Published in final edited form as:
Reprod Domest Anim. 2012 August ; 47(0 4): 338–347. doi:10.1111/j.1439-0531.2012.02096.x.

Epigenetic mechanisms in the actions of endocrine-disrupting chemicals: Gonadal effects and role in female reproduction

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Abstract

There is a heightened interest and concern among scientists, clinicians, and regulatory agencies as well as the general public, regarding the effects of environmental endocrine-disrupting chemicals (EDCs). In this review, we identify the main epigenetic mechanisms and describe key ovarian processes that are vulnerable to the epigenetic actions of EDCs. We also provide an overview of the human epidemiological evidence documenting the detrimental effects of several common environmental EDCs on female reproduction. We then focus on experimental evidence demonstrating the epigenetic effects of these EDCs in the ovary and female reproductive system, with an emphasis on methoxychlor, an organochlorine pesticide. We conclude the review by describing several critical issues in studying epigenetic effects of EDCs in the ovary, including transgenerational epigenetic effects.

Keywords

DNA methylation; histone modifications; non-coding RNA; epigenetics; ovary; female fertility; endocrine disruptors

1. Introduction

Endocrine-disrupting chemicals (EDCs) are synthetic or natural compounds in the environment that can interfere with endocrine functions. The EDCs in the environment can be categorized as pesticides, plasticizers, industrial side products, pharmaceuticals, flame-retardants, phytoestrogens, or heavy metals such as cadmium (Diamanti-Kandarakis *et al.*, 2009; Iavicoli *et al.*, 2009; Kortenkamp, 2011).

2. Developmental exposures to EDCs and the Barker hypothesis

Exposure to EDCs during development is a bigger concern than exposure during adulthood. The health consequences of developmental exposure are mostly permanent or long-lasting while the individuals who are exposed during adulthood usually regain their normal

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Conflicts of interest None to report.

physiological functions after the withdrawal of chemicals. The long-lasting effects of EDCs can be viewed from the perspective of developmental origins of health and diseases (DoHaD) concept, often called the Barker hypothesis. This concept was first developed based on the observation that *in utero* exposure to poor nutrition can lead to various adult disorders, including metabolic and cardiovascular diseases (Barker, 1995). The same concept applies to EDC exposure during the critical window(s) of development, which can alter the programming of the female reproductive organs, including the ovary, and lead to infertility. Abnormal developmental programming occurs when the adverse environmental conditions alter the epigenetic mechanisms which modify gene expression patterns (Heijmans *et al.*, 2009).

3. Epigenetic mechanisms

Epigenetics is the study of mitotically and meiotically heritable changes in the gene function (i.e., gene expression) without changing the DNA sequence. Three common epigenetic mechanisms are DNA methylation, post-translational modifications of histone proteins (histone modifications), and non-coding RNA (ncRNA) (Jirtle and Skinner 2007), and they collectively make up the major components of the epigenome. Exposure to EDCs can alter these three epigenetic mechanisms in the ovary and various female reproductive organs, leading to gene expression changes (Zama and Uzumcu, 2009; Bredfeldt *et al.*, 2010; Luense *et al.*, 2011).

3.1. DNA methylation

Methylation of DNA occurs at cytosine residues in CpG dinucleotides and plays a role in genomic imprinting, suppression of retrotransposons, and X-chromosome inactivation, as well as gene expression regulation (Ehrlich, 2003). CpGs are underrepresented in the genome, except in segments of DNA called CpG islands (CGIs), where the CG content is markedly increased and is associated with the regulatory sequences of the genes (Turner, 2009). Methylation of DNA can interfere with transcription factor binding, leading to a reduction of gene expression. In the absence of DNA methylation, transcription factors can bind to the regulatory sequences leading to upregulation of gene expression (Yagi and Koshland, 1981; Ehrlich, 2003). The DNA methylation alterations in CpGs outside CGIs are now also considered to be important in gene regulation (Suzuki and Bird, 2008), necessitating more genome-wide methylation studies.

3. 2. Histone modifications

Histones are the protein components of the nucleosome, through which genomic DNA is packaged in eukaryotes. Post-translational modifications of histones at lysine, arginine, serine, and threonine amino acids play significant roles in both structural and functional states of the chromatin (Hirose *et al.*, 1985; Turner, 2009). These modifications include acetylation of lysine, methylation of lysine and arginine, and phosphorylation of serine and threonine. Whereas acetylation of lysine residues is associated with the relaxation of chromatin, allowing access to transcription factors and active transcription, deacetylation leads to compaction of the chromatin, preventing access of transcription factors and silencing the loci. Similarly, methylation or phosphorylation can be associated with gene

activation or gene silencing, depending on the position of the amino acid modified (see (Sharma *et al.*, 2005; Yoo and Jones, 2006; Turner, 2009) for more details).

