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Trends Endocrinol Metab. Author manuscript; available in PMC 2010 September 1.

Published in final edited form as:
Trends Endocrinol Metab. 2009 September ; 20(7): 357–363. doi:10.1016/j.tem.2009.03.009.

Endocrine disruptors in female reproductive tract development and carcinogenesis

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Abstract

Growing concerns over endocrine disrupting chemicals (EDCs) and their effects on human fetal development and adult health have promoted research into the underlying molecular mechanisms of endocrine disruption. Gene targeting technology has allowed insight into the genetic pathways governing reproductive tract development and how exposure to EDCs during a critical developmental window can alter reproductive tract development, potentially forming the basis for adult diseases. This review primarily uses diethylstilbestrol (DES) as a model agent for EDCs and discusses the recent progress elucidating how DES and other EDCs affect murine female reproductive tract development and cancer at the molecular level.

Keywords

diethylstilbestrol; uterus; development; epigenetics; cancer

Characteristics of endocrine disruptors

The US Environmental Protection Agency's defines endocrine disruptors as “exogenous agents that interfere with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development, and/or behavior”. Its broad inclusion has led to the recently proposed name change to endocrine toxicants and their various classifications [1]. Nevertheless, the realization that environmental chemicals adversely affect both human fetal development and the adult endocrine system is a tremendous step forward towards improving public health. Several aspects of endocrine disruptors deserve our attention. First, exposure to endocrine disruptors during critical developmental time windows could form the basis for adult diseases including cancer. Second, EDCs not only affect the person exposed, but also the person's offspring through epigenetic modifications. And finally, EDC exposure usually occurs as a complex mixture of compounds affecting multiple endocrine systems, making the understanding of endocrine disruption complicated. Diethylstilbestrol (DES, a prototype of endocrine disruptors) functions as a strong estrogen whose in utero exposure has devastating effects on many organ systems including the male and female reproductive tract [2,3]. Its

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transgenerational effect has also been shown recently [4,5]. This review highlights recent findings gleaned from studies using the mouse DES model and discusses DES's role in female reproductive tract patterning and uterine cancer.

Development of the mouse female reproductive tract

The mouse female reproductive tract (FRT) consists of the oviducts, uterus, cervix and vagina, and is mainly derived from the Müllerian duct (a.k.a. paramesonephric duct). Müllerian duct development commences at embryonic day E11.75 in parallel to the Wolffian duct when the coelomic epithelium of the mesonephros invaginates and extends caudally until it reaches the cloaca by E13.5 [6,7]. According to Dr. Behringer, Müllerian duct formation is divided into three phases: 1) initiation, 2) invagination of the coelomic epithelium into the mesonephros, and 3) elongation of the Müllerian duct to the cloaca [6] (Figure 1). This artificial division is based on analyses of mouse mutants that exhibit developmental arrest of Müllerian duct formation at specific phases. In the first phase, *Lim1* and *Pax2* expressing cells in the coelomic epithelium are specified to form the Müllerian duct at E11.75 [8] (Figure 1a). *Wnt4* likely functions in the first phase downstream of *Lim1* to initiate Müllerian duct invagination [9] (Figure 1b). Once the Müllerian duct reaches the Wolffian duct, its further extension to the cloaca requires cell proliferation at the leading tip [6] or along the entire Müllerian duct epithelium [10], in addition to the presence of the Wolffian duct. *Pax2* is involved in Müllerian duct elongation and maintenance (Figure 1c). In fact, the Müllerian duct initially forms, but then degenerates, in *Pax2* mutant mice [8,11].

The Wolffian duct not only serves as a guide for Müllerian duct extension, but also secretes WNT9b, a canonical Wnt signal required for Müllerian duct extension [12] (Figure 1c). However, the Wolffian duct does not contribute any cells to the Müllerian duct as clearly demonstrated by two recent lineage tracing studies [6,10]. Interestingly, the early Müllerian duct is mesoepithelial in character, and epithelialization of the Müllerian duct occurs from E13.5 to birth in mice [6]. The mesoepithelial nature of the Müllerian duct might facilitate its regression in males, because this is when the Müllerian duct is sensitive to Müllerian inhibiting substance (MIS)-induced regression [6]. Lineage tracing experiments demonstrated that the mesenchymal cells surrounding the Müllerian epithelium also originate from the coelomic epithelium [10]. Interestingly in male embryos, MIS induces epithelial to mesenchymal transition of the coelomic epithelium and migration of these cells into the Müllerian duct mesenchyme [13]. Several other transcription factors including empty spiracles homolog 2 (EMX2), pre B-cell leukemia transcription factor 1 (PBX1), retinoic acid receptor proteins, and transcription cofactors Dachshund 1 and 2 have been shown to also play important roles in Müllerian duct formation [14–17]. However, their exact roles in this process need further investigation.

