Cross-talk between signal transducer and activator of transcription 3 and estrogen receptor signaling

Tetsuya Yamamoto[a,1], Tadashi Matsuda[a,1,*], Akira Junicho[b], Hiroyuki Kishi[a], Fahri Saatcioglu[c], Atsushi Muraguchi[a]

[a] *Department of Immunology, Faculty of Medicine, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-0194, Japan*
[b] *Department of Urology, Faculty of Medicine, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-0194, Japan*
[c] *Biotechnology Centre of Oslo, University of Oslo, Gaustadalleen 21, 0349 Oslo, Norway*

Received 25 October 2000; accepted 15 November 2000

First published online 24 November 2000

Edited by Marco Baggiolini

Abstract Interleukin-6 (IL-6) is a multifunctional cytokine that plays important roles in the immune system, hematopoiesis, and acute phase reactions. Estrogens have significant roles in a variety of biological events, such as the development and maintenance of female reproductive organs, and bone and lipid metabolism. Previous studies demonstrated that estrogens suppress IL-6-induced osteoporosis and the growth of multiple myeloma cells by repressing IL-6 and IL-6 receptor gene expression. Here we present a novel mechanism for the inhibitory effect of estrogens on IL-6 function. IL-6-induced activation of signal transducer and activator of transcription 3 (STAT3) activity and STAT3-mediated gene expression were suppressed by 17β-estradiol (E2) in breast cancer cells. E2-mediated inhibition of STAT3 activation was reversed by tamoxifen, an estrogen receptor (ER) antagonist. We provide evidence that the inhibitory action of ER on STAT3 activity was due to direct physical interactions between STAT3 and ER which represents a novel form of cross-talk between STAT3 and ER signaling pathways.

Key words: Interleukin-6; Signal transducer and activator of transcription 3; 17β-Estradiol; Estrogen receptor; Cross-talk; Transcription

1. Introduction

Interleukin-6 (IL-6) is a pleiotropic cytokine that regulates immune and inflammatory responses [1,2]. IL-6 also acts as a regulator in many malignant tumors, including breast cancer cells [3]. The receptors for the IL-6 family of cytokines share the gp130 molecule through which signals are generated, although the cytoplasmic region of gp130 does not contain any catalytic domain. Instead, the Janus kinase (Jak) family of protein kinases constitutively associate with gp130 and are activated by the IL-6 family of cytokines [4], leading to the tyrosine-phosphorylation and activation of signal transducer and activator of transcription (STAT) family of transcription factors.

One member of the STAT family of proteins is STAT3 which is mainly activated by IL-6 family of cytokines, epidermal growth factor, and leptin [2,5]. Like other members of the STAT family, STAT3 is tyrosine-phosphorylated by Jak kinases, upon which it dimerizes, and translocates into the nucleus to activate target genes [5,6].

Estrogen receptor (ER) is a ligand-activated transcriptional factor that is a member of the nuclear receptor superfamily [7]. Estrogens play an important role in the differentiation and development of various organs, in the maintenance of proper cellular function in a wide variety of tissues and are also characterized as risk factors for breast and endometrial cancer in women [8].

ER activates transcription through interaction with estrogen response elements (EREs) in the enhancer region of target genes and directly regulating their transcription [7].

In the previous studies, estrogens have been shown to inhibit IL-6 functions in osteoblast and multiple myeloma cells by repressing IL-6 production and IL-6 receptor expression [9–12] Consistent with these findings, ovariectomy in IL-6 knockout mice did not cause osteoporosis [13], indicating that IL-6 is essential for the bone loss caused by estrogen deficiency. Recent studies have also demonstrated that the ER can directly interact with the transcription factors NF-IL6 and NF-κB and can thereby inhibit their DNA binding activity [14,15]. This, in turn, might be the molecular basis for repression of IL-6-dependent gene expression by estrogens. However, no direct effect of ER on the downstream molecules of the IL-6 signaling pathway has been documented.

In this study, we provide an evidence for a new mechanism by which estrogens inhibit IL-6 function which is mediated by direct physical and functional interactions between STAT3 and ER.

