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Title: Cross-talk between endocrine-disrupting chemicals and cytokine signaling through estrogen receptors

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Running title: Cross-talk between STAT3 and EDCs

ABSTRACT

STAT3 mainly acts as a signal transducer of IL-6 family cytokines and transcriptionally activates specific target genes. STAT3 has also been demonstrated to mediate cellular transformation and is found in numerous cancers. Endocrine-Disrupting Chemicals (EDCs) are a diverse group of chemicals that bind to estrogen receptors (ERs), mimic estrogenic actions, and may have adverse effects on human health. In our previous study, we demonstrated that estrogens suppressed the STAT3-mediated transcription activity through ERs. In this study, we examined the effects of EDCs on STAT3-mediated signaling through ERs. Surprisingly, some of EDCs enhanced STAT3-mediated transcription activity through ERs. This finding strongly suggests that EDCs may play an important role in the endocrine functions by mimicking cytokine activity by stimulating STAT3 actions through ERs.

Key words: STAT3, cytokine, endocrine-disrupting chemicals (EDCs), estrogen receptor (ER), transcription

INTRODUCTION

In addition to the endogenous hormone estradiol (17 β -estradiol; E2), estrogen receptor (ER) activity can be modulated by chemicals in the environment termed endocrine-disrupting chemicals (EDCs) or xenoestrogens, which include natural plant compounds (phytoestrogens) and industrial byproducts (industrial estrogens). Recently, there has been increasing awareness and concern about the endocrine-disrupting effects of these chemicals and their impact on humans and animals (1, 2, 3). Although the molecular mechanisms of EDCs are not well understood, it is clear that many of these chemicals function by binding ERs and blocking estrogen action (4). In addition to their potential antagonist activities, EDCs are also known to induce activating conformational changes in ERs that enable the receptors to interact with co-activators and to activate target gene transcription (5, 6, 7).

It has been actually shown that the *in vivo* effects of EDCs such as Bisphenol A mimic those of 17 β -estradiol (E2), including the vaginal cornification (8), growth and differentiation of the mammary gland (9), a decreased cholesterol level in serum (10), an increased prolactin level (11), and an increased c-fos mRNA level in the uterus (8), suggesting that EDCs have enough potential to alter reproductive function and endocrine system *in vivo*. However, we do not know whether the EDCs can modify other cellular signaling such as growth factors or cytokines.

One member of the STAT family of proteins is STAT3, which is mainly activated by IL-6 family of cytokines including Leukemia inhibitory factor (LIF) and Leptin (12). Like other members of the STAT family, STAT3 is tyrosine-phosphorylated by Jak kinases, then forms a dimer and translocates into the nucleus to activate target genes (13, 14). It has been shown that the activated STAT3 can mediate cellular transformation (15, 16, 17) and is found in numerous cancers, including prostate and breast cancer (17). Furthermore, STAT3 has been recently shown to act as an oncoprotein (15).

In the present study, we have demonstrated that the EDCs enhances STAT3-mediated signaling through ERs. The enhancement of STAT3 activation by ER/EDCs can be reversed by an anti-estrogen, Tamoxifen or a dominant negative form of STAT3. Furthermore, EDCs activated MAP kinase (MAPK) signaling and a MAPK inhibitor suppressed the enhancement of STAT3 activation by ER/EDCs. Taken together, these results strongly suggest that the EDCs may disrupt endocrine homeostasis not only by directly binding to estrogen receptors but also by influencing the cytokine signaling pathways.

MATERIAL AND METHODS

Reagents and antibodies.

17 β -Estradiol (E2), Genistein, Nonylphenol, Bisphenol A, Methoxychlor, α -Endosulfan, Tamoxifen were purchased from Wako Chemicals (Osaka, Japan). Human recombinant LIF was purchased from INTERGEN (Purchase, NY). Expression vectors, ER α (18), ER β (19), Vit-LUC (18), STAT3-LUC (20), DN-STAT3(20), NF- κ B-LUC(21), ELK-1-LUC(22) were kindly provided by Dr. F. Saatcioglu (University of Oslo, Norway), Dr. T. Hirano (Osaka Univ., Osaka, Japan) and Dr. T. Fujita (Tokyo Metropolitan Inst. Med. Sci., Tokyo, Japan) respectively. Anti-STAT3 and anti-Actin antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Chemicon (Temecula, CA). Anti-Phospho STAT3, anti-Phospho MAPK, anti-MAPK antibodies were purchased from Cell Signaling Technology (Beverly, MA).

