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

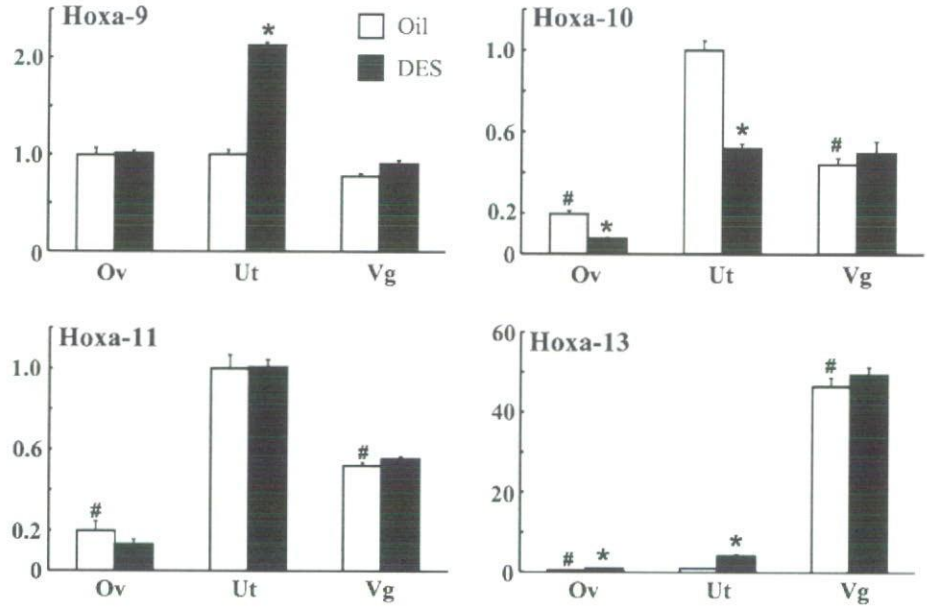

Figure 3. Quantification of Hoxa-9, a-10, a-11, and a-13 mRNA expressions in the oviduct, uterus, and vagina at GD 19 using Q-PCR. Results were normalized by ribosomal L8 expression. Ratios were calculated relative to expression levels in the control uterus. Ov, oviduct; Ut, uterus; Vg, vagina. #$P 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

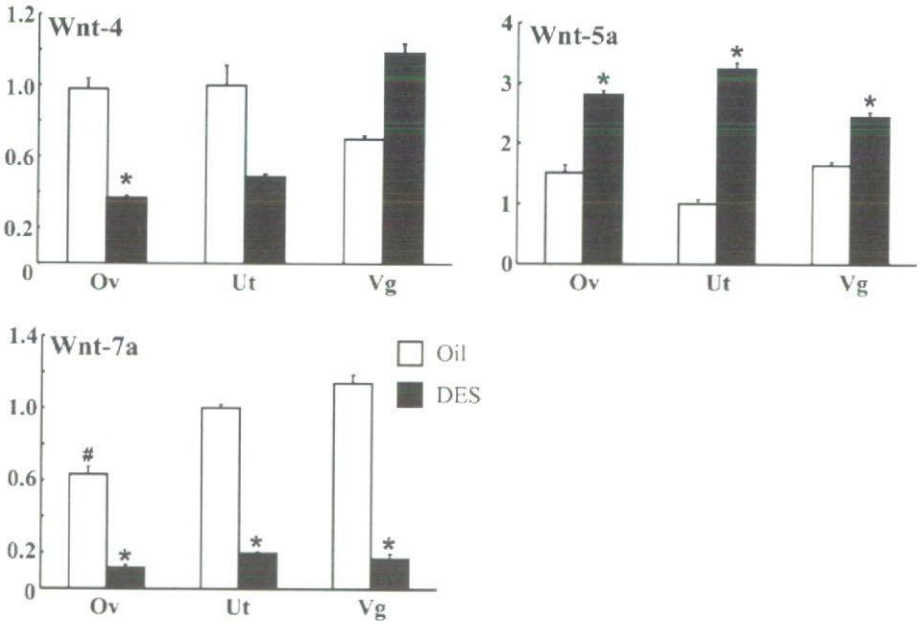

Figure 4. Quantification by Q-PCR of Wnt-4, –5a, and –7a mRNA expressions in the oviduct, uterus, and vagina at GD 19 following exposure to DES at GDs 10–18. Results were normalized by ribosomal L8 expression. Ratios were calculated relative to expression levels in the control uterus. Ov, oviduct; Ut,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

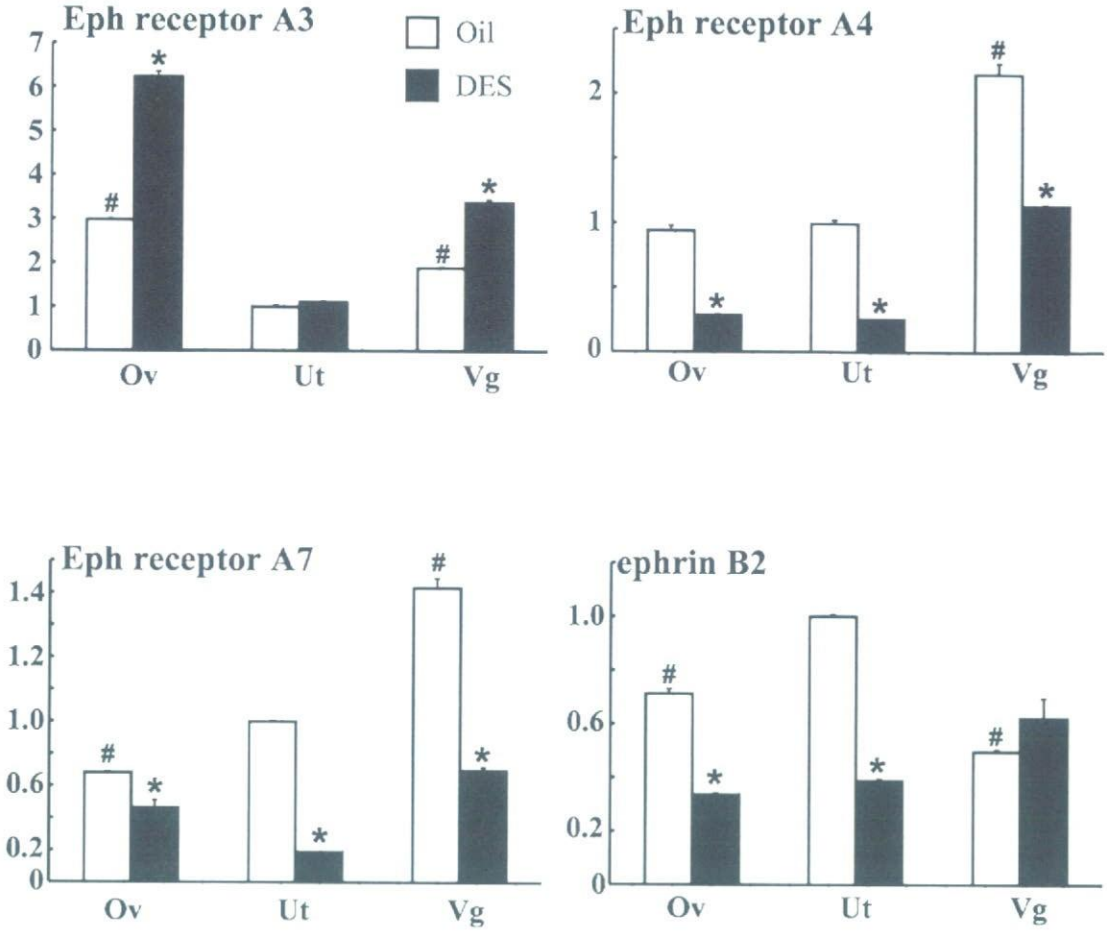

Figure 5. Quantification by Q-PCR of ephrin B2 and Eph receptors A3, A4, and A7 mRNA expressions in the oviduct, uterus, and vagina at GD 19 following exposure to DES at GDs 10–18. Results were normalized by ribosomal L8 expression. Ratios were calculated relative to expression levels in the control 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

