

Chemical Induced Changes in Gene Expression Patterns Along the HPG-axis at Different Organizational Levels Using a Small Animal Model (Japanese medaka)

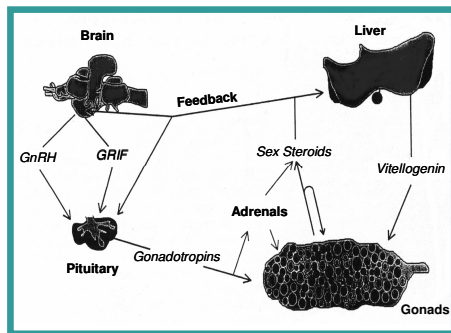
Markus Hecker, Amber Tompsett, June-Woo Park, Paul Jones, John Newsted & John Giesy
U.S. EPA, ORD, Computational Toxicology Research

Project ID - IA-3



Science Question

Recent research on endocrine disruptors has focused on compounds that are direct hormone agonists or antagonists, especially those that interact with the estrogen receptor (ER). Because chemicals can cause both direct (receptor-mediated) and indirect effects (through changes in signal transduction pathways), methods are needed that permit the screening of both types of responses. Furthermore, techniques are needed that can screen for these effects simultaneously in a number of tissues, including during critical windows of development, when tissues may be small and the amount of material available for testing is small and difficult to remove from the organism. Therefore, we are developing a whole animal, *in situ* hybridization (ISH) test system to screen for effects in multiple tissues in the same organism to better understand mechanisms of action of endocrine disrupting chemicals (EDCs).



Tissue	Primary Gene
Brain	CYP19A, CYP17, GnRHs; ERα & β; AR
Pituitary	GnRH Receptors; GnRH I & II; ERα & β; AR; Activin/Inhibin
Adrenals	CYP11A & B; CYP17; 3β-HSD; 17β-HSD; StAR; AR; GnH Receptors; Activin/Inhibin
Gonads	CYP11A & B; CYP17; CYP19A; 3β-HSD; 11β-HSD; 17β-HSD; StAR; ERα & β; AR; GnH Receptors; Activin/Inhibin

Figure 1. Systems approach: Interactions of related genes of interest among target organs.

METHOD DEVELOPMENT

Whole Animal Histology

- Gross dissection: Removal of skull roof, otoliths, and opercula; body cavity longitudinally opened
- Standard tissue processing and embedding
- Sections cut serially at 10 μm and floated onto a 40°C water-bath
- Sections picked up on Superfrost Plus slides
- Slides dried at 40°C overnight



Figure 2: Paraffin embedded sample shows good morphology

Histological Evaluation: Hematoxylin and Eosin staining

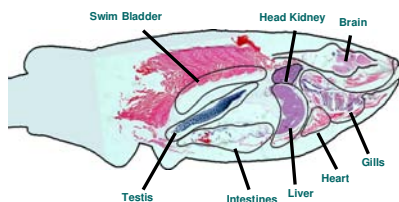


Figure 3: Hematoxylin & Eosin stained male Japanese medaka

Methods/Approach/Results

In situ Hybridization

- Selection of genes
 - Initially, three genes were selected to develop ISH techniques (CYP19A & B, and beta actin (housekeeping)).
 - Entire list of genes to be analyzed in the project is given in Figure 1.
- Synthesis of riboprobes
 - cDNA generated using the primers in Table 1
 - Product sequenced and size checked (Fig. 4)
 - cDNA cloned into an E. coli vector
 - Riboprobes synthesized with RNA polymerase using standard methods

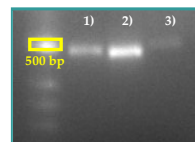


Figure 4. Electrophoresis of PCR products. 1) Beta actin; 2) CYP19A; 3) CYP19B.

Table 1. Primer sequences for synthesizing riboprobes

Primer	Accession #	Sequence (sense/antisense primer)
beta actin	D89627	5'-TTGGCAATGAGAGGTTCC-3' / 5'-AATGAGTGGTCTGCTAGG-3'
CYP19A	D82968	5'-CCTGTTAATGGTCTGGAGTCAC-3' / 5'-GAAGAGCCTGTTGGAGATGTC-3'
CYP19B	U181998	5'-CTTCTCTGCTGGTATGTTTCTGTC-3' / 5'-GTCACCACAATCGCTCTG-3'

In situ Hybridization Results

Tissue Section Hybridization

- Standard hybridization protocols
- Visualization with digoxigenin/alkaline phosphatase system
- Complete optimization of the system in progress
- High specificity for target sequences demonstrated (Figs. 5, 6, & 7)
- Sense probe (negative control) with low/no nonspecific binding

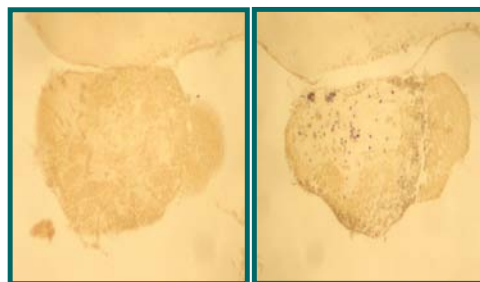


Figure 5. Hybridization with CYP19B riboprobe, adult female medaka: Sense probe (left) shows no staining; Antisense probe (right) shows specific staining in the hypothalamus of the brain. No counterstain.

