Gene Expression Change in the Müllerian Duct of the Mouse Fetus Exposed to Diethylstilbestrol *In Utero*

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In utero **exposure to diethylstilbestrol (DES) induces various abnormalities in the Müllerian duct of the mouse. In order to understand the underlying molecular mechanisms associated with DES-induced abnormalities of the Müllerian duct, gene expression was examined on Gestation Day (GD) 19 in mouse fetuses exposed to DES (67 μg/kg body weight) from GDs 10 to 18. Microarray analysis revealed that 387, 387, and 225 genes were upregulated and 177, 172, and 75 genes were downregulated by DES in the oviduct, uterus, and vagina, respectively. DES exposure *in utero* commonly upregulated 72 genes and downregulated 15 genes in these three organs. The present study demonstrated that organ-specific gene expression patterns in the mouse Müllerian duct were altered by *in utero* DES exposure. DES-induced changes in expression of genes such as *Dkk2*, *Nkd2*, and *sFRP1* as well as changes in genes of the Hox, Wnt, and Eph families in the female mouse fetal reproductive tract could be the basis for various abnormalities in reproductive tracts following exposure to this estrogenic drug.** Exp Biol Med 232:503–514, 2007

Key words: microarray; gene expression; diethylstilbestrol; oviduct; uterus; vagina; mouse

Introduction

Prenatal diethylstilbestrol (DES) exposure induces persistent malformations of male and female reproductive organs in mice. Female mice exposed perinatally to DES showed noncoiled oviducts, uterine metaplasia, disorganization of the uterine circular muscles, and ovary-independent vaginal epithelial stratification and cornification (1–6).

DNA microarray has been successfully used to analyze estrogen-responsive genes in the mouse uterus and vagina and to begin to identify those genes possibly related to persistent vaginal proliferation induced by neonatal DES exposure (7–12). Therefore, we studied global gene expression, including signal transduction and organogenesis genes in the Müllerian duct, after DES exposure *in utero* using microarray at Gestation Day (GD) 19, and we selected several genes for further study. We focused on the expression of ephrin, Eph family, Wnt, Wnt-antagonists, and Hoxa genes, since they showed altered expression by *in utero* DES exposure in the present study.

Hox genes, expressed in spinal cord, limb, and reproductive tracts, determine anterior to posterior body axis patterning. Hox genes are expressed in the Müllerian duct along its axis, with genes *a-9*, *a-10*, *a-11*, and *a-13* exhibiting anterior to posterior expression pattern at GD

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Table 1. Sequences for Primers Using Quantitative RT-PCR

GenBank accession no.	Name	Sequence of forward primer	Sequence of reverse primer	Length (bp)
AB005457	Hoxa9	CTGACTGACTATGCTTGTGGTTCTC	TCTCGGCATTGTTTTCGGA	84
L08757	Hoxa10	ACAATGTCATGCTCGGAGAGC	TGATGAGCGAGTCGACCAAA	61
U20370	Hoxa11	TTCTGCCACAGGCTTTCGA	TAGTCGGAGGAAGCGAGGTTT	72
U59322	Hoxa13	TGTACAGCATTCGTGGCAAAG	ACAGGCGACAGCTCAATGTG	69
BC019952	Nkd2	ACATTTGATGCAGCTGATGGTT	TGGATGACACAGGAGCACGT	50
NM_020265	Dkk2	TGTCTGAAGCACAGGCTGGAT	CTTCTGGAGCCTCTGATGGC	50
U88566	sFRP1	CCAACAGCCTCACTTTGTAATTCC	CCCTGTCTTATGCTGCTGTTCTTT	60
NM_009523	Wnt4	TGTACCTGGCCAAGCTGTCAT	TTTCTCGCACGTCTCCTCTTC	58
NM_009524	Wnt5a	AGTTTCACTGGTGCTGCTATGTCA	CCACAATCTCCGTGCACTTCT	50
NM_009527	Wnt7a	TTACACAATAACGAGGCGGGT	ACACTCCAGCTTCATGTTCTCCT	56
BC026153	Eph receptor A7	TGTTAAACCAGTGATGTTTTC	CCCATCTGAGGGAAGTCCTTAA	50
BB292785	Eph receptor A3	TTTTGTTACAGCCAAGTGCCAA	TTTCTTACTGCTGACAATTTGCAAT	51
BB706548	Eph receptor A4	AATTTGGGCAGATCGTCAACA	TGTTGGGATTGCGGATGAGT	50
U30244	ephrin B2	CTACAGCTTGTTTAACGGCAGTGT	TTTCCTCATTACAGTGCAAAGGG	50
U67771	Ribosomal protein L8	ACAGAGCCGTTGTTGGTGTTG	CAGCAGTTCCTCTTTGCCTTGT	84

15.5 (13). With the positional expression of Hox genes along the anterior to posterior axis, the Müllerian duct differentiates into three distinct reproductive organs: oviduct, uterus, and upper vagina. Lack of positional Hox gene expression results in reproductive abnormalities due to the loss of organ specificity (14, 15). Lack of *Hoxa-13* expression, for example, resulted in the loss of the caudal Müllerian duct (16). DES repressed the expression of *Hoxa-10* and *a-11* in the mouse uterus at GD 17, which is associated with reduced reproductive performance, including embryo implantation, in adult offspring (17, 18). The lack of Hox gene expression leads to disturbed patterning in the body axis, primarily in limbs, spinal cord, hindbrain, and reproductive tracts (14, 15, 19–22).

Hoxa-13 knockout mice showed the downregulation of the *Eph receptor A7* and inhibition of mesenchymal cell adhesion and apoptosis in limbs (23), suggesting possible functions in reproductive tracts. Eph receptors and ephrin ligands regulate cell–cell communication, cellular movement, and mitogenic responses during development *via* the ERK/MAPK cell signaling pathway (24–26).

Epithelial–mesenchymal differentiation in the Müllerian duct is regulated by Wnt signaling correlated with expression of Hox genes. In female reproductive organs, *Wnt-4*, *–5a*, and *–7a* are expressed during development (27). Lack of *Wnt-7a* expression induced uterine metaplasia, an abnormality similar to that reported in mice exposed to DES *in utero* (28). Wnt-7a maintains the expressions of *Hoxa-10* and *a-11*; thus, lack of Wnt-7a is considered to disrupt segmentation of the reproductive organs. Moreover, Wnt-4 is essential for the early development of female reproductive tracts (29).

Wnt signaling regulates vaginal growth and differentiation through epithelial–mesenchymal interactions. Perinatal DES exposure reduced expression of *Wnt-7a*, which resulted in uterine metaplasia (30), suggesting the presence of suppression factors for Wnt signaling. Secreted frizzled related protein (sFRP) was downregulated by 17β-estradiol treatment in the adult mouse uterus and was shown to compete with Wnt and frizzled (Fz) receptors (31, 32). Dicknock (Dkk; Refs. 33–38) and Naked cuticle (Nkd; Ref. 39) have been reported as Wnt antagonists in various developing organs.

