A High Throughput Zebrafish Embryo Gene Expression System for Screening Endocrine Disrupting Chemicals

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ABSTRACT

Reproduction and development in man and animals are essential for survival of species, species diversity, maintenance of ecosystems, and commercial activities. Thus, there is an urgent need for regulators to develop methods to better predict which of the estimated 87,000 chemicals in the environment have the potential to disrupt hormone-dependent processes of development, physiology and reproduction (EDC, endocrine disrupting chemicals). We propose development of an assay using living zebrafish (*Danio rerio*) embryos as a whole animal *in vitro* screening system for simultaneous detection of multiple subsets of EDC: (a) EDC that act via estrogen receptors (ER) to induce brain P450 aromatase (P450aromB) and hepatic vitellogenin (vtg) expression; (b) EDC that act via arylhydrocarbon receptors (AhR) to reduce gonadal aromatase (P450aromA) and increase P4501A1 expression; (c) EDC that interact directly with preformed aromatase enzyme to block aromatization; and (d) EDC that perturb ER and AhR expression *per se*. An automated real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) approach will be used to measure targeted mRNAs in single and multiplex assays. The proposed zebrafish embryo system is a novel alternative to, and extension of, the current EDSP Tier 1 Screening Battery, which includes a mandate for ER binding and reporter assays and an alternative placental aromatase (enzyme) assay, but does not presently include an assay for chemicals that disrupt endogenous estrogen signaling by altering aromatase or ER expression, nor does it include an assay that can detect possible AhR mediated effects on reproductively relevant gene targets, or a screening assay that can simultaneously compare sensitivity and responsiveness of multiple genes to a given chemical. Although the proposed *in vitro* assay minimizes animal and chemical use, it has the advantages of an *in vivo* system for predicting agonist vs. antagonist properties of a chemical without *a priori* knowledge of uptake and accumulation, activating or metabolizing pathways, access to targets, receptor binding and activation, or required coregulators. Resultant data will provide biologically relevant criteria for prioritizing chemicals for further testing and will help to interpret reports of reproductive and developmental effects in wildlife and humans. Validation of a zebrafish embryo gene expression assay for detecting known and suspected ER- and AhR-acting EDC will have immediate applicability for routine chemical screening, and will demonstrate the feasibility of the same approach to detect chemicals that interact with other members of the nuclear receptor superfamily.

ENDOCRINE DISRUPTING CHEMICALS (EDC): AN URGENT ENVIRONMENTAL PROBLEM

The "endocrine disruptor hypothesis" is based on scientific principles; data from laboratory, wildlife and epidemiological studies; weight-of-evidence; and the precautionary principle (See *EDSTAC 1998; Fox et al. 2004).*

Endocrine disrupting chemicals (EDC) are hormonally active agents:

- that mimic or block diverse hormone signaling systems essential for normal development, reproduction, growth, metabolism, homeostasis, etc.);
- are widely distributed in the environment;
- derive from many different human activities (pesticides, industrial byproducts, cancer drugs, fattening agents) & are also found as natural products (human & animal waste; phytoestrogens);
- adversely effect cells, tissues, organs, the organism, its progeny, species fitness & survival, and ecosystems, even at low doses and transient exposures (see Fig. A);
- endocrine disrupting effects cannot be predicted on the basis of chemical structure alone (see Fig. B);
- few of the >87,000 chemicals added to the environment have been tested for endocrine disrupting effects.

Figure A. Examples of adverse effects linked to EDC *(McLachlan, 2001; Fox et al., 2001, 2004)*

Organism	Effects	Pollutant/Chemical
Mammals:		
Human	Gynecomastia, precocious puberty, learning disabilities, decreased sperm count, oligospermia, impotence, increased testicular cancer	DDT, kepone, DES, PCBs
Cattle, sheep	Infertility	Phytoestrogens
Seals	Impaired reproductive functions	PCBs
Mink	Population decline, hormone changes	PCBs, dioxins
Rabbits, guinea pigs	Infertility, failed ovulation	Phytoestrogens
Mice	Infertility Proliferative lesions, tumors	DES, phytoestrogens
Birds:		
Quail	Abnormal reproductive behavior	DDT
Gulls	Abnormal ovarian & oviduct development	DDT
Waterfowl	Egg shell thinning, embryo mortality & abnormal development	DDE, PCBs, dioxins
Reptiles:		
Alligators	Abnormal gonads, decreased phallus size	DDT, DDE
Turtles	Anomalous reproductive tract development	Nonaclor, DDE
Amphibians:		
Frogs	Feminized gonads	Atrazine
Finfish:		
Perch, trout, minnows, etc.	Feminization of testes; masculinization, hermaphroditism, vitellogenin in males, reduced gonadal size, decreased hormone levels, abnormal reproductive tract & secondary sex characteristics	Sewage effluent mixture, dioxins, PAHs, PCBs
Non-vertebrates:		
Snails	Masculinization, imposex, additional female organs, increased oocyte production	Tributyl tin, bisphenol A, octylphenol
Copepods	Advanced sexual maturation, increased egg production	Bisphenol A
Daphnia	Delayed molting time	PCBs
Sponges	Developmental abnormalities and inhibited growth	Bisphenol A, nonylphenol
Bacteria	Inhibited signal exchange with plant host and N_2 fixing symbiosis	PCBs, DDT, malathion

