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# Estrogen receptor subtypes selectively mediate female mouse reproductive abnormalities induced by neonatal exposure to estrogenic chemicals

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ABSTRACT

Perinatal exposure to estrogens such as diethylstilbestrol (DES), and to estrogenic chemicals, induces persistent anovulation caused by alteration of hypothalamic–pituitary–gonadal (HPG) axis, polyovular follicles, uterine abnormalities and persistent vaginal changes in mice. Most activities of estrogenic chemicals are mediated through estrogen receptor α (ERα) and/or ERβ. However, little was known about the relative contribution of the individual ER subtypes in induction of abnormalities. We tested the effects of neonatal exposure to ER selective ligands and DES on female mice. Transactivation assays using mouse ERα and ERβ showed that $10^{-10}$ M DES activated both ER subtypes and that the ERα agonist (propyl pyrazole triol, PPT) and the ERβ agonist (diarylpropionitrile, DPN) selectively activated their respective ERs at $10^{-9}$ M. Neonatal female mice were injected subcutaneously with DES, PPT or DPN and the animals were examined at 13 and 15 weeks of age, respectively. Persistent estrous smears and anovulation were induced in all mice by 0.025–2.5 μg DES and 2.5–25 μg PPT, but not by DPN, suggesting that the observed anovulation was primarily mediated through ERα. Disorganization of uterine musculature and ovary-independent vaginal epithelial cell proliferation accompanied by persistent expression of EGF-related genes and interleukin-1-related genes were also mediated through ERα. In contrast, polyovular follicles were induced by neonatal treatment with both ERα and ERβ ligands, suggesting that ovarian abnormalities are mediated through both ER subtypes.



## 1. Introduction

Estrogens tightly regulate cell proliferation and differentiation particularly in the oviduct, uterus, vagina and mammary gland of the female reproductive tracts. Long-term estrogenic stimulation is a known risk factor for carcinogenesis in humans and laboratory animals (Marselos and Tomatis, 1992a,b). Transplacental exposure to the synthetic estrogen, diethylstilbestrol (DES), which was routinely prescribed to pregnant women for preventing miscarriages in 1940s–1970s, induces vaginal clear-cell adenocarcinoma in young women (Herbst et al., 1971). It has been hypothesized that *in utero* DES exposure influences the incidence of breast cancer, squamous neoplasia of the cervix and vagina, and vaginal clear-cell adenocarcinoma later in life (Herbst, 2000; Hatch et al., 2001; Palmer et al., 2002). Therefore, as generations of women exposed to DES approach menopause, concerns about possible long-term health risks of DES exposure grow.

Rodent models of DES exposure have been developed to understand the mechanistic basis of DES effects on humans. In mice, developmental exposure to estrogens within a critical developmental period elicits various permanent alternations in the female reproductive tract (Herbst and Bern, 1981). For example, neonatal estrogen administration induces persistent epithelial cell proliferation and superficial keratinization in the vagina, even after ovariectomy. This results in hyperplastic lesions later in life, as well as smooth muscle disorganization and epithelial squamous metaplasia in the uterus (Takasugi et al., 1962; Forsberg, 1979; McEwen et al., 1977; Iguchi, 1992).

We previously reported that persistent phosphorylation of estrogen receptor α (ERα), erbB receptors and JNK1 and sustained expression of EGF-like growth factors, interleukin-1 (IL-1)-related genes and IGF-1 mRNA (*Igf1*) contributed to persistent activity of these signaling pathways in the neonatally DES-exposed vagina (Miyagawa et al., 2004a,b). Neonatal treatment of female rats and mice with estrogens and estrogenic chemicals induces anovulation and persistent estrus as a consequence of insufficient phasic

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secretion of gonadotropins from the hypothalamic–pituitary axis (Takewaki, 1962; Takasugi, 1976; Iguchi, 1992; Kato et al., 2003).

Estrogens act primarily through the nuclear estrogen receptors, ERα and ERβ in mammals. ERα and ERβ can be detected in a broad spectrum of tissues. In some organs, both ER subtypes are expressed at similar levels, whereas in others, ERα or ERβ predominate. In addition, both ER subtypes may be present in the same tissue, but in different cell types. ERα is mainly expressed in the uterus, prostate (stroma), ovary (theca cells), testis (Leydig cells), epididymis, bone, breast, various regions of the brain, liver and white adipose tissue. ERβ is expressed in colon, prostate (epithelium), testis, ovary (granulosa cells), bone marrow, salivary gland, vascular endothelial cells and certain regions of the brain (Weihua et al., 2003; Dahlman-Wright et al., 2006).

ERα knockout (αERKO) mice were used to study the action of DES. In wild-type mice, uterine expression of the genes *Hoxa10*, *Hoxa11* and *Wnt7a* exhibited significant decreases shortly after DES treatment (Ma et al., 1998; Kitajewski and Sassoon, 2000), whereas this effect was not observed in the αERKO mice (Couse et al., 2001). Expression was induced in an estrogen dose-dependent manner for most of the genes noted. However, expression of these genes was not altered following estrogen treatment in αERKO mice (Watanabe et al., 2002). Adult αERKO mice were completely resistant to the chronic effects of neonatal DES exposure such as atrophy, decreased weight, smooth muscle disorganization, and squamous metaplasia in the uterus epithelium, proliferative lesions of the oviduct, and persistent vaginal keratinization (Couse et al., 2001). Thus, the lack of DES effects on gene expression and on tissue differentiation in the αERKO mouse provides unequivocal evidence supporting an obligatory role for ERα in mediating the detrimental actions of neonatal DES exposure in the murine reproductive tract. Couse et al. (2003) reported that ERα, but not ERβ, is indispensable in the negative-feedback effects of estradiol that maintain proper LH secretion from the pituitary. ERα appears to be the predominant ER in the adult mouse uterus, vagina, oviduct and mammary gland (Couse et al., 2000; Korach et al., 2003). Immunohistochemical localization of ERβ was demonstrated only in differentiating granulosa cells of the ovary where ERα was observed prominently in interstitial cells. ERα mRNA was expressed in the female reproductive tract at all ages examined with little or no significant levels of ERβ, except on postnatal day 1 when a low level of message appeared (Jefferson et al., 2000). ERβ was detectable in the uterus of both wild-type and αERKO mice, but only at very low levels (Korach et al., 2003). The significance of ERβ in the induction of polyovular follicles by genistein in mice has been reported by Jefferson et al. (2002). Bodo et al. (2006) demonstrated that both ERα and ERβ are involved in the sexual differentiation of the anteroventral periventricular area in the mouse hypothalamus.

On the other hand, estrogenic chemicals in the environment have been concerned to have potential adverse effects on animals and humans exposed during embryonic developmental stage (Damstra et al., 2002). Most of estrogenic chemicals bind to ERα better than ERβ, but some chemicals bind to ERβ better than ERα (Kuiper et al., 1997). Thus, importance of ER subtypes need to be studied in induction of adverse effect by estrogenis chemicals.

Recently, ERα- and ERβ-specific ligands have been synthesized and characterized using transactivation assays (Harris et al., 2002; Frasor et al., 2003; Katzenellenbogen et al., 2003). In this report, we studied the importance of each ER subtype in the induction of anovulation through the hypothalamic–pituitary axis with persistent estrus, permanent vaginal epithelial cell proliferation, disorganization of uterus, and in the induction of polyovular follicles in mice exposed neonatally to selective ER ligands or to DES.

## 2. Materials and methods

### 2.1. Reagents

Diethylstilbestrol (DES), 17β-estradiol ($E_2$) and ethinylestradiol ($EE_2$) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Estrogen receptor α (ERα) specific ligand, 4,4′,4″-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (propyl pyrazole triol, PPT) and ERβ specific ligand, 2,3-bis(4-hydroxyphenyl)-propionitrile (diarylpropionitrile, DPN) were obtained from Tocris Bioscience (Ellisville, MO, USA). Sesame oil and dimethyl sulfoxide (DMSO) were obtained from Kanto Chemical (Tokyo, Japan).

### 2.2. Estrogen receptor transactivation assay

CHO-K1 cells were seeded in 24-well plates at $5 \times 10^5$ cells/well in phenol-red free Dulbecco's modified Eagle's medium (Sigma–Aldrich Corp., St. Louis, MO, USA) supplemented with 10% charcoal/dextran treated fetal bovine serum (Hyclone, South Logan, UT, USA). The cells were transfected overnight with 400 ng of pGL3-Basic-4 × ERE-tk-luc, 100 ng of pRL-tk-luc (as an internal control to normalize for variation in transfection efficiency; contains the *Renilla reniformis* luciferase gene with the herpes simplex virus thymidine kinase promoter; Promega, Madison, WI, USA), and 200 ng of pTARGET-mouse ERα (mERα) or mERβ using Fugene 6 transfection reagent (Roche Diagnostics, Basel, Switzerland) according to the manufacturer-recommended protocol.

After 18 h, doses ranging from $10^{-12}$ to $10^{-6}$ M of DES, PPT, DPN, $E_2$ or $EE_2$ were administered to the culture media. Twenty-four hours after the cultures treated with ligands, the cells were collected and the luciferase activities of the cells were measured by a chemiluminescence assay with Dual-Luciferase Reporter Assay System (Promega). Luminescence was measured using a Turner Designs Luminometer TD-20/20 (Promega). Promoter activity was calculated as firefly (*Photinus pyralis*)-luciferase activity/sea pansy (*R. reniformis*)-luciferase activity (Katsu et al., 2006). All transfection assays were repeated 5 times.

### 2.3. Animals and treatments

Female C57BL/6J mice were maintained under 12 h light/12 h dark at 23–25 °C and fed laboratory chow (CE-2, CLEA, Tokyo, Japan) and tap water *ad libitum*. All procedures and protocols were approved by the Institutional Animal Care and Use Committee at the National Institute for Basic Biology, National Institutes of Natural Sciences.

Three female newborn mice were sacrificed and the hypothalamus, ovary, uterus and vagina were dissected to measure expression levels of ERα and ERβ mRNA. The other female newborn mice were given 5 daily subcutaneous (s.c.) injections of 0.025, 0.25 or 2.5 μg DES/g body weight (BW) dissolved in sesame oil, 0.25, 2.5 or 25 μg/g BW PPT or DPN, dissolved in 5.6% DMSO or the vehicle alone beginning from day 0 (the day of birth). These mice were ovariectomized at 13 weeks and sacrificed at 15 weeks of age.

Vaginal smears were recorded from 11 weeks of age for 4 weeks. After ovariectomy, the dissected ovaries were weighed and fixed in 10% neutral buffered formalin. At 15 weeks of age, 6 mice in each experimental group treated with the highest concentrations of DES, PPT and DPN, and oil controls were given a single injection of 50 μg of BrdU/g BW 2 h before sacrifice. The vagina was cut in half longitudinally and one horn of each uterus was weighed. Half of the tissue was fixed in 10% neutral buffered formalin and the other half was frozen in liquid nitrogen for the analysis of gene expression.