2. Materials and methods

2.1. Reagents and antibodies

Human recombinant IL-6 was a kind gift from Ajinomoto (Tokyo, Japan). Human recombinant leukemia inhibitory factor (LIF) was purchased from Intergen (Purchase, NY, USA). 17β-Estradiol (E2), retinoic acid (RA), dexamethasone (DEX), 1α, 25-dihydroxyvitamin D_3(1,25(OH)$_2$D$_3$) and tamoxifen were purchased from Wako Chemicals (Osaka, Japan). FLAG-tagged STAT3–C [16], human ERα (HEGO), Vit–luciferase (LUC), human VDR, DR3–LUC, human RARα, βRE2LUC [17], C/EBPδ cDNA and STAT3–LUC (18) were

*Corresponding author. Fax: (81)-76-434 5019.
E-mail: tmatsuda@ms.toyama-mpu.ac.jp

[1] These authors contributed equally.

Abbreviations: IL, interleukin; ER, estrogen receptor; STAT, signal transducer and activator of transcription; E2, 17β-estradiol; LUC, luciferase; LIF, leukemia inhibitory factor

0014-5793/00/$20.00
PII: S0014-5793(00)02296-1

kindly provided by Dr. J.F. Bromberg (Rockefeller University, New York, USA), Dr. P. Chambon (Institut de Chimie Biologique, Strasbourg, France), Dr. D.J. Mangelsdorf (University of Texas Southwestern Medical Center, Dallas, TX, USA), Dr. S. Akira (Osaka University, Osaka, Japan) and Dr. T. Hirano (Osaka University, Osaka, Japan), respectively. Anti-ERα antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-FLAG M2 was purchased from Upstate Biotechnology (Lake Placid, NY, USA).

2.2. Cell culture, transfections, and LUC assays

Human breast cancer cell line MCF7 was a kind gift from Cell Resource Center for Biomedical Research (Tohoku University, Sendai, Japan) maintained in DMEM containing 10% FCS. Before stimulation, the cells were cultured for 24 h in DMEM containing 2% TCM (ICN) followed by treatment with IL-6 and/or E2 [19]. MCF7 cells ($2–2.5\times10^5$ in a 6-cm dish) were transfected by using LipoTAXI (Stratagene) following the manufacturer's instructions. 293T cells were transfected in DMEM containing 1% FCS by the standard calcium precipitation protocol. LUC assay was performed as described [20]. The cells were harvested 48 h after transfection and lysed in 200 μl of PicaGene Reporter lysis buffer (Toyo Ink, Tokyo, Japan) and assayed for LUC and β-galactosidase activities according to the manufacturer's instructions. LUC activities were normalized to the β-galactosidase activities. Three or more independent experiments were carried out.

2.3. Northern blot analysis

MCF7 cells were maintained as described above. After serum starvation, cells (1×10^7) were treated with IL-6 (100 ng/ml) and/or E2 (10^{-8}M) for 6 h. Total RNAs were prepared by using Iso-Gen (Nippon Gene) and used in Northern analysis according to established procedures. A nylon membrane (Hybond N^+, Amersham Pharmacia Biotech) and radiolabelled cDNA probes, as indicated, were used.

2.4. Immunoprecipitation and immunoblotting

The immunoprecipitation and Western blotting were performed as described previously [20]. Cells were harvested and lysed in lysis buffer (50 mM Tris–HCl, pH 7.4, 0.15 M NaCl, containing 0.5% NP-40, 1 μM sodium orthovanadate, 1 μM phenylmethylsulfonyl fluoride and 10 μg/ml each of aprotinin, pepstatin and leupeptin). The immunoprecipitates from cell lysates were resolved on 5–20% SDS–PAGE and transferred to Immobilon filter (Millipore, Bedford, MA, USA). The filters were then immunoblotted with each antibody. Immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).

3. Results and discussion

3.1. Estrogens inhibit IL-6-induced STAT3 activation

To examine the molecular basis of the cross-talk between IL-6 and estrogen signaling pathways, we utilized an IL-6-responsive, ER-positive breast cancer cell line, MCF7, and the transient transfection assay. The STAT3-mediated transcriptional responses were measured by using STAT3–LUC, in which the α2-macroglobulin promoter [18] drives expression of the LUC reporter gene. ER activity was monitored by using Vit–LUC in which two copies of an ERE drive expression of the LUC gene. MCF7 cells were transfected with STAT3–LUC and treated with IL-6 and/or E2 and LUC activities were determined. As shown in Fig. 1A, IL-6 stimulated STAT3–LUC activity, whereas E2 alone did not show effect. When cells were treated with both IL-6 and E2, STAT3–LUC activity was decreased by 40–50% compared with the activation by IL-6 alone.

