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Molecular mechanisms of induction of persistent changes by estrogenic chemicals on female reproductive tracts and external genitalia☆

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ABSTRACT

The effects of environmental endocrine-disrupting chemicals (EDCs) are a great and growing concern for human and animal development and life. The reproductive organs are considered as a primary target of EDCs, yet the effects on reproductive organs can extend to other body systems. Perinatal diethylstilbestrol (DES)-exposed mice exhibit various reproductive organ abnormalities. The perinatal DES-exposure model has allowed insight into our understanding of the mechanisms of persistent reproductive organ abnormalities elicited by exposure to estrogens and/or estrogenic EDCs. The persistent changes in the vagina of neonatally DES-exposed mice result from sustained expression of growth factors by ligand-independent transcriptional activation of the estrogen receptor. Developmental regulatory genes, such as Wnt and Hox genes, are also targets of DES during fetal stages and altered gene expression can induce malformations of the reproductive organs. In this review, we focus on the development of female reproductive tracts and external genitalia, and discuss the recent progress in understanding the disruptive effects of estrogens and EDCs on these organs.

1. Introduction

The mammalian female reproductive tract provides the sites for gamete fertilization, implantation and subsequent development of the embryo and delivery of the fetus. The female reproductive tract is derived from the Müllerian duct and urogenital sinus. During normal mouse development, the Müllerian duct forms as a small invagination of the surface epithelium of the mesonephros, located adjacent to the cranial end of the Wolffian duct. The Müllerian duct extends caudally towards the urogenital sinus. Once the Müllerian duct forms, it differentiates into oviduct, uterus, cervix and the upper part of the vagina, whereas the urogenital sinus gives rise to the lower part of the vagina and urinary tract. In males, the Müllerian duct regresses under the action of anti-Müllerian hormone, which is secreted from Sertoli cells in the testis. Androgens are also secreted from Leydig cells in the testis, and consequently the Wolffian duct is maintained, resulting in its differentiation into epididymis, vas deferens and seminal vesicle. Thus, reproductive organ development depends on gonadal development and its secretion of hormones at the correct times and amounts during development [1–3].

Recent studies have shown various forms of sex determination in vertebrates. Sex is genetically determined in the medaka (fish) by the presence or absence of the Y chromosome specific gene DMY and estrogens facilitate and maintain sex differentiation of the ovarian cells and the following female pathway [4–6]. Administration of exogenous estrogens shortly after fertilization causes male to female sex-reversal, with the formation of a functional ovary and reproductive capabilities [7–9]. Successful induction of sex-reversal by sex steroid hormones has been also achieved in various fish species and amphibians [10–12]. In the chicken, sex reversal can be induced experimentally, at least in part, by injecting eggs with estrogens, or by inhibiting estrogen production [13,14], indicating a critical role for estrogen in avian sex determination. Some reptiles, including crocodilians and some turtles and lizards, exhibit temperature-dependent sex determination; sex depends on the temperature at which the eggs are incubated. Incubation temperature can modify the expression and activity of aromatase in the red-eared slider turtle and American alligator [15–17]. In addition, administration of exogenous estrogens to an egg can override the effects of male incubation temperature on sexual differentiation [18,19], suggesting that endogenous estrogen mediates ovarian development as a downstream signaling event in response to environmental temperature. Thus, in these animals, estrogen is critical for gonadal sex differentiation (ovary formation), and the subsequent female reproductive tract development.

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In contrast, the relative importance of sex steroid hormones in sex determination apparently seems to diminish in mammals compared with other vertebrates. Estrogen signaling is indeed required for complete ovarian differentiation and maintenance in adult mice [20–23]. Intriguingly, estrogen receptors (ERs) are necessary to repress the transdifferentiation of an adult ovary to a testis, probably cooperating with forkhead transcription factor Foxl2 [24,25]. However, there is no evidence that endogenous estrogens affect sex determination and ovary formation in mammals. Studies using knockout and mutant mice for ERs and aromatase genes have revealed no fundamental effects of endogenous estrogens on anatomical/morphological development in the reproductive tract s during embryogenesis and neonatal stage [20–23]. Notwithstanding this observation, ERs have been already expressed in developing mammalian reproductive organs from early stages and thus, they respond to estrogenic signals and can be the targets of endocrine-disrupting chemicals (EDCs). Therefore, administration of exogenous estrogens or estrogenic environmental signals that mimic hormones in animals can disrupt its normal development. One of the best-studied cases is a synthetic estrogen, diethylstilbestrol (DES). Beginning in the 1940s, DES was routinely prescribed to pregnant women for the prevention of miscarriages. To date, it is well-known that *in utero* exposure to DES induces vaginal clear-cell adenocarcinoma and various malformations in the reproductive tracts in young women [26–30]. Furthermore, in males exposed *in utero* to DES, reproductive organ abnormalities, including hypospadias, are found more frequently than in non-exposed controls [30–32]. Sexually dimorphic development of external genitalia depends on sex hormone environment during embryogenesis and thus can be strongly affected by the EDCs [33–36].

