# Commonality in Signaling of Endocrine Disruption from Snail to Human

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*Several nuclear receptors have recently been identified as mediators of endocrine disruption as well as steroid hormone receptors. The ubiquitous environmental contaminant tributyltin chloride (TBT) is a ligand for retinoid X receptor (RXR) in rock shell at the nanomolar level, and it acts as a ligand for both the RXR and the peroxisome proliferator-activated receptor γ in the frog* Xenopus laevis *and in humans. TBT, which induces imposex in marine snails and promotes adipogenesis in* X. laevis *and in mice, is an example of an environmental endocrine disrupter that promotes adverse effects, from the snail to mammals, through common signaling. In addition, juvenile hormone agonists used as pesticides showed endocrine-disruptive effects on parthenogenic* Daphnia magna*, lowering rates of reproduction, and inducing 100% male offspring. In this article, we focus on commonality in signaling through nuclear receptors and newly found endocrine disruption in* D. magna.

*Keywords: endocrine-disrupting chemicals, environmental estrogens, organotins, adipogenesis, nuclear receptors*

**Endocrine-disrupting chemicals (EDCs) can act at** multiple sites through multiple mechanisms of action. Using receptor-binding assays and receptor-based functional assays, researchers have shown that some environmental chemicals interact with estrogen receptors (ERs; e.g., nonylphenol, octylphenol, bisphenol A, *o,p*'-DDT, ethynylestradiol), androgen receptors (ARs; e.g., vinclozolin, *p,p*'-DDE), and arylhydrocarbon receptors (e.g., tetrachlorinated dibenzo-p-dioxin, polychlorinated biphenyls, polychlorinated dibenzofurans) (McLachlan 2001, Damstra et al. 2002). The Validation and Management Group (VMG) of the Organization for Economic Cooperation and Development (OECD) is in the process of establishing standardized methods of *in vitro* screening assays (Akahori et al. 2008) and *in silico* three-dimensional (3-D) quantitative structure-activity relationship (QSAR) models using the nuclear magnetic resonance–derived structure of the human ERα (hERα) ligand-binding domain and the structure of chemicals. These models can be used for quick prediction of chemicals that bind to hERαs (Akahori et al. 2005). So far, more than 2000 out of 200,000 chemicals have displayed potential binding to hERα in the 3-D QSAR model, with about 85% accuracy (Japanese Ministries of Economy, Trade and Industry and Health, Labour and Welfare). These results need to be confirmed using transactivation assays and receptor-binding assays to determine the accuracy of the *in silico* models. Other *in vitro* screening systems using ARs and thyroid hormone receptors (THRs) are under construction by the OECD VMG group.

To date, screening of chemicals of potential EDCs has focused mainly on human health, although we recently established transactivation assay systems using ERs and ARs from various animal species, including fish, amphibians, and reptiles (figure 1; Katsu et al. 2006, 2007a, 2007b, 2008). We compared the sensitivity of ERα in six fish species (zebrafish, medaka, fathead minnow, stickleback, roach, and carp) using the transactivation assay, demonstrating that the response to 17β-estradiol is quite similar among fish species. The response to DDT (dichlorodiphenyltrichloroethane) and its metabolites, however, showed species differences. We therefore need to understand the molecular mechanisms underlying species differences in hormone receptor sensitivity in various wildlife species.

Receptor-mediated mechanisms have received the most attention, but other mechanisms (e.g., hormone synthesis, transport and metabolism, activation of nuclear receptors, gene methylation) have been shown to be equally important (figure 2; Damstra et al. 2002, Tabb and Blumberg 2006). For most associations reported between exposure to EDCs and a variety of biological outcomes, the mechanisms of action