3.3. Non-coding RNA

The importance of ncRNAs in biological processes has been recently recognized (Chang *et al.*, 2006). Non-coding RNAs are transcripts without a clear open reading frame; therefore they do not code proteins, but regulate the expression of other genes in *cis* and *trans* manners. The ncRNAs vary in size from microRNAs, or miRNAs (15-21 nucleotides, nt), and small RNAs (100-200 nt) to large RNAs (>200 nt) and are involved in vital functions such as X-chromosome inactivation, genomic imprinting, transposon and virus silencing, and developmental patterning and differentiation (reviewed in (Costa, 2008; Brosnan and Voinnet, 2009)).

The above-mentioned three epigenetic mechanisms are known to influence each other's roles in cellular development, differentiation and function (Li, 2002; Costa, 2008; Namihira *et al.*, 2008). During establishment of the epigenetic marks, histone modifications lead to DNA methylation, whereas in maintenance or inheritance of the marks, DNA methylation plays a primary role. In addition, ncRNAs, histone modifications and DNA methylation work cooperatively in various processes, including X-chromosome inactivation (Avner and Heard, 2001) and genomic imprinting (Bartolomei, 2009; Brosnan and Voinnet, 2009).

4. Major epigenetic modifications in the mammalian life cycle

The epigenome, especially DNA methylation patterns, is stably maintained in somatic cells. However, major epigenetic programming occurs during two stages of development: (1) in the preimplantation embryo, and (2) in primordial germ cells (PGCs) in the bipotential gonad (Reik *et al.*, 2001). In the preimplantation embryo, a genome-wide epigenetic reprogramming occurs through which the epigenetic marks, except on imprinted genes, are eliminated (Santos and Dean, 2004); the zygote gains its totipotency; and embryonic cell lineage-specific methylation occurs, including in the PGCs. After the PGCs migrate from the extragonadal sites to the genital ridge, the DNA methylation patterns of the imprinted genes in the PGCs are also erased in both sexes (Figure 1) (Hajkova *et al.*, 2002), and reestablished in a sex-specific manner. In males, the remethylation starts on embryonic day (E) 14 and is mostly completed by E16-17 (Ueda *et al.*, 2000). In contrast, the female germ cell remethylation is initiated during postnatal day (PND) 1 through PND5 and continues throughout oocyte growth until the pre-antral follicle stage in mice (Obata and Kono, 2002).

The two stages that are described above are likely to be the most vulnerable to the epigenetic effects of EDCs. If EDCs interfere with DNA methylation during the epigenetic reprogramming in the preimplantation embryo, all three embryonic germ layers can be affected, and therefore all tissues of the organism (Dolinoy *et al.*, 2007). If EDCs alter epigenetic reprogramming (i.e., imprinted genes) of the germline, subsequent generations can be affected (Stouder and Paoloni-Giacobino, 2011). Although the effects that occur during the erasure can influence both sexes, the effect during remethylation can be sex-specific.

5. Ovarian development and function

In addition to effects that can occur during the two major epigenetic modifications described above, EDCs can have ovary-specific effects if the exposure takes place during the critical developmental processes in the ovary. In addition, the cyclic processes that occur in adult ovary throughout the female's reproductive life which are closely regulated by local and endocrine factors, including estradiol (E_2), progesterone (P_4), and androgens, can be affected by EDCs that display estrogenic, antiestrogenic, and antiandrogenic actions (Zama and Uzumcu, 2010). The specific ovarian processes are discussed using a timeline that applies to rodent species (Figure 1). We focused on processes that are likely to be affected by EDCs, rather than describing the normal ovarian development and function in detail, which can be found in recent reviews (Edson *et al.*, 2009; Gougeon, 2010).

5.1. Oocyte nest breakdown and primordial follicle formation

Oocyte nest breakdown that leads to the formation of primordial follicles is mediated by oocyte apoptosis starting at E16.5 in mice (Pepling and Spradling, 2001). By PND3-4, the primordial follicles are formed. Each primordial follicle consists of an oocyte at the center surrounded by a single layer of squamous pre-granulosa cells. *In vitro* and *in vivo* experiments have shown that the steroids P_4 and E_2 inhibit primordial follicle formation by inhibiting oocyte nest breakdown, leading to multiovular follicles (MOF; presence of more than one oocyte within a follicle) (Kezele and Skinner, 2003; Chen *et al.*, 2007).