Potential endocrine disruptive effects in wild animals and humans exposed to EDCs during development have been summarized previously [37,38]. Despite this, the molecular mechanisms underlying EDC action remain largely unknown. Animal studies have shown that experimental exposure to estrogens/estrogenic chemicals induces misregulation of the endocrine systems and developmental sequences during embryogenesis. The perinatal mouse model has been used to understand molecular mechanisms of EDC-induced abnormalities in reproductive organs. In particular, DES effects were well recognized and firmly documented as it significantly alters the developing organism and results in persistent effects in the adult. In this review, we focus on the effects of DES on the female reproductive tracts and external genitalia.

2. Estrogen independent activation of ERα and growth factor signalings in mouse vagina exposed neonatally to DES

Estrogen acts via intracellular ERs that are members of the nuclear receptor superfamily of transcription factors. Upon ligand binding, ERs enhance the rate of transcriptional initiation by recruiting and assembling transcription regulatory complexes to the promoter regions of its target genes. Thus, estrogens exhibit acute and transient actions in target organs. In the adult female reproductive tracts, administration of estrogens in the adult increases organ weight and promotes cell proliferation and differentiation, whereas estrogen withdrawal induces rapid involution of uteri and vaginae resulting in atrophy. These specific and reversible effects of estrogens are important in maintaining homeostasis and are required for normal health and reproduction. In contrast, long-term exposure to estrogens induces an imbalance in cell proliferation and increases the risk of cancer of the reproductive organs in rodents and in humans [39,40]. It is also well-known that *in utero* exposure to DES causes vaginal clear-cell adenocarcinoma in a subset of exposed females including humans [28]. This is rare type of tumor, but its epidemiology revealed a clear association with early exposure to DES *in utero*. In addition, as the generation of women exposed to DES become older, concern has arisen about their health risks, because it has been hypothesized that *in utero* DES exposure could also influence the incidence of breast cancer, squamous neoplasia of the cervix, vaginal cancer and potentially other pathologies of the reproductive system [27,41–43]. Like humans, perinatal female mice exposed to DES develop estrogen-independent persistent cell proliferation, stratification and cornification of the vaginal epithelium, resulting in hyperplastic lesions and vaginal cancer later in life [44–46]. Although the evidence for endocrine disruption in humans resulting from exposure to EDCs is limited, animal studies have shown that perinatal exposure of various EDCs reproducibly induces estrogen-independent abnormal phenotypes in vagina. For instance, neonatal exposure of bisphenol A, an EDC exhibiting a weak estrogenic activity, also induces such malformations in mouse vagina. Thus, the animal DES model has been used to advance our knowledge of the potential risk of the carcinogenetic effects of estrogens, including developmental effects of EDCs [47]. Thus, the animal DES model has been used to advance our knowledge of the potential risk of the carcinogenetic effects of estrogens, including developmental effects of EDCs [48,49].

The proliferation and differentiation of mouse vaginal epithelial cells are strongly regulated by ovarian estrogens. The vaginae of ovariectomized mice show an atrophied epithelium of 2-3 cell layers, but estrogen administration rapidly induces epithelial cell proliferation, stratification and superficial cornification. In the uterus and vagina, mitogenic effects of estrogen are mediated by stromal ERα, as shown by recombination experiments with ERα mutant-stroma and wild type-epithelium [50,51]. These data indicate that such stroma-derived growth factors stimulate epithelial cell proliferation and differentiation during normal activity. In fact, previous studies have shown that several growth factors, including EGF-like growth factors, are expressed in the female reproductive organs upon estrogenic stimulation [52–54]. Intriguingly, the vagina in ovariectomized mice exposed DES neonatally also expresses EGF-like growth factors at high levels, even in the absence of endogenous estrogen [55–57]. Furthermore, EGFR and erbB2, receptors for EGF-like growth factors, are activated in such vagina. Serine residues located in the N-terminal activation function (AF-1) domain of ERα were identified as downstream targets of the erbB signaling pathway [57]. In the neonatal DES-exposed mouse vagina, the AF-1 domain of ERα is also phosphorylated even after ovariectomy. It has been shown that phosphorylation of ERα induces transcription activity in a ligand-independent manner through AF-1 [58]. Thus, persistent phosphorylation of ERα via erbB signaling could activate growth factor expression, resulting in formation of an auto-activation loop, which can contribute to the formation of cancerous lesions later in life (Fig. 1). Intriguingly, this activation loop of ERα-EGF-like growth factors-erbBs seems to be established only in the epithelium but not through the stroma, because high expression of EGF-like growth factors is detected in the epithelium only in vaginae from neonatally DES-exposed mice [57]. Failure of the regulatory interactions between the epithelium and stroma could be one of the mechanisms for aberrant activation of vaginal epithelial cell proliferation. In addition to erbB signal transduction, IGF-I signaling also appears to contribute to abnormalities in vagina exposed to DES perinatally [59]. It is currently unknown how the ERα transactivation induced by only AF-1 is maintained in vagina from mice exposed neonatally to DES. Specific modulators that change ERα function could provide further insight into this question.

