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Synthetic chemicals found in the environment have the capacity to disrupt the development and function of the endocrine system in both wildlife and humans. This has drawn public concern since many of these chemicals may bind to estrogen receptors (ERs) and evoke estrogenic effects. Early evidence that exposure to estrogenic chemicals during development could pose a threat to human health came from studies of a synthetic hormone, diethylstilbestrol (DES), which was used to prevent premature birth and spontaneous abortion. Laboratory experiments showed that exposure to sex hormones during critical windows of perinatal life caused the immune and nervous systems, bone, muscle, and the liver of animals to be affected. Although many of these chemicals can bind to ERs in wildlife and humans, the molecular basis for the action of environmental estrogens remains poorly understood. Thus, understanding the molecular mechanisms through which environmental estrogens and sex hormones act during critical developmental windows is essential.

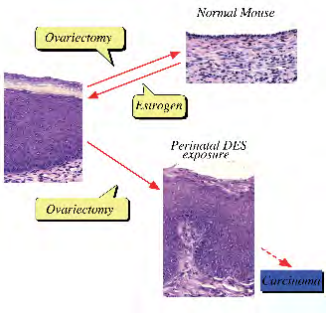

Figure 1. Scheme of estrogen-dependent and -independent vaginal epithelial cell proliferation in mice induced by perinatal estrogen exposure.

I. Developmental origin of adult disease: Perinatal estrogen exposure induces persistent changes in reproductive tracts

The emerging paradigm of the "embryonic/fetal origins of adult disease" provides a powerful new framework for considering the effects of endocrine disrupters on human and animal health. In 1971, prenatal DES exposure was found to result in various abnormalities of the reproductive tract in women. This syndrome was named the DES syndrome. Similar abnormalities have been demonstrated in experimental animals exposed perinatally to estrogens. Developmental estrogen exposure in mice, for example, induces persistent proliferation of vaginal epithelial cells (Figure 1). We found that the persistent changes in the vagina in mice exposed neonatally to estrogens result from the persistent activation of erbBs and ERα, and sustained expression of EGF-like growth factors (Figure 2). Currently, we are analyzing the methylation status in the mouse vagina using a microarray (MeDIP-chip). We found several differentially methylated or demethylated DNA profiles in neonatally DES-exposed mouse vaginae and controls. We thus consider that neonatal DES exposure affects DNA methylation profiles, resulting in persistent abnormalities in mouse reproductive organs. We also found that ERα is indispensable for normal vaginal epithelial cell differentiation in mice.

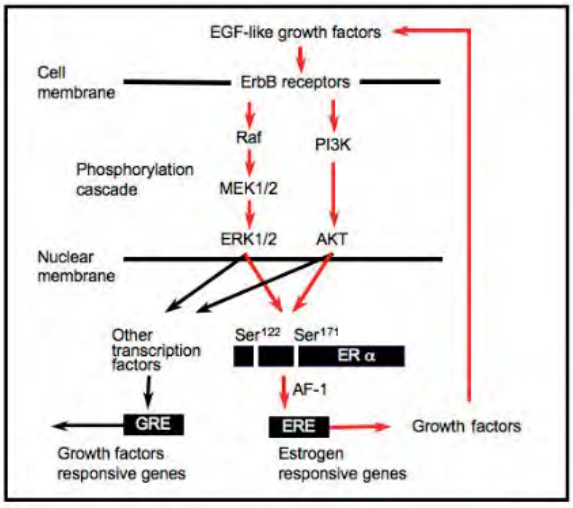

Figure 2. A hypothetical model for the estrogen-independent ER activation pathway in mouse vaginae.

II. Estrogen receptors of birds, reptiles, amphibians, and fishes

Steroid and xenobiotic receptors (SXR) have been cloned from various animal species (fish, amphibians, reptiles, birds, and mammals) by our group and we have demonstrated species-specific differences in their responses to various environmental and endogenous chemicals (receptor gene zoo). Thus, simple predictions of chemical effects based on data from a few established model species are not sufficient to develop real world risk assessments. ER and ER-like genes have been cloned from various animal species including rockshell, *Amphioxus*, lamprey, catshark, whale shark, lungfish, sturgeon, gar, *polypterus*, arowana, roach, stickleback, mosquitofish, mangrove *Rivulus*, Japanese giant salamander, Tokyo salamander, newt, axolotl, toad, *Silurana tropicalis*, American alligator, Nile crocodile, freshwater turtle, Japanese rat snake, Okinawa habu, and vultures. Functional studies showed that the *Amphioxus* ER sequence does not bind estrogen but *Amphioxus* steroid

Note: Those members appearing in the above list twice under different titles are members whose title changed during 2015. The former title is indicated by an asterisk (*).
†: This laboratory was closed on 31 March, 2016.

receptor and lamprey ER exhibited ligand-dependent transactivation, proving that invertebrate and primitive vertebrates, such as the Agnatha, have a functional ER. We found that medaka ER subtypes have their specific functions, and medaka, zebrafish and stickleback ERs are more sensitive to estrogen/estrogen-like chemical exposures than other fishes by reporter gene assay. Thus, these approaches are efficient to evaluate the relationship between species and their sensitivities to chemicals.

