Session 2 Basic Science

Sex Determination and Differentiation in Vertebrates: A Target for Endocrine Disrupting Chemicals

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The sex of most vertebrates is determined by sex chromosomes at the time of fertilization. Various environmental factors such as temperature have also been shown to play a critical role in determining the sex in some non-mammalian species such as some reptiles and fishes. Two sex-determining genes (*SRY/Sry* in mammals and *DMY* in a teleost fish, the medaka, *Oryzias latipes*) were identified in vertebrates and there is no sequence homology between them. Further, phylogenetic analysis indicated that *DMY* acts as the sex-determining gene only in a few species of medaka. It is important to note that *DMY* transgenic XX medaka are fully-functional and fertile males, whereas *Sry* transgenic XX mice are sterile. We will need to continue searching for the elusive genes that underpin sex determination in vertebrate species that determine sex through environmental cues such as temperature and social situation.

Despite the vast diversity of sex-determining mechanisms among vertebrates, the molecular cascades that lead to males and females (gonadal sex differentiation) seem to be relatively conserved. In non-mammalian vertebrates, steroids play a critical role in gonadal sex differentiation. Using all genetic male and female tilapia, we have shown that endogenous estrogens act as the natural inducers of ovarian differentiation, while *DMRT1* may be important for testicular differentiation. The roles of these correlates were further ascertained by gene or hormonal blockade strategy.

Since the sex-determining gene, *DMY* was identified in medaka, we examined the effects of sex steroid hormones on the expression of *DMY* and *DMRT1* using XX and XY medaka. In these experiments, XX and XY fries were treated with androgen or estrogen for several days, respectively. The expression of *DMY* in XX or XY gonads was not affected by either treatment. The expression of *DMRT1* was induced by androgen treatment in XX gonads, but was down-regulated by estrogen treatment in XY gonads. These findings indicate that the sites of actions of sex steroid hormones and endocrine disrupting chemicals are not at the level of the sex-determining gene, but the process of gonadal sex differentiation. The actions of these chemical compounds may be mediated by the actions of somatic cells within gonads. Some of our recent findings on the effects of sex steroid hormones and endocrine disrupting chemicals on gonadal sex differentiation in fish will be presented.

Cross-talk of Estrogen Receptor- and AhR-mediated Signalings

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Environmental contaminants are known to affect a wide variety of biological events in many species, and dioxins, typical environmental contaminants, exert adverse estrogen-related effects. While dioxins are well described to exert anti-estrogenic actions, dioxins are also suggested to exert the possible estrogenic effects. However, the molecular mechanism underlying such estrogen-related actions of dioxins remains largely unknown.

The most actions estrogen (E2) are considered to be exerted through cognate nuclear receptors(ERs) acting as ligand-inducible transcription factors. During the ligand-induced transactivation of ERs, two domains at the N-terminal A/B domains (AF-1) and the C-terminal E domain (AF-2) in ERs are prerequisite by recruiting coactivator complexes (1). The heterodimer of dioxin receptor (AhR) and Arnt, basic helix-loop-helix (bHLH)/PAS family transcription factors, are known to mediate most of dioxins toxic effects.

We have examined a possible cross-talk of two signalings meditaed their cognate receptors (1). Agonists-activated AhR/Arnt heterodimer directly associate with ER? and ER?. This association induced recruitment of unliganded ER with AhR/Arnt and co-activator p300 to estrogen-responsive gene promoters, activating transcription and exerting estrogenic effects, while attenuated function of liganded ER. The estrogenic actions of AhR agonists were detected in wild-type ovariectomized mouse uteri, but were absent in AhR-/- or ER?-/- ovariectomized mice. Our findings uncoverd a novel mechanism whereby

ER-mediated estrogen signaling is modulated by a co-regulatory-like function of the activated AhR/Arnt, giving rise to adverse estrogen-related actions of dioxin-type environmental contaminants.