Although the precise mechanisms have not been established explaining how such an activation loop with persistent gene expression is elicited, one of the possibilities might be an alteration of methylation of specific genes (Fig. 1). It has been shown that prenatal DES exposure alters methylation patterns in the promoter of

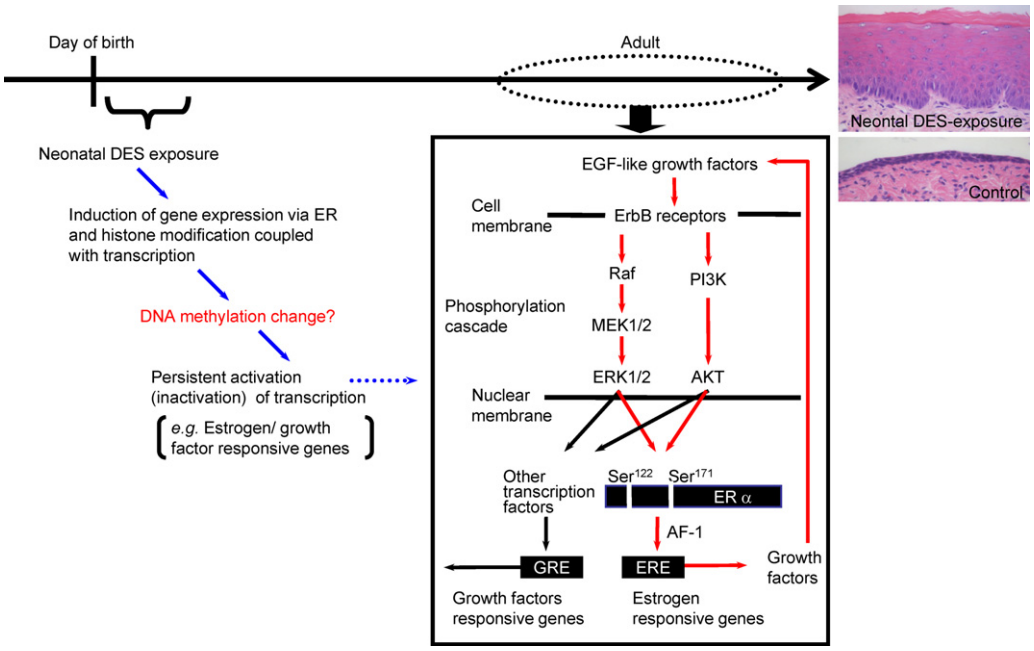

Fig. 1. Hypothetical model for the estrogen-independent estrogen receptor and growth factors activation pathway in neonatally DES-exposed mouse vagina. Histological sections show ovariectomized mouse vagina exposed neonatally to DES or vehicle alone.

some estrogen-responsive genes, including the demethylation of the c-fos and lactoferrin genes in the Müllerian duct [60,61]. c-Fos is a growth promoter that can predispose cells to becoming tumors. Hypomethylation of the nucleosomal binding protein 1 (Nsbp1) gene and its subsequent elevated expression is also reported after neonatal exposure to either DES or genistein [62]. Neonatal estrogen – or bisphenol A – exposure in rats induces hypomethylation of phosphodiesterase type 4 variant 4 (PDE4D4) gene, resulting in elevated expression of this gene in the prostate [63]. PDE4D4 is a crucial regulator for cAMP degradation and suggests a correlation between its expression and development of prostatic intraepithelial neoplasia. Thus, epigenetic changes could lead to altered gene expression and hence to altered tissue differentiation and formation, which could produce an increased susceptibility to disease and dysfunction later in life.