Cell culture, transfections, and luciferase assays.

Human embryonic kidney carcinoma cell line, 293T cells were transfected in DMEM containing 1% FCS by the standard calcium precipitation protocol (23). Human hepatoma cell line Hep3B was maintained in DMEM containing 10% FCS (24). Before stimulation, the cells were cultured for 12 h in DMEM containing 1% FCS followed by treatment with or without LIF and/or E2 or EDCs. Hep3B cells were transfected by using FuGENE6 (Roche Diagnostics GmbH, Mannheim, Germany) following manufacturer's instructions. Luciferase assay was performed as described (23). The cells were harvested 48 h after transfection and lysed in 100 μ l of PicaGene Reporter Lysis Buffer (Toyo Ink, Tokyo, Japan) and assayed for luciferase and β -galactosidase

activities according to the manufacturer's instructions. Luciferase activities were normalized to the β -galactosidase activities. During EDCs' stimulation, cell viability did not changed, when we monitored by Cell Counting Kit-8 (Wako Chemicals, Tokyo, Japan). Three or more independent experiments were carried out for each assay.

Immunoprecipitation and immunoblotting.

Immunoprecipitation and Western blotting were performed as described previously (23). Briefly, the transfected 293T cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 0.3 M NaCl, containing 1% NP-40, 1mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride and 10 μ g/ml each of aprotinin, pepstatin and leupeptin). Aliquots of cell lysates were resolved on 5-20% SDS-PAGE and transferred to Immobilon membrane (Millipore, Bedford, MA). The membranes were then probed with each antibody as indicated. Immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).

RESULTS AND DISCUSSION

Cross-talk between of EDCs and STAT3-mediated signaling through ERs.

We previously demonstrated that estrogens inhibited STAT3-mediated signaling pathway through estrogen receptors (ERs) (18). We then examined whether EDCs has any inhibitory effect on STAT3-mediated signaling through ERs, because it is clearly shown that many of EDCs function by binding ERs and blocking estrogen access (4). EDCs is also demonstrated to induce activating conformational changes in ERs that enable the receptors to interact with co-activators and to activate target gene transcription (5, 6, 7)

To first examine the EDC-mediated signaling through ERs, we carried out transient transfection experiments in 293T cells. ER activity by EDCs was monitored by using Vit-LUC in which two copies of an estrogen response element (ERE) drive expression of the LUC gene (18). As shown in Fig. 1A, Genistein and Nonylphenol, Bisphenol A stimulated Vit-LUC activity in a dose dependent manner in 293T cells. However, Methoxychor and α -Endosulfan showed a very weak stimulation of Vit-LUC activity in 293T cells. To assess the cross-talk between STAT3 and EDCs signaling pathways, we examined whether EDC affects transcriptional activation of STAT3 in 293T cells. Cells were transfected with STAT3-LUC, in which the α 2-macroglobulin promoter drives expression of the LUC gene (20), with or without an expression vector for ER α were stimulated with leukemia inhibitory factor (LIF) (25) in the absence or presence of E2 or EDCs. As shown in Fig. 1B, STAT3-LUC activity was stimulated by LIF, whereas E2 or each EDC alone did not change this activity. In the presence of ER α and LIF, E2 suppressed STAT3-LUC activity in a dose-dependent fashion as we

described previously (18). Genistein and Nonylphenol did not alter LIF-induced STAT3-LUC activity. Interestingly, Bisphenol A, Methoxychlor, α -Endosulfan significantly enhanced LIF-induced STAT3-LUC activity.

We next examined if EDCs has similar effects on STAT3-mediated signaling through the other major ER isoform, ER β (26), in an analogous experiment. As shown in Fig. 1C, LIF-stimulated STAT3-LUC activity was enhanced by Methoxychlor and α -Endosulfan in the presence of ER β , similar to that observed with ER α . These data suggest that both ER isotypes may be involved in enhancement of STAT3-mediated signaling by EDCs.

