lysine; D, aspartic acid; E, glutamic acid; L, leucine) (Figs. 5.13B & 5.13E).

Interestingly, Hela cells transfected with WT+FLAG expressed LHFPL2 in the hair-like structure, which was highly possible to be filopodia (Fig. 5.13G). However, none of cells expressing MUT+FLAG had such a hair-like structure (Fig. 5.13H).
Figure 5.13. Immunocytochemistry of HeLa cells transiently transfected with WT and MUT LHFPL2 FLAG fusion proteins. A-F. Immunocytochemistry detection of WT (A) and MUT (D) FLAG fusion protein, endoplasmic reticulum (ER) marker (B & E) and their merged pictures in Hela cells transfected with either WT or MUT LHFPL2 LFAG expression. G & H. Immunocytochemistry of LHFPL2 FLAG fusion proteins in Hela cells transfected with WT (G) or MUT (H). Yellow arrows, the filopodia.
5.5 DISCUSSION

This study discovers the first gene essential for distal reproductive tract development. The point mutation in Lhfpl2 leads to distal atresia of the vagina in females and vas deferens in males, resulting in 100% female infertility and 70% male infertility. *In vivo* expression studies reveal WT LHFPL2 in the epithelium of postnatal vagina and vas deferens; and *in vitro* transfection in Hela cells shows that WT LHFPL2 is expressed in the endoplasmic reticulum and filopodia but MUT LHFPL2 is only expressed in the endoplasmic reticulum.

Distal vaginal atresia is the reason for vaginal imperforation, which prevents mating activity and thus causes female infertility (Figs. 5.4 & 5.6). Vaginal atresia occurs in 1 of 4000-5000 live female births in humans [183]. Vaginal imperforation in mice might be caused by estrogen signaling deficiency [101]. However, upon 3-day estrogen treatment, MUT vagina did not open despite comparable responsiveness (increased weights; increased heights of epithelium in the uterus and vagina) with that of WT mice, which had vaginal opening (Fig. 5.5). This suggested that estrogen signaling could not be the reason for vaginal imperforation in Lhfpl2 MUT mice. The distal vaginal atresia occurred before puberty (Fig. 5.7) and the absence of distal vagina would make female lose the tissue for making vaginal orifice at the distal part, which would lead to vaginal imperforation. Currently, there are four theories explaining the formation of the distal vagina: 1. the classical model (the “UGS+MD” origin) suggested that upper 2/3 vagina was from the MD and lower 1/3 vagina from UGS [184]; 2. The MD+WD theory suggested that WD contributed to vaginal epithelium and vaginal formation [184]; 3. The MD theory suggested that the vaginal epithelium derived from the MD only but the WD
played a role in the downward growth of the MD-derived vagina and the formation of hymen [185]; 4. The UGS origin thought the whole vaginal epithelium is from the UGS epithelium [184]. A recent study indicated that the distal vagina developed mainly from the MD during PND0-7 with UGS contributing to vaginal orifice and WD present only in the dorsolateral stroma wall [86]. The embryonic development of MD and WD is being investigated in the Lhfpl2 WT and MUT mice.

Distal vas deferens atresia is the reason for sperm transport blockage, causing male infertility in the Lhpfl2 MUT males. Distal vas deferens obstruction, called ejaculatory duct obstruction in humans, is responsible for 1-5% of male infertility [186]. The vas deferens atresia blocked sperm transport in the male reproductive tract, which was clearly suggested by the absence of sperm in the WT females mated with infertile MUT male. The infertile MUT had vas deferens and seminal vesicle blockage (Fig. 5.11) and thus prevented sperm transport as well as secretion of the seminal vesicle fluid into urethra. The seminal vesicle is essential for plug formation [187]. Interestingly, these infertile MUT males had comparable plugging capability with those from WT males (Figs 5C & 5D). This suggested that our ex vivo blue dye injection might not reflect the in vivo sperm transport. Indeed, valves that block retrograde transport were non-functional and the injected dye traveled into bladder while the sperm generally did not reach the bladder in vivo [188]. In fertile MUT males, the sperm traveled through seminal vesicle into urethra and this altered transport route did not alter male fertility (Fig. 5.9B). Taken together, distal vas deferens atresia blocked (~70%) and/or altered sperm transport (~30%) between vas deferens and urethra. The phenotypic variation (i.e. sperm
transport blockage or sperm transport alteration) may be due to genetic heterogeneity [189].

