Application of Ecotoxicogenomics for Studying Endocrine Disruption in Vertebrates and Invertebrates

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Chemicals released into the environment potentially disrupt the endocrine system in wild animals and humans. Developing organisms are particularly sensitive to estrogenic chemicals. Exposure to estrogens or estrogenic chemicals during critical periods of development induces persistent changes in both reproductive and nonreproductive organs, including persistent molecular alterations. Estrogen-responsive genes and critical developmental windows of various animal species, therefore, need to be identified for investigators to understand the molecular basis of estrogenic activity during embryonic development. For investigators to understand molecular mechanisms of toxicity in various species, toxicogenomics/ecotoxicogenomics, defined as the integration of genomics (transcriptomics, proteomics, metabolomics) into toxicology and ecotoxicology, need to be established as powerful tools for research. As the initial step toward using genomics to examine endocrine-disrupting chemicals, estrogen receptors and other steroid hormone receptors have been cloned in various species, including reptiles, amphibians, and fish, and alterations in the expression of these genes in response to chemicals were investigated. We are identifying estrogen-responsive genes in mouse reproductive tracts using cDNA microarrays and trying to establish microarray systems in the American alligator, roach, medaka, and water fleas (*Daphnia magna*). It is too early to define common estrogen-responsive genes in various animal species; however, toxicogenomics and ectotoxicogenomics provide powerful tools to help us understand the molecular mechanism of chemical toxicities in various animal species. *Key words:* **alligator, *Daphnia magna*, ecotoxicogenomics, estrogen receptor, mosquitofish, mouse, roach, xenopus.** *Environ Health Perspect* **114(suppl 1):101–105 (2006). doi:10.1289/ehp.8061 available via *http://dx.doi.org/* [Online 21 October 2005]**

Environmental endocrine-disrupting chemicals (EDCs) such as polychlorinated biphenyls, dioxin, and pesticides as well as plasticizers, pharmaceuticals, and natural hormones can interact with steroid and retinoid receptors (Colborn and Clement 1992; Guillette and Crain 2000; Iguchi et al. 2002a; McLachlan 2001). Those chemicals showing estrogenic activity usually have structural similarities, namely, a phenol ring (Nishihara et al. 2000). Receptor-based functional assays, therefore, have been used to detect putative biological activity of many environmental chemicals (Yamasaki et al. 2002). Mean concentrations of estrogenic chemicals such as nonylphenol (NP), bisphenol A (BPA), estrone, and 17β-estradiol (E_2) in the Tamagawa River in Tokyo were 564, 27, 33, and 4.6 ng/L, respectively (Nakada et al. 2004). Possible endocrine disruption in wild animals and humans exposed to EDCs during embryonic development has been discussed extensively (Damstra et al. 2002; Gulledge et al. 2001; Iguchi 2000; Iguchi and Watanabe 2003).

The perinatal mouse model has been used to demonstrate the long-term effects of early sex hormone exposure on the female reproductive tract (Iguchi 1992; Iguchi et al. 2001; McLachlan 2001; Newbold 2004). Neonatal treatment of female mice with E_2 and a synthetic estrogen, diethylstilbestrol (DES), induces various abnormalities in the reproductive tracts, hypothalamo–hypophyseal–ovarian axis, immune function, and skeletal and muscular tissues. The growth response of perinatally DES-exposed reproductive organs to estrogen is altered, as are levels of estrogen receptor (ER), epidermal growth factor receptor (EGFR), prolactin receptor, and oncogenes and *Hox* genes (Bern 1992; Iguchi et al. 1993, 2002b; Newbold et al. 2004; Yin and Ma 2005). We found that activation cascade, such as persistent phosphorylation of erbB2 and ER-α, and sustained expression of EGF-like growth factors and phosphorylation of JNK1, IGF-I receptor, and Akt, has been found in the vagina of neonatally DES-exposed mice (Miyagawa et al. 2004a, 2004b). Several genes have been cloned in the vagina after neonatal DES exposure (Katsu et al. 2002, 2003). The critical windows for induction of various abnormalities by postnatal exposure to exogenous estrogens varied from 3 to 30 days, thereby indicating the presence of tissue- and organ-specific critical windows in mice (Iguchi et al. 2002b).