To examine the cross-talk between EDCs and STAT3-mediated signaling pathway under more physiological conditions through endogenous ER proteins, we first utilized a LIF-responsive, ER-positive hepatoma cell line, Hep3B (27) , and the transient transfection assay. Hep3B cells were transfected with STAT3-LUC and treated with LIF and/or E2 or EDCs and LUC activities were determined (Fig. 1D). When cells were treated with both LIF and E2, STAT3-LUC activation was decreased by 40-50% compared with the activation by LIF alone. In contrast, Bisphenol A, Methoxychlor, α -Endosulfan enhanced LIF-induced STAT3-LUC activity more than those observed in 293T cells.

A dominant negative STAT3 can reverse the enhanced effect of EDCs on STAT3 activation.

We next assessed whether the observed effects were mediated through STAT3 or some other intermediary factors, we used a dominant negative form of STAT3 (DN-STAT3) (20). A DN-STAT3 significantly inhibited IL-6- or LIF-induced STAT3-LUC expression in a dose-dependent fashion in 293T cells as described previously (27). As

shown in Fig. 2A, a DN-STAT3 down-regulated STAT3-LUC expression induced by LIF and EDCs, suggesting that the effect of EDCs on STAT3-LUC activity is mediated by STAT3.

Tamoxifen can reverse the enhanced effect of EDCs on STAT3 activation.

To further assess the specificity of EDCs function on STAT3 activation, we utilized the anti-estrogen Tamoxifen. STAT3-LUC was transfected into 293T cells in the presence of an expression vector for ER α , cells were treated with LIF in the absence or presence of E2 or EDCs and/or Tamoxifen. Enhanced effect of ER/EDCs on STAT3 activation was reversed by Tamoxifen when cells were treated with increasing concentrations of Tamoxifen (Fig. 2B), whereas an inhibitory effect of ER/E2 on STAT3 activation was reversed by Tamoxifen as described previously (18). These results indicate that the enhanced effect of EDCs on STAT3 activation in 293T cells is mediated by ER α .

Effects of EDCs on LIF-induced phosphorylation of STAT3

We next examined whether EDC treatment has any effect on immediate early STAT3 activation by LIF in 293T cells. We then assessed changes in tyrosine-phosphorylation of STAT3 in 293T cells. To that end, 293T cells were transfected with ER α and either left untreated or treated with LIF, LIF plus E2 or EDCs, and their cell extracts were prepared and subjected to Western blot analysis with an anti-phospho STAT3 (Tyr705) antibody. As shown in Fig. 3A, tyrosine-phosphorylation of STAT3 by LIF had no alteration even in the presence of E2 or EDCs. We also examined Ser727-phosphorylation of STAT3 using an anti-phospho STAT3 (Ser727) antibody. Similarly, serine phosphorylation of STAT3 showed no alteration by EDCs (Fig. 3A). These data show that EDC treatment results in no alteration of STAT3 phosphorylation

in 293T cells.

Involvement of MAP kinase signaling in EDC-mediated enhancement of STAT3 activation.

One of the possible mechanisms that are consistent with the data described above is that there is an alternative pathway to activate STAT3 like MAP kinase (MAPK) signaling. We tested this possibility by reporter assays to monitor other signaling pathways. We then examined the activation of NF- κ B and MAPK signaling by EER/EDCs. 293T cells were transfected with NF- κ B-LUC or ELK-1-LUC with ER α were stimulated with LIF in the absence or presence of E2 or EDCs. As shown in Fig. 3B, NF- κ B-LUC activity was not stimulated by LIF in 293T cells. E2 and Bisphenol A slightly suppressed the basal NF- κ B-LUC activity. Whereas both Genistein and Nonylphenol showed a little bit enhancement on NF- κ B-LUC activity. With the same conditions, the ELK-1-LUC, which reflects MAPK activation (22), was by 4-fold enhanced by Genistein and Nonylphenol (Fig. 3B). We also examined activation of MAPK by Western blot analysis using an anti-phospho MAPK antibody. 293T cells were transfected with ER α and either left untreated or treated with LIF, LIF plus E2 or EDCs, and their cell extracts were prepared and subjected to Western blot analysis with an anti-phospho MAPK antibody. As shown in Fig. 4A, phosphorylation of MAPK by LIF enhanced in the presence of EDCs, whereas expression of MAPK proteins was also induced approximately 2-3-fold by EDCs. We further examined MAPK activation in Hep3B cells. Similarly, both phosphorylation and protein expression of MAPK in Hep3B cells were enhanced by EDCs (Fig. 4A). These data suggest that the EDC treatment resulted in the induction and activation of MAPK in 293T and Hep3B cells. We finally tested the effect of an MAPK inhibitor on the enhancement of STAT3 activation by EDCs.

