Examination of the Estrogen Response Pathways in a New Vertebrate Model

Project Scope

This project investigated estrogen responses in developing zebrafish embryos and young fish, to complement studies conducted using mouse models. Researchers identified and isolated a zebrafish estrogen receptor and investigated estrogen response element (ERE)-driven responses in transgenic fish.

The main objectives of this research were to:

- Examine the responsiveness of various EREs during specific stages of development through transient injections of DNA constructs having EREs coupled to the a simple thymidine kinase promoter driving expression of green fluorescent protein (GFP), or through the production of transgenic fish having these genes incorporated, as new genetic material, into their genome.
- Examine both the appearance of estrogen mRNA during development and the time course of estrogen receptor responsiveness after the administration of various estrogenic endocrine disruptors.

The expression of the zebrafish estrogen receptor during early development was investigated using whole-mount in situ hybridization and fluorescence to localize the estrogen receptor mRNA during embryogenesis and to also examine its expression under the influence of treatment with estrogens. Then, the expression was examined using an introduced estrogen responsive reporter gene that provides a localized fluorescent signal (the GFP gene). With this reporter, researchers aimed to record gene expression within the whole organism and capture this expression using a laser scanning confocal microscope.

Grant Title and Principal Investigator

Examination of the Estrogen Response Pathways in a New Vertebrate Model

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Key Findings and Implications

- Transgenic fish that express a nuclear-targeted, enhanced, green fluorescent protein (eGFP) gene were produced using both pseudotyped retroviral vector infection and DNA microinjection of embryos.
- Technologies were developed to help in the isolation, characterization, and study of a zebrafish estrogen receptor beta (ER-β).
- Researchers developed a YFP reporter that minimized autofluorescence while providing a bright reporter gene product.
- Computerized time-lapse fluorescent images of developing zebrafish covering several days of growth demonstrated the progression of developmental gene expression. Analysis of the images allowed the researchers to visualize gene in relations to specific embryonic events, which could be done with mammalian embryonic models.
- Developmental expression studies found indicates maternal loading with ER-β, followed by degradation and transcription between 24 and 48 hours postfertilization, coinciding with aromatase expression.
- Sequence similarity of other species indicate that ER-β has been highly conserved during evolution and is likely used during later embryogenesis in zebrafish.

Project Period: November 1996 to November 1999

Project Results and Implications

A major part of the research project was the development of a transgenic zebrafish model that indicated reporter gene activity associated with developmentally regulated promoters. Researchers investigated methods to routinely obtain transgene expression in zebrafish and measure the signal from a non-enzymatic reporter gene such as the fluorescent reporter GFP. Because the zebrafish embryo can produce considerable autofluorescence that can obscure the reporter fluorescence, the investigators studied different mutant forms of the GFP gene. They found that the YFP (yellow fluorescent protein) reporter minimized autofluorescence while providing a bright reporter gene product. Embryonic pigmentation appeared within 24 hours of hatching and caused fluorescence quenching of the reporter

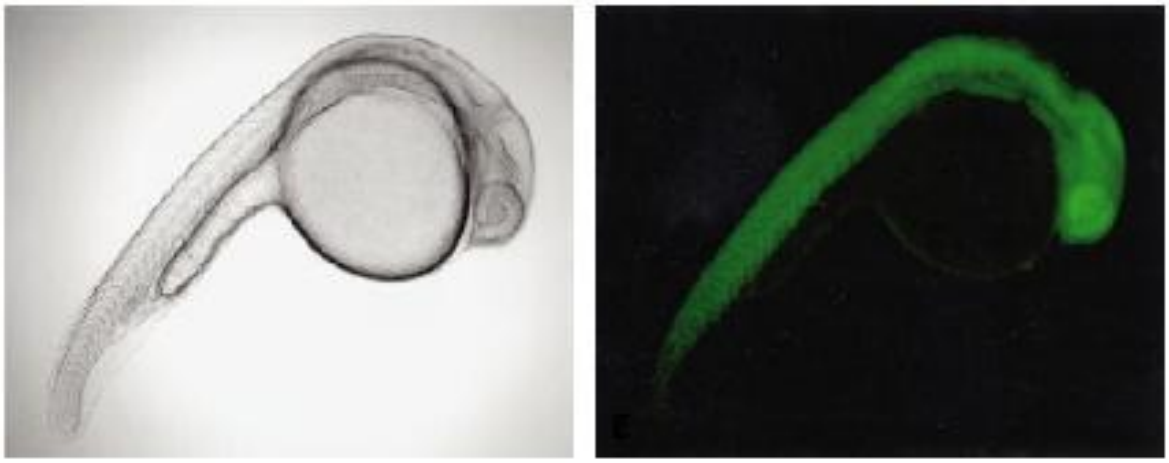
signal. Researchers transferred transgenic lines into zebrafish lines to minimize natural pigmentation and bred the transgenic lines produced into a homozygous albino background strain. The resulting zebrafish cross allowed researchers to follow fluorescence in individual cells in the embryo and larvae for as long as 18 days. Using this resulting transgenic zebrafish model, live embryos could be embedded in agarose, mounted, and imaged. Two-day time-lapse capture studies of developing zebrafish were converted to quick-time movies to demonstrate developmental gene expression. This allowed for visualization of gene expression during embryonic development in relation to specific embryonic events, which was not possible in mammalian embryonic models at the time of this research.