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Chemical Interference with Non-genomic Steroid Actions: A Novel Mechanism of Endocrine Disruption

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There is now convincing evidence that, in addition to the classic genomic mechanism of steroid action via binding and activation of nuclear steroid receptors, steroids also act at the cell surface of target tissues to initiate rapid, nongenomic responses, and that these actions are mediated by steroid membrane receptors. Steroid membrane receptors and rapid steroid actions, including activation of intracellular signaling pathways, have been identified in many tissues including cardiovascular tissues, brain, pituitary, bone, kidney, liver, gonads, and gametes. Several recent studies have shown that nongenomic steroid actions, like genomic ones, are susceptible to interference by xenoestrogens. Recent studies with spotted seatrout and Atlantic croaker have shown that the mechanism of interference with these nongenomic steroid actions involves xenoestrogen binding to the steroid membrane receptors thought to mediate these actions. A variety of xenoestrogens, including Kepone and o,p'-DDD, bind to the oocyte progestin membrane receptor (mPR) in spotted seatrout and also antagonize progestin-induced oocyte maturation in an in vitro bioassay at concentrations of 10^{-6} to10^{-7} M, equivalent to 20-40ppb, a tissue concentration frequently reported in fish from contaminated environments (Das and Thomas, 1999). Moreover, these xenoestrogen effects on receptor binding and oocyte maturation in vitro are reversible (i.e they were not nonspecific toxic effects of the compounds) and both activities were completely restored after washing. Xenoestrogens have also been shown to interfere with the nongenomic action of progestins to increase sperm motility in these two fish species by binding to their mPRs on sperm. The finding that the inhibitory action of a hydroxylated PCB on sperm motility was partially reversed by addition of excess progestin is consistent with a receptor-mediated mechanism of xenobiotic action. Initial experiments show that the binding of several xenoestrogens (methoxychlor, hydroxylated PCBs and DDT derivatives) to the oocyte mPR is dependent on localization of the receptor in the plasma membrane and is related in part to their lipophilicity. These preliminary results suggest that xenoestrogen interactions with plasma membrane and nuclear steroid receptors are qualitatively different and that membrane receptor-mediated steroid actions may be especially susceptible to interference by lipophilic xenobiotic compounds. In addition, xenoestrogens have been shown to bind to a estrogen membrane receptor in croaker testes and mimic the nongenomic inhibitory actions of estrogen on testicular androgen synthesis (Loomis and Thomas, 2000). Recently we have cloned, sequenced and characterized the seatrout oocyte mPR, the first steroid membrane receptor whose structure has been determined in any vertebrate species (Zhu et al., 2003a). The seatrout mPR has 7-transmembrane domains which is characteristic of G-protein coupled receptors (GPCRs). Fourteen similar genes have been identified and partially characterized in other vertebrates, including three in humans (Zhu et al., 2003b). Preliminary results indicate that the recombinant proteins produced in a bacterial expression system transfected with the three human genes also bind progestins and have characteristics typical of mPRs. In conclusion, a new family of steroid receptors has been discovered that is structurally unrelated to nuclear steroid receptors, but instead has characteristics typical of GPCRs. Moreover, evidence has been obtained that xenoestrogens can interact with mPRs belonging to this receptor family. The discovery of the structures of mPRs will enable experimental and theoretical approaches to be developed to determine their potential interactions with xenoestrogens at the molecular level.

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Hormonal Regulation of Oocyte Maturation in Starfish

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After the oocyte completes its growth (vitellogenesis in oviparous animals), it becomes ready for the next phase of oogenesis, i.e., the resumption of meiosis, which is accompanied by several maturational processes in the nucleus and cytoplasm of the oocyte. This process, called oocyte maturation, occurs prior to ovulation and is a prerequisite for successful fertilization. Oocyte maturation has been studies in a variety of vertebrates and invertebrates, including mammals, amphibians, fishes, and starfishes. As a result, it is now established that oocyte maturation in animals is regulated by three major mediators, gonadotropin (GTH) or gonad-stimulating substance (GSS), maturation-inducing hormone (MIH), and maturation-promoting factor (MPF). Several endocrine-disrupting chemicals have been reported to antagonize MIH-induced meiotic maturation of fish oocytes in vitro. More recently, diethylstilbestrol (DES) was shown to induce oocyte maturation in several fishes.