Following its formation, the homogenous Müllerian duct next divides into segments along the anterior-posterior axis with each segment developing into distinct structures (i.e. oviduct, uterus, cervix and upper vagina). The Abdominal B Hox genes play an instructive role in this process. AbdB Hoxa and d genes exhibit nested expression patterns in both male and female reproductive tracts, forming a “Hox” code that provides positional information to specify the identity of different regions [18–20]. Mutation in *Hoxa10* leads to loss of uterotubal junction and infertility, whereas loss of *Hoxa11* leads to uterine hypoplasia and infertility. On the other hand, *Hoxa13* mutant null embryos show agenesis of the posterior portion of Müllerian ducts [21–24]. Classical tissue recombination experiments demonstrated that the Müllerian duct stroma provides the cues for epithelial differentiation until postnatal days 5 to 7 [25,26]. AbdB Hoxa genes are good candidates for this cell-fate determination event. Genetic knock-in experiments replacing the HOXA11 homeodomain with that of HOXA13 led to stratification of the uterine epithelium and a change in the molecular signature to resemble that of vaginal

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epithelium [27]. Since *Hoxa11* is expressed only in the stroma, this result indicates that HOXA11 and HOXA13 normally control the expression of stromal signals that instruct the differentiation of overlying epithelia to adopt uterine and vaginal epithelial fate, respectively. On the other hand, replacing the HOXA11 homeodomain with that of HOXA10 only leads to a moderate reduction in fertility, with no change in uterine cell fate [28].

The identity of the stromal signals that control FRT epithelial fate determination remains elusive, but several growth factors including IGF1, KGF and WNTs have been proposed to exert paracrine functions in the uterus [29]. In particular, members of the WNT signaling family play important roles in FRT differentiation. In addition to its role in Müllerian duct regression in males [30], *Wnt7a* is also required for FRT development along both the A-P and radial axis and to maintain high *Wnt5a*, *Hoxa10* and *Hoxa11* expression in the adult uterus [31]. In fact, both *Wnt7a* and *Wnt5a* are essential for uterine gland formation [31,32], and knocking out β-Catenin, a key signaling molecule in the canonical WNT signaling pathway, in all tissue layers of the uterus using PR-Cre leads to uterine hyperplasia, lack of gland formation and severely compromised fertility [33]. This phenotype likely reflects a requirement for WNT signaling in uterine epithelia, since knocking out β-catenin specifically in the stroma using Amhr2-Cre did not result in an epithelial defect, but instead, led to transformation of uterine smooth muscle cells into fat cells and disrupted oviductal coiling [34,35]. *Wnt5a*, on the other hand, is required for posterior FRT development and for turning off *Wnt7a* expression during adenogenesis [32]. Both Hox and Wnt genes are targets of endogenous estrogen [18,36], and it is becoming increasingly clear that endocrine disruptors such as DES alter FRT development by genetic pathways regulating normal FRT morphogenesis, including but not limited to Hox and Wnt pathways.

Endocrine disruptors on FRT development

Diethylstilbestrol (DES) was the first synthetic estrogen administered to pregnant women from the 1940s to the 1970s in efforts to prevent miscarriage. In the United States, at least four million women and their fetuses were exposed to DES before its teratogenic and oncogenic effects on FRT were discovered in 1971 [2]. Müllerian duct formation does not appear to be sensitive to estrogenic compound exposure as treatment of pregnant mice with DES from E9.5 to E16.5 does not affect Müllerian duct formation in female embryos [37]. Presumably genes important for early Müllerian duct formation are not targets of DES. However, FRT differentiation is sensitive to DES exposure. DES leads to a loss of uterotubal junction, stratification of the uterine epithelium, disorganized uterine muscle layers, delayed and reduced uterine adenogenesis, and vaginal adenosis [37]. DES potently inhibits expression of *Wnt7a*, *Hoxa10* and *Hoxa11* during critical windows of FRT development through a mechanism involving *Wnt5a*, providing a molecular basis for its effect on FRT differentiation [18,32,38, 39] (Figure 2). In addition to DES, in utero exposure to other xenoestrogens including methoxychlor and bisphenol A also perturbs *Hoxa10* expression [40,41]. Likewise, Polychlorinated biphenyls (PCBs) and 17α-ethynyl estradiol treatment led to a dramatic downregulation of *Wnt7a* expression [42,43]. These results indicate that xenoestrogens may perturb FRT development the same way DES does, underscoring the importance of understanding genetic pathways regulating FRT patterning and differentiation.