In order to understand the molecular mechanisms underlying the reproductive tract abnormalities reported in female mice exposed prenatally to DES, we analyzed expression changes in Eph family, Wnt, Wnt-antagonists, and Hoxa genes following exposure to this pharmaceutical estrogen.

Materials and Methods

Animals. Mice of the ICR/Jcl strain were kept under a 12:12-hr light:dark cycle at 23°C–25°C, were given a commercial diet (CE-2; CLEA, Tokyo, Japan) and tap water *ad libitum*. All experiments and animal husbandry protocols were approved by the animal care committee of the National Institutes of Natural Sciences. The day on which a vaginal plug was found was considered GD 0. DES (Sigma Chemical Co., St. Louis, MO) was dissolved in sesame oil. Pregnant mice were given daily subcutaneous injections of DES (67 μg/kg maternal body weight) or the oil vehicle alone from GDs 10 to 18 as described previously (40). These experiments were repeated three times.

DNA Microarray Analysis. Total RNA was extracted from the oviduct, uterus, and vagina (7–12 pups/3 litters) at GD 19 using TRIzol (Invitrogen, Tokyo, Japan) and purified with the RNeasy mini kit (Qiagen, Tokyo, Japan). Total RNA quality was examined with a Bioanalyzer 2100 (Agilent Japan, Tokyo, Japan). Purified RNA was processed according to the manufacturer's protocol to prepare the labeled cRNAs, which were hybridized to the mouse expression array 430A (Affymetrix Japan, Tokyo, Japan). Hybridization, washing, and scanning were performed according to the manufacturer's protocol as

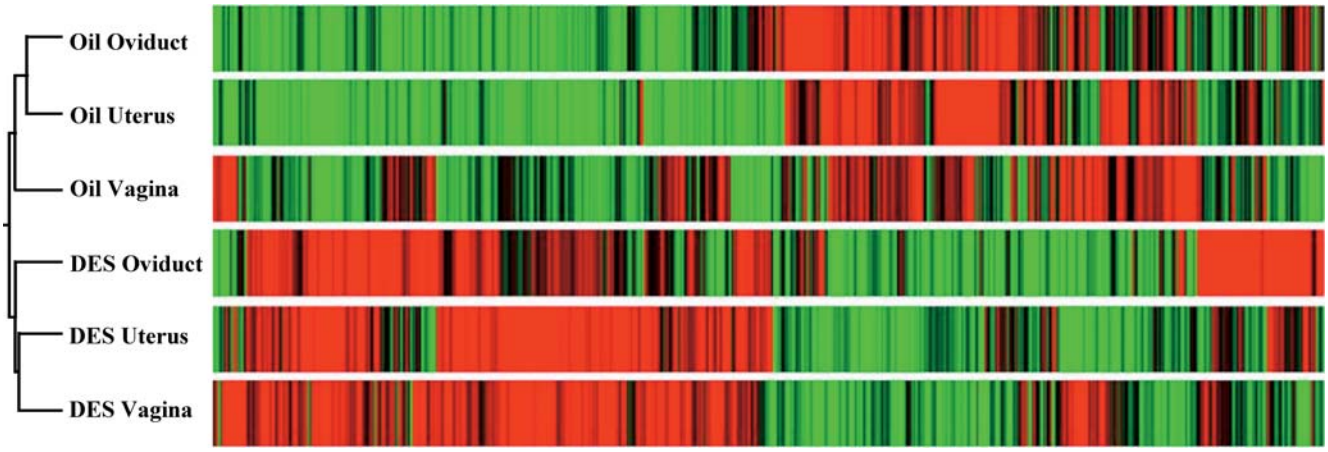


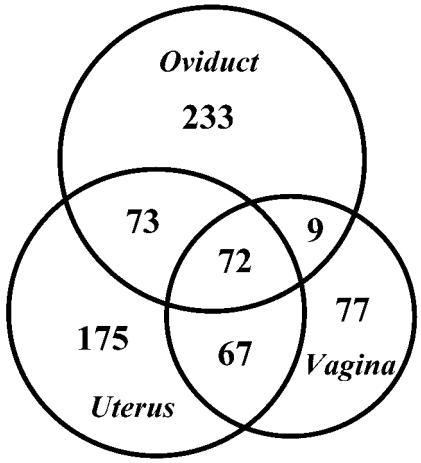
Figure 1. Clustering analysis of DES-responsive genes in the mouse oviduct, uterus, and vagina at GD 19. Each colored bar indicates the expression level of one gene: red, induction; green, repression; yellow, average expression in six groups; gray, not detected. Only genes showing more than a 2-fold change in expression following *in utero* DES exposure are shown here.

described (7). Microarray analysis was performed in triplicate using three different samples.

Data Analysis. Scanned data were analyzed with GeneChip Suit Analysis Software version 5.0 (Affymetrix Japan) to obtain the average intensity of each cell corresponding to each oligonucleotide probe. The averaged fluorescence intensity (2500) of each probe was further analyzed by dChip, a model-based expression analysis program (41), and expression levels were estimated. The PM-only model was used for the analysis, and the estimated values were transferred to the GeneSpring software program (Silicon Genetics, Redwood City, CA) and analyzed. To calculate changes in expression, genes for which average expression levels were more than 1000 fluorescence intensity units under at least one experimental condition were selected, and the average expression values of the treated samples was divided by those from control samples. For the clustering analysis, genes activated more than 2-fold by DES were selected, and similarities between experiments and expression levels were measured by standard correlation using the GeneSpring program as described (7–10). Gene expression changes were estimated by assigning the value of the control uterus as one. These selected genes were listed on http://www.nibb.ac.jp/bioenv1/suzuki/suzukidata004.html. These raw data were loaded into the National Center for Biotechnology Information's Gene Expression Omnibus as the dataset GSE1886 (GEO: http://www.ncbi.nlm.nih.gov/geo/). Categories in DES-regulated genes were determined from the GEO database. Putative target genes were validated by quantitative real-time polymerase chain reaction (Q-PCR).

Quantitative Real-Time PCR. Total RNA was purified as described above. Complementary DNA was synthesized from purified total RNA with Superscript II RT(-) (Invitrogen) and random primers at 42°C for 60 mins. PCR reactions were performed in the PE Prism 5700 Sequence Detection System (PE Biosystems, Tokyo, Japan) with

a. Up-regulated genes



b. Down-regulated genes

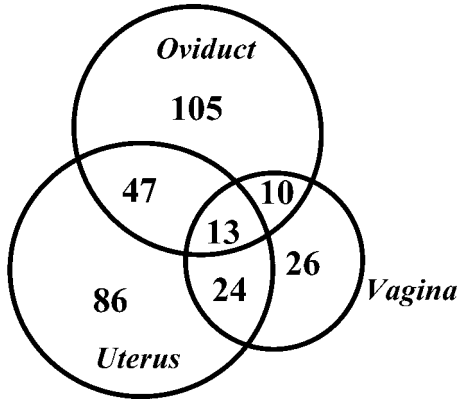


Figure 2. Venn diagrams of the number of upregulated and downregulated genes in the GD 19 oviduct, uterus, and vagina exposed to DES at GDs 10–18. (a) Number of upregulated genes by DES in the Müllerian duct. The number of DES-upregulated genes was the smallest in the vagina. (b) Number of downregulated genes by DES in the Müllerian duct.