To understand the molecular basis of EDCs and endogenous estrogens on developing organisms, we must understand the linkages between exposure levels, genes responsive to EDCs, and the adverse effects induced by these chemicals. Various modes of action of chemicals and nontraditional targets of EDCs recently have been summarized (Fox 2005; Tarrant 2005). However, understanding the toxicology and the molecular biology of the effects of EDCs on various species, from invertebrates to mammals, is still vitally important. The subdiscipline that combines the fields of genomics and mammalian toxicology is "toxicogenomics" (Inoue and Pennie 2002; Nuwaysir et al. 1999). In this context, "ecotoxicogenomics" is defined as the study of gene and protein expression in wild animal species that is important in understanding responses to environmental toxicant exposures (Miracle and Ankley 2005; Snape et al. 2004). The use of DNA microarrays provides a high-throughput diagnostic tool to screen the many variables required to examine gene expression patterns effectively. Profiling of transcripts, proteins, and metabolites can help discriminate classes of EDCs and toxicants and aid in understanding modes of action. Through systematic efforts to generate mechanistic information, diagnostic and predictive assessments of the risk of EDCs and toxic chemicals will be established in model species for ecological risk assessment.

We have begun to identify estrogen-responsive genes in the mouse reproductive tract using cDNA microarrays and to establish microarray systems in the American alligator, roach, medaka, and water fleas (*Daphnia magna)*. Toxicogenomics and ectotoxicogenomics are powerful tools for understanding molecular mechanisms of chemical toxicity; however, it is too early to select common

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estrogen-responsive genes in various animal species. In this short review we provide an overview of our current approach to understanding EDCs.

Mouse

The cDNA microarray method has been developed recently and has been successfully applied to genomewide analysis of gene expression stimulated by hormones and/or chemicals (Inoue and Pennie 2002; Watanabe and Iguchi 2003). Knowledge of the patterns in the expression of estrogen-responsive genes is essential to understanding the action mechanism of estrogenic chemicals on mouse reproductive organs. A large number of genes affected by estrogen were selected from the mouse (Moggs et al. 2004; Watanabe et al. 2002a, 2002b, 2003a) and rat uterus (Daston and Naciff 2005). For most of the selected genes, expression was induced in a dose-dependent manner. Moreover, their expression was not altered following E_2 treatment in ER-α knockout mice, thus confirming the dependency of these genes on ER-α. Activation of these genes suggests a basis for the marked uterotrophic effect observed several days following estrogen administration. Characteristic gene expression patterns were observed for each environmental estrogenic chemical, and these patterns were distinct from that of E_2, thereby suggesting specific mechanisms of action for endocrine disruption that could be different from that induced by endogenous estrogen (Daston and Naciff 2005; Watanabe et al. 2004a). Physiological estrogens (E_2), nonphysiological estrogens (DES), and dioxin have distinct effects on uterine gene expression (Watanabe et al. 2003b, 2004b). In the liver, however, NP and dioxin activated another set of genes that were distinct from estrogen-responsive genes (Watanabe et al. 2004a, 2002b). Thus, these results suggest that only a small number of genes are directly involved in the uterotrophic effects of estrogen treatment, and NP has effects very similar to those of E_2 on gene expression in uterus but not in hepatic tissue. Tissue-specific effects, therefore, should be considered in order to elucidate the distinct effects of various EDCs.