5.2. Primordial-to-primary follicle transition

The transition from primordial to primary follicles, which is also known as "initial recruitment" (McGee and Hsueh, 2000), ensures a steady supply of follicles for further growth and ovulation. Once the primordial follicle is recruited, the oocyte undergoes growth and maturation to gain competence for fertilization, and the pre-GCs differentiate into GCs by growing in size and becoming cuboidal and they proliferate. Most of the growing follicles (99.9%) are destined to become atretic and the rest will ovulate after completing the following follicular stages: primary, secondary; pre-, early-, mid-, and late-antral; and pre-ovulatory (see (Armenti *et al.*, 2008) for a detailed description of follicular stages).

Both E_2 and P_4 also have inhibitory roles in the initial recruitment, and in the ovaries that are exposed to E_2 and P_4 *in vitro*, the transition is inhibited (Kezele and Skinner, 2003). In addition, when newborn rats are injected with estradiol benzoate, their ovaries have more primordial follicles and fewer growing follicles (Ikeda *et al.*, 2001). In contrast, cytochrome P450 aromatase knockout (ArKO) mice, which lack endogenous E_2, have fewer primordial and primary follicles (Britt *et al.*, 2004). These results show that whereas E_2 is needed for early events in folliculogenesis, excessive E_2 inhibits these events. In contrast, androgens stimulate the primordial-to-primary follicle transition but inhibit follicle growth beyond the primary stage (Yang and Fortune, 2006; Yang *et al.* 2009).

5.3. Pre-antral and early-antral follicular growth

Follicular growth during primary and secondary stages is regulated by local factors. Starting with the secondary follicle stage, follicles begin to gain receptors for gonadotropins, follicle-

stimulating hormone (FSH) and luteinizing hormone (LH) (Sokka and Huhtaniemi, 1990). Around this time, follicles also gain a second somatic cell type, thecal cells. The follicular progression becomes dependent on gonadotropins during the pre-antral stage (Kumar *et al.*, 1997). Granulosa and thecal cells cooperate in the production of E_2 under the regulation of FSH and LH, respectively (Magoffin, 2005). Increasing E_2 concentrations cause negative feedback at the hypothalamus and pituitary levels, thereby reducing the secretion of gonadotropins.

Estradiol plays stimulatory a role in later stages of folliculogenesis. This role is the clearest in transgenic mouse strains, in which ERβ is globally deleted via homologous recombination (Carpenter and Korach, 2006) or ERα is deleted in thecal cells (Lee *et al.*, 2009), the primary cell type that expresses ERα. Both mouse strains show inhibition in follicular maturation and ovulation. In contrast, while androgens are stimulatory during the initial stages of folliculogenesis, excess androgen is inhibitory and its receptor is actively down-regulated at the later stages of folliculogenesis (Tetsuka *et al.*, 1995).

5.4. Follicular selection, maturation, and ovulation

To complete the full course of folliculogenesis and ovulation, one follicle within the recruited cohort is selected in monoovulators whereas multiple follicles are selected in polyovulators. The selection process is under complex regulation, which is primarily determined by the elevated gonadotropin responsiveness of the selected follicle(s) despite decreasing gonadotropin concentrations. A whole host of local growth factors, such as insulin-like growth factors (IGFs), play significant roles throughout folliculogenesis including the final maturation (reviewed in (Edson *et al.*, 2009; Sirotkin, 2011). At the preovulatory stage, elevated E_2 exerts positive feedback at the hypothalamus, leading to the preovulatory LH surge. During this stage, GCs also gain responsiveness to LH, which initiates numerous gene expression alterations that prepare the follicle for ovulation (Richards and Pangas, 2010). The oocyte is released during the ovulation along with surrounding cumulus cells – a special subpopulation of GCs that are immediately adjacent to oocyte. The remaining cells of the follicle form the corpus luteum (CL), which is the source of the P_4 that prepares the reproductive tract for the pregnancy. The formation, function, and demise of the CL (in the case of continuous reproductive cycle) or maintenance of CL (in case of pregnancy) are closely regulated events (Stocco *et al.*, 2007), which are affected by EDCs.

Although the ovary plays a central role in female reproduction, its function is closely regulated by the hypothalamus and pituitary. The EDCs can act at the levels of the hypothalamus and pituitary as well as the ovary. However, in this review, we focus on the direct effects of select EDCs in the ovary.