In addition, 8–19 newborn female mice were given 5 daily injections of 2.5 μg DES/g BW, 25 μg PPT or DPN/g BW or the vehicle alone. These mice were used for analysis of polyovular follicles in the ovary at 30 days of age.

### 2.4. Hematoxylin and Eosin (HE) staining and BrdU immunostaining

Tissues were embedded in paraffin, sectioned at 8 μm, following by standard HE staining and analysis of the ovaries, uteri and vaginae. Parts of deparafinized sections were incubated in 0.3% $H_2O_2$ in methanol for 30 min, then immersed in 2N HCl for 20 min in order to denature the genomic DNA. After washing with PBST (PBS in 0.5% Tween), the sections were incubated with anti-BrdU antibody (Boehringer Mannheim, Mannheim, Germany) diluted 1:20 in PBS containing 1% BSA overnight at 4 °C.

The sections were subsequently incubated with 3,3-diaminobenzidine tetrahydrochloride containing hydrogen peroxide. BrdU-labeling index was estimated by counting the number of BrdU-incorporated cells per h in the basal layer of cells in the vaginal epithelium as described previously (Miyagawa et al., 2004a,b). Polyovular follicles containing more than one oocyte in a follicle bigger than 50 μm were histologically examined and counted as described previously (Iguchi et al., 1986).

### 2.5. Real-time quantitative RT-PCR

Changes in gene expression were confirmed and quantified using the ABI Prism 5700 Sequence Detection System (Applied Biosystems, Foster City, USA). Total RNA,

**Table 1**
Sequences of gene primer sets for real-time quantitative RT-PCR.

| Gene | Primer (5′–3′)[a] | Product size (bp) | GenBank accession no. |
|---|---|---|---|
| *Arfg* | F: CATTATGCAGCTGCTTTGGA<br>R: TTTCGCTTATGGTGGAAACC | 124 | NM_009704 |
| *Erfg* | F: CGCTGCTTTGTCTAGGTTCC<br>R: GGGATCGTCTTCCATCTGAA | 122 | NM_007950 |
| *Hbegf* | F: GACCCATGCCTCAGGAAATA<br>R: GGCATTTGCAAGAGGGAGTA | 89 | NM_010415 |
| *Illr2* | F: GTTTATCTCGGCTGCTTACCCA<br>R: CAAAAATCAGCGACACTTCCAC | 101 | NM_010555 |
| *nip* | F: GGGCCAAGGAATCAAAGAGC<br>R: CGGATTCGAAGCTGGAGGTA | 69 | NMO19451 |
| *Esrl* | F: AATGAAATGGGTGCTTCAGG<br>R: AAGGACAAGGCAGGGGTATT | 98 | NM_007956 |
| *Esr2* | F: CTACAGTGTTCCCAGCAGCA<br>R: GCATAGAGAAGCGATGATTGG | 136 | NM_010157 |

*Areg* (amphiregulin), *Erfg* (epiregulin), *Hbegf* (heparin binding-epidermal growth factor), *Illr2* (interleukin-1 receptor type II), *Hip* (IL-1 family, member 5), *Esrl* (estrogen receptor α, ERα) and *Esr2* (ERβ).

[a] F, forward; R, reverse.

isolated with RNeasy kit (QIAGEN, Chatsworth, CA, USA) from each group, was used in RT-PCR reactions carried out with SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and SYBR Green Master Mix (Applied Biosystems) according to the manufacturer's instructions. PCR conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, and 36 cycles of 95 °C for 15 sec and 60 °C for 1 min in 15 μl volumes. Relative RNA equivalents for each sample were obtained by standardization of ribosomal protein L8 levels. Sequences of gene primer sets are given in Table 1. More than three pools of samples per group were run in triplicate to determine sample reproducibility, and the average relative RNA equivalents per sample were used for further analysis. Error bars represent the standard error, with all values represented as fold change compared to the control treatment group normalized to an average of 1.0.

### 2.6. Statistical analysis

Statistical analysis was performed using one-way or two-way of analysis of variances, Fisher's exact probability test, Student's *t*-test or Welch's *t*-test followed by *F*-test as appropriate. Differences with $P < 0.05$ were considered significant.

## 3. Results

### 3.1. Estrogen receptor transactivation assay

Transactivation assays with ERα revealed that DES, $E_2$ and $EE_2$ showed high activity at $10^{-10}$ M, whereas the ERα-specific ligand, PPT, showed the highest transactivation activity at $10^{-9}$ M. The estrogenic activity of PPT toward mERα was 10 times less than DES, $E_2$ and $EE_2$. The ERβ-specific ligand, DPN, showed no significant estrogenic activity to mERα, confirming that PPT is an ERα specific ligand (Fig. 1A).

Transactivation of mERβ showed the highest activity of DES at $10^{-10}$ M, and $E_2$ and $EE_2$ at $10^{-9}$ M. DPN, showed highest transactivation activity at $10^{-9}$ M. As with PPT and ERα, the estrogenic activity of DPN toward mERβ was 10 times less than DES, $E_2$ and $EE_2$. PPT showed no significant estrogenic activity toward mERβ, comfirming that DPN is an ERβ specific ligand (Fig. 1B). Based on these results, the doses of PPT and DPN for neonatal mouse were set 10 times higher than those of DES.

### 3.2. Vaginal smear observation

Vaginal smears were observed daily from 11 to 15 weeks of age (from 2 weeks before ovariectomy until 2 weeks after ovariectomy). Control mice showed regular estrous cycles before ovariectomy

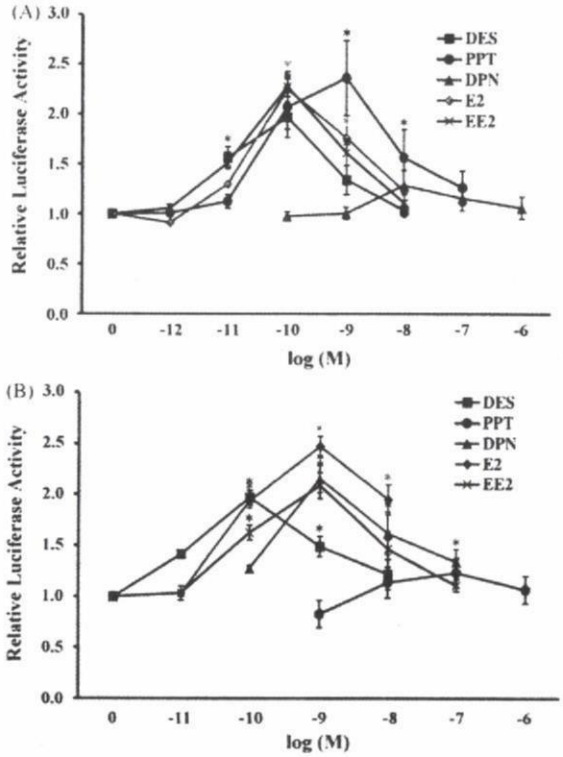


**Fig. 1.** Estrogenic activities of ER selective ligands, natural and synthetic estrogens for mouse ERα (A) and mouse ERβ (B) in reporter gene assays. DES (diethylstilbestrol), PPT (propyl pyrazole triol); ERα specific lignad, DPN (diarylpropionitrile); ERβ specific ligand, $E_2$ (17β-estradiol) and $EE_2$ (ethinylestradiol). *$P < 0.05$ vs controls (two-way ANOVA).

and diestrous type smears after the ovariectomy. All mice given 0.025–2.5 μg DES showed constant estrous smears before ovariectomy and 4 of 11 mice exposed to 0.025 μg, and all of 0.25 and 2.5 μg DES exposed mice showed constant estrous smears even after ovariectomy. Seven of 11 mice exposed to 0.025 μg DES showed diestrous type smears after ovariectomy. Three of 10, 10 of 11 and all 16 mice treated neonatally with 0.025, 0.25 and 2.5 μg PPT, respectively, showed constant estrous smears before ovariectomy. The remaining 7 of 10 and 1 of 11 mice treated with 0.25 and 2.5 μg PPT, respectively, showed estrous cycles before ovariectomy. After ovariectomy, 9 of 10 and 9 of 11 mice at the 0.25 and 2.5 μg doses of PPT showed diestrous smears after ovariectomy. The remaining 1, 2 and 16 mice treated with 0.25, 2.5 and 25 μg PPT, respectively, showed persistent estrous smears even after ovariectomy. In neonatally DPN treated mice, 1 of 10 mice at the 0.25 μg dose, and 5 of 16 at the 25 μg dose showed constant estrous smears. The remaining mice showed regular estrous cycles before ovariectomy. After ovariectomy, 2 of 16 mice at the 25 μg DPN dose showed persistent estrous smears, the rest showed diestrous smears.

### 3.3. Ovarian histology

Ovaries dissected at 13 weeks of age were examined histologically. All control mice showed corpora lutea in the ovary indicating that ovulation had occurred. However, all DES-exposed mice lacked corpora lutea in the ovary, demonstrating anovulation even at the lowest (0.025 μg/g BW) concentration. A significantly higher incidence of anovulation was found in mice exposed neonatally to 2.5 and 25 μg PPT, however, no significant increase in the num-

**Table 2**
Effects of neonatal exposure of ERα- and ERβ-ligands on ovary (13 weeks), uterus and vagina (15 weeks) in mice.

| Treatments (μg/g BW) | No. of mice used | No. of mice with | | |
|---|---|---|---|---|
| | | Ovary without corpora lutea | Uterine muscle disorganization | Vaginal epithelial stratification |
| Oil | 14 | 0 | 0 | 0 |
| 0.025 DES | 11 | 11* | 11* | 4 |
| 0.25 DES | 9 | 9* | 9* | 9* |
| 2.5 DES | 14 | 14* | 14* | 14* |
| 0.25 PPT | 10 | 3 | 3 | 1 |
| 2.5 PPT | 11 | 10* | 9* | 2 |
| 25 PPT | 16 | 16* | 16* | 16* |
| 0.25 DPN | 10 | 1 | 0 | 0 |
| 2.5 DPN | 12 | 0 | 1 | 0 |
| 25 DPN | 16 | 5 | 0 | 2 |

*$P < 0.05$ vs controls (Fisher's exact probability test).

ber of mice showing anovulation was induced by 0.25 μg of PPT or by any dose of DPN exposure (Table 2 and Fig. 2). Hyperplastic interstitial cells and lack of corpora lutea were encountered in the ovaries of mice exposed neonatally to DES (0.025–2.5 μg) and 2.5 and 25 μg PPT. In all treatment groups, mice showed regular estrous cycles before ovariecomy had corpora lutea, whereas, mice showing constant estrous smears lacked corpora lutea in the ovary.