It was reported that DNA methylation changes can induce transgenerational effects, further exacerbating the potential role of EDCs that affect this pathway [64]. Increased level of lactoferrin gene in the uterus is detected in neonatally DES-exposed mice and also in their pups which never received DES-exposure [65]. Furthermore, the pups of prenatally DES-exposed mice have higher risks of reproductive organ abnormalities including tumors [66,67]. The preliminary evidence of the risk of reproductive dysfunction in daughters whose mothers were exposed DES *in utero* is reported [68–70], but further follow-up is needed.

3. Morphological defects of female reproductive tracts by DES

EDCs act in specific ways in tissues to disrupt normal developmental sequences. When EDCs affect relatively early periods of organogenesis, they can lead to congenital anomalies. DES acts both as a carcinogen and teratogen in the developing fetuses and neonates in mammals. For example, DES causes the boundary between the oviduct and uterus (the uterotubal junction) to be lost, resulting in infertility or subfertility [71,72]. DES-exposed mice also exhibit a malformed oviduct that lacks coils, and uterine abnormalities including hypoplasia, epithelial cell stratification, disorganized myometrial muscle and reduced uterine glands [45,73].

The Müllerian duct differentiates into the oviduct, uterus, cervix and upper part of vagina, along an anterior to posterior pattern. The Abdominal B (AbdB) Hox genes are expressed in a nested fashion throughout the Müllerian duct (Fig. 2). Hoxa9 is expressed in the oviductal or cranial part of the Müllerian duct but not in the more caudal region. Hoxa10 expression exhibits a sharp boundary at the junction between the presumptive uterus and oviduct. Hoxa11 is also expressed in the uterus as well as extending caudally in the cervix. Hoxa13 expression is caudally restricted primarily to the vagina in mice [74]. Analyses in knockout mice revealed that AbdB Hox genes play an instructive role on cellular identities along undifferentiated axes. In female Hoxa10 mutants, the anterior part of the uterus exhibits an anterior transformation to the oviduct and abnormalities of the uterotubal junction, and stratification of the distal uterine epithelium [75]. Loss of Hoxa11 results in a narrowing of the entire uterus, which makes it difficult to assess the uterotubal junction, and decrease in the number of uterine glands [76,77]. Importantly, although not identical, these phenotypes are

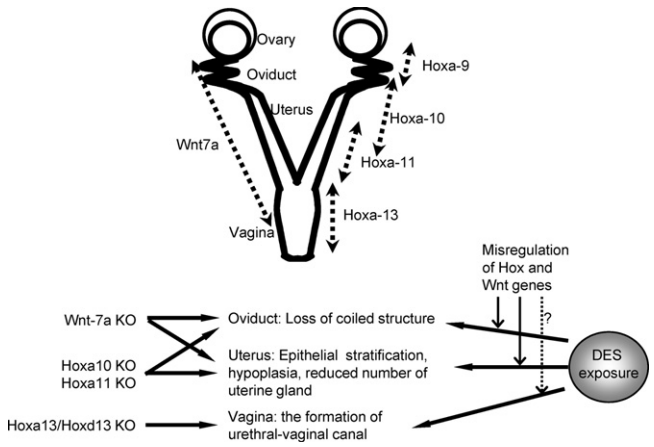

Fig. 2. Expression pattern of Hox genes and Wnt7a in female reproductive tracts during embryogenesis. Mutant mice for those genes exhibit various defects similar to those of prenatal DES-exposed mice.

similar to those of prenatally DES-exposed mice (Fig. 2). In fact, DES exposure can alter the expression of the Hox genes in the Müllerian duct. *In utero* DES exposure shifts Hox9 expression from the oviduct to the uterus and decreases both Hoxa10 and Hoxa11 expression in the embryonic uterus [78,79]. In addition to Hox genes, prenatal DES exposure also reduced Wnt7a expression in the Müllerian duct [80]. Wnt7a plays critical roles in epithelial–mesenchymal interactions during embryonic development [81]. Wnt7a is expressed throughout the entire Müllerian duct in embryos, whereas after birth it becomes restricted to the oviductal and uterine epithelium. Wnt7a mutants exhibit a lack of oviductal coiling and uterine gland formation, and exhibit a multilayered uterine epithelium [82]. It is therefore likely that abnormalities in the prenatally DES-exposed mouse oviduct and uterus are associated with misregulation of morphogenetic genes such as Wnt7a and the Hox genes (Fig. 2). Although mutant mouse studies provide an insight into complex gene networks during female reproductive organ formation, the involvement of endogenous estrogens in the expression of these genes has not been understood, because ER mutant mice do not show distinct altered phenotypes of Müllerian duct formation and differentiation. In contrast, it is obvious that altered expression of Wnt7a and AbdB Hox genes produced by DES exposure are mediated through ERα, because ERα mutant mice fail to induce such abnormalities [73]. Recently, it has been shown that the frequency of DNA methylation in the Hoxa10 intron is higher in prenatally DES-exposed mice when compared with controls [83]. Hoxa10 is associated with a variety of aspects of cellular physiology and women's health. Although altered methylation by DES exposure in humans has not been reported, down-regulation of Hoxa10 expression and aberrant methylation of Hoxa10 gene is associated with endometrial carcinoma and endometriosis in humans [84–86].