To further examine whether estrogens affect IL-6-induced transcriptional activation of cellular genes, we carried out Northern analysis on RNA samples prepared from MCF7 cells which were induced by IL-6 and/or E2. As a cellular target for IL-6/STAT3, we analyzed the expression of C/EBPδ (CCAAT/enhancer binding protein δ) which is a regulator of acute-phase response genes in hepatocytes and is up-regulated by IL-6 treatment [21]. As shown in Fig. 1B, IL-6 treatment induced C/EBPδ expression in MCF7 cells by 5-fold and this activation was decreased by 40% in the presence of E2, whereas E2 alone did not affect C/EBPδ expression. These data show that E2 inhibits IL-6-induced transcription in MCF7 cells.

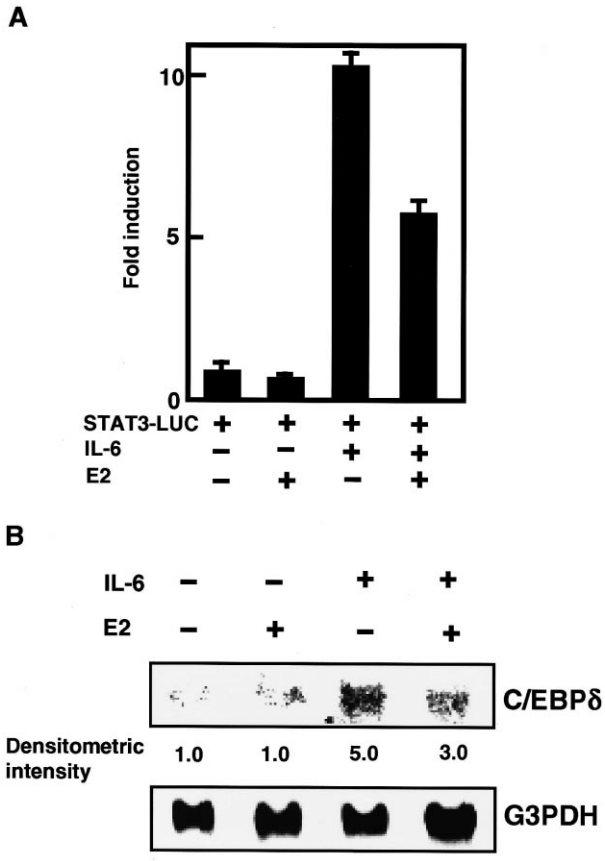

Fig. 1. Estrogens inhibit IL-6 induced STAT3. A: MCF7 cells were grown in a 6-cm dish and transfected with STAT3–LUC reporter (1 μg) and then stimulated with IL-6 and/or E2 as indicated. 48 h after transfection, cells were stimulated for an additional 12 h. Cells were harvested and relative LUC activities were measured. The results are presented as fold induction of LUC activity from triplicate experiments, and the error bars represent the standard deviations. B: Effect of C/EBPδ expression by IL-6 and/or E2 in MCF7 cells. Northern blot analysis of 20 μg of total RNA from LNCaP cells treated with IL-6 (100 ng/ml) and/or E2 (10^{-8}M) for 6 h. The fold induction of C/EBPδ expression was shown as the densitometric intensity. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA is included as a loading control (lower panel).