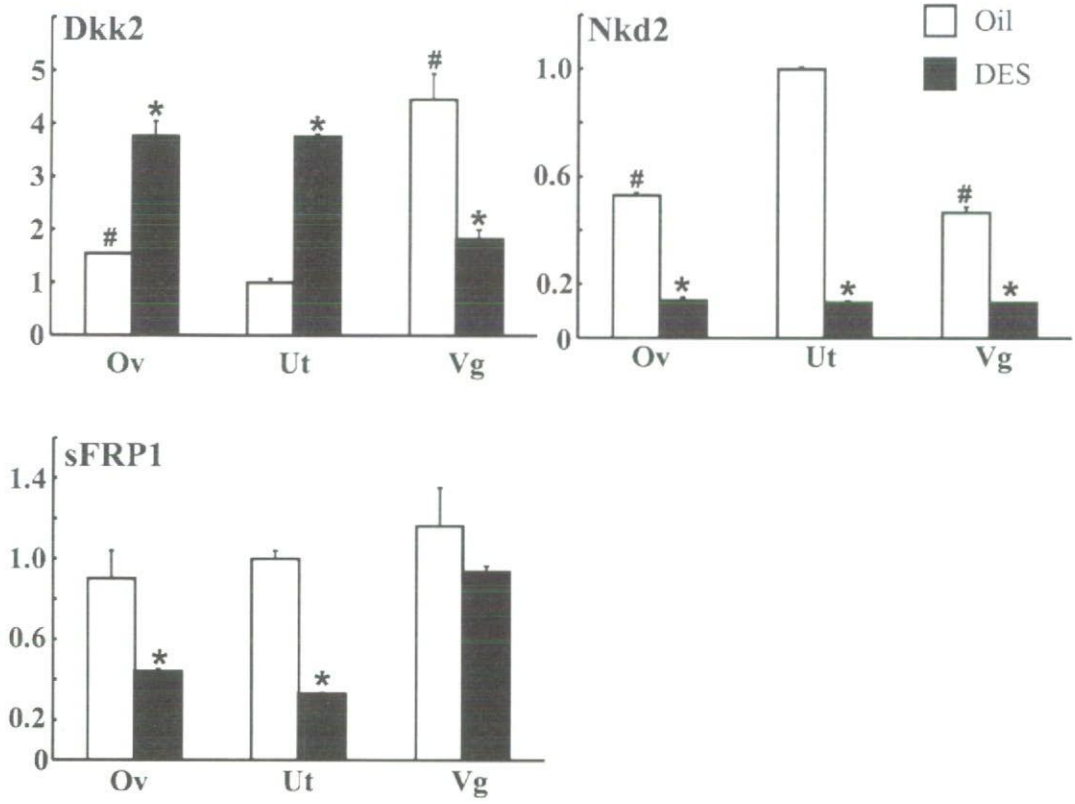

Figure 6. Quantification by Q-PCR of DKK2, Nkd2, and sFRP1 mRNA expression in the oviduct, uterus, and vagina at GD 19 following exposure to DES at GDs 10–18. Results were normalized by ribosomal L8 expression. Ratios were calculated relative to expression levels in the control uterus. #$P < 0.05$ vs.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

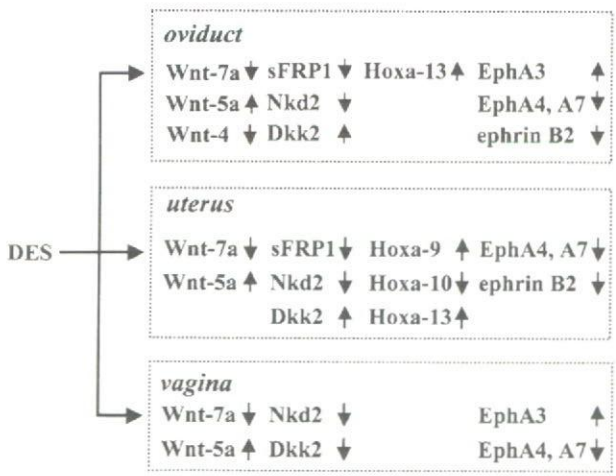

Figure 7. Summary of mRNA expression of Eph family, Wnt, Wnt antagonists, and Hoxa genes in the DES-exposed Müllerian duct. Eph, Ephrin receptor.

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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Endocrine Disrupting Organotin Compounds are Potent Inducers of Imposex in Gastropods and Adipogenesis in Vertebrates

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Abstract

The persistent and ubiquitous environmental contaminant, tributyltin chloride (TBT), induces not only imposex in gastropods but also the differentiation of adipocytes *in vitro* and increases adipose mass *in vivo* in vertebrates. TBT is a nanomolar affinity ligand for retinoid X receptor (RXR) in the rock shell (*Thais clavigera*) and for both the RXR and the peroxisome proliferator activated receptor γ (PPARγ) in the amphibian (*Xenopus laevis*), mouse, and human. The molecular mechanisms underlying induction of imposex by TBT have not been clarified, though several hypotheses are proposed. TBT promotes adipogenesis in the murine 3T3-L1 cell model and perturbs key regulators of adipogenesis and lipogenic pathways *in vivo* primarily through activation of RXR and PPARγ. Moreover, *in utero* exposure to TBT leads to strikingly elevated lipid accumulation in adipose depots, liver, and testis of neonate mice and results in increased adipose mass in adults. In *X. laevis*, ectopic adipocytes form in and around gonadal tissues following organotin, RXR or PPARγ ligand exposure. TBT represents the first example of an environmental endocrine disrupter that promotes adverse effects from gastropods to mammals.

Keywords: TBT, Gastropods, *Xenopus laevis*, Mouse, Human, RXR, PPARγ, Imposex, Adipogenesis

Organotins are a diverse group of widely distributed environmental pollutants. Tributyltin chloride (TBT) and bis (triphenyltin) oxide (TPT), have pleiotropic adverse effects on both invertebrate and vertebrate endocrine systems. Organotins were first used in the mid-1960s as antifouling agents in marine shipping paints, although such use has been strictly restricted in recent years. Organotins persist as prevalent contaminants in dietary sources, such as fish and shellfish, and through pesticide use on high value food crops[1,2]. Additional human exposure to organotins may occur through their use as antifungal agents in wood treatments, industrial water systems and textiles. Mono- and di-organotins are widely used as stabilizers in the manufacture of polyolefin plastics (polyvinyl chloride), which introduces the potential for transfer by contact with drinking water and foods.

Exposure to organotins such as TBT and TPT results in imposex, the superimposition of male sex characteristics in (or "on") female gastropod mollusks[3-5]. Imposex results in impaired reproductive fitness or sterility in severely affected animals and TBT exposure represents one of the most clear cut examples of environmental endocrine disruption. TBT exposure also leads to masculinization of at least two fish species[6,7]. In contrast, TBT exposure results in slight effects on the mammalian reproductive tracts and has not been reported to alter sex ratios[8,9]. Hepatic-, neuro- and immunotoxicity are reported to be the major effects of organotin exposure in mammals[10]. Our current understanding of how organotins disrupt the endocrine system is based on how organotins affect the expression or activity of steroid regulatory enzymes such as P450 aromatase together with less specific toxic effects resulting from damage to mitochondria and immune cells[11-15]. The currently available data do not permit one to draw firm conclusions regarding whether organotins function primarily as protein and enzyme inhibitors *in*

vivo, or instead regulate gene expression in a more direct manner. In this review, current possible hypotheses of imposex induction by organotin compounds and newly identified molecular mechanism of adipogenesis induced by organotins are summarized.

