DIVISION OF MOLECULAR ENVIRONMENTAL ENDOCRINOLOGY

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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 (ER) 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 of animals to sex hormones during critical windows of perinatal life caused irreversible alterations to the endocrine and reproductive systems of both sexes. In the immune and nervous systems, bone, muscle, and the liver were also 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.

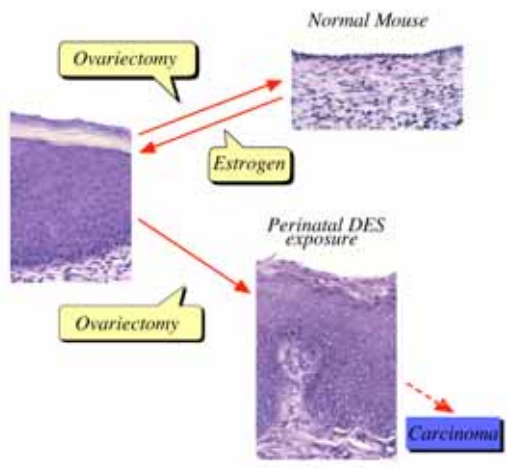

Figure 1. Scheme of estrogen-dependent and -independent vaginal epithelial cell proliferation in mice induced by perinatal estrogen exposure.

I. Perinatal estrogen exposure induces persistent changes in reproductive tracts

Perinatal exposure to sex hormones such as DES induces lesions in the reproductive tracts of female mice. In the early seventies, a close correlation between the occurrence of vaginal clear cell adenocarcinoma in young women and early intrauterine exposure to DES was demonstrated. The possible relevance of the mouse findings to the development of this human cancer has been emphasized. The neonatal mouse model has been especially useful in studying the long-term effects of early sex hormone exposure on the female reproductive tract. Female reproductive tracts in mice exposed prenatally to estrogen show altered expression of Hoxa genes and Wnt genes and knockout mice lacking Hoxa-10 or Wnt7a show uterine hypoplasia. Neonatal treatment of female mice with estrogens induces various abnormalities of the reproductive tract including ovary-independent cervicovaginal keratinization, adenosis, uterine hypoplasia, epithelial metaplasia, oviductal tumors, polyovular follicles and polyfollicular ovaries. The growth response of neonatally DES-exposed reproductive organs to estrogens is reduced, as are ER levels and epidermal growth factor (EGF) receptor levels, in addition to other hormone receptor levels.

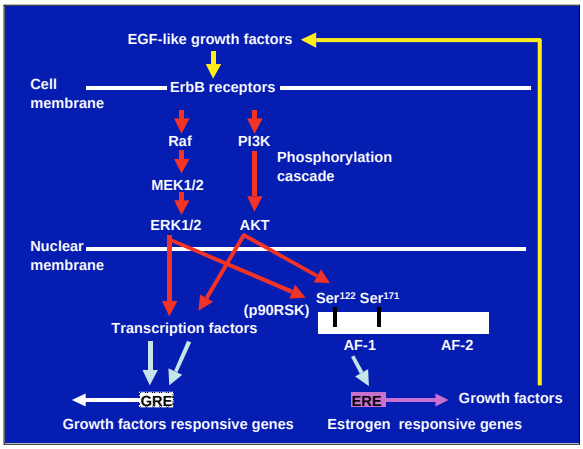

Figure 2. A hypothetical model for the estrogen-independent ER activation pathway in mouse vagina. EGF-like growth factors activate the protein-phosphorylation cascade via erbB receptors. Estrogen receptor is phosphorylated on serine 122 and 171 in AF-1 domain. Furthermore, transcription factors are activated by phosphorylation. These phosphorylations induce the transcriptional activity of ER, and then growth factors are expressed via estrogen-response element (ERE). Growth factors induced by ER activate EGF-receptors.

Growth factor and ER signaling cooperate to play essential roles in cell proliferation, differentiation and tumor progression in mouse reproductive organs, yet the mechanisms underlying the estrogen-dependent and -independent pathways remain unknown. EGFR and erbB2 were activated by estrogen treatment in mouse vaginal epithelium. This activation was also found in vaginae from neonatally DES-exposed mice, along with the expression of EGF, TGF-α, HB-EGF, amphiregulin and neuregulin. Immunohistochemical analysis indicated that erbB2 was primarily expressed in vaginal epithelium.

Serine 118 and 167 located in the AF-1 domain of ERα were phosphorylated in these vaginae. Administration of antagonists for erbB2 (AG825), EGFR (AG1478), and ER (ICI 182,780) blocked proliferation of vaginal epithelium induced by neonatal DES exposure. This suggests that signal transduction via EGFR and erbB2 could be related to the estrogen-induced vaginal changes. Persistent erbB phosphorylation and sustained expression of EGF-like growth factors would lead to ERα activation, resulting in cancerous vaginal lesions later in life in neonatally DES-exposed mice.