3.2. Reconstitution of the cross-talk between STAT3 and ER signaling pathways in 293T cells

To further delineate the mechanisms of cross-talk between STAT3 and ER signaling pathways, we carried out transient transfection experiments in 293T cells. Cells were transfected with STAT3–LUC with or without an expression vector for ERα were stimulated with LIF [19] in the absence or presence of E2. As shown in Fig. 2A, STAT3–LUC activity was stimulated by LIF, whereas E2 alone did not change this activity. In the presence of ERα and LIF, E2 suppressed STAT3–LUC activity in a dose-dependent fashion. These results indicate that the inhibitory effects of E2 on STAT3 transcriptional

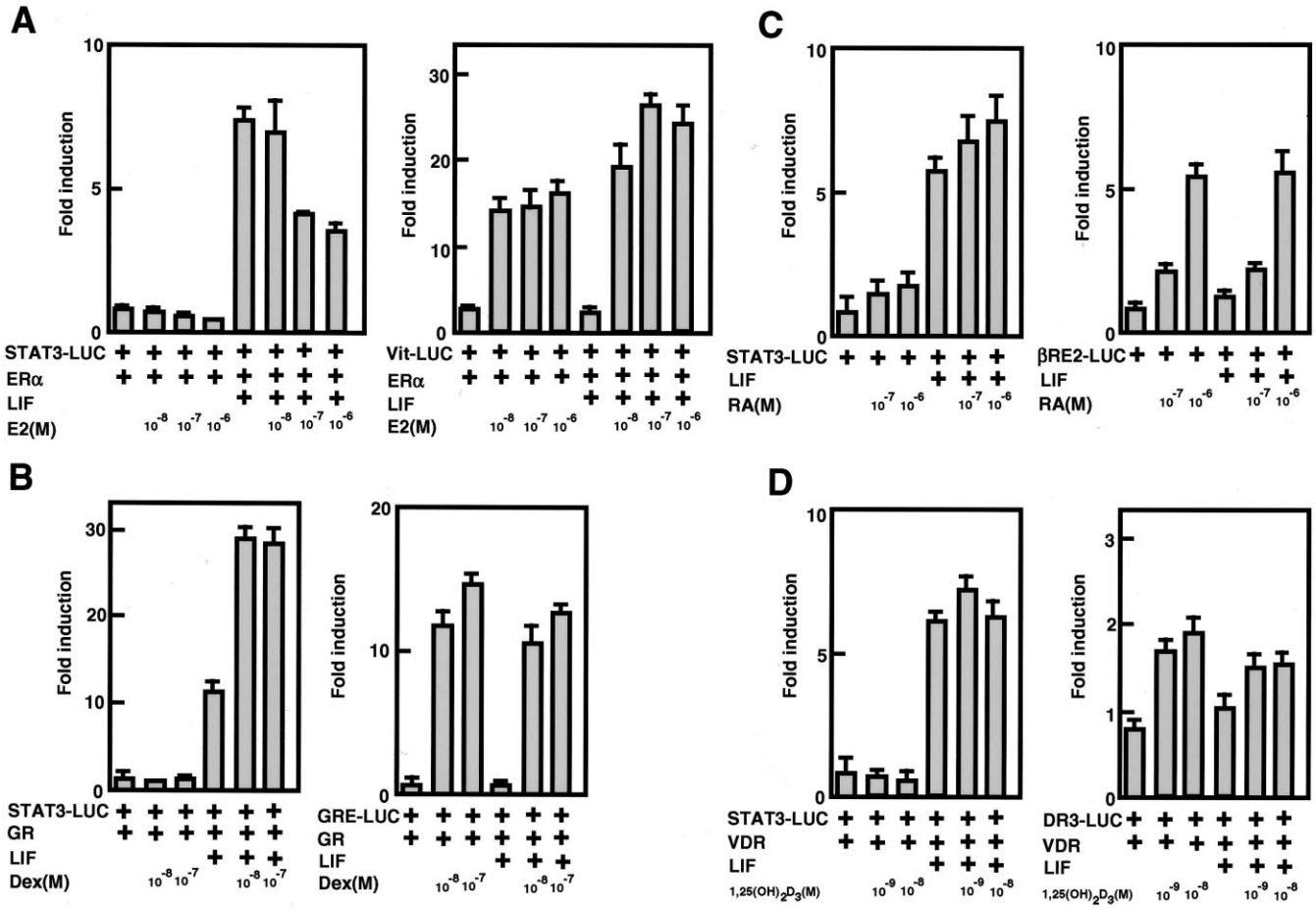

Fig. 2. Reconstitution of cross-talk between STAT3 and ERα signaling in 293T cells and comparison with other nuclear receptors. 293T cells were transfected with 1 μg of reporter constructs STAT3–LUC (A, B, C, D), Vit–LUC (A), GRE–LUC (B), βRE2–LUC (C) or DR3–LUC (D), together with ERα (A), GR (B) and VDR (D) expression construct (1 μg) or control vector (1 μg). 48 h after transfection, cells were stimulated for an additional 12 h with LIF (100 ng/ml) and/or E2(A), DEX (B), RA(C) and $1,25(OH)_2D_3$ (D) as indicated. Cells were harvested and relative LUC activities were measured. The results are presented as the fold induction of LUC activity from triplicate experiments, and the error bars represent the standard deviations.