Table 2. DES-Regulated Genes Related to Signal Transduction and Organogenesis in the Müllerian Duct at GD 19[a]

Gene accession no.	Fold change			Name
	Ovi	Ut	Vg	
BG066967	10.7	2.7	2.1	RAB20, member RAS oncogene family
BQ174703	2.5	5.1	NC	Double cortin and calcium/calmodulin-dependent protein kinase-like 1
NM_010557	5.4	7.6	NC	Interleukin-4 receptor, alpha
NM_013769	3.2	3.4	NC	Tight junction protein 3
NM_008397	2.6	3.0	NC	Integrin alpha 6
BC027196	2.3	2.9	NC	RIKEN cDNA D530020C15 gene
BC003714	2.6	2.0	NC	Calcium and integrin binding 1 (calmyrin)
NM_080795	NC	2.7	2.3	Ligand of numb-protein X 2
AF039601	NC	2.2	2.2	Transforming growth factor-beta receptor III
AA717838	NC	3.2	2.2	Interleukin-6 signal transducer
NM_021475	7.8	NC	NC	ADAM-like, decysin 1
NM_013602	5.0	NC	NC	Metallothionein 1
U42467	3.7	NC	NC	Leptin receptor
NM_008935	3.1	NC	NC	Prominin 1
M68513	2.3	NC	NC	Eph receptor A3
NM_133485	2.3	NC	NC	Protein phosphatase 1, regulatory (inhibitor) subunit 14c
BC011193	2.0	NC	NC	Prostaglandin E receptor 4 (subtype EP4)
NM_029716	2.0	NC	NC	RIKEN cDNA 0710001E19 gene
AF440694	NC	3.1	NC	Insulin-like growth factor–1 (IGF-I)
BC026642	NC	3.0	NC	Expressed sequence AW049765
NM_016798	NC	2.9	NC	PDZ and LIM domain 3
NM_007429	NC	2.7	NC	Angiotensin II receptor, type 2
NM_009365	NC	2.7	NC	Transforming growth factor-beta 1–induced transcript 1
AF350047	NC	2.6	NC	Regulator of G-protein signaling 3
BE307478	NC	2.5	NC	Ectonucleoside triphosphate diphosphohydrolase 1
AI788797	NC	2.1	NC	Utrophin
NM_019417	NC	2.1	NC	Reversion-induced LIM gene
NM_025278	NC	2.0	NC	Guanine nucleotide (G) binding protein gamma 12
NM_007706	NC	2.0	NC	Suppressor of cytokine signaling 2
BC015254	NC	NC	2.3	Chemokine orphan receptor 1
BB447551	NC	NC	2.3	GATA binding protein 5
BB751088	NC	NC	2.0	G-protein–coupled receptor 49
NM_011196	0.2	0.3	0.2	Prostaglandin E receptor 3 (subtype EP3)
NM_013869	0.3	0.2	0.5	Tumor necrosis factor receptor superfamily, member 19
BC026153	0.3	0.2	0.5	Eph receptor A7
BM946869	0.2	0.2	NC	Stathmin-like 2
BB751088	0.2	0.4	NC	G-protein–coupled receptor 49
NM_019583	0.5	0.3	NC	Interleukin-17 receptor B
BB453355	0.5	0.5	NC	Ephrin B2
AK018789	0.4	NC	NC	Neurotrophic tyrosine kinase, receptor, type 2
AF209905	0.4	NC	NC	Calcitonin receptor-like
NM_133248	0.4	NC	NC	Glomulin, FKBP-associated protein
AW493905	0.5	NC	NC	G-protein–coupled receptor 23
NM_007936	0.5	NC	NC	Eph receptor A4
AK018032	0.5	NC	NC	SH3-domain kinase binding protein 1
AK018504	0.5	NC	NC	Ras association (RalGDS/AF-6) domain family 2
NM_013518	NC	0.4	NC	Fibroblast growth factor 9 (Fgf9)
NM_008016	NC	0.4	NC	Fibroblast growth factor inducible 15
U38501	NC	0.5	NC	Guanine nucleotide–binding protein, alpha inhibiting 1
BC005799	NC	NC	0.5	RIKEN cDNA 5830484J08 gene
BC010581	NC	NC	0.4	Stathmin 1
BC005475	NC	NC	0.4	RIKEN cDNA E430018M08 gene
Organogenesis				
NM_007921	2.7	4.6	2.2	E74-like factor 3
NM_015814	4.2	2.2	NC	Dickkopf homolog 3 (*Xenopus laevis*) (Dkk3)
NM_020265	2.8	4.1	NC	Dickkopf homolog 2 (*Xenopus laevis*) (Dkk2)
L13204	5.8	NC	NC	Forkhead box J1 (Foxj1)
NM_010135	4.4	NC	NC	Enabled homolog (Drosophila)
AK006314	2.4	NC	NC	Spermatid perinuclear RNA binding protein

Table 2. (Continued)

Gene accession no.	Fold change Ovi	Ut	Vg	Name
NM_024226	2.3	NC	NC	Reticulon 4
AW538200	2.1	NC	0.3	Filamin, beta
BM119387	2.1	NC	NC	Villin 2
AI462296	NC	2.5	2.2	Forkhead box O1
BB151515	NC	6.0	2.2	Nerve growth factor receptor
BB759833	NC	NC	3.5	Forkhead box C1
NM_009523	NC	NC	2.4	Wnt-4
BF141691	0.3	0.2	0.3	Naked cuticle 2 (Nkd2) homolog (Drosophila)
L42114	0.1	0.5	NC	Growth differentiation factor 10
NM_009152	0.3	0.3	NC	Semaphorin 3A
BQ176610	0.4	0.3	NC	Semaphorin 5A
NM_009526	0.5	0.4	NC	Wnt-6
BC019150	0.3	0.4	NC	Hoxd-9
BI658627	0.4	0.5	NC	Serected frizzled-related sequence protein 1 (sFRP1)
AK004683	NC	0.1	0.1	Wnt-7a
NM_013601	NC	0.5	0.4	Homeobox, msh-like 2 (Msx2)
BC013463	0.1	NC	NC	Hoxd-10
NM_010450	0.5	NC	NC	Hoxa-11
AK007893	0.2	NC	NC	Sclerostin domain containing 1
D78264	0.3	NC	NC	Olfactomedin 1
NM_010698	0.5	NC	NC	LIM domain binding 2
BC016426	NC	0.1	NC	Homeobox, msh-like 1 (Msx1)
NM_021457	NC	0.4	NC	Frizzled homolog 1 (Drosophila)
AK019458	NC	0.4	NC	Myeloid/lymphoid or mixed lineage-leukemia translocation to 3 homolog (Drosophila)
NM_009519	NC	0.5	NC	Wnt-11
AW107802	NC	0.5	NC	Glypican 3
NM_013598	NC	0.5	NC	Kit ligand
NM_010496	NC	NC	0.4	Inhibitor of DNA binding 2
BG065227	NC	NC	0.5	Tripartite motif protein 37
AF153440	NC	NC	0.5	BMP and activin membrane-bound inhibitor, homolog

[a] Fold change means ratio vs. organ-matched oil controls. NC means no change included with less than 2-fold change and more than 0.5-fold change. Ovi, oviduct; Ut, uterus; Vg, vagina.