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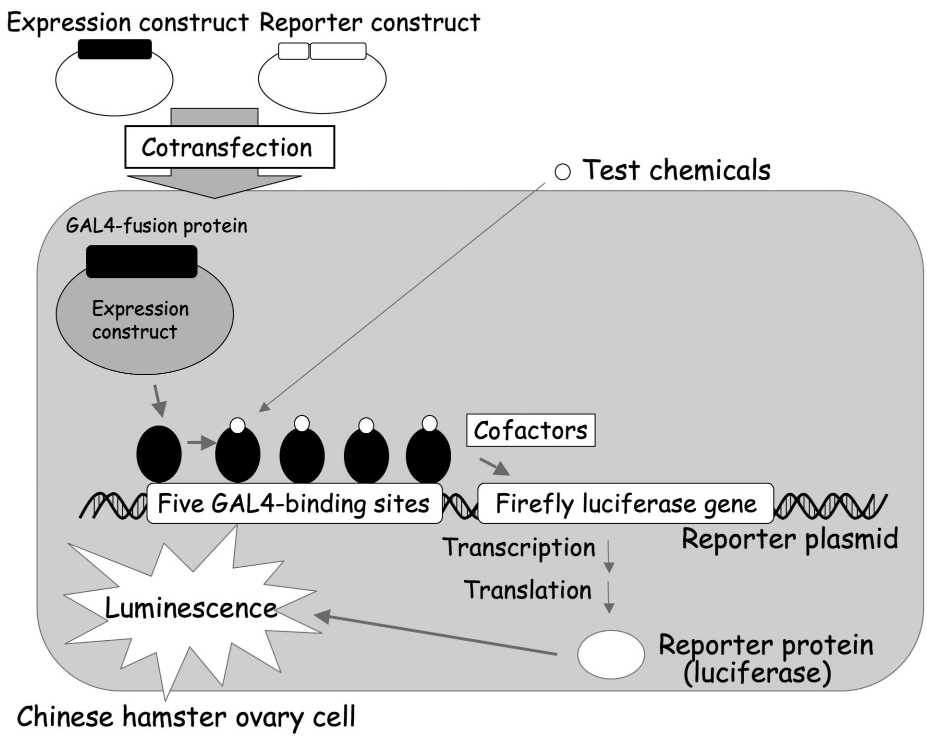


*Figure 1. Transactivation assay using the estrogen receptor (ER). To examine the interactions of a suspected ER ligand with a cloned ER, Chinese hamster ovary cells were transfected with two genetic constructs. One was a reporter gene construct containing five GAL4-binding sites and the firefly luciferase gene. The second was an ER expression construct that expresses GAL4-fusion ER protein and also contains the sea pansy luciferase gene, which is used as a control (not shown). When GAL4-fusion ER protein from the ER expression construct binds to the GAL4-binding sites adjacent to the firefly luciferase gene in the reporter gene construct, it can, together with cofactors, cause transcription of the firefly luciferase gene. Normally it does so at a very low level, but when GAL4-fusion ER protein binds to estrogens or estrogenic chemicals, its structure changes in a way that greatly increases transcription of the firefly luciferase gene. This leads, via translation, to production of firefly luciferase, which acts as a reporter: it can be detected by its luminescence. Various steroid hormones or environmental chemicals that were possible ER ligands were added to the medium containing the doubly-transfected cells at various concentrations. After 44 hours of incubation, the luciferase activity in the cells was measured. The ability of the tested chemicals to act as ER ligands was calculated as the ratio of firefly-luciferase activity to sea pansy–luciferase activity (sea pansy luciferase gene transcription is not affected by binding of possible ligands to GAL4-fusion ER protein).*

are poorly understood. In this article we discuss commonality in signaling through nuclear receptors and newly found endocrine disruption in the invertebrate water flea, *Daphnia magna.*

## Examples of potential endocrine disruptor–related outcomes in wildlife and humans

Several examples of intersex and sex reversal hypothesized to be induced by environmental chemicals have been reported in wildlife (table 1; Colborn and Clement 1992, Damstra et al. 2002, Iguchi et al. 2002). Female marine snails develop vas deferens and a penis after exposure to organotin compounds, including tributyltin (TBT) and triphenyltin. The males of some fish species in rivers in the United Kingdom (see Jobling and Tyler 2008) and the Tama River, Japan (Hara et al. 2007), have elevated plasma vitellogenin levels, which suggests that they have been exposed to estrogenic contaminants. In contrast, androgenic effects have been found in female fish in rivers carrying pulp and paper mill effluents (mosquitofish) and feedlot effluent (fathead minnows) (Orlando et al. 2004, 2007).

Deformed frogs showing various anomalies such as split hind limbs, supernumerary limbs, and duplicated paired limbs have been reported throughout the United States, in some regions of Canada, and in Japan. In alligators and freshwater turtles, sex determination is influenced by the temperature of the nest during incubation, but also is highly sensitive to exogenous estrogenic chemicals in the environment (see Milnes and Guillette 2008).