To gain a global view of how DES affects FRT morphogenesis, several microarray studies were carried out. One microarray study used the prenatal mouse DES model and revealed DES-regulated genes in the oviduct, uterus and vagina [44], identifying a number of interesting region-specific targets as well as a number of common targets (Figure 2). We used the neonatal DES mouse model to identify uterine genes whose expression is altered upon DES treatment relatively early, i.e. before FRT cell-fate determination. The neonatal model is widely believed to mimic the developmental stages of human exposure (Table 1). We found that DES treatment

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forced uterine epithelial cells out of the cell cycle, inhibited apoptosis, and changed uterine cell fate by inducing expression of various differentiation markers [45]. A similar study using the same model system but looking at the prepubertal uterus (P19) showed that most of the gene expression changes observed on P5 were no longer detected [46]. However, 43 common genes were found with our study, ex. *Lactoferrin* (*Ltf*), *Complement component C3*, and *Sprr2f* which could represent those whose expression is permanently changed by DES exposure. This study adds to the current list of genes whose expression could be permanently altered in an ovary-independent manner, suggesting that neonatal DES exposure can reprogram the uterus into a permanently "estrogenized" environment. The molecular basis for DES's permanent effect on gene expression could be attributed to changes in promoter methylation and/or chromatin structure [47].

Consistent with the fact that DES is a strong estrogen, a study directly comparing DES and estrogen targets showed that the two compounds indeed share a majority of their targets [48]. Similarly, many genes (n=173) regulated by neonatal DES exposure are also targets of 17β-estradiol in the adult uterus, including the aforementioned molecules as well as *Msx1*, *Hoxd4* and *Tgfbi* [46,49]. These results also reflect the dynamics of gene expression during uterine differentiation and how molecular changes in response to DES change with time, that is to say many genes that showed an acute response to DES treatment, returned to normal with time. This does not mean that these genes are not important in DES-induced FRT diseases. In fact, changes during the critical period of FRT development may permanently reprogram FRT tissues such that their aberrant differentiation and response to endogenous estrogen after puberty may form the underlying basis for adult diseases. One such example is *Wnt7a*, whose function is so critical for FRT development. Although *Wnt7a* is only transiently repressed by DES, its downregulation may be sufficient to change uterine epithelia from simple columnar to stratified squamous morphology [39].

In the developing cervix and vagina, the cervicovaginal stroma induces p63 expression in the overlying epithelium to initiate stratification and cervicovaginal epithelial differentiation [50]. Neonatal DES exposure disrupts this stromal signal and represses p63 expression. Although most epithelial cells recover from this repression, some do not and will eventually develop glands (cervicovaginal adenosis), believed to be the precancerous lesion leading to adenocarcinoma [50]. DES exposure also stimulates epidermal growth factor signaling pathways which may lead to vaginal cancer [51].

Genetic pathways affected by DES

In the past two decades, with the advent of knockout technology, newly generated gene targeted mouse models have allowed us to ask more specific questions about the molecular mechanisms of DES and/or estrogen function. For example, using ERα knockout mice, Dr. Korach's group showed that most of the effects of DES on the FRT including cancer are mediated through ERα [52]. Currently it is not clear whether ERβ also plays a role in this process. However, it is interesting that ERβ can regulate ERα targets even in the absence of ligands [53]. Thus it is possible that ERβ may help ERα to promote DES teratogenicity. However, ERβ knockout uteri show wild type morphology [3]. Furthermore, DES is able to induce all the phenotypic changes in βERKO male prostate as in wild type mice, whereas αERKO mice are completely resistant [54]. These results argue against the involvement of ERβ in mediating DES toxicity.