SYBR-Green PCR core reagents (Applied Biosystems Japan, Tokyo, Japan) in the presence of the appropriate primers according to the manufacturer's instructions. The primers were chosen to amplify short PCR products of less than 100 base pairs, and their sequences are listed in Table 1.

Each PCR amplification was performed in triplicate using the following conditions: 2 mins at 50°C and 10 mins at 95°C, followed by a total of 40 two-temperature cycles (15 secs at 95°C and 1 min at 60°C). Model 7000 software (Applied Biosystems, Foster City, CA) was used to construct amplification plots from extension-phase fluorescent emission data collected during PCR amplification. Threshold ($C\tau$) values were calculated by determining the point at which fluorescence exceeds a threshold limit.

Gene expression levels were normalized to the expression levels of ribosomal protein L8 mRNA (U67771), and changes in concentration were calculated. Gel electrophoresis and melting curve analyses were performed to confirm correct amplicon size and the absence of nonspecific bands. Quantification of mRNAs was repeated three times with independent mice, and average levels of change were calculated. Statistical analyses of the Q-PCR data were conducted by ANOVA. Q-PCR data were expressed as the relative expression of each gene to that of the control uterus in order to compare differences in gene expression among the three organs derived from the Müllerian duct.

Results

DNA Microarray Analysis. We examined gene expression in the oviduct, uterus, and vagina at GD 19 in DES-exposed mice and oil controls. The correlation coefficients of microarray chips averaged 0.980 (minimum: 0.967; maximum: 0.996). As described above, genes showing at least a 2-fold expression change in DES-exposed mice have been listed at http://www.nibb.ac.jp/bioenv1/suzuki/suzukidata004.html.

To examine the gene expression changes by DES in the three organs of Müllerian duct origin, we analyzed the clustering pattern of DES-regulated genes. Clustering analysis in controls revealed that there was organ specificity in gene expression. Gene expression profiles in the uterus

Table 3. DES-Regulated Common Genes in the Three Organs Derived from Müllerian Duct at GD 19[a]

Gene accession no.	Fold change			Name
	Ovi	Ut	Vg	
Upregulated				
NM_023268	2.0	2.4	2.2	Quiescin Q6
BB284358	2.1	3.4	2.0	EGL nine homolog 3 (*C. elegans*)
BB253720	2.2	2.4	2.1	Procollagen-proline, 2-oxoglutarate 4-dioxygenase, alpha 1 polypeptide
AK009873	2.3	4.8	2.7	RIKEN cDNA 2310047E01 gene
NM_007955	2.4	3.0	2.2	Protein tyrosine phosphatase, receptor type, V
NM_011961	2.4	3.5	2.4	Procollagen lysine, 2-oxoglutarate 5-dioxygenase 2
AV003026	2.4	2.9	2.8	Glutathione *S*-transferase omega 1
AW558468	2.5	2.7	2.4	Natriuretic peptide receptor 2
BG072404	2.5	4.1	2.0	RIKEN cDNA 4631427C17 gene
BC016131	2.5	2.4	2.2	DNA segment, Chr 14, ERATO Doi 813, expressed
NM_008062	2.5	2.6	2.0	Glucose-6-phosphate dehydrogenase X-linked
BB546344	2.5	3.8	3.1	Dehydrogenase/reductase (SDR family) member 8
NM_013650	2.5	4.8	2.9	S100 calcium binding protein A8 (calgranulin A)
NM_013864	2.6	3.5	2.4	*N*-myc downstream regulated 2
BC025020	2.6	2.8	3.1	RIKEN cDNA 2810049G06 gene
BC019434	2.6	3.5	2.1	UDP-glucuronosyltransferase 1 family, member 1
NM_007921	2.7	4.6	2.2	E74-like factor 3
BC026209	2.7	2.8	2.6	Arachidonate 5-lipoxygenase activating protein
NM_009760	2.7	2.8	2.3	BCL2/adenovirus E1B 19-kDa interacting protein 1, NIP3
BC014753	2.7	4.6	2.3	Hydroxysteroid 11-beta dehydrogenase 2
BM209618	2.7	2.1	2.6	Mus musculus cDNA clone C0650E08 3', mRNA sequence.
NM_015786	2.8	3.3	2.3	Histone 1, H1c
NM_013703	2.8	2.7	2.9	Very low-density lipoprotein receptor
BM242294	2.8	2.9	2.4	RIKEN cDNA 6330500D04 gene
NM_019468	2.9	2.7	2.5	Glucose-6-phosphate dehydrogenase X-linked
BB533903	2.9	3.0	2.3	Histone 1, H1c
NM_010050	3.0	12.1	5.9	Deiodinase, iodothyronine, type II
BB114323	3.1	3.3	2.4	3′ similar to X85991 M.musculus mRNA for semaphorin B
NM_013655	3.2	2.3	3.7	Chemokine (C-X-C motif) ligand 12
D87867	3.2	4.0	2.1	UDP-glucuronosyltransferase 1 family, member 1
BC021352	3.3	3.8	3.4	Procollagen lysine, 2-oxoglutarate 5-dioxygenase 2
D87867	3.3	4.6	2.2	UDP-glucuronosyltransferase 1 family, member 1
BM209618	3.3	2.1	2.6	Mus musculus cDNA clone C0650E08 3′, mRNA sequence
BC013477	3.4	9.7	3.7	Alcohol dehydrogenase 1 (class I)
NM_008735	3.4	3.1	2.4	Mus musculus nuclear receptor interacting protein 1 (Nrip1)
BB353211	3.5	18.6	18.3	Inhibin beta-B
BC005486	3.6	2.9	2.6	E26 avian leukemia oncogene 2, 3′ domain
M27695	3.6	3.1	3.1	Urate oxidase
BM239615	3.6	8.5	3.3	Testis-expressed gene 2
NM_011926	3.7	7.2	2.4	CEA-related cell adhesion molecule 1
BM212947	3.7	2.1	2.0	Transmembrane protease, serine 2
AV171622	3.8	3.0	2.3	RIKEN cDNA 3300001H21 gene
NM_011361	4.0	4.1	3.2	Serum/glucocorticoid regulated kinase
BC020531	4.1	2.5	2.1	Spondin 1, (f-spondin) extracellular matrix protein
BC021770	4.2	10.8	4.7	Claudin 10
NM_007799	4.3	3.6	4.7	Cathepsin E
AK012175	4.3	5.1	2.8	RIKEN cDNA 2610528J11 gene
BM207588	4.3	2.2	3.2	Mus musculus transcribed sequences
NM_011488	4.5	2.8	2.6	Signal transducer and activator of transcription 5A
AV171622	4.5	4.3	2.2	RIKEN cDNA 3300001H21 gene
BC024886	4.5	9.0	6.6	Coagulation factor III
NM_053262	4.7	7.5	5.5	Dehydrogenase/reductase (SDR family) member 8
NM_019511	4.8	10.4	12.3	Receptor (calcitonin) activity modifying protein 3
BC022950	4.8	12.1	6.8	RIKEN cDNA 1600029D21 gene
D85596	5.0	6.6	3.4	AMP deaminase 3
BC003705	5.1	8.9	10.3	Surfactant associated protein D
U36502	5.8	4.0	3.2	Signal transducer and activator of transcription 5A
BG069413	6.0	9.3	4.4	Kruppel-like factor 4 (gut)
NM_033648	6.3	5.4	2.9	FXYD domain-containing ion transport regulator 4