6. Epidemiological evidence linking EDCs to female reproductive disorders

Epidemiological studies strongly suggest that EDCs affect both male and female reproduction. One of the earliest reports related to the decline in sperm parameters, such as reduced sperm count and seminal volume in men during the previous 50 years pointed out the harmful effects of EDCs (Carlsen *et al.*, 1992). This time period closely corresponds to

the time of the widespread use of synthetic EDCs. Although this 1992 report is controversial (Fisch *et al.*, 1996; Younglai *et al.*, 1998), there is agreement regarding a geographical region-dependent decline in sperm parameters (Swan *et al.*, 2003), which supports an environmental etiology. For example, several studies have shown that there are clear differences between male fertility parameters of men in Denmark and Finland. These studies show that semen quality parameters are lower while the incidence of testicular cancer, hypospadias, and cryptorchidism is higher in males living in Denmark, including the Denmark-born offspring of immigrants, as compared to males from Finland (Boisen *et al.*, 2004; Jorgensen *et al.*, 2006; Myrup *et al.*, 2008). The human data have been supported by findings from laboratory animals (Gray *et al.*, 2001) and wildlife species (Guillette and Gunderson, 2001). The pathologies in males are collectively named as testicular dysgenesis syndrome (TDS), which has been extensively reviewed (Toppari *et al.*, 2010).

Females also have EDC-originated fertility problems, leading to ovarian dysgenesis syndrome (ODS) (Buck Louis *et al.*, 2011). Although it is not as clearly defined or well accepted as TDS, the use of the term ODS is gaining support (Fowler *et al.*, 2012). The impaired fecundity rate in the U.S. increased from 11 to 15% between 1982 and 2002 (Guzick and Swan, 2006). Although various other confounding factors such as lifestyle changes can contribute to this decline, the role of EDCs are also strongly considered due to supporting studies. The incidence of female reproductive disorders, such as ovarian cancer, is on the rise in a region-dependent manner (reviewed in (Buck Louis *et al.*, 2011)). Furthermore, numerous studies suggest a strong link between exposure to EDCs either during the adult life or during development and increased female reproductive disorders.

6.1. Diethylstilbestrol (DES)

The most convincing human evidence that estrogenic EDC exposure during development can permanently affect female reproduction comes from reports of the effects of DES, a nonsteroidal synthetic estrogen. From the 1940s to the 1970s, DES was prescribed at doses of 5–150 mg⁄day to prevent miscarriages (Smith and Gabbe 1999). Numerous abnormalities in the reproductive, cardiovascular, and immune systems have since been reported in both male and female offspring of women treated with DES, and similar effects have been demonstrated in animal models (see (Newbold, 2004) for specific examples). Some of these effects, such as irregular cylicity or ovarian cancer, are being observed in the granddaughters of DES-treated women as well (Blatt *et al.*, 2003; Titus-Ernstoff *et al.*, 2006). In light of this multigenerational aspect, whether epigenetic mechanisms are involved is a significant question (Newbold *et al.*, 1998).

6.2. Bisphenol A (BPA)

Bisphenol A is a plasticizer used in the manufacture of polycarbonate plastics and epoxy resins, and its total worldwide production exceeds 6 million tons per year. Exposure to BPA may occur through various materials that humans use daily, such as food and drink cans, baby bottles, and carbonless paper (reviewed in (Zama and Uzumcu, 2010)). As a result, 95% of adults who were tested had detectable levels of BPA in their urine (Calafat *et al.*, 2008). Urine BPA levels of women undergoing infertility treatment are negatively correlated

with the number and quality of eggs retrieved, and with serum E_2 levels (Mok-Lin *et al.* 2010; Fujimoto *et al.*, 2011).

6.3. Genistein

Genistein is a well-studied isoflavonoid phytoestrogen derived from soy. Phytoestrogens were originally shown to have endocrine-disrupting potential in domestic animal species: Newborn lambs born to ewes fed clover, a rich source for phytoestrogens, had reproductive abnormalities (reviewed in (Jefferson, 2010)). Soy products are popular because they are lactose-free substitutes for dairy products and are considered to be protective against breast cancer risks (Peeters *et al.*, 2003). However, a major cause for concern is that babies who are fed soy formula consume on average of 6-9 mg/kg body weight, which is many-fold higher than levels approved by the FDA for adults for cardio-protective purposes (Setchell *et al.*, 1998; Johns *et al.*, 2003). It is alarming to know that early life exposure to soy formula is associated with a greater risk of uterine fibroids in adulthood (D'Aloisio *et al.*, 2010).