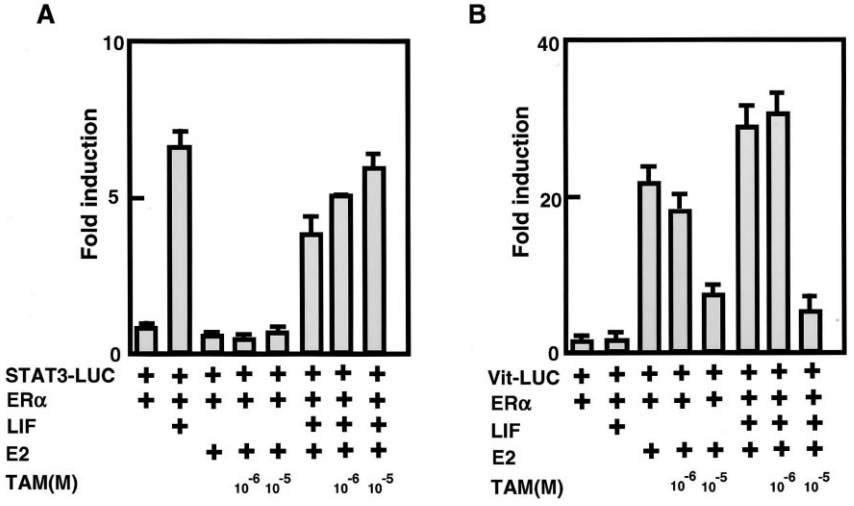

Fig. 3. Tamoxifen reverses the inhibitory effect of E2 on STAT3 activation in 293T cells. 293T cells were transfected with STAT3–LUC (1 μg) (A) or Vit–LUC (1 μg) (B) together with ERα expression construct (1 μg) or empty vector, pSG5 (1 μg). 48 h after transfection, cells were stimulated for 12 h with or without LIF (100 ng/ml) and/or E2 (10^{-7} M), and/or Tamoxifen as indicated. Cells were harvested and relative LUC activities were measured. The results are presented as fold induction of LUC activity from triplicate experiments, and the error bars represent the standard deviations.