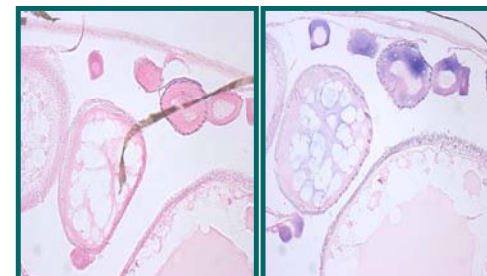


Figure 6. Hybridization with CYP19A riboprobe, adult female medaka: Sense probe (left) shows no staining; Antisense probe (right) shows specific staining (purple) in early stage oocytes. Counterstain: nuclear fast red (pink)

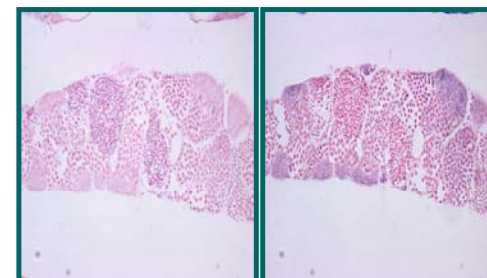


Figure 7. Hybridization with CYP19A riboprobe, juvenile male medaka: Sense probe (left) shows no staining; Antisense probe (right) shows specific staining (purple) in the testis. Counterstain: nuclear fast red (pink)

Conclusions

In an initial approach three genes were selected to develop and validate the ISH approach using Japanese medaka. Sectioning techniques have been optimized, and a standard protocol was developed. A set of three genes consisting of two target genes (CYP19A & B) and one housekeeping gene (beta-actin) have been developed focusing on their specific utilization for ISH. Selective staining of the complementary anti-sense strands with no parallel staining of the sense strands indicates the specificity of the probes. Currently, an exposure experiment using fadrozole, a specific inhibitor of the expression of the aromatase gene, is underway to further validate the ISH methods developed in this project.

Impact and Outcomes

The methods developed in this project will allow for screening of multiple effects in multiple tissues, even at points in development when the tissues are too small to be accurately dissected for use in more traditional molecular techniques. These methods will be applied to a set of "model" and "test" compounds for a set of target genes. Once the methods have been developed and validated, they can be adapted for use with other genes and/or species of interest and used to efficiently and completely screen for endocrine disruptor effects beyond simple receptor binding.

Research Goals

- Development of methods that will permit the identification of changes in the expression profiles of genes that are associated with key aspects along the HPG-axis such as hormone receptors, gonadotropins, steroidogenic enzymes, etc. in the Japanese medaka, as a small animal model (Figure 1).
- Application of these techniques to develop gene expression profiles for "model" compounds of specific modes of endocrine actions (e.g. estrogenic, androgenic), and compare these changes to biologically relevant endpoints such as reproductive performance.
- Application of multivariate statistical pattern recognition techniques to differentiate the effects of different classes of chemicals.
- Test the hypothesis that the identified responses can be used to classify endocrine disrupting "test" chemicals into groups to facilitate risk assessments of multiple chemicals and development of quantitative predictive models based on structure activity relationships (QSARs).

CURRENT ACTIVITIES

Method Validation Study with Fadrozole

- Exposure of 10 male and 10 female four-months-old medaka to 0, 1, 10 and 100 μg/L fadrozole.
- In situ* hybridization of whole fish sections using CYP19A & B, and beta actin riboprobes.
- Confirmation of hybridization results using quantitative RT-PCR techniques.



Figure 8. Fadrozole validation exposure study set up & the MSU medaka team during water renewal.

Future Directions

- Methods for ISH will be optimized based on the results obtained during the initial fadrozole exposure experiment.
- Probes for the genes listed in figure 1 are synthesized to investigate multiple endpoints along the HPG.
- Development of multiplexing methods is underway to allow for the determination of multiple genes in the same section.
- Validate the ISH model using five "model" compounds known to interact with endocrine pathways: Fadrozole, trenbolone, ethinylestradiol, ZM 189,154, vinclozolin.
- Validate effects observed using the ISH model by quantitative RT-PCR.
- Evaluate the relevance of the changes measured at the gene expression level by comparing them to effects at higher levels of organization:
 - Protein (immunohistochemistry)
 - Hormone (ELISA)
 - Organism ("Partial Life-Cycle Test")