6.4. Phthalates

Phthalates are used as plasticizers in polyvinyl chlorides, personal care products, some flooring, car products, medical devices, and insect repellent. Although phthalates have been mostly studied for their effects on male reproduction, there is accumulating evidence that they adversely affect female reproduction as well. Di-(2-ethylhexyl) phthalate and its metabolite mono-(2-ethylhexyl) phthalate (MEHP) are the most commonly studied phthalates (reviewed in (Craig *et al.*, 2011)). There is increasing evidence that exposure to phthalates may affect reproductive health in humans (Jurewicz and Hanke, 2011). The levels of phthalates, especially MEHP, in the urine of women undergoing infertility treatments correlate with significantly higher risk of implantation failure (Ehrlich *et al.*, 2010).

6.5. Methoxychlor (MXC)

Methoxychlor (MXC) is a well-studied organochlorine pesticide used as a replacement for dichlorodiphenyltrichloroethane (DDT). It is an estrogenic compound that demonstrates low-affinity binding for estrogen receptors (Cummings, 1997). The major MXC metabolites, 2,2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) and mono-OH MXC, can function as estrogenic, anti-estrogenic, or anti-androgenic compounds (Gaido *et al.*, 2000), and therefore it is used as a model compound (Uzumcu and Zachow, 2007). Epidemiological studies have shown that there is a strong association between developmental exposure to organochlorine pesticides and subsequent female fertility problems (reviewed in (Zama and Uzumcu 2010)). In addition, in a study that examined pesticide-use data and the breast cancer incidence rates in Hispanic females in California, the amount of MXC used was positively associated with increased breast cancer incidence (Mills and Yang, 2006).

7. Experimental evidence from *in vivo* studies regarding the effects of EDCs in the ovary

Characteristic sets of defects in the ovary are the hallmark of EDC exposure in rodent models. For example, mice injected as little as a single dose of 10 μg/kg DES on E15 and

examined at 7 months of age had numerous atretic follicles, and no corpora lutea (an indicator of ovulation) (Wordinger and Highman, 1984). Other studies in rodents of varying doses of DES administered either *in utero* (E9-16) or neonatally (PND1-5) demonstrated similar defects by adulthood (Haney *et al.*, 1984; Tenenbaum and Forsberg, 1985). Estrous cyclicity was completely disrupted and high levels of testosterone were found. A vacuolated interstitial tissue with lipid droplet inclusions and hemorrhagic cysts were also observed. A recent study proposes that while increased lipid droplet are caused by impaired steroidogenesis due to suppressed LH levels, the hemorrhagic follicles are results of direct effects of DES in the ovary (Kakuta *et al.*, 2012). Furthermore, there was a dose-dependent reduction in the number of litters as well as the number of oocytes ovulated after stimulation with exogenous gonadotropins (McLachlan *et al.*, 1982) with such oocytes when used in IVF showing lower levels of fertilizability, suggesting reduced oocyte quality (Wordinger and Derrenbacker, 1989; Iguchi *et al.*, 1991). Most striking of all, an excessive number of MOFs is found in adult ovaries; such a finding is considered to be an indicator of reduced reproductive lifespan (Iguchi and Takasugi, 1986; Iguchi *et al.*, 1990). The above-mentioned phenotype is mostly recapitulated by exposure to other EDCs, including BPA (Howdeshell *et al.*, 1999; Markey *et al.*, 2005) and genistein (Jefferson *et al.*, 2005; Chen *et al.*, 2007). Phthalates also can decrease E_2 production, prolong the estrous cycle, and cause anovulation (Davis *et al.*, 1994; Lovekamp and Davis, 2001); however, effects of phthalates on the ovarian follicular composition are not known.