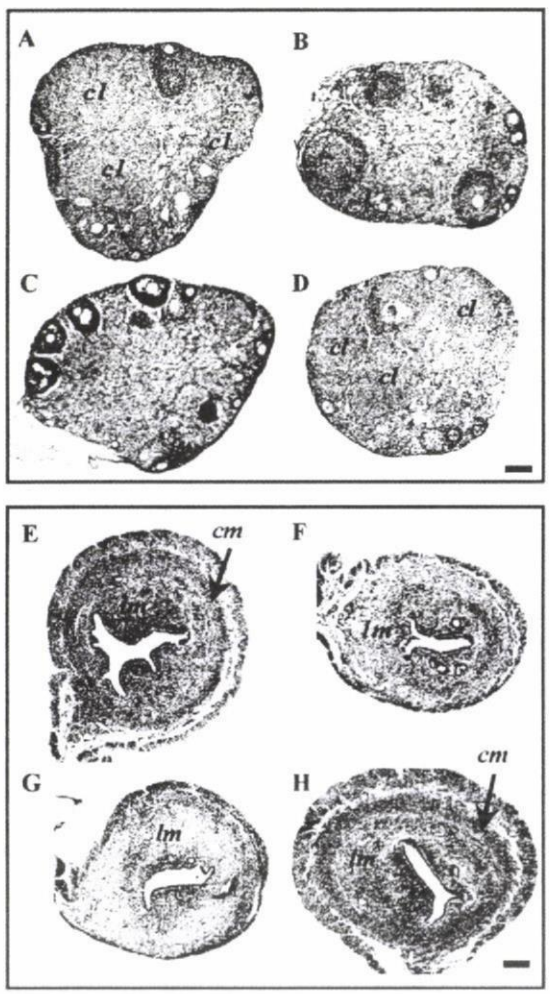


**Fig. 2.** Histology of ovaries in 13-week-old mice exposed neonatally to oil (A), 2.5 μg DES (B), 25 μg PPT (C) and 25 μg DPN (D) for the first 5 days. Note corpora lutea (*cl*) in oil control mouse and DPN-treated mouse. Histology of uteri of 15-week-old, ovariectomized mice exposed neonatally to oil (E), 2.5 μg DES (F), 25 μg PPT (G) and 25 μg DPN (H) for the first 5 days. Note disorganization of muscle layers in DES- and PPT-treated mice. *cm*: circular muscle, *lm*: longitudinal muscle. Bar: 100 μm.

### 3.4. Uterine and vaginal histology

The uterine epithelium was composed of a single layer of low columnar cells with several uterine glands and circular and longitudinal muscle layers in ovariectomized control and 0.25–25 μg DPN-exposed mice. Disorganization of stromal cells and muscle layers, such as hypoplasia of circular muscle and decrease in density of longitudinal muscle, was encountered in mice treated neonatally with 0.025–2.5 μg DES and 2.5 and 25 μg PPT (Table 2 and Fig. 2).

The vaginal epithelium of neonatally oil-injected, 15-week-old ovariectomized control and 0.25–25 μg DPN-exposed mice was composed of 2–3 layers of cuboidal cells. The vaginal epithelium of the age-matched, neonatally 0.25–2.5 μg DES- and 25 μg PPT-exposed, ovariectomized mice exhibited stratification and superficial keratinization (Table 2 and Fig. 3). In the vagina showing ovary-independent epithelial stratification, the basal cells in the epithelium showed high proliferative activity (18–19%), which was confirmed by BrdU immunostaining. In contrast, the basal cells in the vaginal epithelium of control and DPN-exposed ovariectomized animals showed very low incidence (1.7%) of BrdU incorporation (Fig. 3E).

### 3.5. Persistent expression of growth factor and IL-1-related genes

mRNA expression of *Areg*, *Ereg*, *Hbegf*, *Il1r2*, *Il1f5*, *ERα* and *ERβ* in the vagina was analyzed using real-time quantitative RT-PCR in mice exposed neonatally to 2.5 μg DES, 25 μg PPT and 25 μg DPN. The vaginae of mice exposed neonatally to DES and PPT showed persistent expression of these genes, but not the vaginae of mice exposed to oil or to DPN (Fig. 3F). In addition, the expression of ERα mRNA in the vagina of ovariectomized control mice were 1000 times higher than that of ERβ (data not shown), exhibiting that ERα is the predominant estrogen receptor in the vagina. A piece of vagina used for mRNA analysis was also histologically analyzed. We confirmed the epithelial stratification in neontally DES- and PPT-exposed mice, but not in controls and mice exposed neonatally to DPN.

### 3.6. Induction of polyovular follicles

Ovaries dissected at 30 days of age were histologically examined. A high incidence of polyovular follicles (PFs) was found in ovaries of mice exposed neonatally to 25 μg PPT and DPN (3.1% and 4.3%, respectively), although, no significant difference was found

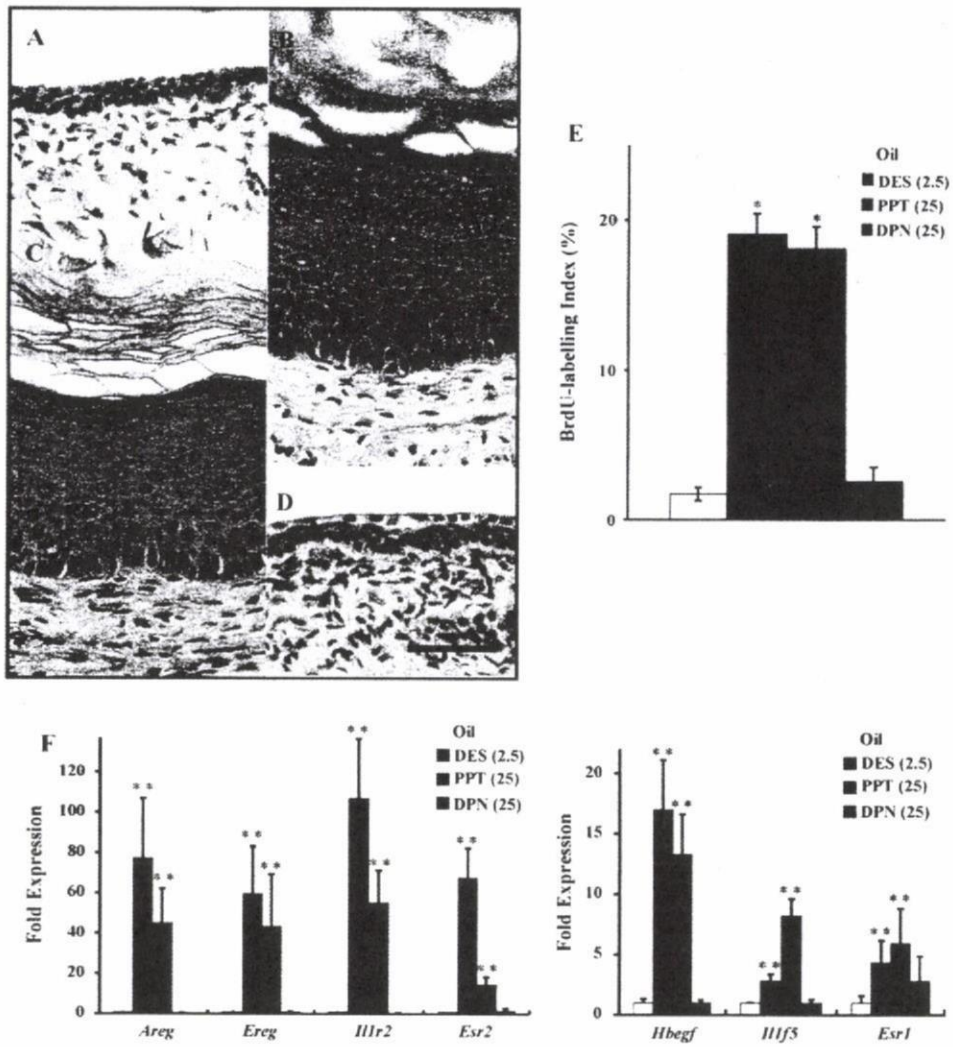


**Fig. 3.** Histology of vaginae of 15-week-old, ovariectomized mice exposed neonatally to oil (A), 2.5 μg DES (B), 25 μg PPT (C) and 25 μg DPN (D) for the first 5 days. Note ovary-independent persistent proliferation of vaginal epithelium in DES- and PPT-treated mice. Bar: 50 μm. Incidence of BrdU-incorporation (%) in basal cells of vaginal epithelium of mice exposed neonatally to oil, 2.5 μg DES, 25 μg PPT and 25 μg DPN. $^{*}P < 0.05$ vs controls (one-way ANOVA) Persistent expression of mRNAs of growth factors, interleukin-1-related genes and estrogen receptors in mouse vagina exposed neonatally to oil, 2.5 μg DES, 25 μg PPT or 25 μg DPN for the first 5 days. Expression of *Areg* (amphiregulin), *Ereg* (epiregulin), *Hbegf* (heparin-binding epidermal growth factor), *Il1r2* (interleukin-1 receptor type II), *Il1f5* (IL-1 family, member 5), *Esr1* (estrogen receptor α, ERα) and *Esr2* (ERβ) in vagina show higher levels in DES- and PPT-treated mice than those of controls and DPN-treated mice. The expression of each mRNA in vagina of the oil-treated control mice was regarded as the basal level (1.0). $^{*}P < 0.05$ vs controls (Student's *t*-test or Welch's *t*-test followed by *F*-test).

in the incidence of PFs between mice exposed to PPT or DPN. Mice exposed to 2.5 μg DES exhibited the highest incidence of PFs in the ovary (14%), showing that DES is the most potent inducer of PFs among chemicals used in this experiment (Table 3 and Fig. 4).

**Table 3**
Incidence of polyovular follicles (PFs) in 30-day-old mice treated neonatally with ERα- and ERβ-ligands.

| Treatments (μg/g BW) | No. of mice examined | PFs frequency (No. of mice with PFs)[a] | Incidence of PFs (%) |
|---|---|---|---|
| Oil | 13 | 77(10) | 0.6 ± 0.14[b] (2)[c] |
| DES (2.5) | 8 | 100(8) | 14.0 ± 0.84* (2–7) |
| PPT (25) | 15 | 100(15) | 3.1 ± 0.43* (2–6) |
| DPN (25) | 19 | 100(19) | 4.3 ± 0.58* (2–3) |

[a] Ratio of mice with PFs (%).
[b] Mean ± S.E.
[c] Range of the number of oocytes/PF in parentheses.
* $P < 0.05$ vs controls (one-way ANOVA).

### 3.7. Expression of ERα and ERβ in various tissues of female newborn mice

The ratio of ERα to ERβ mRNA in the hypothalamus, ovary, uterus and vagina were analyzed using real-time quantitative RT-PCR in newborn female mice. ERα mRNA expression was higher than that of ERβ mRNA in all tissues examined: hypothalamus, 15.0 ± 2.4; ovary, 17.8 ± 4.9; uterus, 33.7 ± 6.1 and vagina, 27.7 ± 5.8 (the value indicates the ratio of ERα mRNA to ERβ mRNA, mean ± S.E.), demonstrating that ERα is the predominant estrogen receptor in these tissues in female newborn mice.