The urogenital sinus, which differentiates into the urinary tract, lower part of vagina and clitoris, is also affected by DES. Neonatal DES exposure induces female hypospadias, the formation of a common urethral-vaginal canal accompanied by a wide cleft clitoris [87]. Compound-induced mutation of Hoxa13 and Hoxd13 results in a common canal of the urinary tract and vaginal lumen [88], although the relationship between female hypospadias induced by DES and Hox genes expression has not been elucidated. Intriguingly, dihydrotestosterone (DHT), or non-aromatizable androgens, also induce female hypospadias [89]. These results indicate that fetal and neonatal stages showing active morphogenesis and development are more sensitive to endocrine disruptive stimulation than are adults.

4. Hormone-dependent development of external genitalia

Prenatally DES-exposed humans and laboratory animals exhibit a range of reproductive organ malformations including hypospadias, microphallus, retained testes and many aspects of the testicular dysgenesis syndrome [90–94]. Hypospadias (in which the urethral meatus is located on the ventral side of the penis) is one of the most frequent human birth defects. The increasing prevalence of hypospadias in humans has been hypothesized to be the result of exposure to EDCs/estrogens during fetal development [95–98]. In laboratory animals, fetuses exposed to DES or 17α-ethinylestradiol (EE2; a synthetic estrogen used in contraceptive pills) exhibit hypospadias-like phenotype with a failure of preputial development in male mice [94]. In addition, permanent dysmorphogenesis of the penis has been observed in adult rats treated neonatally with DES [99].

The sexual dimorphic development of external genitalia depends on the presence or absence of androgens and is, therefore, strongly affected by hormonal environment during embryogenesis. Flutamide, which inhibits androgens binding to the androgen receptor, demasculinize the external genitalia in male rodent offspring [100,101]. Likewise, the fungicides vinclozolin and procymidone, and pesticides DDT and its metabolite *p,p′*-DDE induce defects of androgen pathway with sufficient potency to induce cleft phallus and ambiguous genitalia [102–105]. In contrast, mechanisms of DES and other estrogens-induced external genitalia malformation has not been clarified. ERs are endogenously expressed in the embryonic external genitalia [93,106,107]. Further, ERα mutant mice are resistant to estrogens-induced penile abnormalities [108] indicating that estrogen-exposure could directly perturb male genitalia development.

The external genitalia are typically sexually dirmorphic organs and arise through dichotomous differentiation of common precursor tissues [109,110]. It is well documented that androgen plays a central role in such processes as epithelium-mesenchymal interactions [36,111]. Recently, canonical Wnt signal was shown to regulate the masuculinization of external genital cooperatively with the androgen signal [34]. The bilateral mesenchyme adjacent to the urethral plate epithelium displayed sexually dimorphic activity of the Wnt signal. Loss- and gain-of-function mutants of β-catenin display altered sexual development of the external genitalia, suggesting that the Wnt signaling pathway functions as a locally expressed masculine effector [34]. Mutation of the Hoxa13 gene, which is associated with hand-foot-genital syndrome, causes genital abnormalities including hypospadias [112–114]. The Hoxa13 mutant mouse shows reduced expression of androgen receptors, suggesting a requirement of Hoxa13 for masculinization of external genitalia [115]. As discussed above, ERs signaling can affect the cross-talk with developmental factors such as Wnt and Hox genes. Therefore, estrogen signals could affect such signals as observed in the Müllerian duct.

5. Conclusion

Despite a large number of reports over the last decade on the mechanisms of DES and EDCs on reproductive organ development, much still needs to be understood. An emerging paradigm, the fetal origin of adult disease, is a new framework for considering the effects of EDCs on human and animal life. The plasticity of development in the perinatal period is evolutionarily advantageous for adaptation to the prenatal and early postnatal environment. However, the modern environment has environmental factors, including EDCs that easily influence fetal and neonatal development, resulting in permanent changes in cell differentiation and morphology. The instructive roles of estrogen signaling during embryogenesis are also important issues as an understanding of the precise mechanisms through which EDCs affect developing animals can also help us to understand how normal development is accomplished.

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