American Alligator

Extensive studies on contaminant-exposed and reference populations of American alligator (*Alligator mississippiensis*) have revealed altered steroidogenesis, abnormal circulating hormone levels, hepatic transformation of androgen and endocrine organ morphology in juvenile alligators living in polluted environments, and a number of contaminants in eggs, serum, and body tissues (Guillette and Iguchi 2003). Affinity of some compounds is relatively high for alligator ERs, and many compounds can displace E_2 from the ER (Guillette et al. 2002)

In all species of crocodilians, sex is determined not by a genetic mechanism alone but also by the temperature at which the egg is incubated. In the alligator, the thermosensitive period (TSP) for sex determination is the 7- to 10-day window within stages 21–24 of development (Lang and Andrews 1994). Treating embryos with estrogen during the TSP produces female offspring even at male incubation temperatures. Therefore, estrogens play a role in determining sex in the alligator. However, the mechanisms of estrogen action on sex determination in the alligator are still uncertain. Furthermore, studies of contaminant-exposed alligators have shown alterations in steroid action (Guillette and Iguchi 2003; Guillette et al. 1994). Whether these abnormalities are caused, in part, by alterations in steroid receptor expression remains unclear. To begin to understand the mechanism of steroid action in alligators, the cDNA encoding the ERs and the progesterone receptors (PR) was isolated (Katsu et al. 2004). The ER-α amino acid sequence is similar to that of chicken ER-α (91%). Recently, we cloned Nile crocodile (*Crocodylus niloticus*) ER-α. The ER-α sequence of the Nile crocodile is quite similar to that of the American alligator. The turtle ER-α sequence is closer to that of alligators than to that of the chicken (Katsu et al. 2006).

Several thousand expressed sequence tags (ESTs) from the cDNA library of adult alligators and those of the gonads of embryos incubated at temperatures that produce all males or all females have been sequenced and clustered. We are currently establishing an alligator microarray for the study of the molecular mechanism of sex determination and of the chemical effects on sex determination and the toxic effects of chemicals.

Amphibians

Exogenous chemicals that can interfere with the thyroid hormone axis could pose a significant hazard to human and wildlife health (Colborn 2002; Zoeller 2003). Amphibians represent a suitable model for monitoring reproductive performance, advanced development including metamorphosis, and sexual maturation (Kloas 2002). The influence of NP, BPA, and E_2 on developing *Xenopus laevis* embryos was analyzed. Embryos were exposed to these agents between 3 and 96 hr postfertilization (p.f.). Short body length, microcephaly, flexure, edema, and abnormal gut coiling were induced by 4.4 mg/L NP or BPA or by 2.7 mg/L E_2 at 96 hr p.f. The E_2 effects are consistent with a previous study (Nishimura et al. 1997). Interestingly, the stages of embryos sensitive to BPA and NP were different; BPA affected earlier stages, whereas NP affected later stages (Sone et al. 2004). BPA interferes with the assembly of microtubules (Metzler and Pfeiffer 1995) and causes mitotic arrest and aberrant spindles (Ochi 1999). These BPA actions may affect the susceptibility of embryos, especially at the earliest stages. Insensitivity of *X. laevis* embryos to BPA after 12 hr p.f. may reflect the weak *in vitro* activity of BPA relative to NP or E_2 (Nishikawa et al. 1999). Transcriptional levels of aromatase and ER genes increased from stage 56 in *X. laevis* (Miyashita et al. 2000), although ER mRNA was detected at stage 8 (Nishimura et al. 1997). Considering the ability of estrogen treatment to induce ectopic expression of the ER ligand–binding domain of fused mRNA (Kolm and Sive 1995), it is possible that genes under the regulation of the estrogen–ER pathway are inactive at the developmental stages tested but are capable of transcriptional activation in the presence of an exogenous ligand.

Fish

To establish a model system for studying the effects of EDCs on marine fish, we examined the effects of E_2 on the early development of *Fundulus heteroclitus*. E_2 (2.72 μg/L) reduced hatching and survival rates, and induced malformations with incomplete ossification of bones and 100% females (Urushitani et al. 2002). To clarify the mechanisms contributing to these developmental effects of exogenous estrogen, we cloned *Fundulus* ER-α (fhER-α), which shared 81% identity with medaka (*Oryzias latipes*) ER-α. A receptor binding assay using the fhER-α ligand-binding domain showed that alkylphenols bind to fhER-α 50 times more efficiently than to human ER-α (Urushitani et al. 2003).