We used differential display to identify estrogen-responsive genes related to the proliferation and differentiation of mouse vaginal epithelial cells. A novel c-type lectin that encodes a membrane protein with a c-type lectin domain in the carboxyl-terminal region was identified from this screening. Analysis of mRNA expression showed that this gene is estrogen responsive in the mouse vagina. Furthermore, it is found in epithelial, but not stromal cells, suggesting that this novel c-type lectin may be an important factor in the stratification and/or cornification of the vaginal epithelium of mice. We are continuing efforts to analyze its function during estrogen-induced proliferation and differentiation in the mouse vagina.

Estrogenic compounds such as bisphenol A (BPA) and nonylphenol as well as dioxins and PCBs were found in the human umbilical cord. BPA can easily cross the placenta and enter the fetus in Japanese monkeys and mice. BPA can be found in the fetal brain, testis and uterus when given to pregnant mice and monkeys. Neonatal exposure to a high BPA dose induced ovary-independent vaginal changes, polyovular follciles and infertility lacking corpora lutera. Prenatal exposure to a low BPA dose induced acceleration of vaginal opening in the offspring. This provides further evidence that the developing mammal is sensitive to exposure to estrogenic agents. Neonatal treatment of rats with a high dose of BPA induced infertility in females, but no obvious effects on males.

II. Microarray analysis of estrogen responsive genes

cDNA microarray methods have recently been developed and successfully applied for genome-wide analysis of gene expression stimulated by hormones/or chemicals. Elucidating the expression patterns of estrogen-responsive genes is essential to understanding the mechanisms through which estrogenic chemicals act on mouse reproductive organs. A large number of estrogen-modulated genes were identified in the mouse uterus. Most of these genes were regulated in a dose-dependent manner and their expression was not altered by estrogen treatment in ERα knockout mice. This confirms that expression of these genes is dependent on ERα. Their activation suggests a molecular basis for the marked uterotrophic effect we observed several days following estrogen administration. Intriguingly, characteristic gene expression patterns were observed for each environmental estrogenic chemical and these were distinct from those elicited by estradiol. This suggests that specific mechanisms of action for endocrine disruption exist that could be different from those induced by endogenous estrogens. Physiological estrogens, non-physiological estrogens, and estrogenic dioxins have distinct effects on uterine gene expression. However, nonylphenol and dioxin activate another set of genes in the liver that were distinct from uterine estrogen-responsive genes. These results suggest that only a small number of genes are directly involved in the uterotrophic effects of estrogen treatment, and nonylphenol has very similar effects to estradiol on gene expression in uterine tissue, but not in hepatic tissue. Therefore, potential tissue-specific effects should be considered in order to elucidate the distinct effects of various endocrine disrupting chemicals (EDC) throughout the body.

In order to clarify the molecular mechanisms underlying estrogenic effects, we are studying changes in gene expression patterns induced by perinatal exposure to estrogen and chemicals using differential display and DNA microarray techniques. We have found genes possibly related to the ovary-independent changes by differential display. We also have clustered groups of genes that are responsive to estrogenic stimuli in uterus by using the DNA microarray. Our goals are to understand the molecular background of the critical period during development, the low dose effect of estrogenic chemicals and the molecular metabolism of hormones and hormone-like agents in animals and humans.

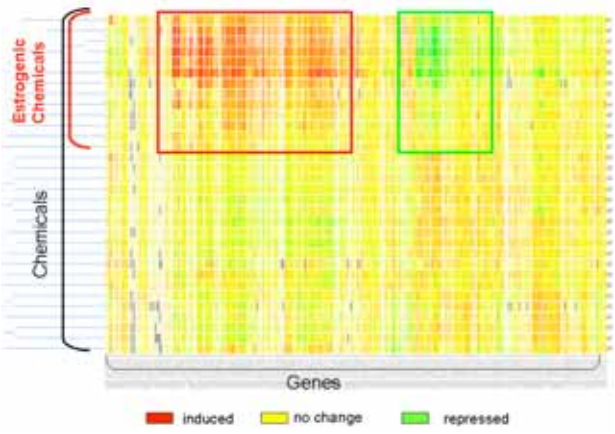

Figure. 3 Scatter plot of average expression levels in control and chemical-treated uterus

III. Effect of estrogen on reptiles, amphibians and fishes

Exogenous estrogen exposure during embryogenesis induces abnormal sex differentiation and abnormal bone formation in the African clawed frog, *Xenopus laevis*, the cyprinodont fish, mummichog (*Fundulus heteroclitus*) and mosquitofish (*Gambusia affinis affinis*). To analyze the estrogen function, we isolated ERα and β cDNA from *F. heteroclitus* and *G. affinis affinis*. Exposure of roach (*Rutilus rutilus* – a common cyprinid fish) to effluents from sewage treatment works containing complex mixtures of EDCs has been shown to alter sexual development and impact negatively on their reproductive capabilities in UK rivers. To unravel the mechanisms of disruption of sexual development in roach exposed to EDCs, we have isolated cDNAs related to sex determination and sex-differentiation such as ERs,

aromatase, StAR, Sox9, vasa, etc., and are examining gene expression during gonadal differentiation with or without EDCs exposure. Furthermore, we have cloned steroid hormone receptors and several oncogenes from the American alligator (*Alligator mississippiensis*), the Nile crocodile (*Crocodilus nicotilus*) and the Florida red-eared slider turtle (*Trachemys scripta*) with the aim of analyzing the ER evolution in reptilian phylogeny. We are also isolating estrogen-responsive genes to understand the molecular physiology of estrogen action with an aim toward understanding temperature-dependent sex determination of alligators at the molecular level.