Here I will review the hormonal regulation of oocyte maturation in starfish. The starfish oocyte maturation has been investigated for nearly 40 years and currently is the most thoroughly characterized model of hormonal regulation of reproduction in invertebrates. In starfish, the ripe ovary contains a huge number of fully grown oocytes of almost equal size. Each oocyte possesses a large nucleus (germinal vesicle, GV), which is arrested in late prophase of the first meotic division. Such immature oocytes are not fertilizable. In 1959, Chaet and McConnaughy found that a water extract of starfish radial nerves can induce the shedding of gametes when injected into the coelomic cavity of ripe animals. The active substance also induces oocyte maturation in isolated ovarian fragment. Since the active substance called as GSS is detectable in the coelomic fluid of starfish during the breeding season, GSS is considered to be a hormone. Recently, we have succeeded in purifying GSS from starfish Asterina pectinifera.

Although GSS is the first mediator in inducing oocyte maturation in starfish, the action of GSS is indirect. This hormone acts on the ovary to produce the second mediator, 1-methyladenine (1-MeAde). Microinjection of 1-MeAde into full-grown immature starfish oocytes was ineffective in inducing oocyte maturation, whereas external application was effective, suggesting that the action of 1-MeAde may be mediated by a membrane-bound receptor protein. Ten synthetic N1-substituted adenines were used to analyze the interaction between 1-MeAde and its stereospecific receptors on the oocyte plasma membranes of the starfish Asterina pectinifera. Our results suggest that regional-specific sterical structures at the N1-site of adenine are important in the interaction between 1-MeAde and its receptors in oocytes. The early steps following 1-MeAde action involve the formation of the major mediator of this hormone, maturation (or M phase)-promoting factor (MPF), which consists of cdc2 kinase and cyclin B. MPF transfer studies have shown that MPF activity is not species-specific and is similar among invertebrates and vertebrates.

Epigenetic Transgenerational Endocrine Disruptor Effects on Male Fertility

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Transgenerational effects of environmental toxins, such as endocrine disruptors, significantly amplify the impact and health hazards of these compounds. The transgenerational nature of the actions of these compounds suggests an epigenetic effect on the germ-line. The current proposal is designed to investigate this transgenerational epigenetic phenomenon on male reproduction. Endocrine disruptors have been shown to influence male reproduction by causing abnormal sperm numbers and fertility. One of the most sensitive periods to endocrine disruptor exposure is during embryonic development. The objective of the research is to investigate the mechanism of action of a model endocrine disruptor on male reproduction with a focus on testis development. A rodent model system is used to provide insight into the mechanistic aspects of endocrine disruptor action. The model endocrine disruptor tested is methoxychlor that has metabolites that are both weak estrogenic and anti-androgenic compounds. Therefore, this model endocrine disruptor allows consideration of both estrogenic and anti-androgenic endocrine disruptor actions. The objective is to obtain insight into the molecular, cellular and physiological actions of endocrine disruptors on male reproduction. **THE HYPOTHESIS TESTED IS THAT TRANSIENT EMBRYONIC IN UTERO EXPOSURE TO AN ENDOCRINE DISRUPTOR INFLUENCES THE EMBRYONIC TESTIS TRANSCRIPTOME AND THROUGH EPIGENETIC EFFECTS (e.g. DNA METHYLATION) RESULTS IN ABNORMAL GERM CELL DIFFERENTIATION THAT SUBSEQUENTLY INFLUENCES ADULT SPERMATOGENIC CAPACITY AND MALE FERTILITY AND THAT THIS PHENOTYPE IS TRANSGENERATIONAL THROUGH THE GERM-LINE.** Previous studies have shown that methoxychlor and vinclozolin can effect embryonic testis development at the time of testis morphogenesis and that this causes an increase in germ cell apoptosis in the adult. Interestingly, observations suggest this abnormal spermatogenesis is transgenerational and may be due to altered DNA methylation of the germ-line through an epigenetic action of the endocrine disruptor. Preliminary studies have also demonstrated that altered gene expression of paracrine growth factors directly influences testis development at the time of endocrine disruptor action. Abnormal testis development and germ cell differentiation caused by endocrine disruptors may in part be due to inappropriate control of the testis transcriptome. into the impact of endocrine disruptors on human development, reproduction and health. The novel observations of transgenerational epigenetic endocrine disruptor actions on male reproduction critically impacts the potential hazards of these compounds as environmental toxins.