## 4. Discussion

We confirmed the selective activation of ER subtypes reviewed previously by Katzenellenbogen et al. (2003) using transactivation assays with mouse ERα and ERβ (Katsu et al., 2006). In the ERα

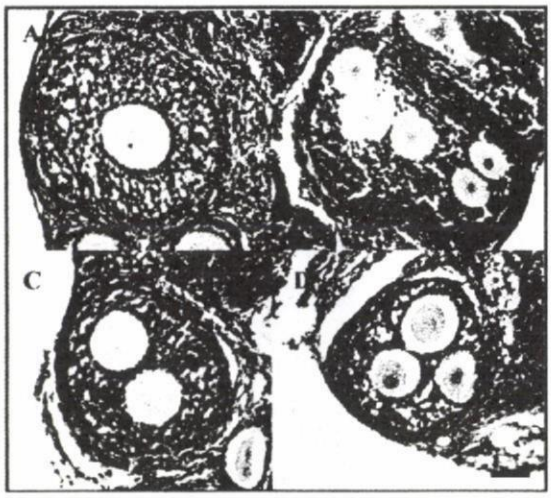


**Fig. 4.** Histology of ovaries in 30-day-old mice exposed neonatally to oil (A), 2.5 μg DES (B), 25 μg PPT (C) and 25 μg DPN (D) for the first 5 days. Note polyovular follicles in ovaries of DES-, PPT- and DPN-treated mice. Bar: 100 μm.

assay, $E_2$ and DES maximally activated the reporter gene at $10^{-10}$ M and PPT activated it at $10^{-9}$ M. In the ERβ transactivation assay, $E_2$, DES and DPN maximally activated the reporter gene at $10^{-10}$ M. Based on these data, the dose of PPT and DPN to be used for *in vivo* studies was set 10 times higher than DES.

In rodents, administration of aromatizable androgen or estrogen to neonatal females induces anovulatory sterility (Barraclough, 1961; Takewaki, 1962; Gorski, 1963; Takasugi, 1976; Iguchi et al., 1988; Aihara and Hayashi, 1989; Kincl, 1990; Iguchi, 1992), whereas castration of neonatal male rats evokes the capacity for sexual cyclicity and lordosis behavior that is characteristic of the female rat. These treatments are effective only during the "critical period" of perinatal life, and the steroids given are considered to masculinize or defeminize the brain (Goy and McEwen, 1980; Iguchi et al., 1988). ERα and ERβ expression has been demonstrated in the mouse brain (Mitra et al., 2003). ERα knockout (αERKO) mice are sterile, βERKO mice are fertile, but the average number of offspring is less than for wild-type mice (Couse and Korach, 1999; Couse et al., 2003). This demonstrates the critical importance of ERα in the normal development of the hypothalamic–pituitary–ovarian (HPG) axis. Couse et al. (2003) further demonstrated that ERα is indispensable to the negative-feedback effects of estradiol that maintain proper LH secretion from the pituitary.

Plastic component, bisphenol-A (BPA) and phytoestrogen genistein bind to ERβ about 7 times better than they do ERα (Kuiper et al., 1997). Neonatal exposure to BPA induced anovulatory sterility in female rats (Kato et al., 2003). Also, neonatal exposure to BPA, or to genistein affected sexual differentiation of the anteroventral periventricular nucleus of the hypothalamus (Patisaul et al., 2006). We, therefore, studied effects of neonatal exposure to ER selective ligands on the hypothalamus. In the present study, vaginal smears of mice exposed neonatally to 0.025–2.5 μg DES and to 2.5 and 25 μg of the ERα specific ligand, PPT, showed persistent estrus. However, mice exposed to the ERβ specific ligand, DPN, showed cyclic smear patterns. Mice showing persistent estrous smear patterns that were exposed neonatally to DES and PPT had no corpus luteum in the ovary, indicating anovulatory sterility. These results clearly suggest that ERα, but not ERβ, mediates most of the estrogenic effects of chemicals on the HPG axis during critical developmental stages.

In newborn mice, ERα is localized in the uterine stromal, but not in the epithelial cells whereas it is expressed in both epithelial and stromal cells in the vagina (Sato et al., 1992). The present study confirmed that ERα is the predominant form of ER in the uterus and vagina as reported previously (Jefferson et al., 2000; Couse and Korach, 2004). The present study demonstrated that the ratio of ERα/ERβ is bigger in the adult vagina than the newborn vagina.

In tissue recombination experiments, ERα-negative uterine epithelium (derived from the αERKO mouse uterus) recombined with ERα-positive stroma, showed proliferation following estrogen stimulation, whereas wild-type epithelium recombined with ERα-negative uterine stroma did not proliferate (Cooke et al., 1997; Buchanan et al., 1998, 1999). These reports suggested that epithelial cell proliferation could be mediated indirectly by ERα in the stroma. In cell culture conditions, estrogen did not stimulate vaginal or uterine epithelial cell proliferation (Iguchi et al., 1983, 1985), however, estrogen stimulated DNA synthesis in human endometrial epithelial cell co-cultured with stroma cells in a transfilter system (Pierro et al., 2001). Estrogen stimulated vaginal and uterine stromal cell proliferation in culture (Inada et al., 2006). Thus, ERα activity in stromal cells is essential for estrogen-mediated epithelial cell proliferation in mouse reproductive tracts. Perinatal treatment with estrogens (e.g., $E_2$, DES, $EE_2$), aromatizable and non-aromatizable androgens, or BPA induce ovary-independent persistent proliferation of vaginal epithelium with superficial keratinization (Takasugi, 1976; Iguchi, 1992; Suzuki et al., 2002; Inada et al., 2006). No such changes in the vagina were induced in the neonatally DES-exposed αERKO mice (Couse and Korach, 2004), indicating the essential role of ERα in the induction of ovary-independent vaginal changes induced by estrogens. Here we showed that persistent vaginal epithelial cell proliferation with the superficial keratinization was induced by neonatal treatment with 0.25–2.5 μg DES or 25 μg PPT, but not DPN. Neonatal treatment with 0.025–2.5 μg DES or 2.5–25 μg PPT induced disorganization of circular muscle in the uterus; however, DPN did not induce this change. These results also indicate that ERα action is essential for induction of uterine muscular disorganization and ovary-independent persistent vaginal epithelial cell proliferation caused by estrogens during the critical developmental stage.

In the persistently proliferating vaginal epithelial cells in mice exposed neonatally to DES, phosphorylation of ERα and erbB2 receptor, and persistent expression of genes related to epidermal growth factor, such as amphiregulin (*Areg*), epiregulin (*Ereg*), heparin-binding EGF (*Hbegf*), interleukin-1 (IL-1) receptor type II (*Il1r2*), IL-1 family member 5 (delta) (*Il1f5*), tumor necrosis factor-α and insulin-like growth factor-I have been reported (Miyagawa et al., 2004a,b). The present results show that the persistent expression of *Areg*, *Ereg*, *Hbegf*, *Il1r2* and *Il1f5* in vagina of mice treated neonatally with DES and PPT, but not DPN, results from ERα action, which is also essential for the induction of persistent molecular changes in the vagina.

Perinatal treatment with estrogens such as $E_2$, DES, $EE_2$ and genistein induces polyovular follicles (PFs) in the ovary (Iguchi, 1985; Iguchi and Takasugi, 1986; Iguchi et al., 1986, 1990; Jefferson et al., 2002; Kirigaya et al., 2006; Kipp et al., 2007). Neonatal treatment with a large dose of BPA also induced PFs in mice (Suzuki et al., 2002). The critical period for induction of PFs is within 3 days after birth in mice (Iguchi et al., 2002). ERα is localized in interstitial and thecal cells, whereas ERβ is localized in granulosa cells (Jefferson et al., 2002). ERβ is the predominant form in the ovary (Jefferson et al., 2002; Couse and Korach, 2004). ERβ is critical in granulosa cell differentiation and the ovulatory response to gonadotropins (Couse et al., 2005). Neonatal exposure of genistein induced PFs in wild-type and αERKO female mice, but not in βERKO females, and the induction of PFs in the ovary is dependent on the presence of

functional ERβ within the ovary (Jefferson et al., 2002). Our results show that neonatal treatment with PPT or DPN equally induce PFs in ovaries; therefore, both ERα and ERβ are involved in the induction of PFs. However, expression of ERα mRNA was higher than that of ERβ mRNA in the ovary of newborn mice. $E_2$, progesterone and genistein disrupt nest breakdown and primordial follicle formation, which may result in the PFs in mouse ovary (Chen et al., 2007). Kipp et al. (2007) showed that PFs induced by neonatal DES or $E_2$ exposure were accompanied by decreased levels of activin β-subunit mRNA and protein. This resulted in loss of phosphorylation of Smad 2 protein, a marker of activin-dependent signaling, in the estrogen-treated ovaries. Therefore, both ER subtypes may be involved in these molecular and histological changes in the newborn mouse ovary.

In conclusion, neonatal treatment with DES or an ERα-selective ligand, but not an ERβ-selective ligand, induced anovulatory sterility, disorganization of uterine circular muscle and persistent proliferation of vaginal changes. We conclude that ERα action is essential for induction of abnormalities in the hypothalamic–pituitary axis and in the uterine and vaginal changes during critical developmental period in mice. In contrast, polyovular follicles were induced by neonatal treatment with both ERα and ERβ selective agonists; therefore, both ER subtypes are involved in induction of ovarian abnormalities induced by neonatal estrogenic chemicals.

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Review

# AhR acts as an E3 ubiquitin ligase to modulate steroid receptor functions

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ABSTRACT

The arylhydrocarbon receptor (AhR) mediates the adverse effects of dioxins, including modulation of sex steroid hormone signaling. The role of AhR as a transcription factor is well described. AhR regulates the expression of target genes such as CYP1A1; however, the mechanisms of AhR function through other target-selective systems remain elusive. Accumulating evidence suggests that AhR modulates the functions of other transcription factors. The ligand-activated AhR directly associates with estrogen or androgen receptors (ERα or AR) and modulates their function both positively and negatively. This may, in part explain the sex steroid hormone-related adverse effects of dioxins. AhR has recently been shown to promote the proteolysis of ERα/AR through assembling a ubiquitin ligase complex, CUL4-$B^{AhR}$. In the $CUL4B^{AhR}$ complex, AhR acts as a substrate-recognition subunit to recruit ERα/AR. This action defines a novel role for AhR as a ligand-dependent E3 ubiquitin ligase. We propose that target-specific regulation of protein destruction, as well as gene expression, is modulated by environmental toxins through the E3 ubiquitin ligase activity of AhR.



## Contents



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*Abbreviations:* AhR, arylhydrocarbon receptor; ERα, estrogen receptor; AR, androgen receptor; XRE, xenobiotic-responsive element; ERE, estrogen-responsive element; bHLH/PAS, basic helix-loop-helix/Per–Arnt–Sim; AF-1, autonomous activation function; $E_2$, 17β-estradiol; 3MC, 3-methylcholanthrene; βNF, β-naphthoflavone; CRL, cullin–RING ubiquitin ligase; SCF, Skp1–CUL1–F-box; CUL4B, cullin 4B; DDB1, damaged-DNA-binding protein 1.