Relevance to ORD's Multi-Year Research Plan

This project contributes to ORD's Multi-Year Plan long-term goal of determining the extent of the impact of endocrine disruptors on humans, wildlife, and the environment (LTG-1)by studying alternative methods for introducing expressing transgenes into the germ line of zebrafish. The research contributed to the understanding of estrogen receptor expression and action in developing animal models. Results provided information on the modes of estrogen action during early development (i.e., in the embryonic stages).

Zebrafish ER-β isolation. Using consensus oligonucleotides for regions shared by estrogen receptors from several different species, RNA from adult female liver and ovaries was used as a target for reverse transcriptase-polymerase chain reaction (RT-PCR) amplification of estrogen receptor sequences. Cloning and sequencing of the product confirmed that at least one clone was estrogen receptor-like. Researchers then used this partial cDNA to isolate a full-length estrogen receptor cDNA from a cDNA library from 30-day-old zebrafish. The receptor isolated was an estrogen receptor (ER)-β whose DNA sequence was very similar to that from the analogous goldfish gene. Transfection experiments confirmed that the gene product was an estradiol inducible receptor. Using RNAse-resistant RNA-RNA hybridization analysis and acrylamide gel electrophoresis, the concentration of this mRNA was found to be very low during embryogenesis, increasing into the larval and adult stages. Antiserum against the carboxyl terminal region of the receptor allowed the identification of ER-β using Western blotting procedures. Genomic sequences from the 5' end of the gene were isolated that appeared to include at least some of the promoter region. The researchers have begun work to identify the complete promoter region so that it can be used to derive transgenic reporter fish displaying fluorescence when the ER-β gene is expressed, as well as to help to identify the DNA controlling elements that mediate its expression.

Zebrafish estrogen response element (ERE) driven transgenic lines. Transgenic zebrafish lines with an estrogen response element coupled to the Herpes simplex virus TK promoter were produced. In zebrafish liver cell lines, transfection of this regulatory sequence coupled to a firefly luciferase reporter gene resulted in estradiol induction of luciferase activity. In the transgenic cell lines, the regulatory sequence was coupled to a GFP gene modified to target the protein to the nucleus. Two transgenic lines were isolated and partially characterized. GFP was expressed in a limited number of cells (20-50) that were detectable 24 hours postfertilization (see photos). The GFP-positive cells appeared to migrate along the pronephric ducts over the course of 14 days and aggregate around the pronephros. While the researchers could not precisely identify the site of aggregation/association, the pattern of GFP expression in this limited number of cells suggest that these migrating cells might be precursors to primordial germ cells. Antiserum to the zebrafish ER-β and to the zebrafish vasa protein (a germ cell marker) were produced,for use in situ to localize ER- and vasa-positive cells and confirm the nature of the GFP-positive cells.



A 24 hour zebrafish embryo expressing GFP derived from a transgenic founder. Source: http://glowfish.mc.duke.edu/about.html

Experimental treatment of the transgenic ERE cell lines with estradiol or anti-estrogens have revealed no distinct change in the GFP fluorescence, but these results are preliminary. Preliminary experiments with administration of estradiol to adult females resulted in an increase of GFP expression in regions of the reproductive tract. Researchers hoped to use quantitative PCR analysis to investigate a change of reporter RNA at an individual embryo level in future projects. The researchers planned to investigate other methods to determine the estrogen responsiveness of these transgenic fish in future projects.

Investigators

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For More Information

Laboratory web page:
http://glowfish.mc.duke.edu/
http://mgm.duke.edu/faculty/linney/index.htm
NCER Project Abstract and Reports:
http://cfpub2.epa.gov/ncer_abstracts/index.cfm/fuseaction/display.abstractDetail/abstract/135/report/0