Such estrogenic actions of these EDCs are mediated via the ER signaling pathway (Kuiper *et al.*, 1998; Drummond and Fuller, 2010; Patisaul and Adewale, 2009). Recent studies have shown that MOFs induced by neonatal exposure to 3 μg/kg DES is mediated by ERβ and not ERα (Kirigaya *et al.*, 2009). DES exposure was shown to reduce oocyte apoptosis (potentially suppressing oocyte nest breakdown) via ERβ signaling mechanisms. Furthermore, it was hypothesized that alterations in the germ cell–to–somatic cell ratio may affect the invasion of pregranulosa cells and basement membrane remodeling during primordial follicle formation (Kim *et al.*, 2009). In contrast to ERβ signaling mechanisms involved in mediating ovarian effects, Couse and colleagues reported that ERα is essential for the mediation of DES effects in the uterus: αERKO female mice exhibited a complete resistance to the effects of DES while βERKO mice did not (Couse and Korach, 2004). Interestingly, *in utero* treatment of βERKO females with low doses of BPA did not enhance the oocyte meiotic defects usually caused by BPA, suggesting that the compound could act via the ERβ signaling pathway (Susiarjo *et al.*, 2007). Furthermore, microarray-based gene expression analysis in the fetal ovary (E12-14.5) after low-dose BPA exposure revealed that mitotic cell-cycle genes are significantly down-regulated, potentially leading to accelerated meiotic entry of the oogonia and maybe eventually leading to a reduced-size oocyte pool after birth (Lawson *et al.*, 2011). Another recent study in rats showed that BPA reduces the pool of primordial follicles by stimulating initial recruitment after a PND1-7 exposure (Rodriguez *et al.*, 2010). Interestingly, Arase and coworkers reported an increase in estrogen production potentially through increase in *Cyp19a1* and *Cyp11a1* expression, in the mouse urogenital sinus specifically in the mesenchymal component after BPA exposure between E9 and E15 (Arase *et al.*, 2011). Thus BPA has a double-edged effect on mitosis and meiosis as well as pre- and postnatal steroidogenesis.

8. Epigenetic mechanisms associated with the female reproductive system

Although there are now well-documented studies of the physiological and morphological effects of EDCs on the ovary, the early research providing evidence of an epigenetic component of EDC actions was predominantly on the uterus. For example, it is well known that DES caused T-shaped uteri and clear cell adenocarcinoma of the uterus, cervix, and vagina in women whose mothers were exposed to DES during pregnancy (Herbst *et al.*, 1971). In the numerous animal studies validating these human reports, developmentally DES-treated mice manifest malformations of the uterus, squamous metaplasia of the luminar and glandular epithelium, endometrial hyperplasia and leiomyomas, and oviductal proliferative lesions (McLachlan *et al.*, 1980; Kitajewski and Sassoon, 2000). Ovariectomized animals when supplemented with E_2 are able to respond by a transient increase in gene expression and concomitant uterine proliferation and growth. When such a stimulus was removed, the uterus returned to its unstimulated state. However, when DES or E_2 is administered during neonatal development, expression of immediate early genes such as *c-fos*, *c-jun*, and *c-myc* as well as *lactoferrin* and *EGF* are upregulated even into adulthood (Nelson *et al.*, 1994; Falck and Forsberg, 1996). This was associated with hypomethylation of the promoter region of the *lactoferrin* gene in the adult uterus (Li *et al.*, 1997). However, if animals were exposed for the same interval during adulthood, no such methylation or expression defects were observed, indicating the importance of developmental exposure. Subsequently, it was also found that exon 4 of the *c-fos* gene was extensively hypomethylated while the promoter region and intron 1 were unaffected, thereby potentially allowing for the upregulation of *c-fos* expression (Li *et al.*, 2003). Furthermore, recent work by Block and coworkers has shown that DES disrupts the regionalization of expression of *Hoxa-9* and *Hoxa-10* and the homeotic anterior transformations associated with hypermethylation in the promoter and intron 1 regions of *Hoxa-10* gene (Block *et al.*, 2000). Additionally, ERα induction is necessary for activation of estrogen-responsive gene expression in the uterus, including that of the *lactoferrin* and *c-fos* genes (Nelson *et al.*, 1994; Falck and Forsberg, 1996). Since many of the above genes are downstream of ER signaling, which is involved in direct actions of EDCs, it is imperative to thoroughly examine the potential role of epigenetic mechanisms in the regulation of ER expression after EDC exposure. In addition, PI3K/Akt signaling downstream of membrane-associated ER signaling caused reduction in trimethylation of the histone H3K27 in response to E_2 and DES exposures. More interestingly, activation of this non-genomic signaling caused reprogramming of the uterine gene expression profile (Bredfeldt *et al.*, 2010). Another example is that of the investigation by Tang and colleagues (Tang *et al.*, 2008), wherein it was demonstrated that neonatal DES/genistein exposure reduced DNA methylation and increased gene expression of *nucleosomal binding protein 1* in adult uteri. These studies highlighted the age-dependent aspect of epigenetic reprogramming and also its interaction with steroid hormones.