Table 3. (Continued)

Gene accession no.	Fold change Ovi	Ut	Vg	Name
NM_133662	6.6	3.5	4.7	Immediate early response 3
AK004847	6.6	10.9	2.7	Ring finger protein 128
NM_019517	6.7	8.1	3.9	Beta-site APP-cleaving enzyme 2
X14607	6.7	27.5	8.2	Lipocalin 2
U20344	6.9	13.0	5.3	Kruppel-like factor 4 (gut)
NM_023270	8.0	11.3	3.2	Ring finger protein 128
NM_009801	9.0	7.6	3.0	Carbonic anhydrase 2
NM_025867	9.8	22.1	12.0	RIKEN cDNA 2310046M08 gene
BG066967	10.7	2.7	2.1	RAB20, member RAS oncogene family
BC026595	11.3	3.6	2.0	Cystathionine beta-synthase
NM_134072	13.9	23.9	10.4	RIKEN cDNA 9030611N15 gene
BC010758	23.3	294.2	15.8	Carbonyl reductase 2
X17502	27.3	17.3	6.7	Branched chain aminotransferase 1, cytosolic
Downregulated				
NM_010780	0.24	0.29	0.31	Mast cell protease 5
X96585	0.37	0.28	0.46	Nephroblastoma overexpressed gene
NM_007731	0.36	0.25	0.35	Procollagen, type XIII, alpha 1
NM_011607	0.36	0.46	0.37	Tenascin C
BF141691	0.26	0.19	0.31	Naked cuticle 2 homolog (Drosophila)
BC019952	0.35	0.21	0.38	Naked cuticle 2 homolog (Drosophila)
NM_013869	0.25	0.23	0.46	Tumor necrosis factor receptor superfamily, member 19
BC026153	0.25	0.24	0.45	Eph receptor A7
AF167554	0.29	0.27	0.40	Tumor necrosis factor receptor superfamily, member 19
NM_011196	0.23	0.29	0.19	Prostaglandin E receptor 3 (subtype EP3)
NM_007548	0.34	0.17	0.41	PR domain containing 1, with ZNF domain
NM_031374	0.16	0.22	0.40	Testis expressed gene 15
AV024662	0.39	0.30	0.38	RIKEN cDNA 3732412D22 gene
BM117827	0.41	0.31	0.37	RIKEN cDNA 3732412D22 gene
BG076147	0.18	0.33	0.48	Lipin 3

[a] Fold change means ratio vs. organ-matched oil controls. Ovi, oviduct; Ut, uterus; Vg, vagina.

were more similar to those of the oviduct than the vagina. However, clustering analysis in DES-exposed mice revealed that DES-regulated genes in the three organs showed less organ specificity compared with controls (Fig. 1).

DES exposure upregulated 387, 387, and 225 genes and downregulated 177, 172, and 75 genes in the oviduct, uterus, and vagina, respectively (Fig. 2). We observed 72 genes that were upregulated and 15 downregulated in common in the three organs. The number of genes showing altered expression by DES was largest in the oviduct and smallest in the vagina (Fig. 2).

We focused on genes related to signal transduction and organogenesis in DES-exposed Müllerian ducts (Table 2). Expressions of *RAB 20* and *E74-like factor 3* were upregulated in all DES-exposed organs. In contrast, expressions of *prostaglandin E receptor 3*, *tumor necrosis factor receptor superfamily member 19*, *Eph receptor A7*, and *naked cuticle 2* (*Nkd2*) were downregulated in all DES-exposed organs (Table 2).

Several organ-specific genes were encountered in DES-exposed mice (Table 2). *Forkhead box J1* (*foxj1*), expressed in ciliated cells in the oviduct (41), was one of the oviduct-specific genes upregulated in DES-exposed oviducts, whereas expression of homeobox, *msh-like 1* (*Msx1*) and *fibroblast growth factor 9* (*Fgf9*) was upregulated and that of *insulin-like growth factor-I* (*IGF-I*) was downregulated in the DES-exposed uterus only. In DES-exposed oviduct and uterus, *Dickkopf* (*Dkk*) *homologs 2* and *3* were upregulated, whereas *ephrin B2*, *growth differential factor 10*, and *secreted frizzled-related sequence protein 1* (*sFRP1*) were downregulated (Table 2).

Hoxa-11 and *Hoxd-10* were repressed in DES-exposed oviducts. Moreover, expression of *Hoxd-9* was downregulated in DES-exposed oviduct and uterus (Table 2). *Wnt-4* gene was induced only in DES-exposed vagina. *Wnt-6*, *Wnt-7a*, and *Wnt-11* genes were commonly downregulated in DES-exposed uterus.

Four genes of the Eph family, *ephrin B2* and *Eph receptor A3*, *A4*, and *A7*, and three Wnt antagonists showed altered expressions in DES-exposed female reproductive tracts (Table 2). Thus, we further studied genes of the Hoxa, Wnt, and Eph families and Wnt antagonists by Q-PCR.

The DES-regulated common genes in the three organs derived from Müllerian duct at GD 19 are listed in Table 3.