Possible Mechanisms in Induction of Imposex by Organotins in Snails

Approximately 150 gastropod species worldwide (including the rock shell) and 39 gastropod species in Japan, were reported to show imposex caused by organotins[5,16,17]. Gastropod imposex was induced by very low concentrations of TBT and/or TPT[18-26]. In severely affected stages of imposex, reproductive failure resulting from either oviduct blockage by vas deferens formation or ovarian spermatogenesis leads to population declines and mass extinction[4,5,22,27]. Imposex in gastropods is also thought to be a clear manifestation of endocrine disruption in wildlife[27].

To date, five possible mechanisms concerning imposex induction in gastropods have been proposed: 1) increased androgen levels caused by aromatase inhibition by TBT in the dog-whelk (*Nucella lapillus*)[28-30]; 2) inhibition of the excretion of sulfate conjugates of androgens by TBT in *Littorina littorea*[31]; 3) disturbance of the release of penis morphogenetic/retrogressive factor from pedal/cerebropleural ganglia by TBT in the sting winkle (*Ocenebra erinacea*)[32]; 4) increase in the neuropeptide APGWamide level caused by TBT in the mud snail (*Ilyanassa obsoleta*)[33,34]; 5) activation of retinoid 'X' receptor (RXR) by TBT induced penis morphogenesis and promoted the development in the rock shell (*T. clavigera*)[35]. These studies were conducted using different species of gastropods; therefore, it may not be possible to derive a common mechanism for the induction of imposex.

Effects of Androgen and Aromatase Inhibitor on the Imposex Development

The proposed mechanism of imposex induction by organotins has been critically reviewed by Horiguchi[17]. No clear correlation has been demonstrated among aromatase inhibition, androgen increase and penis growth to support the aromatase inhibition hypothesis. There is no evidence supporting the presence of either the aromatase gene or the androgen receptor (AR) in gastropods, further calling this model into question. Perhaps the most decisive evidence comes from our studies, which showed that direct injection of androgen into female rock shells did not induce imposex.

We injected live rock shells (*T. clavigera*) collected at Hiraiso (known as a less polluted site by TBT and TPT), Japan with the aromatase ($P450_{Arom}$) inhibitor fadrozole (Fad) and testosterone (T) as well as a positive control triphenyltin chloride (TPTCl). After 1 month, the animals were examined for imposex[24]. The positive control, TPTCl, clearly promoted the development of imposex (Table 1). Fad seemed to have significantly affected penis length, but the penis length was very small and the variance was large. No significant effects of Fad on the development of imposex or the VDS index in the rock shell were observed (Table 1). No significant effect was noted for the combined effect of Fad and T for the development of imposex in the rock shell, based on the incidence of imposex, penis length and VDS index after a month of injections (Table 1). Moreover, Fad (0.3 mg/L) and Fad (0.3 mg/L)+T (0.1, 1, and 10 μg/L) in 3-month flow-through exposure experiments did not significantly promote imposex symptoms in the rock shell (Sugimoto, Horiguchi, Shiraishi, Morita, Takahashi & Miura, unpublished data). Taken together, these results suggest that any expected increase in androgen levels due to inhibition of a postulated $P450_{Arom}$ cannot be a primary factor in the development of gastropod imposex.

The androgen excretion hypothesis is also based on the idea that penis is induced by androgen. However, there is no clear evidence showing androgen induction of penis in gastropods. The penis morphogenetic hypothesis is based on results showing toxic effects of organotins on isolated nervous systems of penis-forming area. No clear evidence of the penis morpho-

Table 1. Imposex incidence, penis length, and vas deferens sequence (VDS) index (mean ± standard deviation) in female rock shells 1 month after injection.

	Ethanol (negative control)	Fadrozole	Fadrozole + testosterone	TPTCl
Incidence (%)	15	35	20	100**
Penis length (mm)	0.02 ± 0.10	0.12 ± 0.19*	0	3.10 ± 3.57***
VDS index	0.20 ± 0.52	0.70 ± 1.03	0.20 ± 0.41	2.80 ± 1.51***

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (χ^2 analysis for the incidence of imposex, and ANOVA for penis length and VDS index)
The amount of injected test solution was calculated, based on estimated (nominal) tissue concentrations, as 5, 0.1, and 1 μg/g wet tissue for Fad, T, and TPTCl, respectively. After injection of the test solution (n=20 females per group, once or twice injections per solution), each group was kept in a 2-L glass beaker with flow-through artificial seawater (10 L/day) and live mussels as food

genetic factor has been demonstrated. Moreover, the reported penis inducing activity of the APGWamide neuropeptide was not strong enough to support this hypothesis[17].

In contrast to the studies regarding androgen synthesis, both TBT and TPT activated the rock shell RXR *in vitro* and natural ligand of RXR, 9-*cis* retinoic acid (RA) induced the development of imposex (penis formation and growth) in the female rock shells *in vivo*, supporting the RXR hypothesis. To date, this is the most realistic hypothesis for the molecular mechanism of imposex induced by organotins. However, detailed experiments will be needed in the future to provide *in vivo* evidence linking RXR activation to penis formation. It will also be necessary to demonstrate which pathways downstream of RXR are required for penis morphogenesis. It should be noted that RXR is required as an obligate heterodimeric partner for other nuclear receptors, both in vertebrates and invertebrates. Therefore, one cannot currently exclude the possibility that imposex might be induced through one or more RXR partners.

Testosterone is Converted to 17β-estradiol in Rock Shell Gonad Extracts

The model that TBT inhibits aromatase to increase T while decreasing 17β-estradiol (E2) requires the presence of an aromatase activity. Since no convincing evidence was available regarding aromatase in the rock shell, we undertook to measure the conversion of testosterone to E2 in the rock shell gonad/digestive gland complex extracts. We found that the gonad extract was able to convert a small amount of testosterone to E2 (Figure 1). This result provides biochemical evidence that the rock shell gonad/digestive gland complex contains a functional aromatase activity that catalyzes production of estrogen from testosterone *in vitro*. However, it should be noted that the production of E2 was relatively low level, compared with that catalyzed by the cloned aromatase from Nile Tilapia (*Oreochromis niloticus*)[36]. This raises the possibility that the rock shell activity may not be a bona fide aromatase, but rather a secondary activity of another enzyme. Further studies, including the cloning of rock shell aromatase and a detailed examination of rock shell steroid levels will be required to clarify this issue.

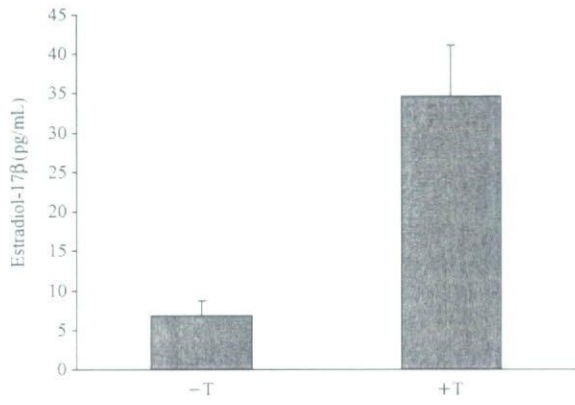


Figure 1. Testosterone (T) is converted to 17β-estradiol (E2) in the extract of the rock shell gonad/digestive gland complex. The rock shell gonad/digestive gland complex were extracted and incubated with T. After incubation, the samples were extracted twice with diethyl ether and evaporated by vacuum centrifugation. E2 in this extract was measured with an Estradiol-17β Enzyme Immunoassay kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions.