Figure B. Known EDC are structurally diverse; effects cannot be predicted on the basis of structure alone.

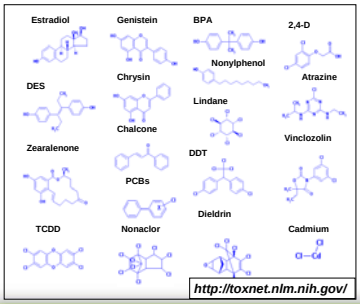
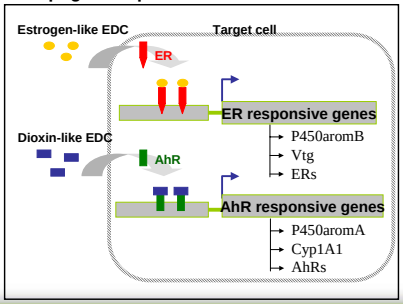


Figure C. Many EDC interact with estrogen- and arylhydrocarbon-receptors (ER, AhR) to disrupt gene expression.

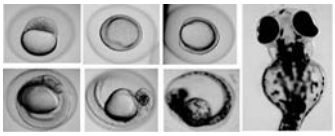


SCIENTIFIC APPROACH

HYPOTHESIS: Altered expression of hormone responsive genes can be used as an endpoint to identify EDC.

Fig. D. The zebrafish embryo is an advantageous animal model for high throughput screening of EDC by gene expression analysis.

- Typical vertebrate hormone systems & development
- 1000's of small, transparent embryos per mating
- Rapid ex utero development facilitates in vitro testing with the added value of a whole organism
- Genome sequence available



WORKFLOW

Computational Biology	Developmental & Molecular Biology
Identify hormone responsive genes	Optimize embryo treatment protocols
Determine gene organization/ sequence	Test chemicals, mixtures, doses, exposures
Design, validate & optimize PCR primers	Isolate/reverse transcribe embryo RNA

Quantitative real-time polymerase chain reaction (qPCR) analysis

Microsoft Excel-based software (Q-Gene)

INITIAL RESULTS

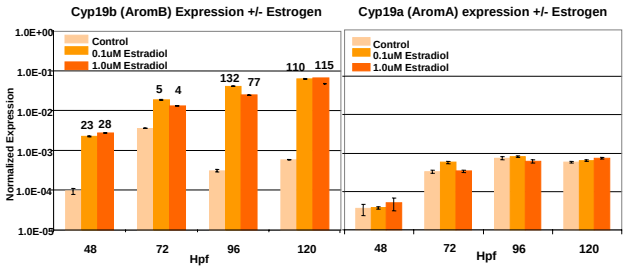


Fig. E. Both estrogen-responsive (cyp19B) and non-responsive (cyp19A) genes are developmentally programmed. Results show a dramatic dose- and time-related induction (up to 115-fold) of expression of the estrogen-responsive target gene (AromB; left figure) with estrogen exposure. By contrast, estrogen exposure has no effect on expression of a closely related control (estrogen unresponsive) gene (AromA; right figure). Numbers above bars show fold-increase with estrogen relative to corresponding control.

IMPACT AND OUTPUTS

- Addresses an important problem in environmental sciences: namely, identification of chemicals with endocrine disrupting activity and the need to assess the extent of the impact of these contaminants on the health of man, animals, ecology and ecosystems.
- Uses a mechanistic approach, and methods of computational, developmental and molecular biology, to develop a new tool for routine chemical screening of, and identification of EDC among, the >87,000 chemicals added to the environment.
- Focuses on EDC that act on ER and AhR responsive genes, but designed as a proof-of-principle study with applicability to chemicals that act via any other nuclear receptor-mediated hormone regulatory pathway.

PARTNERSHIPS

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