Ultraviolet screens and preservatives are increasingly being used in cosmetics and pharmaceuticals. We characterized the estrogenicity of 4-methylbenzylidene, camphor, octylmethoxycinnamate, and propyl paraben (*n*-propyl-*p*-hydroxybenzoate) using medaka vitellogenin (VTG) plasma concentration, VTG, and choriogenin mRNA expressions (Inui et al. 2003). We are currently establishing a medaka microarray containing known genes related to steroidogenesis, sex development, degradation of chemicals, and estrogen-responsive genes.

The occurrence of intersexuality has been reported in wild roach (*Rutilus rutilus*) (Jobling et al. 1998), gudgeon (*Gobio gobio*) (van Aerle et al. 2001), and flounder (*Platichthys flesus*) (Allen et al. 1999) in the United Kingdom and in flounder (*Pleuronectes yokohamae*) in Japan (Hashimoto et al. 2000). Endocrine disruption of roach is thought to be caused by estrogenic agents in sewage effluents. In intersex roach, sexual maturation, gamete production, and fertility are reduced (Jobling et al. 2002a, 2002b). To understand the molecular mechanisms of intersex in roach that are induced by substances in sewage effluents, we have cloned genes of ER-α, ER-β, (androgen receptor

(AR), PR, aromatase brain type, aromatase gonad type, DMRT-1, and other genes related to steroidogenesis. We are now establishing a roach microarray system.

A number of studies have documented endocrine disruption derived from estrogenic responses caused by exposure to pesticides (Wester 1991), surfactants (White et al. 1994), pulp mill effluent (Pelissero et al. 1991), industrial wastewater (Jobling et al. 1995), and sewage effluent (Jobling and Sumpter 1993) in fish. Induction of secondary sex characteristics such as malelike coloration in female guppy (*Poecilia reticulata*), development of malelike gonopodium, and altered reproductive behavior in female mosquitofish (*Gambusia affinisi*) have been reported following exposure to pulp mill effluent (Drysdale and Bortone 1989; Howell et al. 1980). These data indicate the existence of contaminants with androgenic activity, which mimic or block endogenous androgen by interacting with the receptor in the aquatic organisms (Durhan et al. 2002; Parks et al. 2001). However, compared with our knowledge of estrogenic environmental chemicals, the substances and mechanisms of androgenic action remain unclear (Gray et al. 2001). Recent studies with wild fathead minnows (*Pimephales promelas*) have suggested the presence of potent androgenic substances in feedlot effluent (Orlando et al. 2004; Soto et al. 2004). Together with other wastewater contaminants, feedlot effluent has become a major ecological health concern.

Trenbolone acetate, an androgenic and anabolic steroid, is a potent agonist of AR, and it has been used extensively as a growth promoter for beef cattle in the United States. We have cloned mosquitofish (*Gambusia affinis affinis*) AR-α and AR-β, and studied the effects of 17-trenbolone (TB), a hydoxylated active compound of trenbolone acetate, on adult and newborn mosquitofish. TB induced masculinization of the anal fin, accompanied by a transient up-regulation of AR-α and AR-β in adult females. TB also induced differentiation of the anal fin into the gonopodium in fry at 0.3–10 μg/L and stimulated precocious spermatogenesis in males and the formation of ovotestis in females at 1–10 μg/L (Sone et al. 2005).