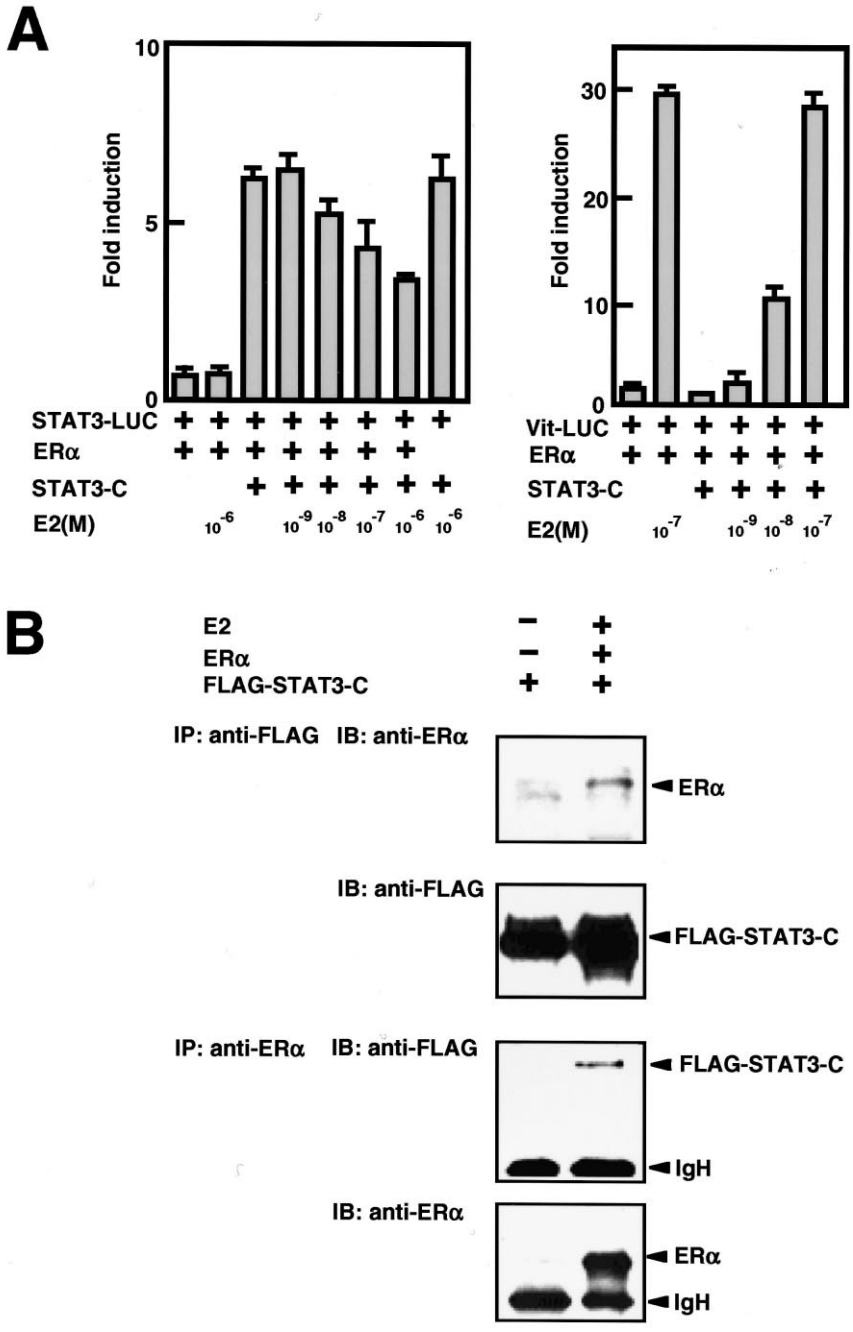

Fig. 4. E2 inhibits a constitutively active form of STAT3 and evidence for physical interactions between STAT3 and ERα. A: 293T cells were transfected with STAT3–LUC (1 μg) (A) or Vit–LUC (1 μg) (B) together with ERα and/or STAT3–C expression construct (1 μg) or the empty vector (1 μg). 48 h after transfection, cells were stimulated for 12 h with or without E2 as indicated. Cells were harvested and relative LUC activities were measured. The results are presented as fold induction of LUC activity from triplicate experiments, and the error bars represent the standard deviations. B: 293T cells were transfected with FLAG-tagged STAT3–C (5 μg) alone or together with ERα (10 μg). 48 h after transfection, cells were treated with E2 (10^{-8} M) for 12 h. Cell lysates were then immunoprecipitated and immunoblotted with anti-FLAG or anti-ERα antibodies as indicated.

activity can be reconstituted in 293T cells similar to those observed in MCF7 cells.

We then assessed the reverse situation for the effects of STAT3 on ER activity in 293T cells, using Vit–LUC as a reporter gene. When ER was expressed in 293T cells, E2 treatment resulted in a robust increase in Vit–LUC activity (Fig. 2A). E2-induced Vit–LUC activity was modestly augmented by LIF in 293T cells, whereas LIF alone did not affect reporter activity.

We also examined the cross-talk between STAT3 and retinoic acid, 1α, 25-dihydroxy vitamin D3, and glucocorticoid receptors (RAR, VDR, and GR) in the reconstituted system in 293T cells (Fig. 2B–D). As previously reported [22], GR stimulated STAT3 activation in the presence of DEX, whereas GR activity, when assessed by GRE–LUC, was not affected by STAT3 in 293T cells (Fig. 2B). Neither RAR nor VDR affected STAT3 activation by LIF treatment in 293T cells in the presence of their respective ligands. Similarly, the tran-

scriptional activities of these receptors assessed by appropriate reporter constructs, βRE2–LUC and DR3–LUC, respectively, were not affected by STAT3 activation (Fig. 2C,D). These data suggest that inhibition of STAT3 activation in 293T cells is not a general phenomenon of nuclear receptors, but highly specific for ER.