Less is known about genetic pathways downstream of ERα. Presumably some DES targets are directly regulated by ERα binding to its promoters, whereas others may be secondary targets of transcription factors and growth factors downstream of ERα. Dr. Sassoon's group showed that DES requires *Wnt5a* to repress *Wnt7a*, *Hoxa10*, and *Hoxa11* [32] (Figure 2). In our microarray study, we found that DES represses *Msx2*, a homeobox gene with important

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functions in uterine epithelial-mesenchymal signal transduction [45,55,56]. In contrast, KLF4, a Krüppel-like Zinc finger transcription factor with important functions in skin barrier formation, is strongly induced by DES, suggesting a novel differentiation pathway induced by DES [45]. We showed that MSX2 normally functions to maintain proper uterine differentiation because in its absence, several differentiation markers are abnormally expressed. Moreover, expression of *Wnt5a*, which is mainly expressed in the uterine stroma, becomes predominantly epithelial in DES-treated *Msx2*−/− uteri, much more so than in wild type DES-treated uteri [45]. Similarly in the vagina, *Msx2* maintains vaginal epithelial differentiation through *Tgfb2* and *3* and is required for the induction of *Aquaporin* (*Aqp*) *3* and *4* expression by DES [56]. These studies led to a proposed model for DES-affected genetic pathway in uterine epithelial differentiation [45]. Likewise, DES treatment failed to repress *Hoxa10* and led to extensive cell death in the uterine epithelium in *Wnt7a*−/− uteri [57]. Together, these studies demonstrate important functions of *Msx2* and Wnt genes in mediating the effects of DES on gene expression and show that it is possible to use gene specific knockout models to further dissect the genetic pathways affected by DES and/or estrogen.

Capacitors in endocrine disruption

One interesting finding from the above studies is that *Msx2*−/−, *Wnt5a*−/− and *Wnt7a*−/− uteri all exhibit a more severe morphological response to DES treatment [32,45,57]. In the case of *Msx2* mutants, DES exposure induces extreme dilation of both the uterus and vagina, with the vaginal epithelium reverting to a simple columnar morphology, likely a result of complete p63 repression [45,56]. The dilated uterine and vaginal lumens are likely caused by abnormal water imbibition associated with failure to upregulate the expression of water transport molecules, namely *Aqp 3* and *4* [56]. Similarly, both *Wnt5a* and *Wnt7a* mutant uteri exhibit abnormal water imbibition when treated with DES; however, it is not clear whether aquaporins are abnormally regulated in these mutants. More interestingly, *Wnt7a*+/− uteri also show increased sensitivity to PCB treatment [42]. These data are puzzling because if DES elicits FRT defects through *Msx2* and *Wnt7a* repression and by shifting *Wnt5a* expression from stroma to epithelium, then DES should not cause more harm in the mutant background. Yet, not only are the DES-induced phenotypes more severe, but also its effects on gene expression, such as the molecular switch *p63* and *Wnt5a* are augmented in *Msx2*−/− mice. These data thus suggest that a set of genes antagonizes or dampens exogenous (or endogenous) estrogenic signals and therefore in their absence, DES can elicit a much more dramatic response. This is similar to a group of genes functioning as capacitors during evolution, which explains why most species carry abundant genetic variation while experiencing a vast range of environmental conditions, and are somehow able to maintain relatively low phenotypic variations [58]. The prime example of one such gene is Hsp90, which is a chaperone protein targeting a large number of signal transduction proteins. In its absence, diverse phenotypic variations were observed in both Drosophila and Arabidopsis [59,60]. Similarly, it makes sense for nature to select for organisms with a good capacitor that can buffer against environmental variations. In this context, perhaps *Msx2* and Wnt genes buffer the FRT response to exogenous estrogen exposure, acting as “endocrine capacitors” to dampen the body's hormonal response. In this sense, if the endocrine capacitor function is lost, the organism may become hypersensitive to certain hormonal stimulus including endocrine disruptors. Therefore, responses to endocrine disruptors can be highly variable depending on the genotype of an individual.