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## 1. Introduction

Dioxin-type environmental contaminants, such as tetrachloro-dibenzo-*p*-dioxin (TCDD), exert toxic effects [1]. Some of these toxicities are estrogen- and androgen-related actions [2–7]. The arylhydrocarbon receptor (AhR) is a ligand-dependent transcription factor belonging to the basic helix-loop-helix/Per–Arnt–Sim (bHLH/PAS) family. AhR possesses a variety of biological and toxicological functions [8–11] (Figs. 1 and 2). AhR mediates the toxicological effects of dioxins. In addition, AhR plays a physiological role in various tissues such as the reproductive and immune systems. The transcriptional activity of AhR is regulated by direct binding of its ligands [12,13] (Figs. 1 and 2A). The unliganded AhR is sequestered in the cytosol by interacting with the Hsp90/XAP2 (also called as ARA9 or AIP) chaperon complex [8–11]. Ligand binding to the PAS-B region of AhR is thought to induce conformational changes and subsequent translocation of the AhR complex to the nucleus [8–10]. AhR then dimerizes with the AhR nuclear translocator (Arnt) in the nucleus after dissociating from the chaperon complex, recognizes the xenobiotic-responsive element (XRE), and recruits co-activators such as the histone acetyltransferase p300/CBP, chromatin remodeling factor Brg1, and the mediator (DRIP/TRAP) complex to activate transcription [8–10] (Fig. 1). The AhR/Arnt heterodimer induces the expression of target genes, such as CYP1A1, CYP1A2, and glutathione-S-transferase [1].

The actions of the direct target genes of AhR alone do not fully explain its toxicological and physiological effects. Accumulating evidence suggests that the AhR exhibits its regulatory functions by modulating the function of other transcription factors [2,11], including estrogen receptor (ERα and ERβ) [14–19] and androgen receptor (AR) [18,19] (Fig. 1). These cross-talk pathways are important mediators of the functions of endogenous and exogenous AhR ligands. The liganded AhR recently has been shown to promote the ubiquitination and proteasomal degradation of ERs and AR by assembling a ubiquitin ligase complex, $CUL4B^{AhR}$ [18,19]. Thus, complexes of the AhR with ERs or AR appear to regulate transcription as functional units by multiple mechanisms. In this review, we will summarize a novel role for AhR as a component of an E3 ubiquitin ligase complex, which mediates cross-talk of AhR with sex steroid receptors through promotion of proteolysis.

## 2. Cross-talk of AhR with ERs or AR

### 2.1. *Transcriptional regulatory mechanism involving nuclear receptors*

ERs and AR belong to the nuclear receptor superfamily of transcription factors [20–22] (Fig. 2). Nuclear receptors, by acting as ligand-dependent transcription factors serve as

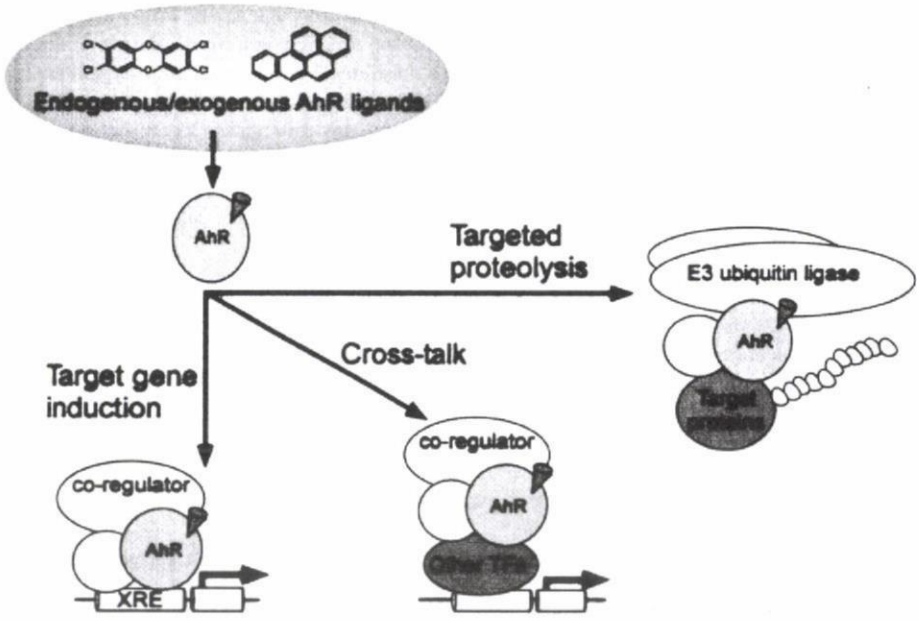


**Fig. 1 – Different modes of the AhR signaling pathways. Molecular pathways for AhR-mediated biological actions. AhR may exhibit its biological actions through different modes of pathways as illustrated. Typically, AhR directly binds to its target gene promoters and induces expression of these genes. In addition, cross-talk of AhR with other transcription factors, as well as the function of AhR as an E3 ubiquitin ligase, is considered important for AhR biology. XRE, xenobiotic-response element; TF, transcription factor.**

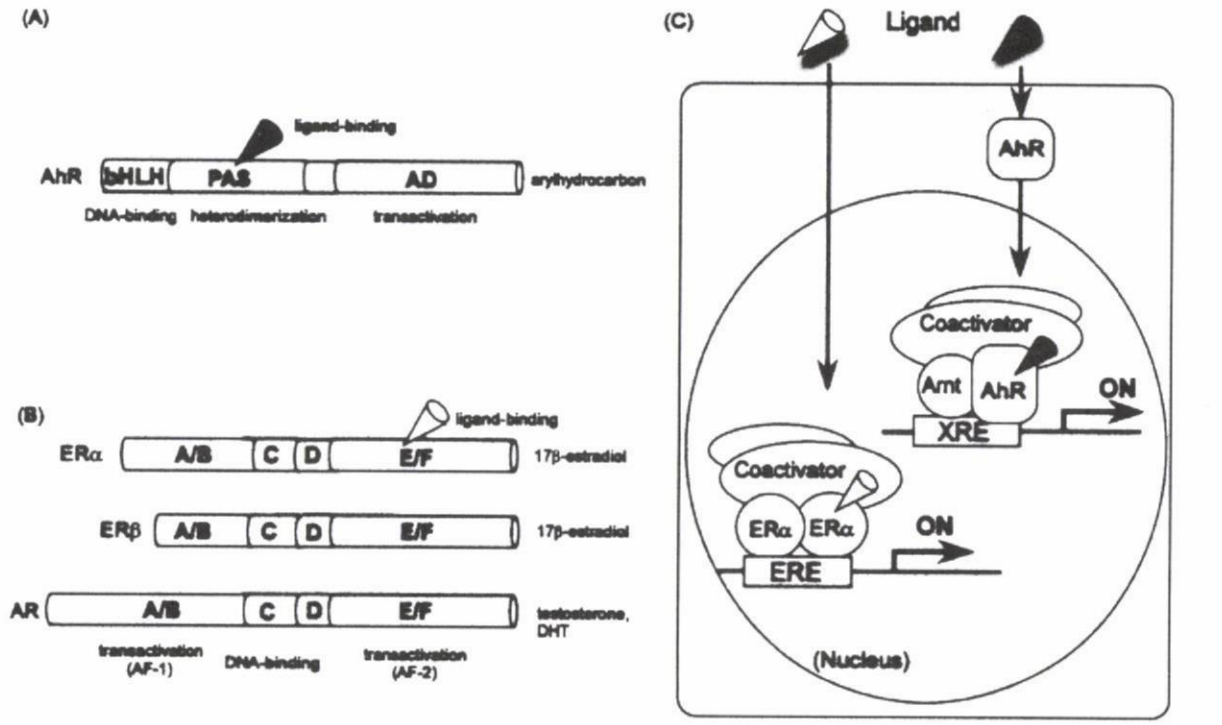


**Fig. 2 – Structure and molecular mechanism of AhR and nuclear receptors. A and B domain structures of AhR (A) and nuclear receptors (B). Domain architectures and cognate ligands for these receptors are illustrated. bHLH, basic helix-loop-helix; PAS, Per–Arnt–Sim domain; AD, activation domain; AF, activation function; DHT, dihydrotestosterone. (C) Mechanisms of gene regulation mediated by AhR and nuclear receptors. ERE, estrogen-response element.**

sensors for low molecular weight, fat-soluble ligands such as steroids/thyroid hormones, and vitamins A and D [20,21]. Members of the nuclear receptor gene superfamily share a common domain structure with distinct functional domains, designated A–E [21] (Fig. 2B). The ligand-binding domain is located in the C-terminal E domain. The most conserved C domain, located in the middle of the receptor, serves as the zinc finger-type DNA-binding domain. This domain specifically recognizes its cognate response elements in the target gene promoters. The N-terminal A/B domain and the C-terminal E domain are required for ligand-induced nuclear receptor transactivation functions. The autonomous activation function (AF-1) in the A/B domain is constitutively active but is presumably masked in the absence of ligand. The autonomous activation function (AF-2) in the ligand-binding E domain is, in contrast, dependent on ligand binding through the ligand-dependent conformational change of helix 12 and subsequent formation of a hydrophobic surface for the interaction with co-regulators [20] (Fig. 2).

Ligand-bound nuclear receptors recruit a number of transcriptional co-regulators and co-regulator complexes to the target gene promoters to mediate ligand-dependent transcriptional control [21,22] (Fig. 2). These complexes can be classified into three categories according to their functions. The first class of co-regulator complexes modifies histone tails covalently [23]. The amino-terminal tails of histones are subjected to various covalent modifications such as acetylation, methylation, phosphorylation, and ubiquitination by specific histone-modifying enzymes. These post-translational histone modifications are thought to serve as a 'histone code' that fine-tunes the transcriptional state through chromatin structure rearrangement [23]. The second class of complex mediates ATP-dependent dynamic remodeling of chromatin structure [22]. Chromatin remodeling complexes use ATP hydrolysis to rearrange nucleosomal arrays in a non-covalent manner. These chromatin remodeling complexes support the accessibility of co-regulator complexes and transcription factors to specific promoter regions. The last co-regulator complex class, the mediator complex, directly regulates transcriptional control by physically interacting with general transcription factors and RNA polymerase II. Recent evidence suggests that numerous co-regulators and nuclear receptors are recruited onto the promoters in an ordered manner, associating and dissociating transiently [24,25]. Nuclear receptors, as well as other transcription factors, serve as specific adaptors that connect co-regulator complexes and specific promoter regions.

The ligand-dependent nuclear receptor function is also regulated by other classes of signal transduction pathways. Such cross-talk pathways include at least two mechanisms: functional modulation through post-translational modification, and the association with other classes of transcription factors. MAPK, activated by EGF, phosphorylates ERα at serine 118 [26]. This in turn potentiates the ligand-dependent transactivation function of ERα [26] as well as its rapid turn-over. Phosphorylation-mediated functional modulation has been reported for a number of nuclear receptors to date.