Small freshwater fish used widely for toxicology research include the Japanese medaka, the fathead minnow, and the zebrafish. To date, very little has been done to apply genomics technologies to ecological risk assessment of aquatic species such as fish. This paucity is caused largely by the lack of genome information. Current genome sequencing efforts for several fish models such as zebrafish, medaka, and fathead minnow make these fish potential candidates for large-scale efforts to incorporate genomics technologies in an effort to understand the mechanistic toxicity pathways for environmental stressors (Miracle and Ankley 2005). In addition, genome sequencings are under way in other species such as European flounder, sheepshead minnow (*Cyprinodon variegates*), large mouth bass (*Micropterus salmoides*), rainbow trout (*Onchorhynchus mykis*), and the three-spined stickleback (*Gusterosteus aculeatus aculeatus*). Therefore, it is likely that the dynamic picture of various biological systems will be understood in the near future.

Mechanism of Induction of Imposex in Marine Snails

In contrast to the relatively large effort to examine the estrogenic/antiestrogenic and androgenic/antiandrogenic action of various environmental chemicals in vertebrates, there has been relatively little research on the tremendous array of invertebrates that inhabit fresh and marine water environments. Detailed information concerning the effects and mechanisms of action of industrial chemicals in invertebrates has been obtained from only a few invertebrate species, although invertebrates represent more than 95% of the known species in the animal kingdom (deFur et al. 1999). The masculinizing effects, known as imposex and characterized by development of a vas deferens and a penis in females, of organotin compounds, such as tributyltin (TBT), on snails have been found in about 150 species of mollusks (Gibbs and Bryan 1986; Horiguchi et al. 1997). The mechanism by which TBT induces imposex in marine snails is still unknown, although TBT has been shown to inhibit aromatase activity (Bettin et al. 1996). TBT exposure at 1 pg/L, an environmentally relevant concentration, for 4 weeks induced imposex in rockshells (*Thais clavigera*); however, injection of the aromatase inhibitor, fadrozole, alone or in combination with testosterone (T) did not induce imposex (Horiguchi T, Katsu Y, Ohta Y, Iguchi T, unpublished data). Aromatization of [^{3}H]-T to [^{3}H]-E_2 was encountered in the rockshell gonad extract (Katsu Y, Horiguchi T, Iguchi T, unpublished data). These results suggest that neither inhibition of aromatase by TBT nor androgen action by TBT is the principal cause of imposex in rockshells.

We have cloned a full-length sequence of ER-like gene. The cells transfected with mouse ER-α showed estrogen-dependent reporter gene activation. However, cells transfected with a rockshell ER-like sequence showed ligand-independent reporter gene activation (Katsu Y, Horiguchi T, Iguchi T, unpublished data), which suggests that the rockshell ER-like sequence has a specific unknown function in the rockshell, but it is unlikely that it acts like vertebrate ER. In the freshwater snail, *Marisa cornuarietis*, BPA and octylphenol (OP) at concentrations as low as 1 μg/L induced development of an additional vagina, enlargement of the accessory pallial sex glands, and enhancement of oocyte production. In the marine prosobranch, *Nucella lapillus,* the same concentrations of BPA and OP reduced the length of the penis and the size of the prostate gland (Oehlmann et al. 2000). Such results suggest that these snails have a functional ER and, thus, that estrogenic chemicals could have a negative impact on these snails.

RXR, one of the nuclear receptors, from humans and from *Xenopus* has been activated by TBT in reporter gene assay systems (Grun F, Watanabe H, Zamanian Z, Maeda L, Arima K, Chubacha R, et al., unpublished data). TBT and 9-*cis* retinoic acid have been shown to activate the rockshell RXR, and 9-*cis* retinoic acid has been shown to induce imposex in the rockshell (Nishikawa et al. 2004). These results suggest that future research to examine the mechanism of action of EDCs in invertebrates needs to focus on other nuclear hormone receptors distinct from the ER and the AR.

Male Production in Daphnids by Juvenile Hormone Agonists

Reproductive, acute, or chronic toxicity tests on daphnids have been used widely for aquatic toxicology. Conflicting results on the molting frequency of *Daphnia magna* following exposure to estrogenic chemicals have been reported (Caspers 1998). A xenoestrogen-induced reduction in the molting frequency of *D. magna* (Niederlehner et al. 1998; Zou and Fingerman 1997) could not be confirmed for BPA (Tatarazako et al. 2002).

