FROM BASIC STUDY FOR ENDOCRINE DISRUPTORS TO ENVIRONMENTAL SEX DETERMINATION IN REPTILES AND DAPHNIDS

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Estrogens and androgens play fundamental roles in regulating reproductive activities through the estrogen receptor (ESR) and androgen receptor (AR) in vertebrates. Developmental estrogen exposure induces female development in teleosts, amphibians and some of reptile species. Most vertebrates have two ESR subtypes (ESR1 and ESR2) and one AR, whereas teleost fish have at least three ESRs (ESR1, ESR2a and ESR2b) and two ARs (ARα and ARβ) after the teleost specific whole genome duplication. Thus, intricate functionalization has been suggested among ESR and AR subtypes. To date, distinct roles of ESR and AR have been characterized in only a limited number of species such as mice and rats.

ARα showed a unique intracellular localization with a higher transactivation response than that of ARβ in medaka (*Oryzias latipes*). We identified two key substitutions generating a new functionality of euteleost ARα. The substitution in the hinge region contributes to the unique intracellular localization of ARα. The substitution on helices 10/11 in the ligand-binding domain possibly modulates hydrogen bonds that stabilize the receptor-ligand complex leading to the higher transactivation response of ARα. The substitutions generating a new functionality of teleost ARα were fixed in teleost genome after the divergence of the Elopomorpha lineage.

We used custom developed *in vitro* ESR1 reporter gene assays for nine fish species to analyze the ligand- and species-specificity for 12 environmental estrogens. Transcriptional activities mediated by estradiol-17β (E2) were similar in ESR1 sensitivity among species. Responses of the different fish ESR1s to weaker environmental estrogens varied, and for some considerably. Medaka, stickleback, bluegill and guppy showed higher sensitivities to 4-nonylphenol (NP), octylphenol, bisphenol A (BPA) and the *o,p′*-DDT compared with cyprinid ESR1s. In addition, ligand-binding domain (LBD) plays a significant role in accounting for ligand sensitivity of ESR1 in different species. Then, we applied *in vitro* reporter gene assays for three ESR subtypes in five fish species to investigate the ESR subtype-specificity for better understanding the signaling pathway of estrogenic chemicals. Responses to BPA, NP and

o,p'-DDT varied among ESR subtypes, and the response pattern of ESRs was basically common among the different fish species. LBD of the different ESR subtypes generally plays a key role in conferring responsiveness of the ESR subtypes to estrogenic chemicals.

We are currently analyzing ESRs knockout and ARs knockout medaka for understanding function of each receptor subtype.

During studies of environmental chemicals effects on wildlife, we found that exposure of estrogenic chemicals to eggs during critical developmental stage induces females even incubated at male producing temperature (MPT) in American alligators (*Alligator mississippiensis*) having temperature-dependent sex determination system, and exposure of juvenile hormone (JH) agonists to *Daphnia magna*, usually producing female offspring as parthenogenesis, induces male offspring production. Therefore, we decided to analyze molecular mechanism of environmental sex determination in alligators and Daphnia.

Temperature-dependent sex determination (TSD), commonly found among reptiles, is a sex determination mode in which the incubation temperature during a critical temperature sensitive period (TSP) determines sexual fate of the individual rather than the individual's genotypic background. In the American alligator, eggs incubated during the TSP at 33 °C (male producing temperature: MPT) yields male offspring, whereas incubation temperatures below 30 °C (female producing temperature: FPT) lead to female offspring. Many of the details of the underlying molecular mechanism remains elusive, and the molecular link between environmental temperature and sex determination pathway is yet to be elucidated. We found the alligator TRPV4 ortholog (AmTRPV4) is activated at temperatures proximate to the TSD-related temperature in alligators, and using pharmacological exposure, we demonstrated that AmTRPV4 channel activity affects gene expression patterns associated with male differentiation. This is the first experimental demonstration of a link between a well-described thermo-sensory mechanism, TRPV4 channel, and its potential role in regulation of TSD in vertebrates, shedding unique new light on the elusive TSD molecular mechanism.

In crustacean *Daphnia magna* and *D. pulex*, we identified a gene, *Doublesex 1*, which is responsible for the production of males. This gene is homologous to the *Doublesex* gene that is functionally conserved in animal species that use genetic sex determination. Expression of *Doublesex* gene was increased primarily in male-specific structures. Gain- and loss-of-function analyses established that Daphnia *Doublesex 1* gene is a major effector that regulates the male phenotype in Daphnia. Five species of Daphnids express *Doublesex 1* gene during male development induced by JH agonists infer that there is an ancient, previously unidentified link between genetic and environmental sex determination. We are currently working on gene cascade before and after the synthesis of JH (methyl farnesoate) in Daphnids.

Keywords: estrogen receptor, androgen receptor, medaka, alligator, daphnids