Complex formation-based cross-talk mechanisms are also seen in several nuclear receptors including the glucocorticoid receptor (GR) [27]. GR ligands have an anti-inflammatory action, which is mediated through ligand-dependent repression of AP-1 activity through direct association. More recently, the exchange of different classes of co-regulator complexes has been reported to underlie the signal cross-talk pathway. Ligand-activated PPARγ typically assembles co-activator complexes on its cognate promoters. In the repression of NF-κB activity, PPARγ forms a complex with NF-κB, and this complex stably associates with an NCoR co-repressor complex by inhibiting the degradation of NcoR [28]. A current view of signal cross-talk at the transcription levels is that signal/ligand-dependent transcription factors associate with each other to assemble diverse types of co-regulator complexes. These exchange dynamically and regulate transcription in a manner specific for each cross-talk pathway [22].

### 2.2. *Molecular mechanisms of cross-talk of AhR with estrogen or androgen receptors*

Signal cross-talk pathways are important mediators of the functions of AhR ligands in various tissues. Dioxin-type environmental contaminants exert both estrogen- and androgen-related effects [1–3,5–7,29–32] (Fig. 3). Dioxins have well-described anti-estrogenic effects, such as the inhibition of estrogen-induced uterine enlargement, MCF-7 cell growth, and target gene induction [3,7]. However, there is also evidence to the contrary as dioxins have also been shown to have estrogenic effects including the stimulation of uterine enlargement [29], induction of estrogen-responsive genes such as *VEGF*, *c-fos*, and *TERT*, and a similar pattern to estrogen of transcriptional regulation in a genome-wide study [6]. In addition, AhR-deficient mice exhibit impaired ovarian follicle maturation [33]. Using AhR-deficient cells, the importance of AhR in the proliferation of mammary cells has been confirmed [34]. These findings suggest that AhR, activated by its endogenous ligand, may modulate the estrogen signaling pathway. Similarly, dioxins exert both androgenic and anti-androgenic effects on prostate development in an age-specific manner [5]. As is true for other cross-talk pathways [22], the AhR appears to modulate estrogen/androgen signaling both positively and negatively depending on cellular context.

The molecular mechanisms of AhR modulation of ERα have been extensively studied, and both direct and indirect regulatory mechanisms have been proposed. First, TCDD/AhR either increases or decreases estrogen levels through an indirect mechanism [2,35]. TCDD promotes the clearance of estrogen, thereby repressing ER transcriptional activity [35]. AhR-deficient mice have decreased estrogen production due to impaired induction of aromatase (CYP19) gene expression [33]. Another indirect mechanism involves competitive DNA binding of AhR and ER on the responsive promoters [2]. AhR and ER, each bound to its own target promoter recruits transcriptional co-regulators

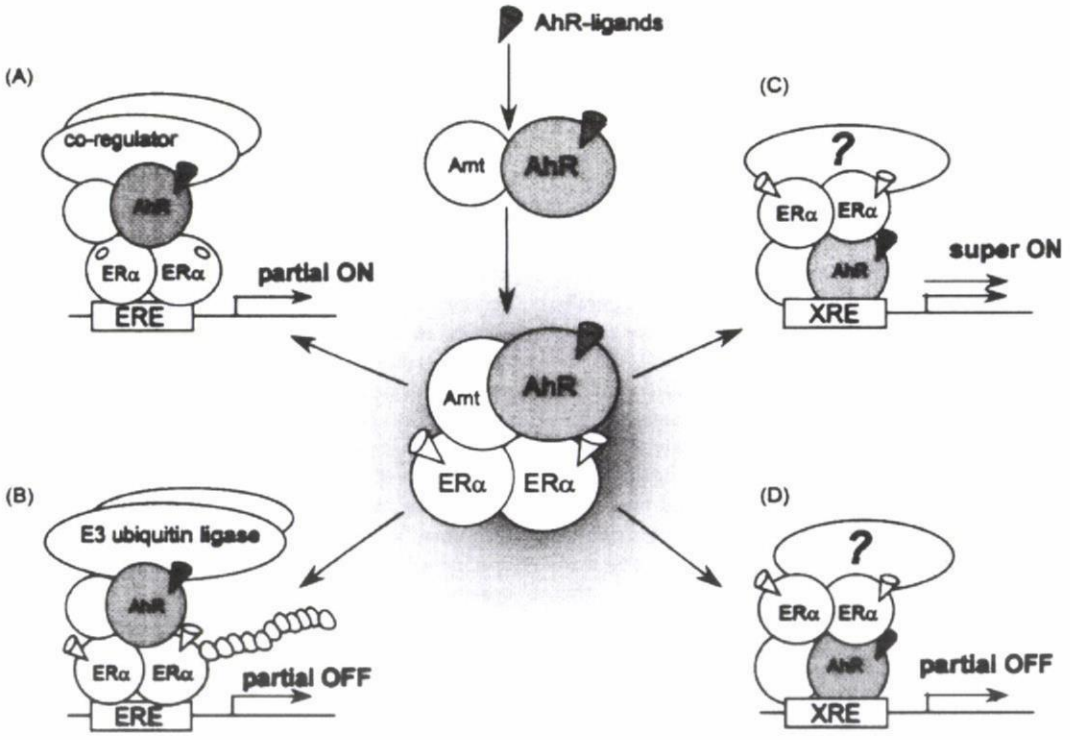


**Fig. 3 – Cross-talk of AhR with ERα through direct association. Ligand-bound AhR directly associates with estrogen or androgen receptors (ERα, ERβ, or AR) in the nucleus. This association leads to different types of cross-talk between AhR and ERs/AR, as illustrated (see text for details). (A) Ligand-bound AhR associates with unliganded ERs upon ERE and recruits transcriptional co-activators. (B) Ligand-bound AhR forms E3 ubiquitin ligase complex and recognizes ERs for proteolysis. (C) Ligand-bound ERα associates with AhR and activates transcription through XRE. (D) Association of ERα with AhR results in repression through XRE.**

in a competitive manner. This mechanism may be limited to specific genes and conditions since not all of the estrogen-responsive promoters contain XRE.

More recently, direct association of AhR with ERs has been independently reported. Ligand-activated AhR/Arnt associates with ERα and ERβ through the N-terminal A/B region within ERs [14–18] (Fig. 3). By means of this association, the liganded AhR potentiates the transactivation function of 17β-estradiol ($E_2$)-unbound ERα (Fig. 3A), while it represses $E_2$-bound ERα-mediated transcription upon the estrogen-responsive element (ERE) [14] (Fig. 3B). The interaction of AhR/ER is induced by different AhR ligands, such as TCDD, 3-methylcholanthrene (3MC), and β-naphthoflavone (βNF). The activation of AhR is thought to be sufficient for the interaction with ERα, as a constitutively active form of AhR [12] modulates ERα function in the absence of AhR ligand [19]. These results suggest that the cross-talk of AhR with ER is initiated primarily through stimulation of AhR. Supporting this, ERα is predominantly located in the nucleus, whereas AhR translocates to the nucleus upon ligand stimulation. The association of AhR/ERα has been shown by several independent approaches, including *in vitro* [36], *in vivo*, and biochemical methods [18]. Moreover, AhR/ERα cross-talk in the transcriptional regulation of ERα-responsive genes is abolished in AhR-deficient mice [10,33], confirming the specificity of the molecular pathway *in vivo* [14]. Reciprocally, $E_2$-bound ERα associates with XRE-bound AhR to either potentiate [15] (Fig. 3C) or repress [16] (Fig. 3D) AhR-mediated transcription. Considered together, the AhR/ERα complex may be able to bind to either XRE or ERE through the attachment functions of AhR or ERα, respectively. Alternatively, different complex subtypes that contain AhR/ERα may control promoter selectivity (Fig. 3). Reflecting this functional cross-talk, Arnt also acts as a co-regulator for both ERα and ERβ [37].

The proposed mechanism of AhR/ER association is a reasonable explanation for dioxin/estrogen cross-talk. First, this mechanism explains the functional AhR/ER cross-talk irrespective of differences in target gene promoters. Second, ligand-dependent AhR/ER association may result in a rapid cellular response to dioxins in terms of ER activity. The responses of ER transcriptional activity to AhR ligands are observed within a few hours in cultured cells as well as in mice, which supports the existence of direct cross-talk mechanisms. Third, variations in the AhR/ER containing co-regulator complexes may result in the complex, bi-phasic consequences of AhR/ER cross-talk. Given that complexes containing different classes of transcription factors can recruit co-regulator complexes distinct from their cognate associating complexes [22], it is possible that the AhR/ER complex, acting as a functional unit, may recruit different types of complexes depending on the cellular context. A current area of interest is the identification of the molecular determinants by which the activity of the AhR/ER complex is controlled.

## 3. Ubiquitin ligase activity of AhR

### 3.1. *The ubiquitin–proteasome system*

The transcriptional regulatory system and the ubiquitin–proteasome system are two major target-selective systems that control intracellular protein levels in response to various cellular contexts in metazoans (Fig. 4A). Whereas the transcriptional regulatory system is targeted by environmental fat-soluble ligands, the involvement of the ubiquitin–proteasome system in the adverse effects of these environmental toxins remains largely unknown. The target selectivity of these systems depends on the recognition of specific DNA elements by sequence-specific transcription factors [20–22] and recognition of degradation substrates by E3 ubiquitin ligases [38–41] (Fig. 4B). These transcription factors and E3 ubiquitin ligases primarily serve as specific adapters to subsequently recruit enzymes such as transcriptional co-

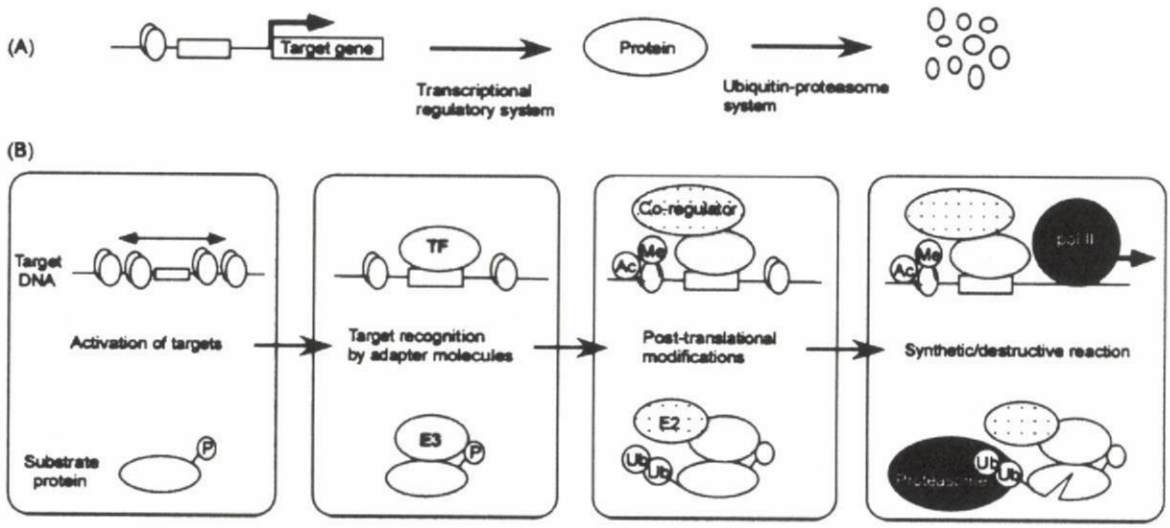


**Fig. 4 – The ubiquitin–proteasome system. (A) The transcriptional regulatory system and the ubiquitin–proteasome system are two major target-selective systems that control intracellular protein levels. (B) The transcription factors and E3 ubiquitin ligases primarily serve as target-specifying adapters in these systems. Ub, ubiquitin; P, phosphorylated serine/threonine; Ac, acetylated lysine; Me, methylated lysine; Pol-II, RNA polymerase II.**

regulators and E2 ubiquitin-conjugating enzymes, respectively, to appropriate targets. Considering the functional analogy of E3 ubiquitin ligase and transcription factors, it is possible that E3 ubiquitin ligase also serves as a target of environmental toxins.