Enhanced effect of ER/EDCs on STAT3 activation was partly inhibited in a dose-dependent manner by a MAPK inhibitor, PD98059, when cells were treated with increasing concentrations of PD98059 (Fig. 4B). These results suggest that the enhanced effect of EDCs on STAT3 activation is partly mediated by MAPK signaling pathway.

Concluding remarks

We have shown here that the EDCs enhance STAT3-mediated signaling through ERs. The enhancement of STAT3 activation by ER/EDCs can be reversed by an anti-estrogen, Tamoxifen or a dominant negative form of STAT3. Furthermore, EDCs activated MAPK signaling and a MAPK inhibitor suppressed the enhancement of STAT3 activation by ER/EDCs.

The later half of the previous century witnessed tremendous industrial development leading to the pollution of air, water, and food. These environmental pollutants severely affect the human and animal bodies in health. Especially, environmental estrogens (EDCs/xenoestrogens) are a diverse group of chemicals that bind to estrogen receptors, mimic estrogenic actions, and may have adverse effects on human health (1, 2, 3). Moreover, most of them can easily access the human body by ingestion or absorption through the skin and mucosal membranes, since those are lipophilic. EDCs have been also suggested to disrupt the internal signaling network that regulates reproductive development and function in animals and humans. In males, environmental exposure to EDCs has been associated with a high incidence of reproductive tract problems such as cryptorchidism, decreased sperm count, hypospadias, and testicular cancer (28, 29, 30). Furthermore, the carcinogenic potential role of EDCs in development of breast cancer in women has been also discussed, but is still questionable (30, 31).

Activation of STAT3 is required for cell transformation by oncogenic Src and by a constitutively active form of Go, a heterotrimeric G-protein subunit (15, 16). In addition, STAT3 is found to be active in several cancer including breast cancer and prostate cancer cells (17). Furthermore, STAT3 has been recently shown to act as an oncoprotein (15). These results may suggest the possibility that enhancement of STAT3 activation by EDCs triggers STAT3-mediated oncogenesis.

In this study, we demonstrated that EDCs enhance cytokine signaling pathway through ERs. It is also noteworthy that MAPK signaling is involved in this effect of EDCs on cytokine signaling. This implies that EDCs-activated MAPK signaling may stimulate phosphorylation of STAT3 to activate, although we could not detect any enhancement of phosphorylation of STAT3. There may be also the possibility that some conformational change of ER by EDCs is involved in this effect of EDCs. Further work would be required to assess the detailed mechanisms of this cross-talk. This study also suggest that EDCs should be evaluated to determine whether they can be subdivided in separate classes of EDCs based on this novel function, which affects cytokine signaling. This knowledge may provide new targets for the prevention of hormonal carcinogenesis or provide rationalization of the carcinogenic potential of EDCs.

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Figure Legends

Fig 1. Transcriptional cross-talk between STAT3 and EDCs.

(A) 293T cells (12-well plate) were transfected with Vit-LUC (0.3 μ g) and/or ER α (0.3 μ g) as indicated. 48 h after transfection, cells were stimulated for 12 h with E2 or each EDC as indicated. Cells were then harvested and relative luciferase activities were measured. The results are presented as fold induction of luciferase activity from triplicate experiments, and the error bars represent the standard deviations.

(B) 293T cells (12-well plate) were transfected with STAT3-LUC (0.3 μ g) and/or ER α (0.3 μ g) as indicated. 48 h after transfection, cells were stimulated for 12 h with LIF(100ng/ml) and/or E2 or each EDC as indicated. Cells were then harvested and relative luciferase activities were measured. The results are presented as fold induction of luciferase activity from triplicate experiments, and the error bars represent the standard deviations.