3.3. Tamoxifen can reverse the inhibitory effect of E2 on STAT3 activation

To further assess the specificity of E2 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 ERα, cells were treated with LIF in the absence or presence of E2 and/or Tamoxifen. The inhibitory effect of ER/E2 on STAT3 activation was reversed by Tamoxifen when cells were treated with increasing concentrations of Tamoxifen (Fig. 3A). On the other hand, E2-induced Vit–LUC activity was inhibited by Tamoxifen in a dose-dependent manner (Fig. 3B). These results indicate that the inhibitory effect of E2 on STAT3 activation in 293T cells is mediated by ERα.

3.4. Estrogens inhibit STAT3 activation by an active form of STAT3

Several studies demonstrated that estrogens downregulate IL-6 production and its receptor expression by acting on their transcription activities [9–12]. To avoid these effects on inhibition of STAT3 activation by E2, we used a constitutively active form of STAT3, STAT3–C [16]. 293T cells were transfected with STAT3–LUC and expression vectors for ERα and/or STAT3–C and the cells were either left untreated or stimulated with E2. As shown in Fig. 4A, STAT3–LUC activity was stimulated by STAT3–C, whereas E2 alone did not change this activity. In the presence of ERα, STAT3–LUC activation by STAT3–C was inhibited when cells were simultaneously treated with increasing amounts of E2. These results indicate the presence of a direct cross-talk between ER and STAT3 in 293T cells.

3.5. STAT3 and ER physically interact in vivo

One of the mechanisms that is consistent with the data described above is that there are direct physical interactions between ER and STAT3. We tested this possibility by co-immunoprecipitation experiments. Expression vectors encoding wild-type ERα and/or FLAG epitope-tagged STAT3–C were transiently transfected into 293T cells. Cells were either left untreated or treated with E2, lysed, and subjected to immunoprecipitation with either an anti-ER monoclonal antibody or anti-FLAG antibody. Immunoprecipitates were then used in Western blot analysis with the appropriate antibodies. As shown in Fig. 4B, ERα physically associated with STAT3 in the presence of E2, regardless of which one was immunoprecipitated first. These data indicate that STAT3 and ER physically interact in vivo.

3.6. Conclusive remarks

In the present study, we have shown that E2 negatively regulates IL-6 signaling mediated by STAT3 in an IL-6 responsive, ER-positive breast cancer cells and that the reconstituted ER signaling in 293T cells suppresses STAT3-mediated transcription in the presence of E2. Inhibitory effects of E2 on STAT3 activation were reversed by the anti-estrogen, Tamoxifen. Furthermore, we demonstrated that active ER directly associates with and acts as a transcriptional co-factor for STAT3.

RA was also shown to inhibit the IL-6-induced growth inhibition and apoptosis in a radiation-induced murine hematopoietic cell line, Y6, without suppressing the IL-6-induced junB gene expression [23]. However, our results indicated no cross-talk between STAT3 and RAR (Fig. 2C). Similarly, the cross-talk between STAT3 and VDR was not observed in the reconstituted 293T cells (Fig. 2D), although both IL-6 and $1,25(OH)_2D_3$ induce the macrophage differentiation in murine myeloid leukemia cells (M1) [24]. Interestingly, the cross-talk between STAT3 and GR was observed in the reconstituted 293T cells (Fig. 2B). It was previously reported that the interaction between STAT3 and GR enhances GR-mediated transcription but not STAT3-mediated transcription, suggesting that STAT3 is a transcriptional co-activator for GR [22]. However, our results suggest another form of interaction between STAT3 and ER, which leads to the repression of transcription at the STAT3 binding site. Further understanding of biochemical effects between STAT3 and ER may provide a clue to develop new drugs for osteoporosis, multiple myeloma and breast cancer.

Acknowledgements: We thank Dr. J.F. Bromberg, Dr. P. Chambon, Dr. D.J. Mangelsdorf, Dr. S. Akira and Dr. T. Hirano for their kind gifts of reagents. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture in Japan, and Norwegian Cancer Society.

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