Expression of Hox and Wnt Genes in Female Reproductive Tracts by Q-PCR. At GD19, Q-PCR

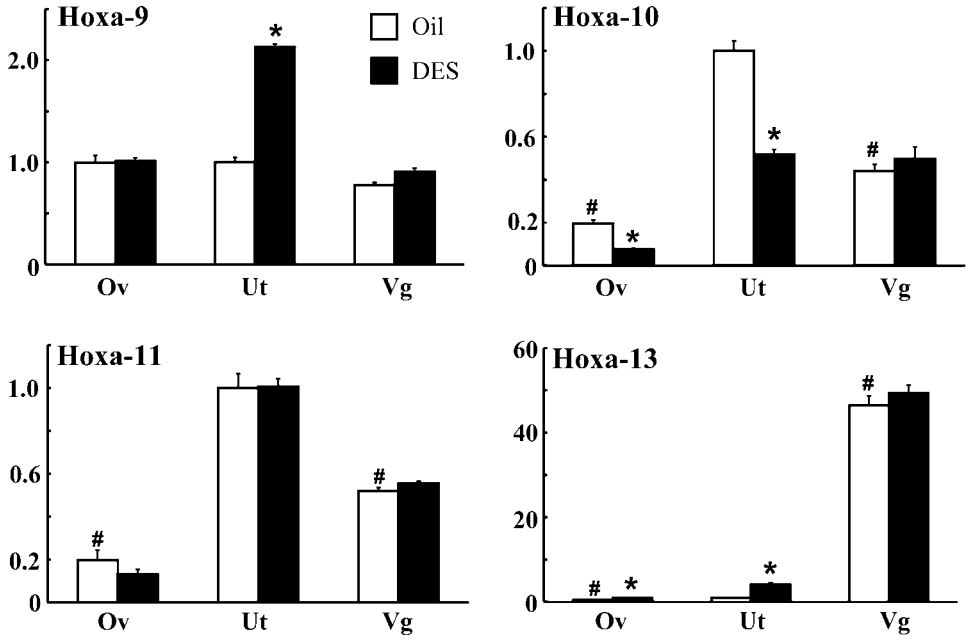


Figure 3. Quantification of Hoxa-9, a-10, a-11, and a-13 mRNA expressions in the oviduct, uterus, and vagina at GD 19 using Q-PCR. Results were normalized by ribosomal L8 expression. Ratios were calculated relative to expression levels in the control uterus. Ov, oviduct; Ut, uterus; Vg, vagina. #$P < 0.05$ vs. control uterus; *$P < 0.05$ vs. organ-matched control groups.

revealed that DES downregulated *Hoxa-10* mRNA in the oviduct (Fig. 3), whereas in the uterus *Hoxa-9* mRNA was upregulated but *Hoxa-10* was downregulated. DES did not alter the expression of *Hoxa-11* in any of the organs studied, nor did we observe a change in the expression of *Hoxa-13* in the vagina. Upregulation of *Hoxa-13* mRNA expression was observed in the oviduct and uterus following DES exposure. Expression of *Wnt-7a* mRNA was repressed by DES. Expression of *Wnt-5a* mRNA was elevated by DES in all organs. The expression of *Wnt-4* was repressed in the oviduct. In the DES-treated uterus, the expression of *Wnt-4* showed a tendency toward repression, but it did not reach statistical significance ($P = 0.06$ vs. organ-matched control). Although upregulation of *Wnt-4* mRNA expression was

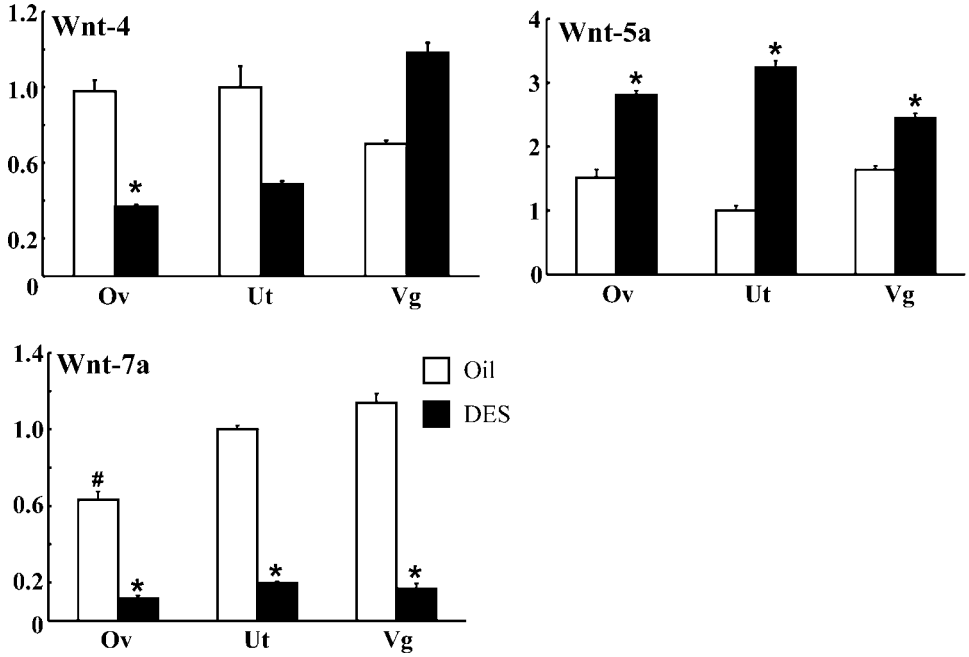


Figure 4. Quantification by Q-PCR of Wnt-4, –5a, and –7a mRNA expressions in the oviduct, uterus, and vagina at GD 19 following exposure to DES at GDs 10–18. Results were normalized by ribosomal L8 expression. Ratios were calculated relative to expression levels in the control uterus. Ov, oviduct; Ut, uterus; Vg, vagina. #$P < 0.05$ vs. control uterus; *$P < 0.05$ vs. organ-matched control groups.

