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.

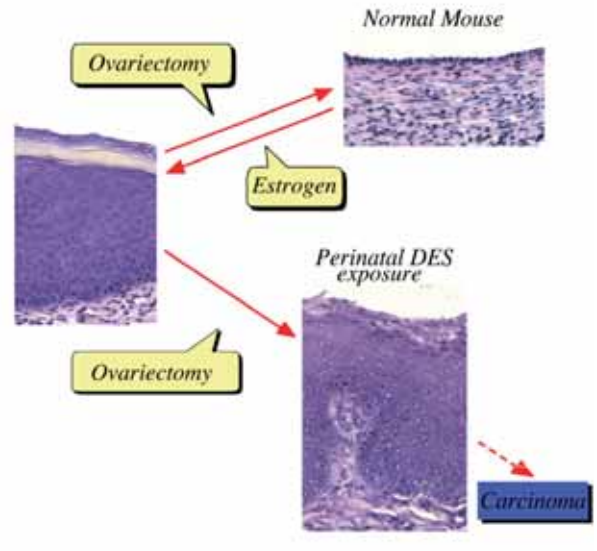

Figure 1. Scheme of estrogen-dependent and -independent vaginal epithelial cell proliferation in mice induced by perinatal estrogen exposure.

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.

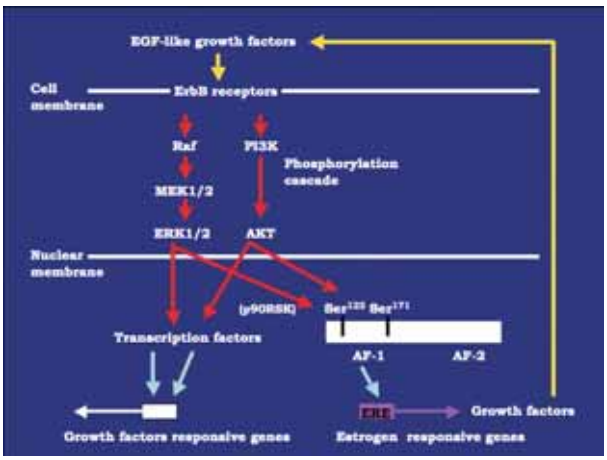

Figure 2. A hypothetical model for the estrogen-independent ER activation pathway in mouse vaginae.

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. 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.

II. Microarray analysis of estrogen responsive genes

cDNA microarray methods have been 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. Most of the estrogen-modulated genes were regulated in a dose-dependent manner and their expression was not altered by estrogen treatment in ERα knockout mice. This confirms that the 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. 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 are 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 that 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.

We identified the estrogen response element in the promoter regions of the adrenomedulin gene and aquapolin 5 gene using the chromatin-immunoprecipitation method and confirmed that these genes are estrogen responsive genes.

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. Using differential display we have found genes possibly related to the ovary-independent changes. We have also clustered groups of genes that are responsive to estrogenic stimuli in the 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.

III. Steroid hormone receptors of reptiles, amphibians and fishes

Exogenous estrogen exposure during embryogenesis induces abnormal sex differentiation in animal species. To analyze the estrogen function, we isolated ERs cDNA from *Gambusia affinis affinis* and *Kryptolebias marmoratu*. Exposure of roach (*Rutilus rutilus* ñ 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, aromatases, StAR, Sox9, vasa, etc.. Furthermore, we have cloned steroid hormone receptors from the American alligator (*Alligator mississippiensis*), the Nile crocodile (*Crocodilus nicotilus*), the Florida red belly slider turtle (*Pseudemys nelsoni*) and the Japanese giant salamander (*Andrias japonicus*) with the aim of analyzing the ER evolution. We are also isolating estrogen-responsive genes to understand the molecular physiology of estrogen action with an aim toward understanding the temperature-dependent sex determination of alligators at the molecular level.

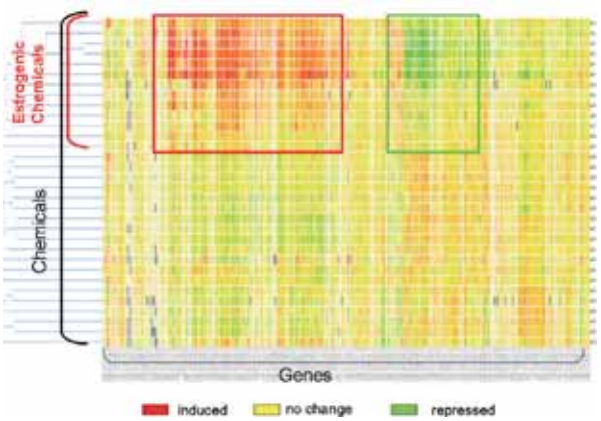

Figure. 3 Scatter plot of average expression levels in control and chemical-treated uterus

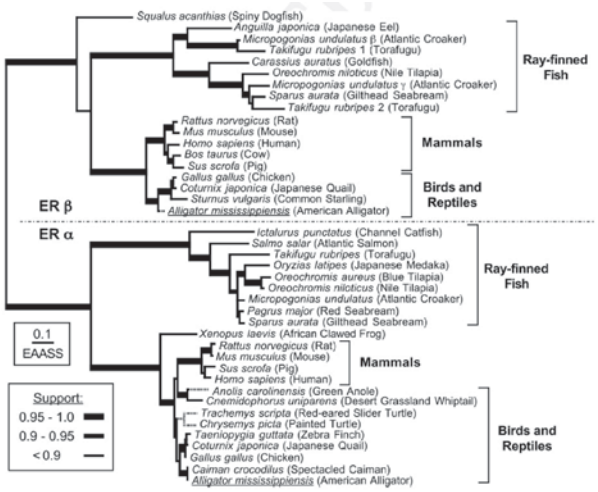

Figure. 4 Evolutionary relationships of estrogen receptor sequences

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 the 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

We found that the persistent and ubiquitous environmental contaminant, tributyltin chloride (TBT), induces the differentiation of adipocytes *in vitro* and increased adipose mass *in vivo*. TBT is a dual noanomolar affinity ligand for both the retinoid ëXí receptor (RXR) and the peroxisome proliferators activated receptor γ (PPARγ). TBT promotes adipogenesis in the murine 3T3-L1 cell model and perturbs key regulators of adipogenesis and lipogenic pathways *in vivo*. Moreover, *in utero* exposure to TBT leads to strikingly elevated lipid accumulation in the adipose depots, liver, and testis of neonate mice and results in increased epididymal adipose mass in adults. In the amphibian *Xenopus laevis*, ectopic adipocytes form in and around gonadal tissues following organotin, RXR or PPARγ ligand exposure. TBT represents the first example of an environmental EDC that promotes adipogenesis through RXR and PPARγ activation. Developmental or chronic lifetime exposure to orgaotins may therefore act as a chemical stressor for obesity and related disorders.

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