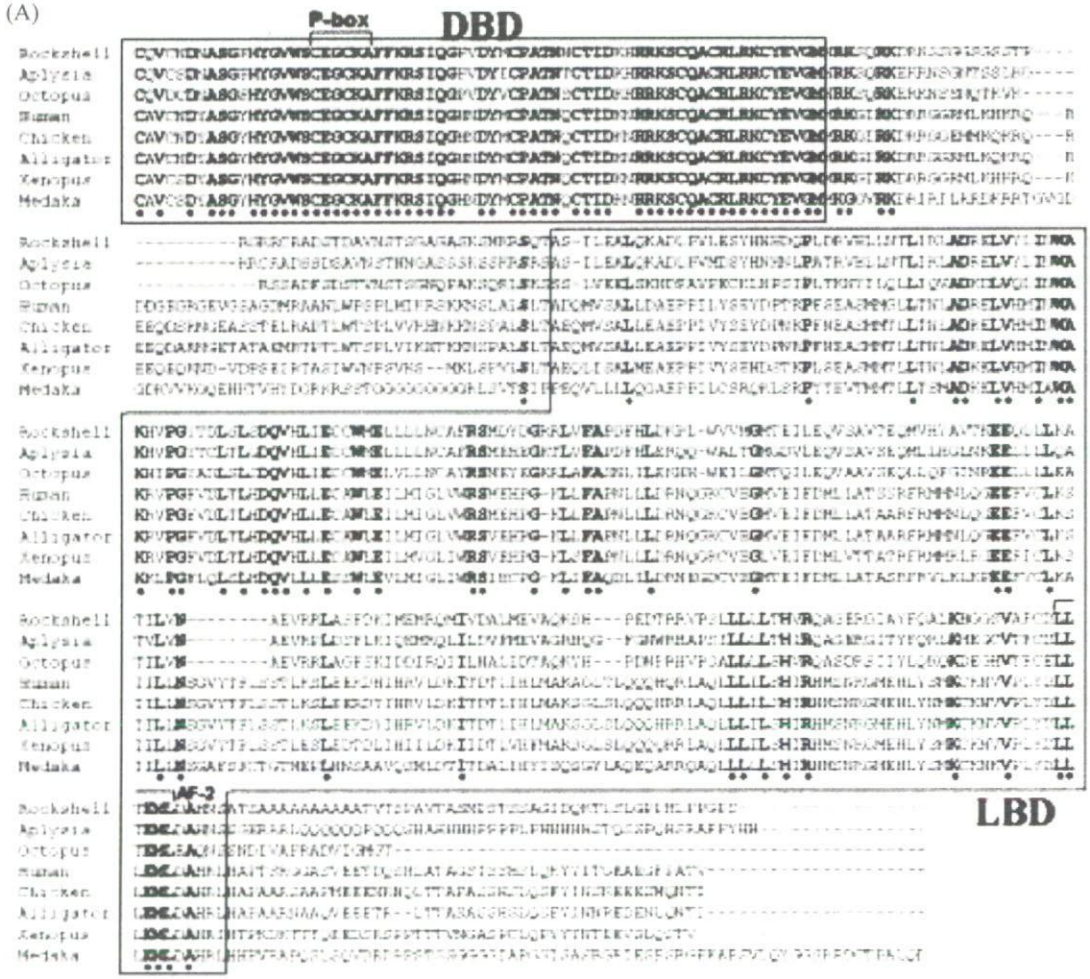
Rock Shell ER-like Protein cDNA

Recently, Kajiwara *et al.*[37] cloned a gene encoding a rock shell estrogen receptor (ER)-like protein. We independently cloned an ER-like sequence from the rock shell (Figure 2). Using the nomenclature of Krust *et al.*[38], the rock shell ER-like protein sequence can be divided into five domains, the N-terminal region, DNA binding domain (DBD), hinge, ligand binding domain (LBD) and C-terminal extension, based on its sequence similarity to other steroid hormone receptors. In the DBD, the rock shell ER-like protein has the highest protein sequence identity to the aplysia (*Aplysia californica*) ER (93%), octopus (*Octopus vulgaris*) ER (93%), human ER (89%), chicken ER (89%), alligator (*Alligator mississippiensis*) ER (89%), *Xenopus* ER (87%) and medaka (*Oryzias latipes*) ER (86%), and much lower similarity to other steroid receptors, human AR (59%), human progesterone receptor (56%), human glucocorticoid receptor (57%) and human mineralocorticoid receptor (57%). In addition, in the P-box of the DBD, a highly conserved motif for DNA recognition[39,40], the rock shell protein has the signature sequence of ER, but not other receptors (Figure 2).

Transactivation of the Rock Shell ER-like Protein

We employed several molecular assays to determine the function of the rock shell ER-like protein. Since the sea hare ER and octopus ER are ligand-independent for transactivation in reporter gene assays, we first tested whether the transactivation of the rock shell ER is ligand-depend or ligand-independent. We prepared a fusion construct of the GAL4-DBD with the rock shell ER-like protein, including C, D, E and F domains, and expressed it in CHO-K1

(A)



(B)

	P-box	DBD (%)	LBD (%)
rockshell	CEGCKA	-	-
aplysia	CEGCKA	93	74
octopus	CEGCKA	93	60
human ER	CEGCKA	89	37
human ERR	CEACKA	68	31
human AR	CGSCKV	59	26
human PR	CGSCKV	56	26
human GR	CGSCKV	57	25
humnan MR	CGSCKV	57	25

Figure 2. Amino acid sequence of the rock shell estrogen receptor (ER)-like protein (rER). (A) The rER and ERs of other animals were aligned with CLUSTAL W software. The DNA binding domain (DBD) and ligand binding domain (LBD) are enclosed within a box. The P-box and AF-2 region are indicated with lines. The closed circles indicate residues conserved in all ER members. (B) The similarity of the rER and the ERs of other species. The amino acid sequences of the P-box in the DBD of the rER are compared with other steroid hormone receptors. The percent of the alignment amino acid that is identical between the rER and other steroid hormone receptors in the DBD and LBD.

