Xenoestrogen Effects During Premeiotic Stages of Spermatogenesis Development in an *In Vitro* Test System and Molecular Markers of Action

Project Scope

The objective of this project was to test the hypothesis that environmental estrogen-like chemicals (xenoestrogens) act directly on cells of the testis to disrupt estrogen-dependent mechanisms of cellular growth control during spermatogenesis. The study used the dogfish shark, an animal model in which successive stages of spermatogenic development and different cell populations were isolated. This allowed for both direct observation and experimental manipulation to establish causal relationships and mechanisms. The focus was on early (premeiotic) spermatogenic stages when nuclear estrogen receptor concentrations are highest.

The main objectives of this research were to:

- Identify elements of the estrogen-activated gene response cascade for use as molecular markers of estrogen action;
- Characterize estrogen's role in regulating cellular proliferation, survival, and development during spermatogensis.

The dogfish shark (*Squalus acanthias*) was identified as a useful model organism for a stage-by-stage analysis because of its cystic mode of spermatogenesis and the simple linear arrangement of developing spermatocysts across the diameter of the testis. In response to the agonistic or antagonistic activity of particular xenoestrogens, disruption of this natural developmental scheme was characterized based on altered profiles of molecular markers and either a deficit in the number of cells that complete spermatogenesis (infertility, sterility) or uncontrolled expansion of the cell population (testicular cancer).

Grant Title and Principal Investigator

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Key Findings

Findings and Accomplishments:

- Developed an animal model for stage-by-stage analysis of factors and mechanisms controlling spermatogenesis, based upon the testis of the dogfish shark (*Squalus acanthias*).
- Documented apoptosis and mitosis *in vivo* and *in vitro*.
- Data confirmed that hormonal estrogen and xenoestrogens preferentially target stem cell and early spermatogonial generations.
- RT-PCR analysis with gene-specific primers showed that androgen receptor (AR) expression in testis is stage-dependent (PrM > M > PoM).
- Data indicate the testis is a direct target of certain arylhydrocarbon receptor (AHR)-binding xenobiotics, and that growth control processes in early developmental stages are targeted.
- Established the utility of the shark testis model for identifying novel unknown stage-specific/dependent and toxicant sensitive genes.
- Eight new shark cDNAs were isolated and characterized, contributing significantly to available resources and the body of knowledge in the field. All of the identified genes have homologs in other vertebrates, including man.

Publications include seven peer reviewed journal articles.

Project Period: February 1997 to February 2000

Relevance to ORD's Multi-Year Research Plan

This project contributes to ORD's Multi-Year Plan long-term goal of providing a better understanding of the science of underlying effects of endocrine disruptors. The study evaluates potential approaches to assessing risks in humans and wildlife, focusing on the effects of endocrine-disrupting pesticides during premeiotic stages of spermatogenesis and the development of an *in vitro* testing model. More specifically, the research helped to elucidate the effects exposure to estrogen-like chemicals during different stages of spermatogeneis and their *in vivo* and *in vitro* mechanisms of action.

Project Results

Aim 1: Stage-by-Stage Documentation of Apoptosis and Mitosis during *In Vivo* and *In Vitro* Spermatogenesis Criteria for staging spermatocysts (follicle-like units comprising a single germ cell clone and second clonal population of Sertoli cells) in the developmental progression were established using light microscopy. Eight recognizable sub-stages, from stem cell to mature spermatid stages of development (based on distance from germinal ridge, cyst diameter, presence/absence of a lumen, *etc*.) were identified. Month-by-month analysis of a complete annual cycle provided to the following data: (1) the absolute number and fraction of spermatocysts in given stages reflecting mitoses and apoptosis in preceding generations; (2) the percentage of proliferating-cell nuclear antigen (PCNA) and terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) immunostained cysts (mitotic and apoptotic indices) varied independently by stage of development, time of year, cell type, and specific toxicant exposure, *e.g*., estradiol, cadmium, TCDD (see details below); (3) mature (approaching meiosis) spermatogonial generations were most susceptible to seasonal (hormone withdrawal) and toxicant effects (as measured by an increased percentage of apoptotic clones); (4) germ cells, but not Sertoli cells, were affected by apoptosis; (5) cessation of mitoses in Sertoli cells preceded that in germ cells by several generations; and (6) within susceptible spermatogonial generations, apoptotic cell death was directly correlated with cell cycle activity (*i.e*., the death pathway is entered through cell cycle). The results showed that overproduction of germ cell clones is the normal condition, and that the final germ cell number is determined by programmed cell death in spermatogonial generations 9 through 13. Although results indicate the utility of the shark testis model for obtaining new information of general relevance, they also underline the necessity of substage-by-substage analysis (rather than analysis of mixed stages) to identify and quantify toxicant effects on apoptosis/mitosis.