A crossed-bill deformity observed in cormorants in the Great Lakes and the phenomenon in which two females form a "mating" pair and share a nest were associated with elevated levels of dioxins, polychlorinated biphenyls (PCBs), and pesticides, as well as other chlorinated compounds. The beluga whales of the St. Lawrence Seaway, which drains the Great Lakes of North America, have greatly elevated PCB concentrations in their bodies. These animals exhibit a greater incidence of all cancers, early mortality, and decreased immunity. The detoxification functions of the hepatic enzyme systems of marine mammals such as seals, dolphins, and whales are inferior to those of terrestrial animals (Tanabe et al. 1994). Furthermore, these animals have thick layers of blubber that can easily accumulate lipophilic contaminants such as PCBs and DDT and its metabolites.

In humans, lowered sperm density and increased rates of hypospadias, cryptorchidism, and testicular dysgenesis syndrome have been reported (Skakkebaek et al. 2001). Cryptorchidism rates are correlated to presumed pesticide exposure in areas with intensive farming in Spain (García-Rodríguez et al. 1996). An increased risk of cryptorchidism has been detected in sons of women employed in the gardening industry (Weidner et al. 1998). An increased risk of hypospadias has been reported in the sons of Dutch women exposed to a synthetic estrogen, diethylstilbestrol (DES) *in utero* (4 of 205), as compared with sons of non–DES-exposed women (8 of 8279 boys) (Klip et al. 2002). Rates of testicular cancer and early menarche have increased in several countries over the past several decades (Bergstrom et al. 1996). Menarche occurs much earlier than expected in US girls (Herman-Giddens et al. 1997). Shorter anogenital distance in boys exposed to phthalates *in utero* has also been reported (Swan et al. 2005), as predicted by observations from laboratory rodent exposure studies.

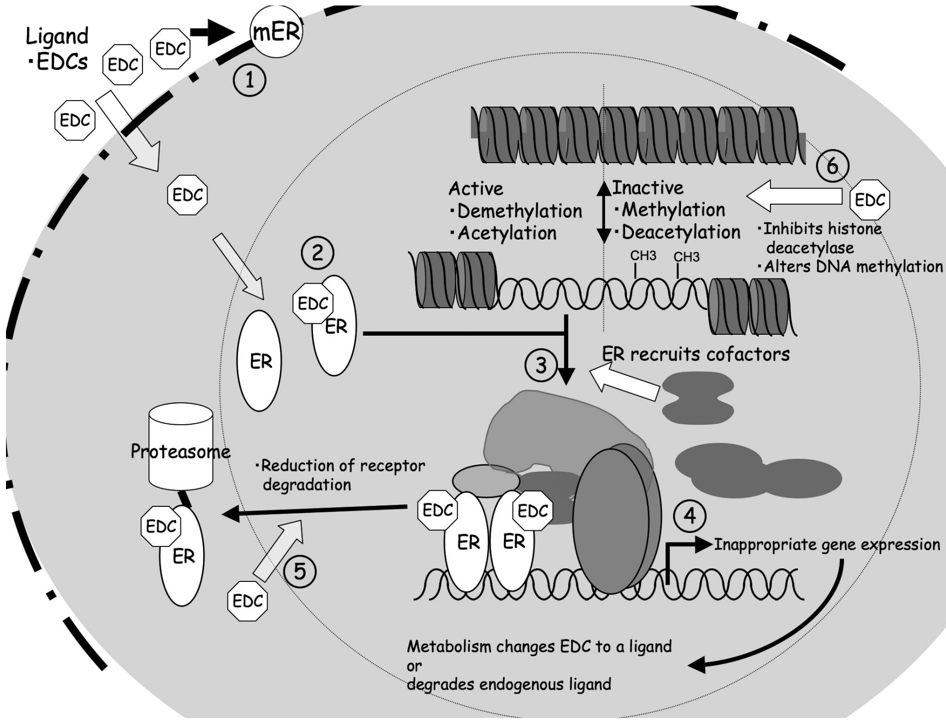


***Figure 2. Endocrine-disrupting chemicals (EDCs) may operate by a variety of mechanisms. When EDCs arrive at a cell membrane (top left) they may bind to a membrane estrogen receptor (mER) (1), or pass through the membrane and bind to a nuclear estrogen receptor (ER) in the nucleus (2). When the complex of EDC and ER binds to a gene containing an estrogen-responsive element (3), it recruits molecules that help cause gene expression by boosting gene transcription by the RNA polymerase complex (large ovals), and so may cause gene expression at inappropriate times (4). EDC/ER complexes can also bind to proteasomes, which can lead to a reduction of the normal process of degradation of ER (5). DNA is shown near the top of the figure wrapped around histone complexes, in which form it is inactive. In the middle of the figure, DNA is shown unwrapped from the histone complexes, which exposes it to molecules that boost transcription. EDCs may cause methylation of DNA or deacetylation of histone (as shown by the methyl groups on the right side of the gene), both of which reduce gene expression. Alternatively, EDCs may cause demethylation of DNA or, by inhibiting the enzyme histone deacetylase, lead to the acetylation of histone (left side of the gene). The second two effects both induce gene expression (6).***