Epigenetics and DES

Currently it is believed that early exposure to EDCs permanently reprograms FRT development and affects FRT's responses to endogenous hormones such as 17β-estradiol later in life [61]. A classic example of this is uterine Ltf expression. Ltf expression is dramatically elevated by DES/estrogen in the neonatal uterus, persisting well into adulthood even in the absence of

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endogenous estrogen [62]. The persistent activation of Ltf is associated with demethylation of five CpG islands in the Ltf proximal promoter [47]. However, although *Hoxa10* and *Hoxa11* also exhibit chronic suppression by neonatal DES, no change in CpG methylation was found in these genes [63]. Recently, a global survey of CpG methylation using methylation-sensitive restriction fingerprinting revealed that 14 genes, including *Nsbp1*, had altered methylation patterns in their promoter regions when exposed to neonatal DES and/or genistein [64]. NSBP1, a protein with structural similarity to the high-mobility-group proteins, is thought to be involved in chromatin remodeling which may participate in DES-induced uterine carcinogenesis [64]. It is possible that the transgeneration effect seen in DES and other EDC-treated animals also results from epigenetic changes induced in the genome.

DES and endometrial cancer

Greater than 90% of CD-1 pups neonatally exposed to DES or genistein develop endometrial cancer by 18 months of age. Interestingly, C57BL/6 mice are resistant to this model [65]. This two-step tumorigenesis model involves the initiation event (neonatal DES exposure), followed by a tumor promoting phase (stimulation of endometrial growth by endogenous estrogen) [66]. Ovariectomy prior to puberty of neonatally DES-exposed animals prevents uterine cancer development. Laser-capture-microdissection and microarray identified gene expression changes in DES-induced endometrial cancer compared to adjacent non-cancerous uterine epithelium [43]. *Decorin*, *Hoxa11* and *Pten* expression were suppressed in cancer tissues which offered a starting point to investigate the role of estrogen in endometrial cancer development. In rats, for example, DES upregulates IGF-II expression and at the same time prevented negative feedback of its receptor IRS-1which may account for the development of endometrial hyperplasia which is the precursor of endometrial cancer [67].

Type I endometrial cancer is clearly associated with elevated estrogen exposure [68]. In addition to its well-established role in promoting endometrial proliferation, estrogen also increases cell survival by inhibiting uterine epithelial apoptosis. How DES/estrogen inhibits apoptosis in the uterine epithelium is poorly understood. We showed recently that members of the baculoviral inhibitors of apoptosis repeat-containing 1 (Birc1) family play important roles in mediating estrogen suppression of apoptosis [69]. *Birc1* gene expression is induced by DES as well as by 17β-estradiol prior to apoptosis suppression; furthermore, in *Birc1a* knockout mice, a subset of uterine epithelial cells escaped apoptotic suppression by DES [69]. ERα is required for *Birc1* induction and apoptotic suppression, and BIRC1 proteins work by subjecting active caspases to ubiquitination and proteasome degradation (Figure 2). Other studies have shown that normal apoptosis in the cervical epithelium is mediated by the P_2X_7 receptor and a Ca^{2+}-dependent mitochondria pathway, and estrogen can suppress apoptosis through modulating this pathway by new protein synthesis [70].

DES treatment also leads to uterine leiomyoma development [71]. Recently, a microarray experiment comparing DES-induced uterine leiomyoma with normal endometrium in rats revealed 171 differentially expressed genes among which 112 are estrogen targets [72]. Six candidates (Gdf10, Car8, Calbindin D9k, Dio2, Gria2, and Mmp3) were identified whose expression was reprogrammed by neonatal DES exposure [72]. These genes play important roles in diverse biological functions such as growth factor and hormone signaling, cell proliferation, calcium uptake, and extracellular matrix remodeling. In sum, neonatal DES exposure can reprogram uterine differentiation by transiently affecting genetic pathways regulating uterine morphogenesis and/or by permanently altering gene expression, particularly in uterine stem cells. With DES promoting cell proliferation and inhibiting apoptosis, these altered uterine stem cells may gain an added survival advantage and respond to endogenous estrogen in a tumor-promoting fashion which eventually leads to cancer development.

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Future directions

Despite the amount of data generated in the past few years on the mechanism of how endocrine disruptors including DES disrupt FRT development and lead to carcinogenesis, much still needs to be learned. Since reproductive tissues are very dynamic, theoretically only stem cells would retain the “memory” of such exposure during the neonatal period. In this sense, only changes made to stem cells would affect adult FRT function and cancer development. Some progress has been made in the study of uterine stem cells. Label-retaining cells have been identified in the epithelium and stroma of the adult uterus [73], and putative stem cell populations have been isolated by fluorescence activated cell sorting; however, definitive markers for uterine stem cells are still lacking [73]. Once uterine stem cells are identified and isolated, we can determine how EDC exposure alters gene expression, DNA methylation and cellular behaviors and how these changes persist into adulthood and lead to cancer formation.