(C) 293T cells (12-well plate) were transfected with STAT3-LUC (0.3 μ g) and/or ER β (0.3 μ g) as indicated. 48 h after transfection, cells were stimulated for 12 h with E2 or each EDC as indicated. Cells were then harvested and relative luciferase activities were measured. The results are presented as fold induction of luciferase activity from triplicate experiments, and the error bars represent the standard deviations.

(D) Hep3B cells (6-well plate) were transfected with STAT3-LUC (1 μ g). 48 h after transfection, cells were stimulated with LIF(100 ng/ml) and/or each EDC as indicated for an additional 12 h. Cells were harvested and relative luciferase activities were measured. The results are presented as fold induction of luciferase activity from triplicate experiments, and the error bars represent the standard deviations.

Fig. 2. A dominant negative STAT3 or Tamoxifen can reverse the enhanced effect of EDCs on STAT3 activation.

(A) 293T cells (12-well plate) were transfected with STAT3-LUC (0.3 μ g) and ER α (0.3 μ g) and/or an increasing amounts of DN-STAT3(0.05-0.15 μ g) as indicated. 48 h after transfection, cells were stimulated for 12 h with each EDC as indicated. Cells were then harvested and relative luciferase activities were measured. The results are presented as fold induction of luciferase activity from triplicate experiments, and the error bars represent the standard deviations.

(B) 293T cells (12-well plate) were transfected with STAT3-LUC (0.3 μ g) and ER α (0.3 μ g). 48 h after transfection, cells were stimulated for 12 h with E2 or each EDC together with or without an increasing amounts of Tamoxifen (TAM; 10^{-6} - 10^{-7}) as indicated. Cells were then harvested and relative luciferase activities were measured.

The results are presented as fold induction of luciferase activity from triplicate experiments, and the error bars represent the standard deviations.

Fig. 3. Effect of EDCs on LIF-induced phosphorylation of STAT3 and on NF- κ B or MAPK signaling.

(A) 293T cells (10-cm dish) were transfected with ER α (5 μ g). 48 h after transfection, cells untreated or treated with LIF and/or E2 (10^{-6} M) or each EDC (10^{-4} M) for 1 h. Cells were then lysed and total extracts (20 μ g) immunoblotted with anti-Phospho STAT3(Tyr705) or anti-Phospho STAT3(Ser727) antibody (upper panel), or anti-STAT3 antibody (lower panel).

(B) (C) 293T cells (12-well plate) were transfected with ER α (0.3 μ g) and NF- κ B-LUC (0.3 μ g)(B) or ELK-1-LUC (0.3 μ g)(C). 48 h after transfection, cells were stimulated for

12 h with LIF (100ng/ml) and/or E2 or each EDC as indicated. Cells were then harvested and relative luciferase activities were measured. The results are presented as fold induction of luciferase activity from triplicate experiments, and the error bars represent the standard deviations.

Fig. 4. Involvement of MAPK signaling in EDC-mediated enhancement of STAT3 activation.

(A) 293T cells (10-cm dish) were transfected with ER α (5 μ g). 48 h after transfection, cells untreated or treated with LIF and/or E2 (10^{-6} M) or each EDC (10^{-4} M) for 12 h. Cells were then lysed and total extracts (20 μ g) immunoblotted with anti-Phospho MAPK(Thr202/Tyr204) (upper panel) or anti-MAPK antibody (middle panel), or anti-Actin antibody (lower panel).

(B) 293T cells (12-well plate) were transfected with ER α (0.3 μ g) and ELK-1-LUC (0.3 μ g). 48 h after transfection, cells were stimulated for 12 h with LIF (100ng/ml) and/or E2 or each EDC in the absence or presence of an increasing amounts of PD98059 (1.0-3.0 μ M) as indicated. Cells were then harvested and relative luciferase activities were measured. The results are presented as fold induction of luciferase activity from triplicate experiments, and the error bars represent the standard deviations.