Other projects related to steroids and physiology are in progress. The Japanese tree frog (*Hyla japonica*) absorbs water through ventral skin. We found that sex steroids and EDCs interfere with water absorption through ventral skin in frogs. Trenbolone, a potent androgenic and anabolic steroid, induced masculinization of anal fin and sperm production in the ovary of mosquitofish. Using the amphibian and fish as model animals we aim to analyze the effects of numerous chemicals released into the environment on endocrine system function in wildlife.

Figure. 4 Evolutionary relationships of estrogen receptor sequences

IV. Male production in Daphnids by juvenile hormones

We found that ten juvenile hormone analogs induce production of males in *Daphnia magna*. Daphnids are susceptible to the male-sex determining effects of juvenoids during oogenesis and the effect of juvenoids is reversible. In order to understand the molecular functional mechanism of juvenoids in induction of male offspring, we are analyzing juvenile hormone binding protein and establishing a microarray system for *D. magna*.

V. Gene zoo

We have established cDNA libraries from various animal species of interest including the Japanese giant salamander (*Andrias japonicus*). Identifying essential genes is indispensable for the basic study and conservation of animal species. We are establishing cDNA library banks of animal species in collaboration with the University of Pretoria, South Africa, the University of Florida, U.S.A., and the Asa Zoo in Hiroshima.

VI. Molecular target search

Abnormalities caused by EDC have been reported but the molecular mechanisms underlying their effects are not well known. Although the ER is one of the most likely candidates responsible for the endocrine disrupting function of many chemicals, ER alone cannot explain the variety of phenomena induced by EDC. Therefore, we are also looking for new target molecules that may be involved in endocrine disruption. We are also studying the ligand-binding mechanisms of nuclear receptors to hormones and other chemicals.

Publication List:

Original papers

Mitui, N., Fujii, T., Miyahara, M., Oka, T., Kashiwagi, A., Kashiwagi, K., Handa, H., Urushitani, H., Santo, N., Tooi, O., and Iguchi, T. (2005). Development of metamorphosis assay using *Silurana tropicalis* for the detection of thyroid hormone disrupting chemicals. Ecotoxicol. Environ. Safety, in press.

Oda, S., Tatarazako, N., Watanabe, H., Morita, M., and Iguchi, T. (2005). Production of male neonates in 4 cladoceran species exposed to a juvenile hormone analog, fenoxycarb. Chemosphere *60*, 74-78.

Oda, S., Tatarazako, N., Watanabe, H., Morita, M., and Iguchi, T. (2005). Production of male neonates in *Daphnia magna* (Cladocera, Crustacea) exposed to juvenile hormones and their analogs. Chemosphere, *61*, 1168-1174.

Sone, K., Hinago, M., Itamoto, M., Katsu, Y., Watanabe, H., Urushitani, H., Tooi, O., Guillette, L.J.Jr., and Iguchi, T. (2005). Effects of an androgenic growth promoter 17β-trenbolone on masculinization of mosquitofish (*Gambusia affinis affinis*). Gen. Comp. Endocrinol. *143*, 151-160.

Todaka, E., Sakurai, K., Fukuta, H., Miyagawa, H., Uzuki, M., Omori, M., Osada, H., Ikezuki, Y., Tsutsumi, O., Iguchi, T., and Mori, C. (2005). Fetal exposure to phytoestrogens – the difference in phytoestrogen status between mother and fetus. Environ. Res. *99*, 195-203.

Watanabe, H., Tatarazako, N., Oda, S., Nishide, I., Uchiyama, M., Morita, M., and Iguchi, T. (2005). Analysis of expressed sequence tags of the waterfrea *Daphnia magna*. Genome *48*, 606-609.

Review articles

Ankley, G.T., Black, M.C., Garric, J., Hutchinson, T.H., and Iguchi, T. (2005). A framework for assessing the hazard of pharmaceutical materials to aquatic species. In Human Pharmaceuticals-Assessing the Impacts on Aquatic Ecosystems. (ed.) Williams, R.T. SETAC Press, Pensacola, FL, U.S.A. pp. 183-227.

Okada, A., Sato, T., Ohta, Y., and Iguchi, T. (2005). Sex steroid hormone receptors in the developing female reproductive tract of laboratory rodents. J. Toxicol. Sci. *30*,

75-89.