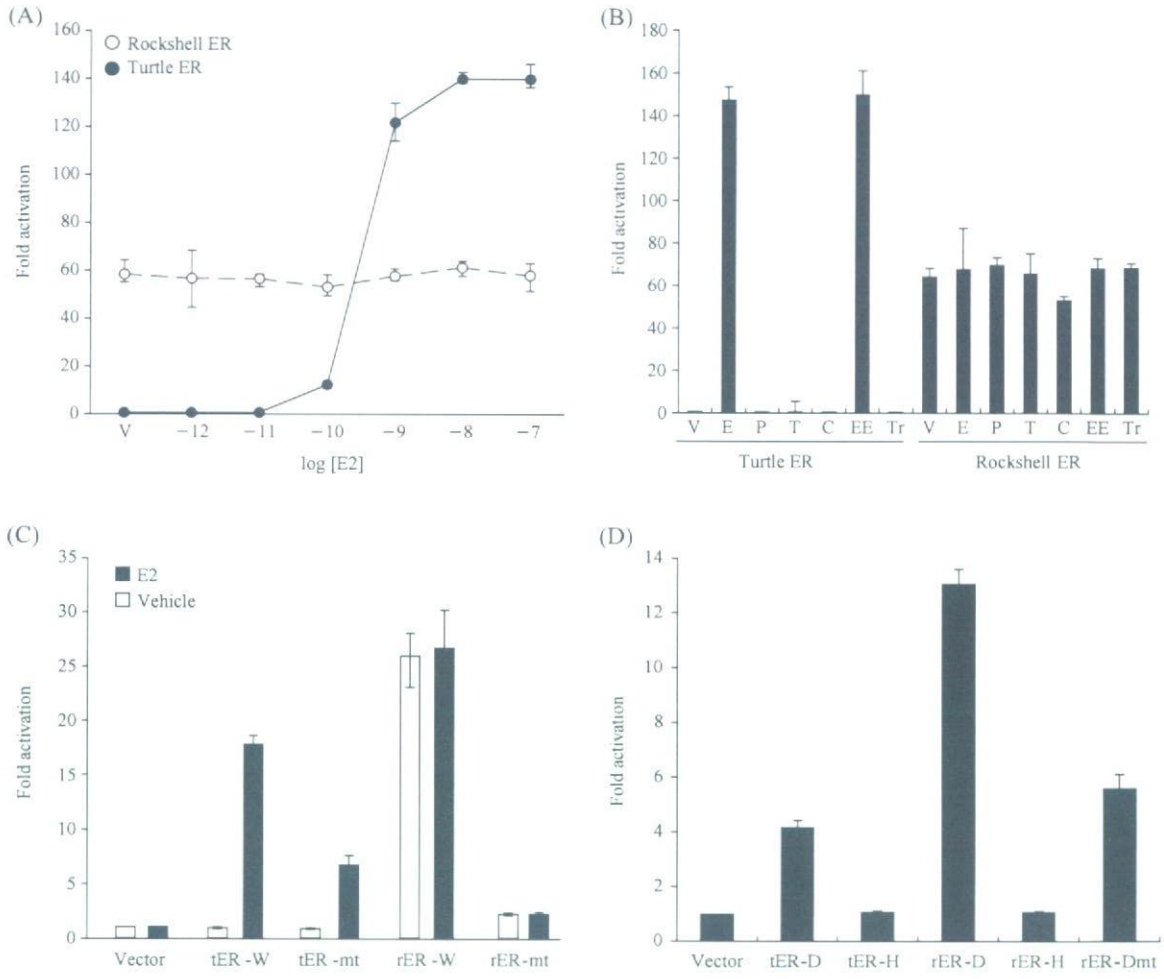


Figure 3. Transcriptional activities of the rock shell ER-like protein (rER). For A and B, the rER or freshwater turtle ER (tER) were expressed in CHO-K1 cells as fusion proteins with a GAL4-DBD. The fold activation indicates luciferase activity relative to the vector control containing GAL4-DBD. Mean ± SE of 3 replicates is shown. (A) The rER is constitutively active and does not respond to increasing concentrations of E2. Cells were treated with vehicle (DMSO) or with E2. (B) The rER does not respond to steroid hormones. Cells were treated with 10 nM of various hormones or with vehicle (DMSO). E, estradiol-17β; P, progesterone; T, testosterone; C, cortisosterone; EE, ethinylestradiol; Tr, Trenbolone. (C) AF-2 mutation of the rER abolished the transcriptional activity. Wild-type and mutated in AF-2 region of rER and tER were expressed in CHO-K1 cells. Cells were treated with 10 nM E2 or with vehicle only (DMSO). Each bar represents the mean of triplicate determinations, and vertical bars indicate the mean ± S.E. (D) Interaction of rER with estrogen response elements (EREs). The rER and tER were expressed in CHO-K1 cells as a fusion protein with the herpes simplex virus VP16 activation domain, along with 4xERE-driven luciferase reporter. Three types construct were used for assay: ER-D containing DBD, hinge and LBD; ER-H containing hinge and LBD, ER-Dmt containing DBD, hinge and AF-2 mutated LBD. Fold activation indicates luciferase activity relative to the vector-only control.

cells with a pG5-luc reporter construct, using charcoal-stripped serum to eliminate the potential for spurious ligand activation. As expected, there was no activation above background by the ERα from the freshwater turtle (*Pseudemys nelsoni*) in the absence of ligands. Increasing the concentration of E2 (1 pM-100 nM) revealed a concentration-dependent reporter activation. In contrast, the rock shell ER-like protein was constitutively active. It activated transcription 60-fold above background when no ligand was added. Addition of E2 had no further effect on reporter activity (Figure 3A). We also treated cells with various steroids (each at 10 nM). The turtle ERα showed the expected activation by estrogens, E2, and ethinyles-

tradiol (EE2). In contrast, reporter activity in response to the rock shell ER-like protein was not changed by the estrogens (E2 and EE2), progesterone, androgens (testosterone and trenbolone), or corticosteroid (corticosterone) (Figure 3B). The rock shell ER-like protein's constitutive activity is not an artifact of saturating assay conditions because increasing the quantity of the rock shell ER-like protein expression plasmid led to an increase in levels of reporter expression when all other assay conditions were held constant (data not shown). Furthermore, we found that the full-length rock shell ER-like protein also constitutively activat-ed an ERE-driven luciferase reporter and did not respond to E2 treatment (data not shown).

Next, we examined the necessity of activation function-2 (AF-2) domain for the transactivation of rock shell ER-like protein (Figure 3, C, D). ER contains two distinct activation regions, AF-1 and AF-241-43. AF-1 is located in the N-terminal A/B domain and exerts ligand-independent transcriptional activity in re-sponse to phosphorylation[44-46]. AF-2 is located in the C-terminal ligand-binding domain and mediates ligand-dependent transcriptional activity[47]. AF-1 and AF-2 activate transcription independently or synergistically and act in a promoter-specific and cell-specific manner[48]. To date, two classes of nuclear receptor coactivator complexes have been identified that directly interact with AF-2 in a ligand-dependent manner. The first contains CBP/p300, the p160 nuclear receptor coactivator family (SRC-1/TIF2/AIB1), an RNA coactivator (SRA), and probably other unknown components[49,50]. This complex facilitates decondensation of the chromatin via the histone acetyltransferase activity of several of its components[51,52]. The second coactivator complex includes proteins of the SMCC/TRAP/DRIP/ARC/Mediator class, and allows the physical link between ER and the general transcription apparatus, facilitating the activation of polymerase II[53]. Cell-type specific activity of both AFs was suggested by results from a specific expression of distinct coactivators[54]. However, the majority of the coactivators are widely expressed at similar levels in most cells[53,54]. Several of the coactivators primarily identified as AF-2 specific have now been shown to also interact with the N-terminal region of ER and to mediate AF-1 activity[48,55]. Therefore, despite considerable advances in understanding the mechanisms allowing the receptor to modulate the transcription of a target gene, no clear scheme is emerging with regard to the differential sensitivity of cell types to AF-1 and AF-2.

Overall, it is difficult to draw firm conclusions regarding the mechanism of organotin action in gastropods, due to deficiencies in our understanding of their endocrinology and developmental biology. An important first step will be the elucidation of the key steroid hormones involved in sex determination, information regarding the enzymology of steroid hormone synthesis and identification of the steroid hormone receptors and their mode of action.

Adipogenesis Stimulation by Organotins in Vertebrates

Recent work has shown that aromatase mRNA levels can be down-regulated in human ovarian granulosa cells by treatment with organotins or ligands for the nuclear hormone receptors, RXR or peroxisome proliferator activated receptor gamma (PPARγ)[56-58]. Furthermore, as mentioned above, the gastropod *T. clavigara* RXR homolog is responsive to 9-*cis* RA and TBT, and 9-*cis* RA can also induce imposex, suggesting a conserved transcriptional mechanism for TBT action across phyla[35]. RXR and PPARγ are ligand-modulated transcription factors that belong to the nuclear hormone receptor superfamily. This is a group of ~150 members (there are 48 human genes) that includes the ER, AR, GR, thyroid hormone receptor (TR), vitamin D receptor (VDR), retinoic acid receptors (RARs and RXRs), PPARs and numerous orphan receptors. We found the similar effects of TBT and RXR/PPARγ ligands on mammalian aromatase mRNA expression intriguing. This led us to hypothesize that TBT could be acting as a nuclear receptor ligand to exert some of its biological effects as a transcriptional regulation of gene expression.