9. Epigenetic effects of methoxychlor in the ovary

Early studies that exposed females to MXC for 6-10 weeks during both *in utero* and early postnatal development periods, show that exposed females have acceleration of the vaginal opening (sign of puberty), acceleration of the onset of the first estrus, irregular cycles with

persistent vaginal estrus, reduced pregnancy rate and litter size despite apparent mating, and early reproductive senescence (Gray *et al.*, 1989; Chapin *et al.*, 1997; You *et al.*, 2002). Serum E_2 and P_4 levels were altered with increased FSH levels (Chapin *et al.*, 1997). The effects on the ovary were dramatic, with both folliculogenesis and ovulation being inhibited. In our more recent study, female rats were treated for 12 days during fetal and neonatal development (E19-PND7) with 100 mg/kg/day MXC, higher than the dose normally encountered by humans or animals on a daily basis, but similar to doses used in the above studies. The exposed females displayed similar abnormalities in reproductive parameters and in ovarian morphology by adulthood despite the fact that the treatment period was much shorter (Armenti *et al.*, 2008). A close examination of follicle composition showed that developmental MXC treatment did not affect the total number of follicles or follicles at primary and secondary stages in adult females. However, the number of pre-antral and early antral follicles was increased and the number of CL was reduced, with numerous cystic follicles. Immunohistochemical staining and quantification of expression patterns of important regulators of ovarian functions revealed that while LHR, CYP11A1, and CYP19A1 levels were reduced, levels of anti-Mullerian hormone and androgen receptor were increased, and levels of StAR and ERα were unchanged (Armenti *et al.*, 2008). Especially noteworthy was that ERβ level was unchanged in primary and secondary follicles, yet decreased dramatically in peri-antral stage follicles, which are very responsive to gonadotropins (unpublished observations). These data suggest that hormone-responsive follicles are most affected by EDC exposure.

Epigenetic analyses using bisulfite-sequencing PCR and methylation-specific PCR showed that MXC caused hypermethylation in multiple CpGs in two CGIs in ERβ promoter sequences (Zama and Uzumcu, 2009). Further analysis has shown that the DNA methylation levels in the promoter regions of these genes were unchanged in neonatal ovaries (PND7) immediately after the exposure (Zama and Uzumcu, unpublished). These data demonstrate the age-dependence and hormone responsiveness of the epigenetic changes, which has also been shown in other tissues (e.g., uterus) with other compounds (e.g., DES, genistein) (Tang *et al.*, 2008). The global DNA methylation analysis using methylation-sensitive arbitrarily primed PCR (AP-PCR) showed that there were multiple loci that were hypermethylated in MXC-treated ovaries (Zama and Uzumcu, 2009). The majority of candidates were those encoding transcription factors or ribosomal proteins. Our most recent studies have involved genome-wide methylation analyses. Since assays such as AP-PCR depend on the availability of restriction enzyme sites, they may not capture all possible methylation events. Furthermore, since DNA methylation is dynamic and reversible (Bhutani *et al.*, 2011), we hypothesized that genome-wide methylation analyses at both ages would be highly informative. Therefore, we examined ovaries: (1) immediately after exposure, at PND7; and (2) after animals reached adulthood, at PND60. The methylated DNA immunoprecipitation protocol (MeDIP), which employs an antibody against the 5-methyl cytosine moiety, was conducted followed by hybridization to microarrays that included probes spanning the promoter regions as well as the CpG islands of all RefSeq genes in the rat genome. This was in an effort to examine not only individual gene methylation patterns but also potential gene networks that were exhibiting altered methylation patterns in response to EDC exposure, immediately after exposure as well as in adulthood, long after the exposure ends. We have

now found that multiple loci belonging to critical ovarian signaling pathways (e.g., PTEN signaling, IGF signaling) were hypermethylated and their gene expression was suppressed. These pathways are essential for early folliculogenesis as well as follicular maturation and ovulation.

10. Conclusions and future studies

Several aspects should be considered when the effects of EDCs in the ovary are evaluated. The ovary is the major source of the hormones that are mimicked by EDCs. The same hormones that the ovary produces also regulate the ovarian functions. Thus, the effects of EDCs in the ovary can be twofold. In addition, the ovary of a reproductive-aged adult consists of follicles in various developmental stages as well as corpora lutea. Therefore, both developing and adult ovaries require involvement of numerous factors that are spatio-temporally regulated. It is likely that these factors are regulated by various epigenetic mechanisms, including DNA methylation (Vanselow *et al.*, 2010), histone modifications (LaVoie, 2005), and ncRNAs (Carletti *et al.*, 2010; Torley *et al.*, 2011; da Silveira *et al.*, 2012).