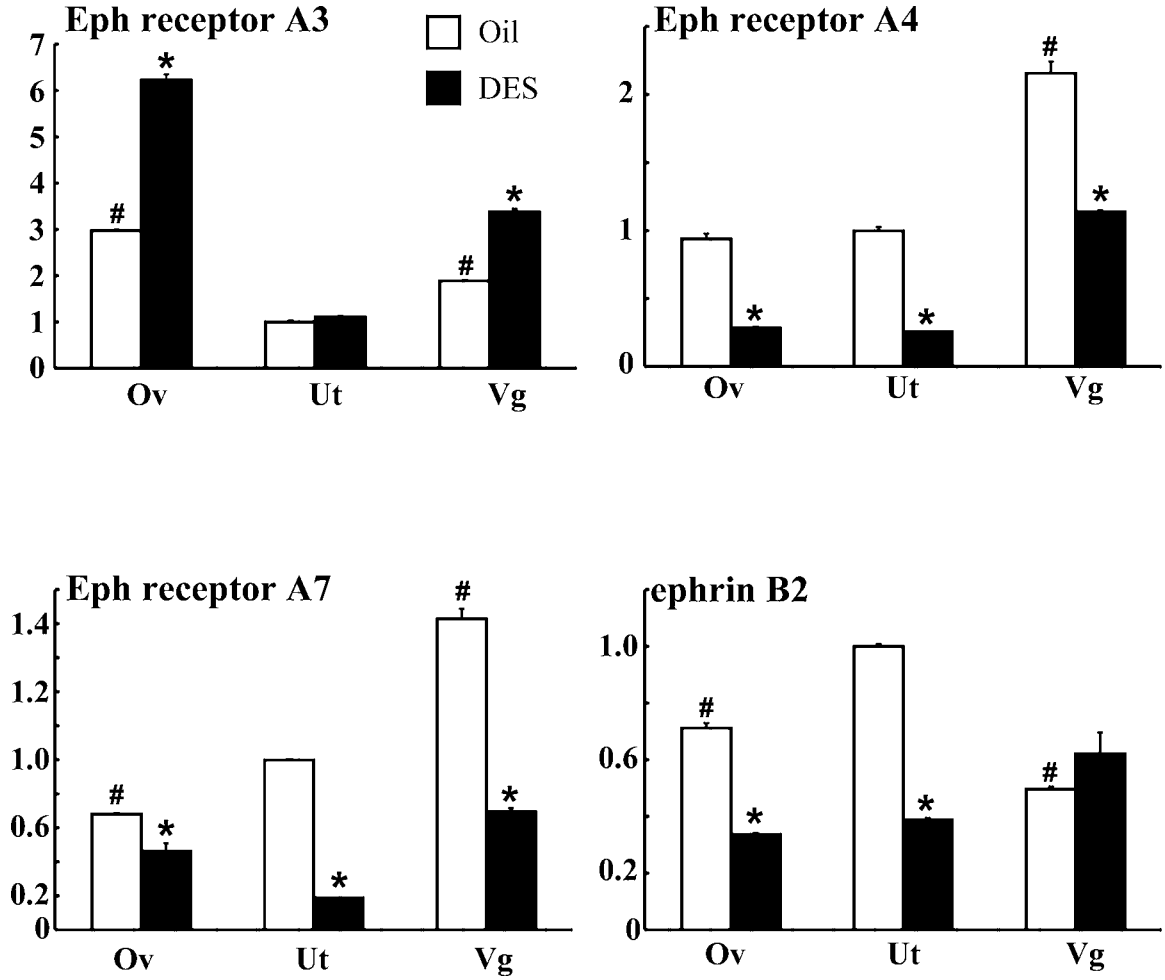


Figure 5. Quantification by Q-PCR of ephrin B2 and Eph receptors A3, A4, and A7 mRNA expressions in the oviduct, uterus, and vagina at GD 19 following exposure to DES at GDs 10–18. Results were normalized by ribosomal L8 expression. Ratios were calculated relative to expression levels in the control uterus. Ov, oviduct; Ut, uterus; Vg, vagina. #$P < 0.05$ vs. control uterus; *$P < 0.05$ vs. organ-matched control groups.

observed in the DES-treated vagina, it also did not reach statistical significance ($P = 0.06$ vs. organ-matched control; Fig. 4).

Gene Expression of Eph Family and Wnt Antagonists in the Müllerian Duct. After DES exposure, *ephrin B2* mRNA was downregulated in the oviduct and uterus, whereas *Eph receptor A3* expression was upregulated in the oviduct and vagina. *Eph receptor A4* and *A7* mRNA were downregulated by DES in all organs studied (Fig. 5).

In DES-exposed mice, expression of *Dkk2* mRNA was upregulated in the oviduct and uterus but downregulated in the vagina (Fig. 6). Expression of *Nkd2* mRNA was downregulated by DES in all organs studied, whereas expression of *sFRP1* mRNA was downregulated in the oviduct and uterus only (Fig. 6).

Some of the microarray (Table 2) and Q-PCR (Figs. 5 and 6) data were not consistent for *Eph receptor A3*, *Eph receptor A4*, *Nkd2*, and *sFRP1*. Therefore, we relied on the Q-PCR data for this discussion.

Discussion

In utero exposure to DES has been repeatedly observed to induce various reproductive abnormalities in mice and humans (40, 43, 44). DES-induced malformations of the reproductive organs have been hypothesized to be caused by a disruption in the expression of *Hoxa* genes along the anterior to posterior axis of the developing Müllerian duct (17). Furthermore, Wnt signaling regulates and maintains Hoxa gene expression in the Müllerian duct (45). DES-induced repression of *Wnt-7a* gene has been linked to developmental effects on the mouse reproductive tract (17, 46, 47).

Previous studies reported downregulation of *Hoxa-10* and *a-11* at GD 17 in the DES-exposed uterus and downregulation of *Hoxa-9* in the DES-exposed oviduct (17, 18). In the present study, we confirmed the decrease in *Hoxa-10*, but we did not observe a change in uterine *Hoxa-11* expression following DES exposure. Four antisense cDNAs for *Hoxa-11* have been described in a cDNA library from the mouse embryonic limb (48). It may be that changes

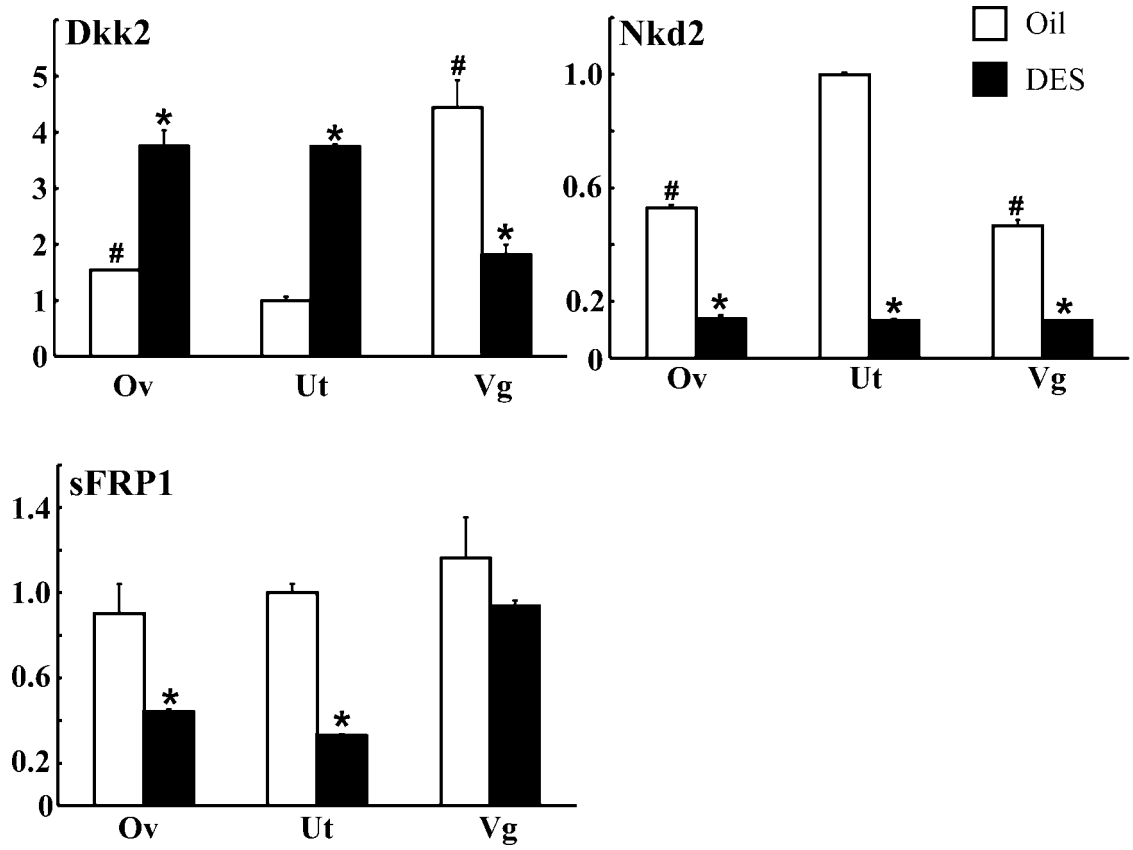


Figure 6. Quantification by Q-PCR of DKK2, Nkd2, and sFRP1 mRNA expression in the oviduct, uterus, and vagina at GD 19 following exposure to DES at GDs 10–18. Results were normalized by ribosomal L8 expression. Ratios were calculated relative to expression levels in the control uterus. #$P < 0.05$ vs. control uterus; *$P < 0.05$ vs. organ-matched control groups.

in uterine *Hoxa-11* mRNA were not detected by our Q-PCR because of the presence of an antisense strand DNA.