## Various mechanisms of endocrine disruption

The endocrine systems of vertebrates largely share molecular mechanisms such as the ability of particular chemicals to bind to steroid receptors. However, the physiological consequences of these mechanisms—for instance, for sex differentiation—differ in different classes of vertebrates. Sex is determined by the *sry* gene in mammals and the *dmy* gene in medaka fish, whereas temperature-dependent sex determination is common in crocodilians and turtles. Estrogen is quite important in the development of ovaries in fish, amphibians, and reptiles, and very likely plays a role in birds as well. Likewise, critical developmental windows of sensitivity—periods when hormonal or xenobiotic chemicals can act during development—differ among vertebrate species.

For invertebrate species, information on the endocrine system and the hormone receptor system is limited. Juvenile hormone agonists used as pesticides induced a reduction of reproduction and resulted in 100% male offspring in parthenogenic *D. magna* (Tatarazako et al. 2003). One of these agonists provides an example of endocrine disruption in an invertebrate species without chemical stress: it induced 100% male offspring production without reducing reproduction (Oda et al. 2005). The ecdysone receptor has been cloned in *D. magna* (Kato et al. 2007), but no juvenile hormone receptor or binding protein has been identified in *D. magna.* Chemicals that affect hormonal activities in vertebrates also affect several invertebrate species, such as *Hydra vulgaris,* copepods, barnacles, nematodes, freshwater mudsnails, and sea urchins (Fox 2005). ER homolog genes identified in *Aplysia,* octopus, and a marine snail (rock shell; *Thais clavigera*) showed no ligand binding, but they did display ligand-independent gene activation (Thornton et al. 2003, Keay et al. 2006, Iguchi et al. 2007). Thus, functional nuclear-type ER may not be present in invertebrates. Membrane ERs have been found in vertebrates and act as an acute response system to estrogens. Therefore, we cannot rule out the possibility that membrane ERs could be present in invertebrate species.

Nuclear receptor subfamily 1, group I, member 2 (NR1I2), commonly known as the steroid and xenobiotic receptor in

*Table 1. Examples of adverse effects found in wildlife and humans and possible mechanisms.*

| Hypothetical relationship | | Evaluation factor | | | |
|---|---|---|---|---|---|
| **Examples** | **Chemicals** | **Association** | **Recovery** | **Hypothesis** | **Possible mechanism** |
| Imposex in marine gastropods | TBT | **** | **** | Strong | RXR? |
| Vitellogenin induction in fish exposed to sewage treatment plant effluents in England | Estrogenic contaminants | **** | ** | Strong | ER<br>Hormone synthesis |
| Developmental abnormalities and reproductive failure in Lake Ontario lake trout | Dioxins and coplanar PCBs | **** | **** | Strong | AhR |
| Reproductive alterations in fish exposed to bleached Kraft mill effluent in Ontario hormone synthesis | Bleached Kraft mill effluent | **** | *** | Strong | AhR,<br>Hormone synthesis |
| Reproductive abnormalities in Lake Apopka alligators | Dicofol and agricultural pesticides | *** | ** | Moderare | ER, AR, TR? AhR?<br>Hormone synthesis |
| GLEMEDS in birds | PCBs | **** | **** | Strong | AhR |
| Eggshell thinning in colonial waterbirds | DDE and other DDT metabolites | **** | **** | Strong | ER, prostaglandin |
| Decreased reproductive function in Baltic seals | PCBs | ** | *** | Strong | AhR? |
| Endometriosis in humans | TCDD, PCBs | * | ND | Weak | ER? AhR? |
| Impaired neurobehavioral development in humans | PCBs | *** | ND | Moderate | TR? AhR? |
| Perturbed immune function | PCBs, TCDD | **** | * | Moderate | AhR? |
| Incidence of breast cancer in humans | DDT, DDE, PCBs | * | ND | Weak | ER? |

AhR, arylhydrocarbon receptor; AR, androgen receptor; DDE, dichlorodiphenyldichloroethylene; DDT, dichloro-diphenyl-trichloroethane; ER, estrogen receptor; GLEMEDS, Great Lakes embryo mortality, edema, and deformities syndrome; ND, no relevant data; PCB, polychlorinated biphenyls; RXR, retinoid X receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; TR, thyroid hormone receptor.