The female and male phenotypes converge on the idea of the developmental defect in the distal reproductive tract in the MUT mice. During early embryo development, the distal WD segment opens into the UGS part of the cloaca (the cloaca will separate into the anterior UGS and posterior rectum by a urorectal septum. Both MD and WD will attach to UGS). On D10.5-11.5, the ureteric bud sprouts from distal WD and invades into mesenchyme, which will form the ureter and the collecting renal system of kidney [190]. On D11.5, the WD and the ureter indirectly connect to UGS via the common nephric duct (CND). Around D13.5, CND undergoes apoptosis and disappears; the WD is separated from the ureter while both of them are fused with the UGS [190]. The MD elongation requires the WD. On D12.0-D12.5, the MD elongates to the UGS; by D13.5, the MD, possibly guided by the fused WD, contacts and fuses with UGS [191]. The fusion process of the WD and the MD with the UGS is essential for forming a complete tract. It is hypothesized that the defect in fusion of the distal reproductive tract with the UGS causes distal reproductive atresia. Because the WD is the first to be fused with the UGS and it is essential for MD elongation to the UGS, the separation of WD with the UGS may explain the distal reproductive tract atresia in both male and female MUT mice. It was suggested that the ureter and the WD contacted and fused with UGS together [87]. Interestingly, the Lhfpl2 MUT mice have normal kidney and urination (data not shown), suggesting normal ureter tract fusion with the UGS. It is possible that the WD in MUT mice might reach the UGS but fails to fuse with it.
Another finding in this study is that WT but not MUT LHFPL2 is expressed in filopodia (Fig. 5.13). Filopodia are hair-like, slender cytoplasmic projections and may play many cellular functions [192]. The preliminary data from our collaborator suggested LHFPL2 might play a role in epithelial-to-mesenchymal transition (EMT) (Personal communication with Dr. Shuang Huang at Georgia Regents University). Recent studies indicated that formation of filopodia might mediate the acquisition of motility and invasive capacities in mesenchymal cells during EMT [193]. LHFPL2 might be involved in assembling regulatory subunits of ion channels similar as LHFPL5 [84]. LHFP, LHFPL2, 3 and 4 might play similar roles in cells [81].

Taken together, this study reveals that LHFPL2 is essential for distal reproductive tract development. This finding provides novel insight into the genetic basis of distal reproductive tract development and potential genetic causes for infertility related to distal reproductive tract atresia. This will provide more knowledge to clinicians for better diagnosis, treatment and prevention of the birth defects in reproductive tracts.

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CHAPTER 6

CONCLUSION

Reproduction is essential not only for our continued existence but also for our happiness. About 1.2 billion people will enter their child-bearing years by 2020. It is estimated that 15% of couples may have the risk of infertility. Therefore, investigating risk factors for infertility is critical in addressing global reproductive problems.

Mammalian reproduction can be influenced by environmental exposure to toxicants and genetic factors, such as endocrine disruptors and gene mutation. Endocrine disruptors are exogenous chemicals that can interfere with the endocrine system and could potentially cause reproductive problems such as polycystic ovarian syndrome, endometriosis and fibroids in women; and testicular dysgenesis syndrome in men. Therefore it is important to investigate the impacts of estrogenic endocrine disruptors on reproduction systems. Mutations in some genes have been reported to cause developmental and/or functional defects in the female reproductive system to impair fertility. However, the molecular and cellular mechanisms of female reproductive development, especially distal reproductive tract development, are still not well understood.

The first part of my dissertation focuses on environmental exposure on female reproduction. Specifically, it investigated effects and mechanisms of zearalenone (ZEA) on female pubertal onset and early pregnancy in C57BL6J mice (Chapter 2 & 3), and postweaning exposure at different timings to (diethylstilbestrol) DES, on early
pregnancy in CD-1 mice (Chapter 4). Pre-mating, postmating and peripubertal exposure to the two estrogenic endocrine disrupting chemical (EEDCs) all affected early pregnancy events and their collective event, embryo implantation. However, different exposure periods affected the embryo implantation via different mechanisms (pre-mating exposure affected fertilization; postmating exposure delayed embryo transport, embryo development and embryo implantation; peripubertal exposure affected ovulation). Extended ZEA exposure over multiple generations had cumulative effects on female reproduction. Cession of exposure before mating (Chapters 2 & 4) as well as exposure termination in F3 generation at weaning (Chapter 3) alleviated the effects of EEDCs on embryo implantation. All findings in Chapters 2-4 suggest that timing of exposure is a key factor for effects and mechanisms of EEDCs on early pregnancy. The early pregnancy period is the most sensitive window for EEDCs affecting pregnancy establishment. It is highly recommended that women planning to get pregnant shall minimize exposure to EEDCs.

Another finding in the first part is advanced puberty by EEDCs. Girls in the United States are maturing earlier than those 30 years ago. EEDCs is part of reasons for advanced pubertal onset in human. This dissertation shows that peripubertal period is the most sensitive window for advancing pubertal onset so it is advised that girls around adolescence avoid foods potentially containing EEDCs, such as soy milk with genistein.

The second part of my discussion focuses on a genetic factor on female reproductive tract development, specifically, a point mutation in Lhfpl2 on distal reproductive tract development in C57BL6J mice (Chapter 5). Lhfpl2 is the first gene identified to be essential in both distal vagina and distal vas deferens development in
mice. Similar congenital defects in human have been concerns for women and men reproductive health, e.g. 1 in 4000-5000 live female births has vaginal atresia and about 1-5% of male infertility cases are caused by distal vas deferens defects. The Lhfpl2 mutant mouse can be a good model to study the physiology and pathology of infertility due to the distal reproductive tract developmental defects, as well as to investigate the cellular and molecular mechanisms involved in the distal reproductive tract development.