In the present study, DES did not downregulate expression of *Hoxa-13* mRNA in the vagina at GD 19, yet the same DES treatment *in utero* has been reported to induce ovary-independent vaginal stratification and cornification in mice (40). Thus, the ovary-independent vaginal changes may not be related to changes in *Hoxa-13* expression. Interestingly, cluster analysis performed here revealed that the pattern of gene expression in the vagina, either in the control or DES-treated animals, differed significantly from those of the oviduct and uterus. *Hoxa-10* expression is required for oviductal formation and uterine growth (14, 15). The molecular mechanisms of growth and differentiation in the caudal Müllerian duct are apparently different from the other regions of the Müllerian duct–derived reproductive system.

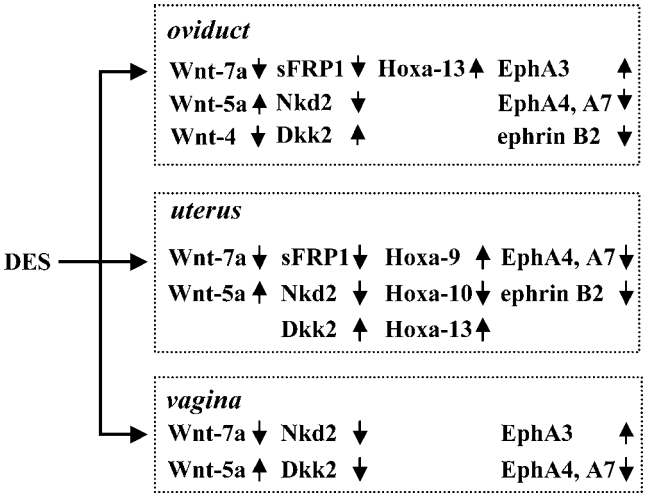


Figure 7. Summary of mRNA expression of Eph family, Wnt, Wnt antagonists, and Hoxa genes in the DES-exposed Müllerian duct. Eph, Ephrin receptor.

Dkk2 acts as an antagonist of Wnt signaling to induce endocytosis of the Wnt-Fz receptor complex and is activated by β-catenin (49). In the present study, DES downregulated *Dkk2* expression in the vagina but upregulated it in the oviduct and uterus. Wnt signaling regulates vaginal growth and differentiation by epithelial–mesenchymal interaction. The importance of epithelial–stromal interaction has been previously reported as an important factor mediating the developmental effects of estrogens, including DES, in the vaginal epithelium of the neonatal mouse, which is mediated through the action of stromal estrogen receptors (50, 51). Loss of *Wnt-7a* expression caused vaginal adenosis and concretions (30), whereas loss of *Wnt-5a* expression caused

the absence of the vagina (52). During the perinatal period, the developing vagina expressed *Wnt-5a* and *–7a* but not *Wnt-4*. Expression of *Wnt-7a* in the vagina disappeared by 10 days of age, and adult vagina expressed *Wnt-4* and *–5a* genes in the epithelium only (27). In the normal neonatal vagina, Wnt-7a regulates the reduction of *Wnt-4* expression (27). However, in DES-exposed vagina, the reduction of *Wnt-7a* expression may cause a reduction of *Dkk2* expression. Thus, vaginal epithelial cells in the DES-exposed fetus differentiate into squamous cells like those seen in an adult, followed by repression of *Dkk2*.

DES repressed expression of *Nkd2* and *sFRP1* in the oviduct and uterus, and *Nkd2* in the vagina. This is the first report showing expression of Wnt antagonists and their estrogen regulation in organs derived from the Müllerian duct. Further studies are needed to clarify the role of Wnt antagonists during development of the Müllerian duct.

Eph receptor–ephrin signaling is a trigger regulating developmental patterning (26). Eph family genes are downstream genes of Hox genes (23, 53). Hoxa-9 directly regulates the transcription of *Eph receptor B4* in endothelial cells, followed by increased cell migration and tube formation (53). In embryo limb, misexpression of *Hoxa-13* caused downregulation of the *Eph receptor A7*, resulting in an inhibition of apoptosis (23). In the present study, DES-induced downregulation of *ephrin B2* mRNA, as well as Hoxa genes, was found in the oviduct and uterus. Moreover, DES exposure downregulated *Eph receptor A4* and *A7* expression in all three organs. The Eph family of proteins may regulate pattern development in the Müllrerian duct by inducing changes in cytoskeleton dynamics, mitogenesis, and integrin signaling, as reported in other organ systems (25).

In Figure 7 we summarized the expression change of Eph families, Wnt, Wnt antagonist, and Hox genes induced by DES *in utero* in the three organs derived from the mouse Müllerian duct. Further studies are needed to understand the functional relationship among these genes in the developing mouse reproductive tract and their relationship to reproductive tract abnormalities induced by DES.

Some of the microarray data and Q-PCR data were not consistent for *Hoxa-11* in the oviduct, *Eph receptor A3* in the vagina, *Eph receptor A4* in all organs, *Nkd2* in the vagina, and *sFRP1* in the oviduct. Therefore, we relied on the Q-PCR data for discussion. Recently, a new microarray method has been proposed that uses a "per cell" normalization method for mRNA measurement (54), which will provide a more consistent correlation between data sets derived from microarray and Q-PCR analyses.

In conclusion, microarray analysis revealed the presence of organ-specific changes in gene expression profiles in the oviduct, uterus, and vagina following DES exposure, thus providing for future study candidate genes that could be related to the reproductive abnormalities reported following embryonic or neonatal exposure to this estrogenic pharmaceutical agent. About 400 genes were upregulated and 200 genes were downregulated in the oviduct and uterus by DES exposure *in utero*. The vagina showed changes in fewer than half the number of DES-regulated genes than those found in the oviduct and uterus. Downregulation of *ephrin B2*, *Eph receptors A4* and *A7*, and *Nkd2*, accompanied with changes in Hox and Wnt gene expression, could lead to abnormalities of segment-related positional identity in the upper part of the Müllrerian duct following DES exposure. In addition, the downregulation of *Nkd2* and *Dkk2* in DES-exposed vagina needs to be studied in more detail to determine whether these factors contribute to the persistent vaginal epithelial stratification seen in DES-exposed females.

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