*Note*: This table summarizes the overall strength of evidence for each criterion of the framework (evaluation factors) developed to present the potential effects of endocrine-disrupting chemicals. Each criterion has been ranked from weak (*) to strong (****).

*Source*: Modified from Damstra and colleagues (2002).

humans, is a key ligand-dependent transcription factor responsible for regulation of xenobiotic, steroid, and bile acid metabolism. The ligand-binding domain is principally responsible for species-specific activation of NR1I2 in response to xenobiotic exposure. We have demonstrated interspecies variation in NR1I2 activation by various ligands using *in vitro* NR1I2 activation assays. Species differences should therefore be taken into account when choosing animal models for assessing environmental health risk (Milnes et al. 2008).

Clarifying the molecular basis of the action of EDCs and endogenous estrogens on developing organisms is essential if we are to understand the linkages among exposure levels, timing of exposure, genes responsive to these chemicals, and adverse effects (Iguchi et al. 2006). It is vital to understand the effects of EDCs on various species, from invertebrates to mammals, at the level of molecular biology to aid in explaining observations at the cellular and organismal levels.

## Possible common signaling through retinoid X receptors: Organotins as a model system

Organotins were first used in the 1960s as antifouling agents in marine shipping paints, although such use has been restricted in recent years. Organotins persist as prevalent contaminants in human dietary sources, such as fish and shellfish, and through pesticide use on high-value food crops (Golub and Doherty 2004). The use of organotins as antifungal agents in wood treatments, industrial water systems, and textiles can also increase human exposure.

Exposure to organotins results in imposex, the abnormal induction of male sex characteristics in female gastropod mollusks (Gibbs and Bryan 1986, Horiguchi 2006). Imposex, one of the clearest examples of environmental endocrine disruption, results in sterility or impaired reproductive fitness in the affected animals. TBT exposure also leads to masculinization of at least two fish species (McAllister and Kime 2003, Shimazaki et al. 2003), but TBT is reported to have only modest adverse effects on mammalian reproductive tracts (Ogata et al. 2001).

Instead, hepatic-, neuro- and immunotoxicity appear to be the predominant effects of organotin exposure in mammals (Boyer 1989). Hence, current mechanistic understanding of the endocrine-disrupting potential of organotins is based on their direct actions on the levels of aromatase or on the activity of aromatase and more general toxicity mediated through damage to mitochondrial functions (Powers and Beavis 1991, Heidrich et al. 2001). However, it remains an open question whether organotins act primarily as protein and enzyme inhibitors, or rather mediate their endocrine-disrupting effects at the transcriptional level.

Recent work has shown that production of aromatase messenger RNA (mRNA) can be decreased in human ovarian granulosa cells by organotins or ligands for nuclear hormone receptors, retinoid X receptors (RXRs), or peroxisome proliferator-activated receptor gamma (PPARγ; Mu et al. 2001). Furthermore, the RXR homolog in gastropod mollusks is found (using the yeast two-hybrid assay) to be responsive to 9-*cis* retinoic acid (RA), and TBT and 9-*cis* RA can induce imposex (Nishikawa et al. 2004, Castro et al. 2007), suggesting a conserved transcriptional mechanism for TBT action across phyla. These ligand-dependent transcription factors belong to the nuclear hormone receptor superfamily—a group of approximately 150 members, represented by 48 human genes—that includes the ER, AR, glucocorticoid receptor, TR, vitamin-D receptor, RA receptors (RARs and RXRs), PPARs, and numerous orphan receptors.

*Daphnia magna* has an RXR homolog, called ultraspiracle (USP), which heterodimerizes with the ecdysone receptor (Kato et al. 2007). Research by one of the authors of this article (T. I.) with Shigeto Oda demonstrates that TBT induces reduction of reproduction in *Daphnia* at 1 ppb (part per billion), but has no effect on molting. Thus, TBT may not affect the ecdysone and USP system in the water flea (table 2).

We have shown that TBT is a potent inducer of adipogenesis *in vitro* in 3T3L1 cells and *in vivo* in developing *Xenopus laevis* and mice, by acting as a novel, high-affinity xenobiotic ligand for RXRα and PPARγ (Grün et al. 2006). TBT is a potent and efficacious ligand for both RXRs and PPARγ that interacts, at least partially, with the same receptor-binding sites as other high-affinity ligands and promotes the necessary cofactor interactions required for agonist activation. Thus, TBT binds to RXRs and activates signaling in gastropods, amphibians, and mammals.