The ubiquitin–proteasome system, which regulates cellular protein degradation, plays a pivotal role in cellular homeostasis [38–41]. Ubiquitin is a 76 amino acid polypeptide that is highly conserved among eukaryotes. Ubiquitin is covalently attached to lysine (Lys) residues of substrate proteins. Ubiquitination of proteins is catalyzed by sequential reactions involving ubiquitin activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin protein ligase (E3). Ubiquitin is conjugated either as one molecule (mono-ubiquitination) or as a tandem polymer (poly-ubiquitination). Poly-ubiquitination can occur at any of seven lysine residues in the ubiquitin molecule. The Lys48-linked poly-ubiquitin chain is then recognized by the 26S proteasome for subsequent proteolysis (Fig. 4B).

Among E1, E2, and E3 enzymes, the E3 ubiquitin ligases are most diverse and therefore possess substrate specificity. E3 acts as a bridge between E2 and the substrate, maintaining the appropriate distance. E2 then conjugates ubiquitin to the substrate [38–41]. Of the RING-type E3s, the largest class is comprised of the cullin–RING ubiquitin ligases (CRLs) [40–44]. CRLs are multisubunit complexes that include a cullin (CUL1, 2, 3, 4A, 4B, or 5) subunit, a RING finger protein Rbx1/Roc1 or Rbx2/Roc2, and a substrate-recognition subunit. Cullin serves as a scaffold protein, binding to the substrate-recognition subunit or adapter protein at its N-terminus while binding to Rbx1 at its C-terminus [41]. Rbx1 binds to E2 enzymes through RING finger to support efficient conjugation of ubiquitin to the substrates. Their diverse substrate-recognition subunits enable CRLs to target numerous substrates. The best characterized CRLs are the SCF (Skp1–CUL1–F-box) complexes. In SCF complexes, F-box proteins function as a substrate-recognition subunit by binding to Skp1, which is bound to the N-terminal region of CUL1. F-box proteins and other types of substrate-recognition subunits serve as adapters for target-specific substrates. Therefore, any protein binding to E3 core components can potentially act in a manner similar to substrate-recognition subunits. More interestingly, F-box proteins and other types of substrate-recognition subunits are rapidly degraded through an auto-catalytic mechanism once they are integrated into the CRL core complexes [42]. In this way, CRLs can efficiently ubiquitinate different substrates by associating with different substrate-recognition subunits. This raises the possibility that F-box and F-box 'equivalent' proteins act either as substrates or as adapter components, as in the case of DDB2 in the CUL4-based CRL complex [45–50].

### 3.2. *AhR is an E3 ubiquitin ligase*

As discussed above, dioxins, through activating the AhR, have well-described effects on the transcriptional regulatory system. TCDD is also reported to decrease the uterine ERα protein level in the rat [51], suggesting that AhR may also be involved in the control of protein stability. Somewhat unexpectedly, our own study has shown that in a ChIP analysis, the ligand-bound AhR does not block co-activator recruitment of liganded ERα. In addition, repression of ERα transcriptional activity by AhR is not observed when ERα is over-expressed in transient reporter assays (Ohtake et al., unpublished data). These observations imply that the ligand-activated AhR has an additional molecular role beyond transcriptional regulation, at least in the modulation of sex hormone signaling.

Exploring the functions of AhR in sex hormone signaling, we found that upon activation of AhR by binding of AhR ligands such as 3MC and βNF, as well as by expression of constitutively active AhR, protein levels of endogenous ERα, ERβ, and AR, were drastically decreased without alteration in mRNA levels [19] (Fig. 5). Since ligand-bound AhR and ERα proteins are ubiquitinated for proteasome-mediated degradation [52–57], we tested whether the functional modulation of ERs and AR by activated AhR is related to this degradation system. 3MC-enhanced degradation of sex steroid receptors is attenuated in the presence of a proteasome inhibitor MG132, and 3MC-enhanced poly-ubiquitination of ERα is consistently observed irrespective of E2 binding. MG132 treatment abrogates the transcriptional modulation of liganded sex steroid receptor function by activated AhR. This indicates that the ubiquitin–proteasome system mediates the repressive AhR–ER cross-talk pathway.

These experiments provide evidence that AhR acts as an E3 ubiquitin ligase component. First, FLAG–AhR immunoprecipitated complexes exert a self-ubiquitination activity in an E1/E2 enzyme-dependent manner *in vitro*. Second, 3MC-dependent recognition of ER and AR by AhR [14] appears to induce ubiquitination of ER/AR. Third, degradation of AhR itself is accelerated upon activation of degradation of sex steroid receptors, which is a typical sign of self-ubiquitination of the E3 component [42]. Taken together, these properties of AhR resemble that of classical adapter components of the E3 ubiquitin ligase complex such as F-box proteins in the SCF complex [39,42], DDB2/CSA in the CUL4A complex [45–49], and VHL in the CUL2 complex [58]. Therefore, we reasoned that activated AhR might serve as an E3 ubiquitin ligase component.

Supporting this idea, an AhR associating ubiquitin ligase complex has been biochemically purified [59] from HeLa cells. This complex includes cullin 4B (CUL4B) [39,60], damaged-DNA-binding protein 1 (DDB1) [61,62], and Rbx1 [39] together with subunits of the 19S regulatory particle (19S RP) of 26S proteasome as well as Arnt and transducin-beta-like 3 (TBL3) (Fig. 5). The core complex appears to constitute a CRL-type E3 ligase, and therefore is referred to as $CUL4B^{AhR}$. Although the typical CUL4B-type CRL complex contains substrate-recognition components having a WDXR/DWD motif [45–49], no such component has been identified in this complex. AhR directly interacts with the N-terminal region of CUL4B in GST pull-down assays. Together with the direct interaction of AhR with ER, it appears that AhR may act as a substrate-recognition component in the $CUL4B^{AhR}$ complex. Using an *in vitro* reconstituted ubiquitination assay, the E3 ubiquitin ligase activity of $CUL4B^{AhR}$ for ERα is dependent only on 3MC, and not on $E_2$. This suggests that $CUL4B^{AhR}$ has the unique property of being able to respond to ligand signals by complex assembly and ubiquitin ligase activity (Fig. 5). The importance of the $CUL4B^{AhR}$ components for the promotion of ERα ubiquitina-

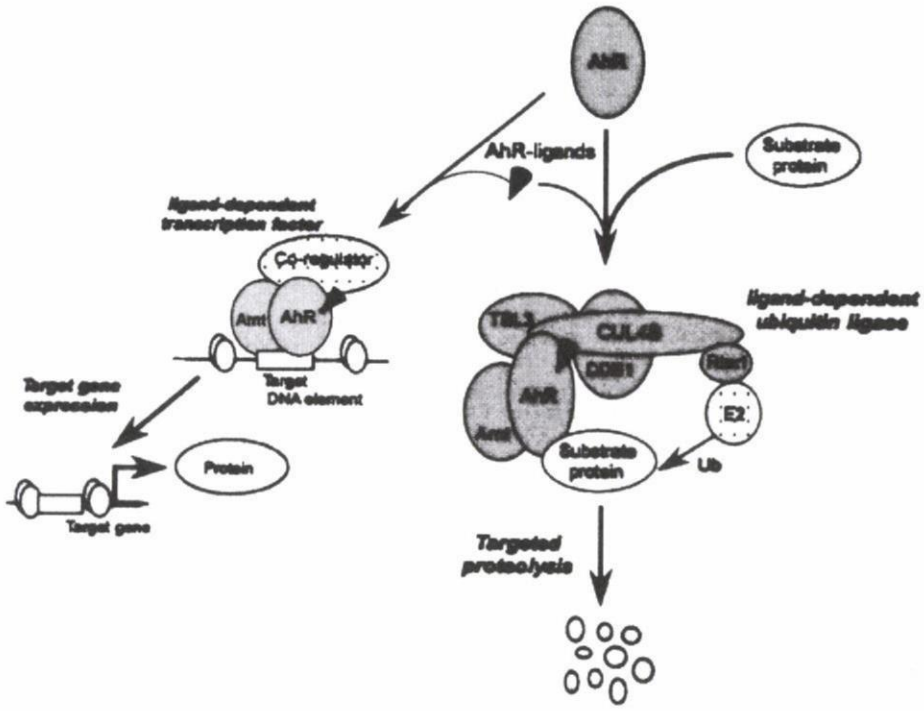


**Fig. 5 – An E3 ubiquitin ligase activity of AhR. Ligand-bound AhR assembles a CUL4B-based atypical E3 ubiquitin ligase complex, $CUL4B^{AhR}$, to mediate a non-genomic signaling pathway of fat-soluble ligands. AhR serves as a ligand-dependent ubiquitin ligase, as well as a transcription factor (see text for details). DDB1, damaged-DNA-binding protein 1; TBL3, transducin-beta-like 3.**

tion and degradation has been demonstrated in knock-down experiments. Degradation of ERα or AR in the uterus and prostate is inducible by treatment with AhR ligands. Such degradation of ERα or AR is not seen in AhR-deficient mice [10,33]. This confirms that the AhR has E3 ubiquitin ligase activity *in vivo*. The anti-estrogenic effects of AhR ligands on estrogen-dependent uterine cell proliferation [14] appear to be mediated by the E3 ubiquitin ligase activity of AhR.

### 3.3. *Perspectives on the E3 ubiquitin ligase activity of AhR in cross-talk pathways*

Although it is well established that AhR is a key factor in mediating the adverse effects of dioxin-type compounds [8–10], the underlying mechanisms for this remain elusive. The putative functions of the previously identified target genes for AhR appear unlikely to fully explain the diverse range of biological actions of AhR ligands [11] (Fig. 1). The discovery of $CUL4B^{AhR}$ suggests that the adverse effects of AhR ligands in sex hormone signaling are, at least in part, attributable to the enhanced degradation of sex steroid receptors through E3 ubiquitin ligase activity of AhR [18,19] (Fig. 5). Target selectivity of the transcriptional regulatory system and the ubiquitin–proteasome system depends on specificity conferred by sequence-specific transcription factors and E3 ubiquitin ligases. To date, however, no single factor has been shown to function as a specificity factor in both target selection systems. Therefore, AhR is the first sequence-specific transcription factor identified that acts as an E3 ubiquitin ligase that also targets substrates for accelerated protein degradation. It is possible that other transcription factors, such as nuclear receptors, also function as E3 ubiquitin ligase components in some cellular contexts. Fat-soluble ligands for nuclear receptors are reported to have 'non-genomic' actions independent of transcriptional regulation-mediated effects. Considered together, ubiquitin ligase-based signaling mechanisms may possibly be involved in these non-genomic actions of various fat-soluble ligands.