Our results demonstrated that TBT induces adipogenesis, *in vitro* and *in vivo*, through its ability to act as a novel, high-affinity ligand for RXRα and PPARγ[59]. Analysis of structure-activity profiles revealed distinct structural preferences for organotins in their ability to activate both receptors. This analysis also showed that organotins of relatively diverse 3-D structures (e.g., TBT and TPT) efficaciously activate these receptors. The ability of the same compounds to bind to, and activate both RXR and PPARγ is surprising, and not easy to reconcile with the classical models of ligand binding for these receptors. It is possible that organotins will interact somewhat differently than previously described RXR/PPARγ ligands. However, the strong binding affinity of organotins for the receptors, coupled with the ability of organotins to displace high-affinity RXR and PPAR ligands suggest that organotins are potent and efficacious ligands for both RXRs and PPARγ. We also note that TBT activates both receptors at nanomolar concentrations, whereas other mechanisms of toxicity, e.g., direct inhibition of aromatase activity, typically occur at micromolar levels. This also supports the model

that organotins may be more likely to affect transcription than other cellular processes at environmentally-relevant concentrations.

Another interesting point is the ability of TBT to act as a dual ligand for permissive heterodimers such as RXRα : PPARγ. These heterodimers can be activated by specific ligands for either receptor individually. Additive or synergistic effects have been observed for permissive heterodimers when ligand for both partners is added together. This raises the possibility that TBT might itself elicit additive or synergistic effects on receptor activation, perhaps even in conjunction with natural ligands. The ability of organotins to activate permissive RXR heterodimeric partners suggests that organotins may have even wider effects on nuclear receptor signaling and endocrine disruption, e.g. LXR and NURR1, suggests that organotins may act more widely to disrupt multiple nuclear receptor mediated hormonal signaling pathways.

The effects of organotin activation of the RXR : PPARγ signaling pathway are predictable and reflect known aspects of RXR/PPARγ biology. The RXR : PPARγ heterodimer plays a central role in regulating adipocyte differentiation and lipid storage and is a key regulator of whole body lipid metabolism. Activation of PPARγ promotes the expression of genes that increase fatty acid storage and inhibits expression of genes that induce lipolysis in white adipose tissue[60]. PPARγ ligands such as the anti-diabetic thiazolidinediones increase insulin sensitivity through these effects on the adipocyte, sensitizing muscle and liver to insulin and thereby reversing insulin resistance in the whole body[61]. An important and undesirable consequence of this increase in whole body insulin sensitivity is that fat mass is increased through the promotion of triglyceride storage in adipocytes coupled with depot-specific remodeling and increase in adipocyte numbers increase following thiazolidinedione treatment[62-64]. Therefore, PPARγ agonists comprise a class of pharmaceutical therapies for type 2 diabetes that can also promote obesity by increasing fat storage. The ability of thiazolidinediones to increase adipocyte number and fat mass suggests that TBT exposure, which activates the same receptors, could affect obesity at any time in life. It is currently an open question whether the increased adiposity resulting from organotin exposure is due to an increase in adipocyte precursor cell number, enhanced adipocyte differentiation from the same number of precursors, an increase in adipocyte size without an increase in number or some combination of these.

The conventional wisdom suggests that high calorie modern diets, coupled with reduced physical activity are the major, or only cause of the dramatic rise in obesity rates worldwide[65]. Although the role played by genetic components is not completely clear, there is little doubt that genetic variation affects individual weight gain. However, it is difficult to imagine a scenario where genetic variation could underlie the rapid worldwide increase in obesity. It is more reasonable to suggest that interaction with the modern environment exposes underlying genetic differences that affect obesity. The Barker hypothesis postulates that *in utero* fetal nutritional status is a potential risk factor for metabolic syndrome diseases[66-70]. Developmental programming of a thrifty phenotype limits the range of subsequent responses to environmental factors such as diet and exercise[71]. Experiments in animal models support this hypothesis[72]. Plausible mechanisms include imprinting of obesity sensitive hormonal pathways or changes in cell type and number e.g. adipocytes, established during development.

An alternative model suggests that the environment plays another role in obesity. The increase in obesity rates parallels the rapid growth in the use of industrial chemicals over the past 40 years. Therefore, it is plausible to associate exposure to environmental chemicals in utero, or throughout ones lifetime with the obesity epidemic. We developed an "obesogen" model that predicts the existence of xenobiotic chemicals that inappropriately regulate lipid metabolism and adipogenesis to promote obesity. Several recent studies provide "proof-of-principle" for the obesogen hypothesis. In addition to organotin exposure, treatment with environmental estrogens such as bisphenol A and nonylphenol can promote adipocyte differentiation or proliferation in murine cell lines[73,74]. Furthermore, epidemiological studies link maternal smoking during pregnancy to an elevated risk of childhood obesity[75-79].

Discussion

Organotins such as TBT and TPT act as RXR activators, resulting in the development of imposex in the rock shell. They also act as chemical stressors or "obesogens" that activate RXR : PPARγ signaling to promote long term changes in adipocyte number and/or lipid homeostasis following developmental or chronic lifetime exposure in vertebrates.

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Chapel Hill bisphenol A expert panel consensus statement: Integration of mechanisms, effects in animals and potential to impact human health at current levels of exposure

Keywords: Bisphenol A; *In vitro*; *In vivo*; Rat; Mouse; Aquatic animal; Cancer; Low dose; Non-monotonic dose–response curves; Developmental programming

1. Introduction

This document is a summary statement of the outcome from the meeting: "*Bisphenol A: An Examination of the Relevance of Ecological, In vitro and Laboratory Animal Studies for Assessing Risks to Human Health*" sponsored by both the NIEHS and NIDCR at NIH/DHHS, as well as the US-EPA and Commonweal on the estrogenic environmental chemical bisphenol A (BPA, 2,2-bis(4-hydroxyphenyl)propane; CAS# 80-05-7). The meeting was held in Chapel Hill, NC, 28–30 November 2006 due to concerns about the potential for a relationship between BPA and negative trends in human health that have occurred in recent decades. Examples include increases in abnormal penile/urethra development in males, early sexual maturation in females, an increase in neurobehavioral problems such as attention deficit hyperactivity disorder (ADHD) and autism, an increase in childhood and adult obesity and type 2 diabetes, a regional decrease in sperm count, and an increase in hormonally mediated cancers, such as prostate and breast cancers. Concern has been elevated by published studies reporting a relationship between treatment with "low doses" of BPA and many of theses negative health outcomes in experimental studies in laboratory animals as well as *in vitro* studies identifying plausible molecular mechanisms that could mediate such effects. Importantly, much evidence suggests that these adverse effects are occurring in animals within the range of exposure to BPA of the typical human living in a developed country, where virtually everyone has measurable blood, tissue and urine levels of BPA that exceed the levels produced by doses used in the "low dose" animal experiments.

Issues relating to BPA were extensively discussed by five panels of experts prior to and during the meeting, and are summarized in five reports included in this issue: (1) human exposure to bisphenol A (BPA) [1]; (2) *in vitro* molecular mechanisms of bisphenol A action [2]; (3) *in vivo* effects of bisphenol A in laboratory animals [3]; (4) an ecological assessment of bisphenol A: evidence from comparative biology [4]; (5) an evaluation of evidence for the carcinogenic activity of bisphenol A [5]. Further discussion occurred at the meeting where participants from the panels were reorganized into four breakout groups. The consensus statements from the meeting are presented below.