Acknowledgments

This work was supported by National Institutes of Health grant ES014482. I would like to thank members of the Ma laboratory for comments on the manuscript and Dr. Yung-Chieh Tan for art work. I have nothing to declare.

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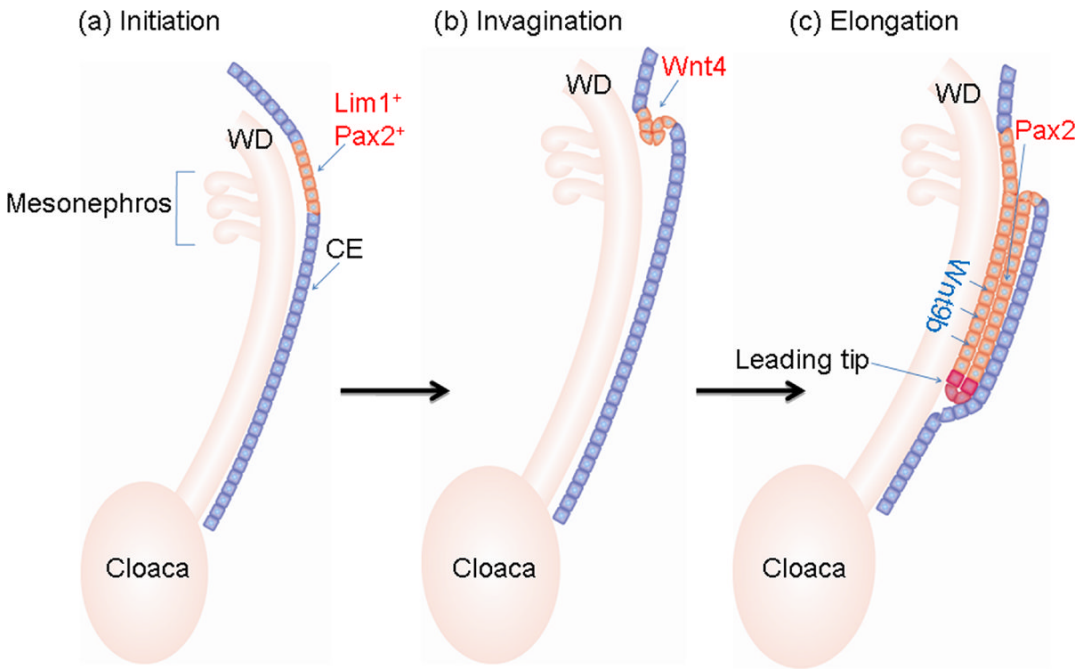

Figure 1.

A schematic diagram depicting Müllerian duct formation. (a) In the first phase, initiation, *Lim1* expressing cells (orange) in the coelomic epithelium (CE) are specified to Müllerian duct fate. (b) In the second phase, invagination, *Wnt4* functions downstream of *Lim1* possibly to induce Müllerian duct invagination to reach the Wolffian duct (WD). (c) In the third phase, elongation, the leading tip cells (pink) proliferate and deposit cells to form the Müllerian duct (orange) until it reaches the cloaca (more specifically the urogenital sinus). WD serves as a guide and secretes WNT9b to promote Müllerian duct elongation. *Pax2* is required for elongation and Müllerian duct maintenance. Genes specifically expressed in the MD (red), WD (blue) or both (purple) are shown.