***Table 2. Possible common mechanisms of action of organotin compounds in various animal species.***

| Species | Nuclear receptors | Possible effect |
|---|---|---|
| Human | RXR, PPARγ | Adipogenesis |
| Amphibian (*Xenopus laevis*) | RXR, PPARγ | Adipogenesis |
| Fish | ? | ? |
| Marine snails (*Thais clavigera*) | RXR | Imposex, formation of penis |
| Water flea (*Daphnia magna*) | RXR (ultraspiracle) | ? |

PPARγ, peroxisome proliferator-activated receptor gamma; RXR, retinoid X receptor.

*Note*: Ultraspiracle, an RXR homolog, shows heterodimerization with ecdysone receptor in *Daphnia magna*.

## Adipogenesis stimulation by organotins in vertebrates

The ability of TBT to act as a dual ligand for permissive heterodimers such as RXRα:PPARγ, which can be activated by specific ligands for either receptor, as demonstrated using transfected Cos7 cells, also raises the possibility for additive or synergistic effects that might increase the potency of these compounds *in vivo* at low doses for this specific signaling pathway. Of note is that receptor activation occurs at nanomolar concentrations, whereas other mechanisms of toxicity and endocrine disruption (e.g., direct inhibition of aromatase activity) typically occur in the micromolar range. Furthermore, the activation of other permissive RXR heterodimeric partners (e.g., LXR [liver X receptor] and NURR1 [nuclear receptor–related 1]) suggests that organotins could act more widely to disrupt multiple nuclear receptor–mediated hormonal signaling pathways.

The RXR:PPARγ pathway plays a key role in adipocyte differentiation and energy storage, and is central to the control of whole body metabolism (Auwerx 1999). PPARγ activation increases the expression of genes that promote fatty acid storage and represses genes that induce lipolysis in adipocytes in white adipose tissue (Ferre 2004). PPARγ ligands, such as the thiazolidinediones, can modulate insulin sensitivity as a result of their effects on the adipocyte, reversing insulin resistance in the whole body by sensitizing the muscle and liver tissue to insulin (Day 1999). However, a consequence of this increase in whole body insulin sensitivity is that fat mass is increased through the promotion of triglyceride storage in adipocytes. Fat cell–specific remodeling and an increase in adipocyte numbers follow thiazolidinedione treatment (Hallakou et al. 1997). Therefore, PPARγ agonists comprise a class of pharmaceutical therapies for type 2 diabetes that could also promote obesity by increasing fat storage. Likewise, RXR ligands act as insulin-sensitizing agonists in rodents (Mukherjee et al. 1997), underscoring the permissive nature of the PPARγ:RXR heterodimer complex and the potential effects on diabetes and the obesity of both PPARγ and RXR agonists.

Our data (Grün et al. 2006) are consistent with recent studies reporting that organotins can mediate some of their endocrine-disruptive effects by transcriptional regulation through nuclear receptors, in particular RXR:PPARγ signaling (Mu et al. 2001, Nishikawa et al. 2004). Consequently, TBT exposure can promote adipocyte differentiation in the same manner as other RXR or PPARγ ligands *in vitro,* using the standard murine 3T3-L1 cell model, and *in vivo* through increased adiposity following intrauterine organotin exposure in newborn mice (table 2).

The environment may play a significant role in obesity. Since the increase in obesity rates parallels the rapid growth in the use of industrial chemicals over the past 40 years, it is plausible and provocative to associate *in utero* or chronic lifetime exposure to chemical triggers present in the modern environment with this epidemic. Hence, an "obesogen" model predicts the existence of xenobiotic chemicals that inappropriately regulate lipid metabolism and adipogenesis to promote obesity. Several recent studies serve as "proof-of-principle" for this hypothesis. Environmental estrogens such as bisphenol A and nonylphenol can promote adipocyte differentiation or proliferation in murine cell lines (Masuno et al. 2003), and perinatal estrogen exposure induces obesity in mice (Newbold et al. 2007). Furthermore, epidemiological studies link maternal smoking during pregnancy to an elevated risk of childhood obesity (Hill et al. 2005).

Organotins such as TBT and its congeners act as RXR activators, resulting in 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 or lipid homeostasis following developmental or chronic lifetime exposure in vertebrates. Research in our laboratory will continue to examine the molecular basis of chemical function on developing animal species, including vertebrates and invertebrates.

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