III. Evolutionary history and functional characterization of androgen receptor genes in jawed vertebrates

Vertebrates show diverse sexual characteristics which are regulated by androgens. To elucidate the evolutionary history and functional diversification of androgen receptor (AR) genes in vertebrates, we cloned the AR cDNAs from a shark, basal ray-finned fishes (Actinopterygii), namely bichir and sturgeon (Acipenseriformes), and teleosts including a basal teleost, arowana (Osteoglossiformes). Molecular phylogenetic analysis revealed that a gene duplication event gave rise to two different teleost ARs (α and β) and likely occurred in the actinopterygian lineage leading to teleosts after the divergence of Acipenseriformes but before the split of Osteoglossiformes. Functional analysis revealed that the shark AR activates the target gene via the androgen response element by classical androgens. The teleost ARα showed unique intracellular localization with a significantly higher transactivation capacity than that of teleost ARβ. These results indicate that the most ancient type of AR, as activated by the classic androgens as ligands, emerged before the Chondrichthyes-Osteichthyes split and the *AR* gene was duplicated during a teleost-specific gene duplication event (Figure 3).

IV. Papillary process formation in medaka

Androgens play key roles in the morphological specification of male type sex characteristics and reproductive organs, whereas little is known about the developmental mechanisms. Medaka show a prominent masculine sexual character, papillary processes in the anal fin, which has been induced in females by exogenous androgen exposure. We have identified androgen-dependent expressions of *Bmp7* and *Lef1* are required for the bone nodule outgrowth leading to the formation of the papillary process in the postal region of the anal fin. We have also developed a testing method for screening of chemicals having androgen and anti-androgenic activity using the anal fin in juvenile medaka.

V. Environmental sex differentiation in Daphnids and American alligators

Daphnia magna has been used extensively to evaluate the organism- and population-based responses of toxicity or reproductive toxicity tests. These tests, however, provide no information about the mode of action of the tested compounds. Therefore, we applied an ecotoxicogenomic assessment of *D. magna*. We established a *Daphnia* EST database and developed an oligonucleotide-based DNA microarray

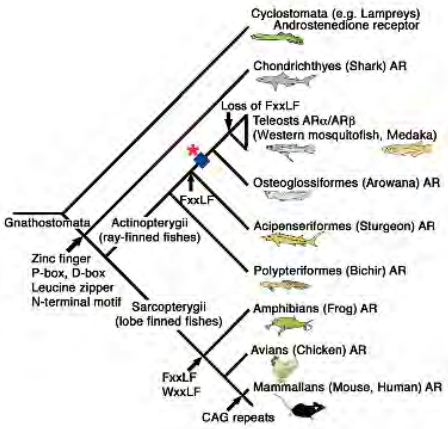

Figure 3. Evolutionary relationships of androgen receptor sequences.

with high reproducibility and demonstrated the usefulness of the array for the classification of toxic chemicals as well as for the molecular understanding of chemical toxicity in a common freshwater organism. *D. magna* and *D. pulex* reproduce asexually (parthenogenesis) when they are in an optimal environment for food, photoperiod and population density. Once environmental conditions become suboptimal, they alter their reproductive strategy from asexual to sexual reproduction (Figure 4). Chemicals are able to affect the sex determination of daphnids and we found that juvenile hormone (JH) agonists (insect growth regulators), for example, induce the production of male offspring. The molecular basis of environmental sex determination is largely unknown in daphnids. To understand the molecular mechanisms of this phenomenon, we isolated sex determination-related genes. Also, we have developed a method to inject genes into *D. magna* and *D. pulex* embryos which will allow us to study gain- and loss-of function analyses in more detail in these species. Using these techniques, we demonstrated that DSX1 (double sex 1), one of the DM-domain genes, is essential for male differentiation in *D. magna*. We have developed an RNAi method and a TALEN method using *D. pulex*. To further explore the signaling cascade of sexual differentiation in *D. magna*, a gene expression profile of JH-responsive genes is essential. We are identifying JH-responsive genes in the ovary of *D. magna* and *D. pulex* exposed to JH agonist and methyl farnesoate (JH identified in decapods) at the critical timing of JH-induced sex determination in *D. magna* and *D. pulex*. We have identified a JH receptor (heterodimer of methoprene-tolerant and steroid receptor co-activator) in daphnids and the function of ecdysone in the molting and ovulation in *D. magna*.

Sex determination mechanisms can be broadly categorized

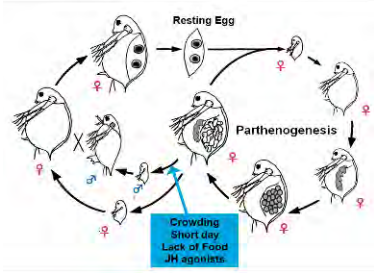

Figure 4. Life cycle of *Daphnia*.

by either a genotypic or environmentally driven mechanism. Temperature-dependent sex determination (TSD), an environmental sex determination mechanism most commonly observed among vertebrates, has been observed especially among reptiles from 1966. However, the temperature-dependent triggering mechanism of TSD and the subsequent differentiation cascade has long remained unknown. We have isolated and cloned the thermosensitive cation channel, TRP vanilloid subtype 4 (TRPV4) as a male-cascade trigger for the American alligator, *Alligator mississippiensis*, in response to high environmental temperature, and demonstrated its thermal activation at temperatures proximate to TSD-related temperatures in the alligator. Furthermore, using pharmacological exposure to manipulate TRPV4 channel activity, we have demonstrated that TRPV4 channel activity has a direct relationship with male differentiation gene expression, suggesting that AmTRPV4 is involved in the male differentiation cascade, and proposed a novel mechanism for the sex determination pathway.

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