Vit-LUC

Fold induction

200

100

0

++
10⁻⁹ 10⁻⁸
10⁻⁶ 10⁻⁵
10⁻⁶ 10⁻⁵
10⁻⁵ 10⁻⁴
10⁻⁵ 10⁻⁴
10⁻⁵ 10⁻⁴
10⁻⁵ 10⁻⁴

Ra
2(M)
enistein(M)
onyphenol(M)
sphenol A(M)
ethoxychlor(M)
Endosulfan(M)

Chemical Concentrations	Fold induction
Control (all 10 ⁻⁶ M)	~1
10 ⁻⁶ M dithionite	~1
10 ⁻⁶ M phenol A	~1
10 ⁻⁶ M glyphosate	~1
10 ⁻⁶ M alachlor	~1
10 ⁻⁶ M alachlor + 10 ⁻⁶ M dithionite	~22
10 ⁻⁶ M alachlor + 10 ⁻⁶ M phenol A	~12
10 ⁻⁶ M alachlor + 10 ⁻⁶ M glyphosate	~12
10 ⁻⁶ M alachlor + 10 ⁻⁶ M dithionite + 10 ⁻⁶ M phenol A	~18
10 ⁻⁶ M alachlor + 10 ⁻⁶ M dithionite + 10 ⁻⁶ M glyphosate	~20
10 ⁻⁶ M alachlor + 10 ⁻⁶ M dithionite + 10 ⁻⁶ M phenol A + 10 ⁻⁶ M glyphosate	~18
10 ⁻⁶ M alachlor + 10 ⁻⁶ M dithionite + 10 ⁻⁶ M phenol A + 10 ⁻⁶ M glyphosate + 10 ⁻⁶ M alachlor	~20
10 ⁻⁶ M alachlor + 10 ⁻⁶ M dithionite + 10 ⁻⁶ M phenol A + 10 ⁻⁶ M glyphosate + 10 ⁻⁶ M alachlor + 10 ⁻⁶ M dithionite	~55
10 ⁻⁶ M alachlor + 10 ⁻⁶ M dithionite + 10 ⁻⁶ M phenol A + 10 ⁻⁶ M glyphosate + 10 ⁻⁶ M alachlor + 10 ⁻⁶ M dithionite + 10 ⁻⁶ M phenol A	~22
10 ⁻⁶ M alachlor + 10 ⁻⁶ M dithionite + 10 ⁻⁶ M phenol A + 10 ⁻⁶ M glyphosate + 10 ⁻⁶ M alachlor + 10 ⁻⁶ M dithionite + 10 ⁻⁶ M phenol A + 10 ⁻⁶ M glyphosate	~105
10 ⁻⁶ M alachlor + 10 ⁻⁶ M dithionite + 10 ⁻⁶ M phenol A + 10 ⁻⁶ M glyphosate + 10 ⁻⁶ M alachlor + 10 ⁻⁶ M dithionite + 10 ⁻⁶ M phenol A + 10 ⁻⁶ M glyphosate + 10 ⁻⁶ M alachlor	~22
10 ⁻⁶ M alachlor + 10 ⁻⁶ M dithionite + 10 ⁻⁶ M phenol A + 10 ⁻⁶ M glyphosate + 10 ⁻⁶ M alachlor + 10 ⁻⁶ M dithionite + 10 ⁻⁶ M phenol A + 10 ⁻⁶ M glyphosate + 10 ⁻⁶ M alachlor + 10 ⁻⁶ M dithionite	~90

LF								+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ER α	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
E2(M)		10^6								10^{-7}	10^6											
Genistein(M)			10^5									10^6	10^5									
Nonylphenol(M)				10^5										10^6	10^5							
Bisphenol A(M)					10^4											10^6	10^5					
Methoxychlor(M)						10^4												10^5	10^4			
α -Endosulfan(M)							10^4													10^5	10^4	

Fig. 1

C **D** **Fig. 1**

A**STAT3-LUC** **B****STAT3-LUC** **Fig. 2**

A

1 No stimulation

2 LIF

3 LIF plus E2

4 LIF plus Bisphenol A

5 LIF plus Methoxychlor

6 LIF plus α -Endosulfan**B**NF- κ B-LUC

LIF
ER α
E2(M)
Bisphenol A(M)
Methoxychlor(M)
 α -Endosulfan(M)

C

ELK-1-LUC

LIF
ER α
E2(M)
Bisphenol A(M)
Methoxychlor(M)
 α -Endosulfan(M)

Fig. 3

A

B

Fig. 4