We found that styrene dimers and trimers, leached from disposable polystyrene cups, reduced the number of offspring in *Ceriodaphnia dubia*. Styrenes (0.04–1.7 μg/L), ecdysones (0.1–1.08 μg/L), and juvenile hormone agonists (1.05 μg/L) reduced fertility, whereas E_2 and BPA had no effect on the reproduction of *C. dubia*. NP (280 μg/L) influenced daphnids via membrane damage (Tatarazako et al. 2002). We have cloned a full-length sequence of an ecdysone receptor from *D. magna* and established an ecdysone reporter gene assay (Watanabe et al. 2005).

We and others have revealed that exposure of adult daphnids to juvenile hormones and their analogs induces parthenogenetically reproducing *D. magna* to produce male neonates (Olmstead and LeBlanc 2002, 2003; Tatarazako et al. 2003). Ten juvenoids [pyriproxyfen, fenoxycarb, methylfarnesoate, juvenile hormone I (JH I), JH II, JH III, methoprene, kinoprene, hydroprene, and epofenonane] have induced male neonate production (Oda et al. 2005b; Tatarazako et al. 2003). In addition, daphnids are susceptible to the male sex–determining effect of

juvenoids during early oogenesis (Olmstead and LeBlanc 2002; Tatarazako et al. 2003), and the effect of juvenoids is reversible (Tatarazako et al. 2003). Although there was a wide range of sensitivity to fenoxycarb (0.6–9.3 μg/L), the production of male neonates in all four species (*Moina macrocopa*, *Moina micrura*, *C. dubia*, and *C. reticulata*) demonstrates that this phenomenon is a common response to juvenoids (Oda et al. 2005a). These findings suggest that juvenile hormone agonists, including some insecticides, affect the chemical signaling responsible for inducing the production of male offspring. We constructed a cDNA library of *D. magna* and characterized the ESTs of over 7,000 clones (Watanabe et al. 2005). To understand the molecular functional mechanism of juvenile hormone agonists in the induction of male offspring, we are currently analyzing juvenile hormone binding protein and establishing a micoroarray system for *D. magna*.

Future Research Needs

The developing embryo is fragile and sensitive to estrogenic agents (Bern 1992). Much of the literature to date on the EDC issue focuses on steroid hormone receptor–mediated toxicity. Therefore, information on gene and protein expression mediated by hormone receptors is essential for understanding chemical effects. Species differences with respect to the interaction of various chemicals with ERs and with regard to the metabolism of chemicals have been observed. Therefore, we are currently cloning receptors of various steroid hormones, and steroid and xenobiotic receptors (SXR), from various animal species, including alligator, quail, and various fish species, in order to find species that are sensitive to EDCs. We are also focusing on orphan nuclear receptors that may provide new insights into the mechanisms of chemical action, as shown with RXR activation by TBT in gastropods and even in *Xenopus* and mice.

Analyses of transgenerational effects of xenobiotic agents are also required in order to estimate and confront potential dangers to human and wildlife populations. There are also species differences in the response of ER and SXR to chemicals, in degradation of chemicals, in critical sensitive windows, and in development. To clarify the adverse effects of chemicals, we need to understand the timing of gene expression (critical developmental window), the amount of gene expression (amount of chemicals) in specific organs, the degradation ability of chemicals, and the normal range of various biomarkers in each species. By the application of omic technologies (genomics, transcriptomics, proteomics, and metabolomics) in the study of EDCs, we will understand the detailed mechanisms of action of chemicals in the future. In this review, we have focused primarily on receptor-mediated gene expression; however, it is critical to broaden the spectrum of hormonal disruption in the hypothalamic–pituitary–end gland axes, and to include the ability of animals to cope with stress or chemical communication (Propper 2005). Further basic biological understanding of comparative molecular endocrinology, genomics, and toxicology in animal species is essential in order to be able to apply omics technologies to the study of wildlife species.

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