The definition of "low dose" of BPA at this meeting used the same two criteria established at a prior NIH meeting concerning the low dose endocrine disruptor issue [6]: (1) for laboratory animal studies "low doses" involved administration of doses below those used in traditional toxicological studies conducted for risk assessment purposes. For BPA the lowest dose previously examined for risk assessment purposes was 50 mg (kg^{-1} day^{-1}) in studies with rats and mice. The 50 mg (kg^{-1} day^{-1}) dose is the currently accepted lowest adverse effect level (LOAEL) that was used to calculate the current US-EPA reference dose (the daily dose that EPA calculates is safe for humans over the lifetime) of 50 μg (kg^{-1} day^{-1}). The current reference dose is thus based on "high dose" experiments conducted in the 1980s [7]. (2) "Low dose" also refers to doses within the range of typical human exposure (excluding occupational exposures). For purposes of this meeting, the published literature that was reviewed met both of these criteria for being considered within the "low dose" range.

Hundreds of *in vitro* and *in vivo* studies regarding the mechanisms and effects of low doses of BPA, as well as studies of biomonitoring and sources of exposure, have been published in peer reviewed journals over the last 10 years, since the first "low dose" BPA *in vivo* studies were published [8–10]. The meeting was convened specifically to integrate this relatively new information. This task required the combined expertise of scientists from many different disciplines, and care was taken to ensure that participants covered these diverse areas.

BPA is a high-volume (>6 billion pounds per year) production chemical used to make resins and polycarbonate plastic [11]. Of particular concern is the use of BPA in food and beverage plastic storage and heating containers and to line metal cans. In addition, potential environmental sources of BPA contamination are due to use in dental fillings and sealants [12], losses at the production site [13], leaching from landfill [14,15], and presence in indoors air [16].

BPA has become a chemical of "high concern" only in recent years, even though BPA was shown to stimulate the reproductive

Abbreviations: ADHD, attention deficit hyperactivity disorder; BADGE, bisphenol A diglycidyl ether; BIS-DMA, bisphenol A dimethacrylate; BIS-GMA, bisphenol A glycerolate dimethacrylate; BPA, bisphenol A; ER, estrogen receptor

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system in female rats and thus to be an "environmental estrogen" in 1936 [17], long before it was used as the monomer to synthesize polycarbonate plastic and resins in the early 1950s. However, more recent evidence has shown that BPA also exhibits other modes of endocrine disruption in addition to binding to estrogen receptors, such as alterations in endogenous hormone synthesis, hormone metabolism and hormone concentrations in blood. BPA also results in changes in tissue enzymes and hormone receptors, and interacts with other hormone-response systems, such as the androgen and thyroid hormone receptor signaling systems. While BPA was initially considered to be a "weak" estrogen based on a lower affinity for estrogen receptor alpha relative to estradiol [18], research shows that BPA is equipotent with estradiol in its ability to activate responses via recently discovered estrogen receptors associated with the cell membrane [19–22]. It is through these receptors that BPA stimulates rapid physiological responses at low picogram per ml (parts per trillion) concentrations.

2. Purpose and organization of the BPA meeting

2.1. Topic-focused expert panels

To address the strength of the evidence regarding the published BPA research, an organizing committee was formed, and five panels of experts from different disciplines were established. Each panel had a chair or co-chairs and included a scientist who agreed to be primarily responsible, along with the chair, for preparing a preliminary draft of the panel's report. A web site was established on which all of the available electronic files of articles concerning BPA were posted, along with other pertinent information relating to the meeting. Prior to the meeting, the panel members began working on draft reports and communicated via electronic media and telephone conference calls. The resulting preliminary report from each panel was posted on the web site and distributed at the meeting for all participants to read. After the meeting, each panel completed a manuscript that is a part of this meeting report. These five panel reports were peer reviewed using the normal manuscript submission process to *Reproductive Toxicology*.The following specific concerns about BPA led to the five expert panels being established:

(1) Leaching of BPA occurs from the resin lining of metal cans and from plastic food and beverage containers under conditions of normal use. BPA is also detected in water and air samples.
(2) Parts per billion (ppb) levels of BPA that are unconjugated (not metabolized and thus biologically active) are detected in human blood and tissues in different countries, and these levels appear to be higher than blood levels that would be present in animals exposed to the US-EPA reference dose.
(3) BPA causes a wide range of adverse effects at "low doses" that are below the US-EPA reference dose in animals, both terrestrial and aquatic.
(4) There is evidence from *in vitro* mechanistic studies that indicates the potential for disruption of human and animal cell function at concentrations of BPA far below unconjugated levels typically found in human blood and tissues.
(5) There is evidence that at very low doses, BPA may be carcinogenic or increase susceptibility to cancer in animals.

The five panels each addressed a different topic related to their specific area of expertise with BPA and prepared a panel report that included documentation of the relevant published studies:

Panel (1) Sources and amounts of human exposure to BPA as well as pharmacokinetics.
Panel (2) *In vitro* studies related to the molecular mechanisms that mediate responses to BPA with an emphasis on studies using low doses.
Panel (3) *In vivo* studies of BPA at "low doses" in laboratory animals.
Panel (4) *In vivo* studies of BPA in aquatic wildlife and laboratory animals.
Panel (5) Relationship of BPA to cancers.

The purpose of the 3-day meeting was to provide an opportunity for members of the different panels to interact with each other to integrate information from different disciplines concerning low dose effects of BPA after each panel of experts had prepared a report in its specific area. The agenda of the meeting was designed to allow the members of the five panels to have time to discuss the information in their panel reports and finalize statements about the strength of the evidence for the literature that the panel had reviewed.

2.2. Integration of information by breakout groups

For the second part of the meeting the focus was on integrating the information from each of the panel reports. This was accomplished by assigning panel members to one of four breakout groups. The four replicate breakout groups were established using the following criteria, such that each breakout group should have

(1) At least two members from each of the five panels.
(2) A person from each panel who had published on BPA.
(3) A person with general knowledge of endocrine disruption research or endocrinology, but who had not necessarily published on BPA.
(4) A person with experience in the process of reaching consensus.
(5) A mixture of junior and senior investigators.

The charge to the replicate breakout groups was to individually integrate the information relating to the following four issues:

Issue (1) Determine the degree to which the findings on BPA mechanisms of action identify mechanisms and bioactive doses that explain results of the studies reported by the panel on *in vivo* laboratory animal studies. Determine the strength of the evidence for plausible mechanisms mediating *in vivo* effects at low doses. In

addition, identify any *in vivo* findings that are unexpected based on the *in vitro* literature.

Issue (2) Assess the degree to which ecological studies with wildlife are consistent with laboratory studies in similar and different species. For example, determine the similarity of exposure levels and types of responses seen in wildlife and laboratory animals.

Issue (3) Discuss the degree to which the low doses of BPA used in laboratory animal studies relate to the levels detected in human serum and tissues (including urine).

Issue (4) Assess the importance of life stage in the pharmacokinetics of BPA, levels of exposure to BPA, and the health effects of BPA in animals and humans.

3. Findings submitted by the four breakout groups

The reports from the breakout groups are presented below. The four breakout groups conducted a critical examination of the published research on BPA in relation to the four topics described above. Each of the breakout groups identified areas of knowledge and research gaps and made suggestions for future directions of research. In addition, each group identified which of the following two categories applied to specific outcomes:

- "We are confident of the following": this category applied when there were findings reported in multiple papers from multiple labs that were in agreement. There should have been no papers reporting conflicting findings, unless there were flaws in those papers, in which case the flaw(s) should have been identified.
- "We believe the following to be likely but requiring confirmation": This category applied when there were multiple consistent findings from one lab, or there may have been some conflicting reports along with reports of significant findings.

4. Levels of confidence for published BPA findings

The responses from the four different breakout groups were integrated together and organized based on levels of confidence. The criterion for a statement being included in a category was that there had to be consensus among all four of the breakout groups about the statement.