One of the most pressing issues in the field of epigenetics and EDCs is the transgenerational (TG) epigenetic effects. All epigenetic actions in the ovary have the potential to affect the subsequent generation(s) via the female germ cells. The effects that are transmitted through epigenetic mechanisms to an unexposed generation are described as epigenetic TG inheritance (Youngson and Whitelaw, 2008). There have been reports in males that EDCs can cause TG epigenetic effects in experimental animals (Anway *et al.*, 2005; Guerrero-Bosagna *et al.*, 2010). Since then, there have been additional reports concerning the effects of EDCs in males (Salian *et al.*, 2009; Stouder and Paoloni-Giacobino, 2011). Although the described effects are also transmitted by the female germline, the impacts are less obvious. The potential reasons for the less prominent effects are not known but can be related to the timing of the treatment and the nature of the chemicals. Importantly, there are some reports in humans regarding TG effects of the environment (for examples, see Section 6.1 and (Titus-Ernstoff *et al.*, 2006).

Additional key questions or challenges related to studying the epigenetic effects of EDCs in the ovary include the following:

- Follicular stage–specific effects in the ovary: Tissues in the body are composed of different cell types differentiations of which are believed to be primarily due to epigenetic modifications specific to a given cell type. Therefore, the epigenetic analysis in a whole tissue consisting of various cell types is considered to be problematic. An additional complexity in the postnatal ovary is the presence of the follicles at different stages of their development. When epigenetic analysis in the ovary is being considered, not only should different cell types be examined individually but also these cell types should be separated based on the follicle stage from which they are obtained.
- EDC mixture effects: Most studies examining the effects of EDCs in the ovary as well as in other organs are conducted with single chemicals. However, individuals

are normally exposed to mixture of these chemicals in daily life. Therefore, studies that are examining the effects of mixtures with various approaches have been intensifying. These studies in other organs have shown that when chemicals are combined their impact increases synergistically—much more dramatically than additive effects of individual compounds (Jacobsen *et al.*, 2010; Rider *et al.*, 2010). The mixture studies further show the complexity of the potential harmful effects of the EDCs as mixtures (Kortenkamp *et al.*, 2007).

- Epigenome-wide effects: The genome is relatively more resistant to the effects of environmental factors that individuals are likely to encounter daily, such as EDCs. In contrast, the epigenome can be vulnerable to such effects. In addition, as compared to the primary DNA sequence, the epigenome is much complex and dynamic. Furthermore, the importance of epigenome in the developmental origins of health and diseases is widely accepted. Therefore, a comprehensive understanding of the effects the EDCs requires studying not only the methylome but also whole epigenome (including histone modifications and ncRNAs, especially miRNA). Such a broader and more precise understanding of epigenetic effects of EDCs will enable the development of effective prevention and therapy.

Acknowledgments

The authors thank Dr. Kathy Manger for her assistance in the preparation of this manuscript. The research results from our laboratory described in this review supported in part by NIEHS grants ES013854 and ES017059 and the NIEHS-sponsored UMDNJ Center for Environmental Exposures and Disease grant NIEHS ES005022. Dr. Elif Oruc, visiting scholar from Cukurova University, Turkey, is supported in part by TUBITAK-BIDEB International Postdoctoral Research Scholarship Program.

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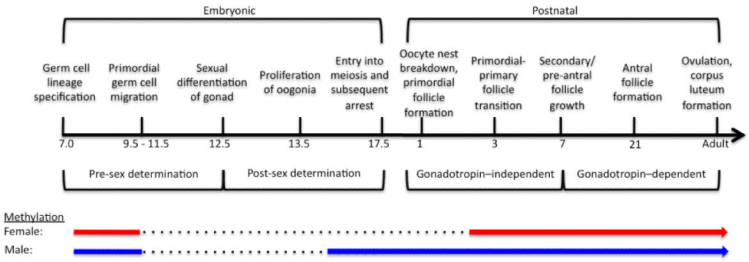

Figure 1. The stages of gonadal development in females (see the text for details)

One of the processes that are particularly vulnerable to the epigenetic effects of endocrine-disrupting chemicals is epigenetic reprogramming (i.e., DNA demethylation and remethylation) in primordial germ cells (PGCs). During the genome-wide epigenetic programming in the preimplantation embryo, the DNA methylation patterns in both male and female genomes are eliminated (not shown), except in imprinted genes (color solid lines) whose methylation patterns are maintained, including in PGCs until their migration to the gonad. After the migration, the patterns are erased (dotted lines) and reestablished (color solid lines) in a sex-specific manner. The timeline shown is primarily based on the events in mice. Adapted from (Zama and Uzumcu, 2010).