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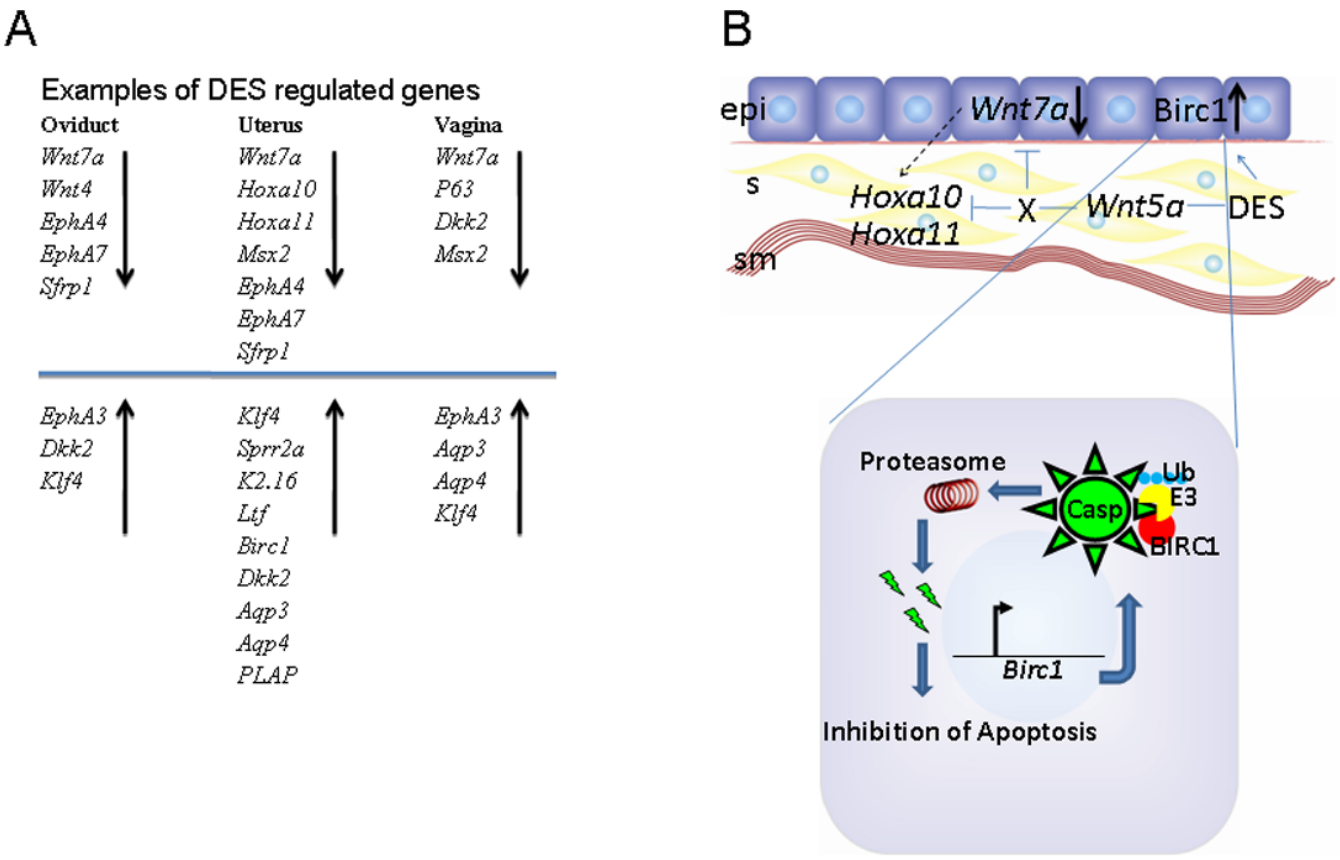

Figure 2.

A summary of DES-regulated genes in the mouse FRT. (a) DES exposure alters gene expression along the developing Müllerian duct. Subsets of DES-regulated genes are listed in different regions of the reproductive tract which are extracted from several publications [32, 44,45,69]. (b) DES induces *Birc1* gene expression in the uterine epithelium to inhibit uterine epithelial apoptosis. BIRC1 proteins ubiquitinate active caspases for proteasome-mediated degradation. Abbreviations: epi, epithelium; s, stroma; sm, smooth muscle; E3, ubiquitin ligase; Ub, ubiquitin; Casp, activated caspases.

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Table 1

Comparison between different DES mouse models

DES dosage Treatment	Prenatal 100 μg/kg/day E9.5–E16.5*	Neonatal 1 mg/kg/day PND 1–5
FRT phenotypes		
Oviductal defects (lack of coiling)	Yes	No
Uterine atrophy	Less common	Yes
Squamous metaplasia of uterine epithelium	Yes	Yes
Smooth muscle disorganization	Yes	Yes
Abnormal urethral openings	Yes	No
Enlarged vagina	Yes	Yes
Vaginal adenosis	Less common	Yes
Persistent vaginal epithelial cornification	Yes	Yes
WD remnant	Yes	Yes
Genital tract tumors	Yes	Yes
Relevance to human exposure	A bit early	Mimic

* Other regimens include treatment from E15–E18 at a dose of 200 μg/day pregnant mother or E10–E18 at a dose of 67 μg/kg/day