The first and second parts of this dissertation are connected in three aspects.

1) The vision of my research is to promote human reproductive health, which can be affected by both nurture and nature factors.

2) Both parts investigate development of the vaginal opening. Postweaning exposure to dietary ZEA and DES all accelerates the timing of vaginal openings. As an important indicator for pubertal onset and a critical reproductive event, the molecular mechanism of vaginal opening is largely unknown. I searched online to find a suitable mouse model for investigating the mechanism. Interestingly, a spontaneous point mutation in Lhfpl2 caused permanent closure of the vagina, suggesting that Lhfpl2 might play a role in vaginal opening. At first, I intended to use Lhfpl2 MUT mouse to study vaginal opening. However, it turned into a different story, which was the second part of my dissertation.

3) Both indicate the significance of early events either during pregnancy (Chapter 2-4) or during the reproductive organ development (Chapter 5) for successful pregnancy. Disrupted early pregnancy and defective distal reproductive tract
development could both impair fertility. This suggested the importance of each event during embryonic development and early pregnancy for successful pregnancy outcome.

In conclusion, my studies used mouse models to reveal the reproductive toxicity upon dietary EEDC exposure on female puberty onset and early pregnancy and the consequence of a point mutation in Lhfpl2 on the distal reproductive tract development. These results will increase our knowledge about effects and mechanisms of estrogenic endocrine disruptors on female reproduction; as well as a genetic basis of reproductive system development. I believe that this dissertation has provided us with more knowledge on developing better strategies for diagnosis, treatment and/or prevention of human reproductive problems. The research covered in this dissertation is a positive contribution to the fields of Reproductive Toxicology and Reproductive Biology.
REFERENCES


APPENDICES

Appendix A. Supplementary data in Chapter 2.

A

![Graph A: Body Weight vs. Postnatal Day]

B

![Graph B: Weekly Water Consumption per Mouse](Weekly Water Consumption per Mouse)

C

![Graph C: Weekly Food Consumption per Mouse](Weekly Food Consumption per Mouse)
Supplementary Figure S2.1. A. Postweaning body weight. Two-way repeated-measures ANOVA followed by Student-Neuman-Keuls (SNK) multiple comparison test. B. Weekly water consumption per mouse. C. Weekly food consumption per mouse. B & C. One-way ANOVA followed by SNK multiple comparison test. A & B. * P<0.05 when 50 ppb compared with control group.

Supplementary Figure S2.2. Days between mating setup and plug detection. Error bar, standard deviation.
Supplementary Figure S2.3. Gestation day (A) and litters size (B) in females mated by 0 ppm and 40 ppm treated males. N=2 in 0 ppm control group; N=5 in 40 ppm treated group. Error bar, standard deviation.
Appendix B. The perinatal parameters during lactation in Chapter 3.

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# P<0.05 4 ppm vs 20 ppm. One-way ANOVA on Ranks with Dunnett’s test. F0c and F1c pups were collected on PND7, PND14 and PND21 so there was no record in litter c.

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Appendix C. Age at vaginal opening in F1 and F2 females from different litters.

Error bar: standard deviation

* compared with 0 ppm in F2 a and b females
In other treatment, comparable among different litters.
Appendix D. Mating parameters in Chapter 3

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a: one mouse was sacrificed due to severe kidney problem. b: one mouse was sacrificed since it never delivered pups for 4 months; c: one mouse was dead due to parturition disorder; d: two were sacrificed due to abnormal anal and mammary morphology. e: only one mouse recorded. NR: Not recorded.
Appendix E. Litter size and gestation period in different litters in Chapter 3

![Graphs showing litter size and gestation period for different treatments and periods.](image)

**F1**
- **Litter size**
  - Error bar: standard deviation
- **Gestation period**

**F2**
- **Litter size**
  - Error bar: standard deviation
- **Gestation period**

**F3**
- **Litter size**
  - Error bar: standard deviation
- **Gestation period**

Error bar: standard deviation
Appendix F. Fertility in 40 ppm treated males

A. The body weight during 4-week treatment.

B. Absolute testis weight. N=3 in each group.

C. Sperm count per cauda. N=3 in each group.

D. Days from cohabitation to 1st plug. N=3 in each group.

Error bar: standard deviation.
Appendix G. Embryo implantation in females sired by 40 ppm treated males. A. Implantation rate. 4 out of 5 plugged females had implantation sites in 0 ppm control group; 5 out of 6 in 40 ppm treated group. B. The average number of implantation sites. Error bar: standard deviation. Only females with implantation sites were included.
Appendix H. Predicated secondary structure of mutant LHFPL2 (Red circle, the point mutation)