A second component of this specific aim was the optimization/standardization of an *in vitro* spermatogenesis system. Effects of serum (FBS), estrogen, and other additives on pre-meiotic cysts were determined for apoptotic and dead cells by staining with acridine orange and propidium iodide, respectively, and incorporation of 3H-thymidine (DNA synthesis) as end points. The total RNA was prepared from selected treatment groups for reverse transcription-polymer chain reaction (RT-PCR) and PCR-differential display (DD). The results indicated that serum increased both mitosis and apoptosis (see conclusion 6 above); however, steroid effects were both stimulatory and inhibitory, depending on dose and the presence or absence of serum.

Aim 2: Stage-Specific Expression of Nuclear Estrogen Receptors (ER) and Cytochrome P450 Aromatase (Genbank accession #AF147746 and AF203106, respectively) To characterize the intratesticular estrogen signaling pathway (source and target of estrogen) during spermatogenesis, a sequential PCR cloning strategy was designed to isolate shark testis specific ER and cytochrome P450 aromatase cDNAs. An 1812 bp ER cDNA which included the full open reading frame was obtained, and gene specific probes and primers were designed for Northern analysis and RT-PCR. Phylogenetic analysis showed the shark ER was of the β-subtype with a single 7.1 kb transcript in testis. No evidence for a second ER (α-subtype) was obtained, suggesting that the β-isoform is the ancestral vertebrate molecule. Although low in abundance, ER mRNA was highest in testis, kidney, and liver. Within testis, the expression was stage-dependent: germinal zone (GZ) > pre-meiotic (PrM) >> meiotic (M) = post-meiotic (PoM). The data confirmed our earlier estrogen binding studies and reinforce the view that hormonal estrogen and xenoestrogens target stem cells and early spermatogonial generations. The ER mRNA levels also were enriched in zone of degeneration (ZD) cysts containing Sertoli cells only (and degenerate

germ cells), implying that Sertoli cells are the ER-expressing cell type in spermatogonial stages that contribute to ZD.

Additionally, a 2118 bp P450 aromatase cDNA was isolated from the shark ovary using a PCR cloning strategy. Phylogenetic analysis and sequence comparisons showed that the shark aromatase was neither a β- nor an α-isoform as shown in teleost fish, but instead is more closely related to the ancestor that gave rise to the single copy avian and mammalian aromatase genes. Northern analysis confirmed the results of RT-PCR (PrM > M = PoM), a stage-related distribution that corresponded to our previously measured aromatase enzyme activity. As with ER, aromatase mRNA was enriched in ZD, indicative of preferential localization of aromatase in Sertoli cells.

Aim 3: Identification of Known and Unknown mRNAs that Correlate with Xenoestrogen Effects, Stage of Development and/or Cell-Type (Genbank Accession #AF421550 and 421555) The regulation of spermatogenesis occurs through an interaction of ER- and androgen receptor (AR)-mediated signaling pathways and through the interaction of certain environmental toxicants with AR. Thus a PCR cloning strategy based on degenerate and sequence-specific primers was applied to obtain a ≈3 kb AR cDNA. The isolated cDNA fragment encodes a protein that begins at the DNA binding domain at the 5'-end and extends through the poly-A tail of the 3' UTR, which was very long. A RT-PCR analysis with gene-specific primers showed that AR expression in testis was stage-dependent (PrM > M > PoM), agreeing with the earlier androgen binding assays. Within the PrM region, AR mRNA increased progressively from stem cell to late spermatogonial substages, indicating the early stages of development are targeted by the effects of hormonal androgen and toxicants that bind to AR.

Ligands that bind to the arylhydrocarbon receptor (AHR), such as dioxin and other polycyclic aromatic hydrocarbons (PAHs), are known to be spermatotoxicants, and induce testicular cancer or interfere with estrogen signaling pathways. In collaboration with Mark Hahn (Woods Hole Oceanographic Institution, WHOI, who has cloned fragments of two AHR cDNA variants [1 and 2] from qualus), RT-PCR was used to show the high testicular AHR expression (relative to liver). It was observed that each isoform had a unique stage-related distribution pattern. Pre-meiotic stage spermatocysts cultured with AHR ligands (TCCD, BNF, 33'44'TCB) showed ligand-specific, dose-response effects on apoptosis (AO staining) and DNA synthesis. The data indicates that the testis is a direct target of certain AHR-binding xenobiotics, and that growth control processes in early developmental stages are preferentially targeted.