From a mechanical point of view, AhR appears to be a unique and atypical type of substrate-specific component in cullin-based E3 complexes. AhR does not bear the reported signature motifs such as F-box [39], but directly associates with CUL4B. Substrate recognition by the other substrate-specific components in ubiquitin ligase complexes is usually evoked by substrate modifications such as phosphorylation [38–41] and hydroxylation [43,44,58]. However, recognition and subsequent ubiquitination of sex steroid receptors by AhR requires dioxin-type ligands, and does not occur following normal modifications of sex steroid receptors. Thus, it is plausible that activation of atypical E3 complexes may be a strategy of sensors for environmental stresses to respond to these stresses (Fig. 6). Supporting this, Hsp70 acts as an atypical substrate-specific adapter within the CHIP E3 complex in response to heat shock stress [63]. Hsp70 interacts with misfolded proteins and promotes their degradation. It later undergoes auto-catalytic degradation through CHIP [63]. In response to DNA damage, an atypical E3 complex alters the stability of TIP60, which in turn regulates ataxia-telangiectasia

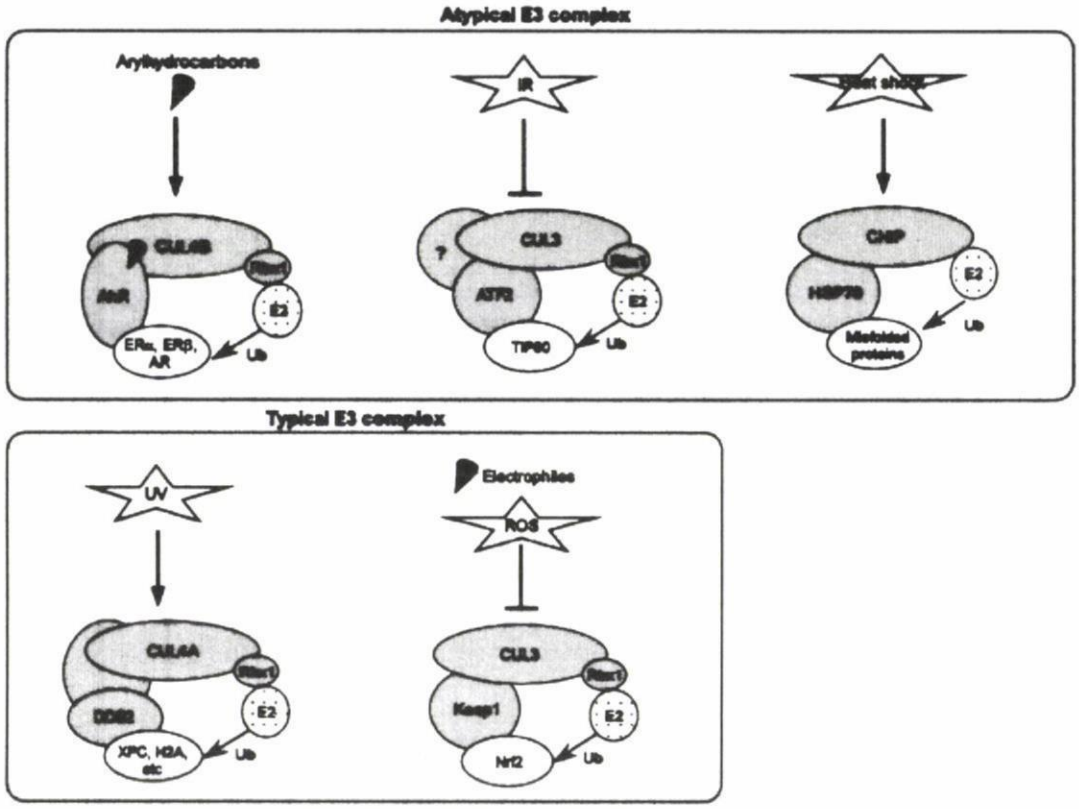


**Fig. 6 – Atypical E3 complexes as sensors for environmental stresses. Several examples of E3 ubiquitin ligase-based perception of environmental stresses are illustrated. In the top panel, signal-responsive factors serve as atypical components of E3 complexes. In the bottom panel, canonical E3 components with conserved signature motif act as signal-responsive factors. ATF2, activating transcription factor-2; TIP60, tat interactive protein 60; CHIP, C-terminus of Hsp70 interacting protein; Hsp70, heat shock protein 70; XPC, xeroderma pigmentosum group C; H2A, histone H2A; Keap1, Kelch-like ECH-associated protein 1; Nrf2, NF-E2-related factor 2; IR, ionizing radiation; ROS, reactive oxygen species.**

mutated (ATM) activation in DNA repair [64]. Activating transcription factor-2 (ATF2) promotes the degradation of TIP60 by assembling a CUL3-based complex under non-stressed conditions. ATF2 dissociates from TIP60 in response to ionizing radiation (IR), resulting in enhanced TIP60 stability and activity [64]. Functional regulation of E3 components is also seen with the CUL3-based component Keap1 in the oxidative stress response [65], and CUL4A-based components DDB2 and CSA in the DNA damage response [61]. Considered together, E3 components that respond to environmental stress may be more diverse than initially believed (Fig. 6). It is possible that $CUL4B^{AhR}$ may cross-talk with these stress-responsive E3 ligases to modulate their functions. As WDXR/DWD motif containing components, including DDB2 and CSA, also bind to CUL4B [46], it is possible that AhR may associate or interfere with these CRL subunits.

The E3 ubiquitin ligase activity of AhR and the transcriptional activity of AhR appear to be responsible for a distinct set of biological events induced by AhR ligands (Fig. 5). As substrate-specific adapters of ubiquitin ligase complexes are capable of recognizing a number of proteins, identification of other $CUL4B^{AhR}$ substrate proteins may reveal new molecular links between AhR-mediated signaling and other signaling pathways and cellular events. In this regard, it is of interest that AhR interacts with various transcription factors [11], such as Rb/E2F1 [66], SF1/Ad4BP [33], and NF-κB [67], to modulate their functions. AhR has recently been shown to regulate the differentiation of Th17 and $T_{reg}$ cells [68–70]. This may be mediated by a functional interaction with STAT1 [70]. In addition, although the underlying mechanisms remain unknown, AhR also modulates the function of transcription factors [71] such as GR and RAR [72,73]. Considering the evolutionary conservation of AhR, it is likely that the intrinsic function of AhR is to mediate the signal transduction of endogenous ligands in cross-talk pathways. A current area of interest is the identification of candidate degradation substrates for AhR which are abnormally stabilized in AhR-deficient mice. In summary, several lines of recent evidence define a novel role for AhR as a ligand-dependent E3 ubiquitin ligase to regulate target-specific protein destruction. The ubiquitin ligase activity of AhR, together with the cross-talk of AhR with nuclear receptors through direct association, provides an additional layer of complexity for AhR biology. Characterization of these new molecular aspects of AhR function may lead to a greater understanding of the diverse biological actions induced by endogenous and exogenous AhR ligands.

## Conflict of interest

The authors declare no competing financial interests.

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# Switching of chromatin-remodelling complexes for oestrogen receptor-α

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**The female sex steroid hormone oestrogen stimulates both cell proliferation and cell differentiation in target tissues. These biological actions are mediated primarily through nuclear oestrogen receptors (ERs). The ligand-dependent transactivation of ERs requires several nuclear co-regulator complexes; however, the cell-cycle-dependent associations of these complexes are poorly understood. By using a synchronization system, we found that the transactivation function of ERα at G2/M was lowered. Biochemical approaches showed that ERα associated with two discrete classes of ATP-dependent chromatin-remodelling complex in a cell-cycle-dependent manner. The components of the NuRD-type complex were identified as G2/M-phase-specific ERα co-repressors. Thus, our results indicate that the transactivation function of ERα is cell-cycle dependent and is coupled with a cell-cycle-dependent association of chromatin-remodelling complexes.**

Keywords: oestrogen receptor; cell cycle; chromatin-remodelling complex

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## INTRODUCTION

Sex steroid hormones exert their biological actions by directly binding to and activating their cognate nuclear receptors (Mangelsdorf *et al*, 1995). Steroid receptors are members of the nuclear receptor superfamily that function as sequence-specific and hormone-inducible transcriptional regulators in target gene promoters. Distinct classes of co-regulators and multiprotein co-regulator complexes are indispensable for the chromatin reorganization that occurs during the hormone-dependent transcriptional control by nuclear receptors (Mellor, 2005; Rosenfeld *et al*, 2006). These complexes seem to modify chromatin configuration in a highly regulated manner by controlling nucleosomal rearrangement and enzyme-catalysed modifications of histone tails (Workman, 2006; Li *et al*, 2007).

Two main classes of chromatin-modifying complexes that co-regulate nuclear receptors have been well characterized (Rosenfeld *et al*, 2006). One class is a histone-modifying complex (Bannister & Kouzarides, 2005; Klose & Zhang, 2007; Lee & Workman, 2007) and the other class is an ATP-dependent chromatin-remodelling complex (Berger, 2007; Kouzarides, 2007). The latter complex uses ATP hydrolysis to rearrange nucleosomal arrays in a non-covalent manner to facilitate or to prevent the access of nuclear receptors to nucleosomal DNA. These ATP-dependent chromatin-remodelling complexes have been classified into three subfamilies depending on which core catalytic components have DNA-dependent ATPase activity. Brg-1/Brm is a core component of the SWI/SNF-type complex, SNF2h is a component of the ISWI-type complex and Mi2 is a component of the NuRD-type complex (Kitagawa *et al*, 2003; Fujiki *et al*, 2005; de la Serna *et al*, 2006; Denslow & Wade, 2007). The combination of the catalytic ATPase subunits with the components of the complex defines the role of each complex in transcriptional control. The SWI/SNF- and ISWI-type complexes potently co-activate the function of nuclear receptors (Belandia & Parker, 2003), whereas the NuRD-type complex is assumed to co-repress the function of nuclear receptors, owing to the presence of histone deacetylase (HDAC) in the complex (Denslow & Wade, 2007; Manavathi *et al*, 2007).

Dynamic structural changes of the chromosome are evident during the cell cycle and control global gene regulation (Martinez & Danielsen, 2002; Swedlow & Hirano, 2003; Narayanan *et al*, 2005; Groth *et al*, 2007; Takezawa *et al*, 2007). The role of chromatin-remodelling complexes in the alteration of chromatin structure over much of the cell cycle has been studied (Baumann *et al*, 2007; Groth *et al*, 2007). However, the role of chromatin-remodelling complexes in the cell-cycle-dependent transcriptional function of nuclear receptors during the S–M cell-cycle stages is unknown.

Oestrogen stimulates both cell proliferation and cell differentiation of target cells (Deroo & Korach, 2006), presumably by means of cell-cycle-dependent co-regulator function. Therefore, the transactivation functions of oestrogen receptors (ERs) and also their co-regulators at

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