4.1. Based on existing data we are confident of the following

4.1.1. Issue 1: In vitro mechanistic research—laboratory animal research connection

1. *In vitro* studies have provided two routes of plausibility for low dose *in vivo* effects of BPA. These include binding to nuclear estrogen receptors that regulate transcription as well as estrogen receptors associated with the cell membrane that promote calcium mobilization and intracellular signaling. Receptors associated with the cell membrane are more sensitive to BPA than the nuclear receptors. Actions mediated by membrane associated receptor signaling may underlie much of the low dose BPA phenomena (effects have been reported at doses as low as 1 pM or 0.23 ppt). This increases the plausibility of effects at low doses, which are within the range of environmentally relevant doses (human and wildlife levels of exposure).
2. *In vitro* mechanistic information has informed us that exposing tissues to only an extremely narrow range of doses of BPA may lead to erroneous conclusions. Non-monotonic dose–response curves are encountered frequently in basic endocrinological research, and numerous examples have been reported for BPA reviewed in Refs. [18,23,24]. Because of this animal experiments on unstudied systems must avoid narrow dose ranges, especially the use of only a few very high doses. Thus, testing one or two doses and concluding that there are no effects is inappropriate. At somewhat higher doses than are required for estrogen receptor (ER)-mediated responses, BPA also interacts with androgen and thyroid hormone receptors, making predictions of effects at different doses very complex.
3. *In vitro* studies can dissect mechanisms of complicated effects observed *in vivo*. The proposed potential mechanisms acting *in vitro* and *in vivo* are the same, involving estrogen receptor mediated (nuclear- and membrane-associated) actions. However, specific effects are dose and cell/tissue specific. In addition, there are *in vivo* processes that are not reflective of currently known mechanisms that have been identified *in vitro*. This is due to previously unknown mechanisms as well as the complexity (due to interactions among cell and tissue types) of *in vivo* systems.

4.1.2. Issue 2: Wildlife—laboratory animal research connection

1. BPA is found in the environment: aquatic, terrestrial and air.
2. Studies of wildlife demonstrate estrogenic responses that are similar to responses seen in laboratory animals. Specifically, reductions in spermatogenesis are seen in wildlife at ecological concentrations of BPA, and these effects are also seen in controlled laboratory studies with BPA. In addition, vitellogenin response is a common biomarker in non-mammalian wildlife and laboratory species for BPA-induced estrogen receptor activation as well as activation by other estrogens.
3. BPA exposure induces similar effects in reproductive systems in wildlife and experimental animal model systems, but concentrations used in experiments involving wildlife species are often higher than environmental exposures. There are conditions in the environment, such as landfill leachates and effluent outflow that cause episodic exposure of field populations to elevated doses of BPA.
4. Responses in a variety of vertebrate wildlife species are qualitatively consistent with controlled laboratory studies with BPA. Thus, animals in the wild show evidence of harm, and controlled laboratory studies with model aquatic animals (i.e., medaka, zebrafish, and fathead minnows) are consistent with observations made in wildlife species. Low dose effects of BPA (low ppb range) have been observed in many of these animals.

5. The similar effects observed in wildlife and laboratory animals exposed to BPA predict that similar effects are also occurring in humans.

4.1.3. Issue 3: Laboratory animal research—human exposure connection

1. Human exposure to BPA is widespread.
2. Human exposure to BPA is variable, and exposure levels cover a broad range [central tendency for unconjugated BPA: 0.3–4.4 ng ml^{-1} (ppb)] in tissues and fluids in fetuses, children and adults.
3. Because the current published literature states that there is a linear relationship between administered dose and circulating levels of BPA in animal studies, this allows circulating levels at lower administered doses to be predicted in experimental animals based on the results from studies in which higher doses were administered.
4. All of the currently published metabolic studies in rats predict circulating BPA levels after acute low dose oral exposures at blood levels less than or equal to 2 ng ml^{-1} (ppb), which is the approximate median and mean unconjugated circulating BPA level in humans. Therefore, the commonly reported circulating levels in humans exceed the circulating levels extrapolated from acute exposure studies in laboratory animals.
5. BPA levels in the fetal mouse exposed to BPA by maternal delivery of 25 μg kg^{-1}, a dose that has produced adverse effects in multiple experiments, are well within the range of unconjugated BPA levels observed in human fetal blood.

4.1.4. Issue 4: Life stage—relationship to exposure pharmacokinetics and health effects

1. Sensitivity to endocrine disruptors, including BPA, varies extensively with life stage, indicating that there are specific windows of increased sensitivity at multiple life stages. Therefore, it is essential to assess the impact of life stage on the response to BPA in studies involving wildlife, laboratory animals, and humans.
2. Developmental windows of susceptibility are comparable in vertebrate wildlife species and laboratory animals.
3. BPA alters "epigenetic programming" of genes in experimental animals and wildlife that results in persistent effects that are expressed later in life [25]. These organizational effects (functional and structural) in response to exposure to low doses of BPA during organogenesis persist into adulthood, long after the period of exposure has ended. Specifically, prenatal and/or neonatal exposure to low doses of BPA results in organizational changes in the prostate, breast, testis, mammary glands, body size, brain structure and chemistry, and behavior of laboratory animals.
4. There are effects due to exposure in adulthood that occurs at low doses of BPA. Substantial neurobehavioral effects and reproductive effects in both males and females have been observed during adult exposures in laboratory animals.
5. Adult exposure studies cannot be presumed to predict the results of exposure during development.
6. Life stage impacts the pharmacokinetics of BPA.

4.2. We believe the following to be likely but require confirmation

4.2.1. Issue 1: In vitro mechanistic research—laboratory animal research connection

1. BPA metabolism occurs in cell culture systems, and although there are differences between cell types, there is less variability than in the entire animal. Metabolism is an important issue for humans and wildlife field populations with large genetic variability. Individual differences in BPA pharmacokinetics allow for underlying variability within a population, and may allow for the identification of sensitive and insensitive subpopulations.
2. The activity of various enzymes involved in drug, chemical, and hormone metabolism, as well as protection against oxidative stress, are programmed by hormone levels during sensitive periods in development. Developmental alterations in hormonal programming (activation or inhibition) may thus affect metabolism of BPA and other hormones and chemicals. Direct interaction of BPA with enzymes in cells has only been reported at higher doses than expected for human exposures.
3. The set of genes regulated by BPA is expected to differ among doses. Therefore, different doses of BPA do not produce different effects only due to a quantitative difference in the expression of the same set of genes.
4. Differential expression of estrogen receptor subtypes (α/ß; variant isoforms), and protein–protein interactions (estrogen receptor homo- and hetero-dimer formation, co-regulators, etc) modulate the cellular response to BPA. Direct actions of BPA on intracellular signal transduction modulate some cellular responses, which are similarly dependent on differential expression and protein–protein interactions.
5. Bioactive doses can be mathematically modeled, but further model refinement and experimental confirmation is required.
6. Other mechanisms (androgen receptors, thyroid hormone receptors) may be relevant for BPA action, but at higher doses than for estrogen responsive mechanisms.

4.2.2. Issue 2: Wildlife—laboratory animal research connection

1. The effects observed in laboratory animals could be present in wildlife, because the low doses being studied in laboratory animals are now relevant to environmental exposure levels of wildlife. The similarities in mechanisms that have been observed between different species suggest that field populations will respond to the same low levels.
2. Measurements of vitellogenin production in fish have established that there are exogenous estrogenic signals in the their environment. BPA may be contributing to this phenomenon as it enters natural water systems after leaching from landfills and due to plastic debris in water.
3. Delayed spawning is seen in male and female fish, which may relate to observed changes in estrous cyclicity in mammals in laboratory experiments.