To evaluate direct and indirect effects of diverse toxicants on germ cells, a shark testis specific homolog of vasa (a known germ cell marker and member of the DEAD box family of helicases) was cloned. Vasa was expressed at the highest levels in the PrM and M stages. Additionally, a shark β-tubulin cDNA was cloned, and sequence-specific primers and probes were generated for normalizing RT-PCR and Northern analyses of all genes of interest. The results indicated that the number and size of multiple β-tubulin mRNAs varied in a stage-specific manner.

To establish the utility of the shark testis model for identifying novel unknown stage-specific/dependent and toxicant-sensitive genes, the PCR-DD method of mRNA fingerprinting was applied. Five primer sets were used to obtain a total of 49 stage-dependent and cadmium (Cd) regulated bands. In the initial experiments, Cd was used because it is a known mammalian spermatotoxicant which interferes with ER actions, and accumulates in ER expressing stages in shark testis where it increases the number of apoptotic cysts. Of the identified bands, three were subjected to further analyses (*e.g.*, sequence analysis, cDNA library enrichment and screening, 5' and 3' RACE, RT-PCR, or Northern analysis). In one cDNA, which was enriched in GZ/PrM stages and upregulated five-fold *in vivo,* Cd was identified as a ~400 bp fragment of the control region of mitochondrial (mt) DNA, implying that it is a marker of ongoing transcriptional activity on the H strand. It may be relevant that, of the 12 proteins encoded by mtDNA, the cytochrome oxidase subunits appear to be involved in caspase activation leading to apoptosis. They also are reported to be stage-dependent and androgen-responsive in rodent testis. A second band was ultimately identified as the shark homolog of BRCA1-associated protein 2 (BRAP2, Acc. # AF421550). The high degree of sequence identity between the shark, human, and bacterial BRAP2 genes implies an important conserved function. There is good evidence that BRAP2 is a novel cytosolic protein that specifically binds to nuclear localization signals, thereby, regulating cytosolic-nuclear shuttling of transcription factors and other nuclear proteins. In shark testis, BRAP2 expression increased with spermatocyst maturation but decreased after Cd exposure *in vivo*, suggesting a possible toxic mechanism for observed effects on apoptosis in PrM stages and additional, still to be identified effects on transcriptional regulation in maturing and mature stages of development. A third PCR-DD identified band was found to be S-100 (Acc. # AF421551), one of a large family of Ca++ binding protein with diverse

functions. In shark testis, it was observed that the S-100 expression increased progressively with maturation and was Cd-regulated.

Aim 4: Applicability and general relevance of the Shark Testis Model Findings of this project clearly demonstrated the utility of modern methods of cellular and molecular biology as applied to the favorable anatomy of the shark testis model for identifying developmentally programmed and toxicant-sensitive genes. Eight new shark cDNAs were isolated and characterized, contributing significantly to available resources and the body of knowledge in the field. All of the identified genes have homologs in other vertebrates, including man. Indeed, sequences are remarkably conserved, signifying important functions and adaptive value. Although the mRNA and protein products of BRAP2 and S-100 have not previously been studied in testis, results of these studies suggest that they could serve as molecular markers of spermatotoxicant effect and point to new hypotheses for testing in laboratory mammals. While results at the start of this project indicated that spermatocysts in defined stages would have utility for high throughput *in vitro* toxicant testing, the following factors necessitated a focus on mainly *in* vivo treatment paradigms: the seasonality of spermatocyst condition, functional variability from one substage to another within a given preparation of staged cysts, and the inability to optimize and stabilize the spermatogenic condition and progression *in vitro* (as measured by apoptosis, mitosis, and mRNA yield and quality) within the time frame of this project. The experiments were especially successful with Cd; in contrast to endogenous estrogen, Cd is virtually undetectable in controls, thereby facilitating recognition of treatment effects in shark testis.

Investigators

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For More Information

Laboratory web page:

http://www.bu.edu/biology/Faculty_Staff/gvc.html

NCER Project Abstract and Reports:
http://cfpub2.epa.gov/ncer_abstracts/index.cfm/fuseaction/display.abstractDetail/abstract/136/report/0