ORIGINAL ARTICLE

DUSP22/LMW-DSP2 regulates estrogen receptor- α -mediated signaling through dephosphorylation of Ser-118

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In the previous study, we demonstrated the involvement of dual specificity phosphatase 22 (DUSP22/LMW-DSP2) in regulating the leukemia inhibitory factor/interleukin-6/signal transducer and activator of transcription 3-mediated signaling pathway. In this study, we show β -estradiol (E2)-induced DUSP22 mRNA expression in estrogen receptor α (ER α)-positive breast cancer cells, whereas E2-induced phosphorylation and activation of ER α was suppressed by overexpression of DUSP22 but not catalytically inactive mutants. Furthermore, small-interfering RNA-mediated reduction of DUSP22 expression enhanced ER α -mediated transcription and endogenous gene expression. In fact, DUSP22 associated with ER α *in vivo* and both endogenous proteins interacted in ER α -positive breast cancer T47D cells. These results strongly suggest that DUSP22 acts as a negative regulator of the ER α -mediated signaling pathway.

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Introduction

Estrogen receptor- α (ER α) is a ligand-activated transcriptional factor that is a member of the nuclear receptor superfamily (Mangelsdorf *et al.*, 1995). Estrogens play important roles in the differentiation and development of various organs, maintenance of proper cellular function in a wide variety of tissues, and are also risk factors for breast and endometrial cancer (Couse and Korach, 1999). The ER α interacts with estrogen response elements (EREs) in the target gene promoters and directly regulates their transcription (Mangelsdorf *et al.*, 1995). The ER α has two well-characterized

transcriptional activation functions (AFs): AF-1, which is located in the N-terminal A/B region and may be activated in a ligand-independent manner, and AF-2, which is located in region E and whose activity is ligand dependent. AF-1 and AF-2 can activate transcription independently and synergistically act in a promoter- and cell type-specific manner (see Figure 4, Tora *et al.*, 1989; Mangelsdorf *et al.*, 1995).

The ER α , like other members of the steroid hormone receptor superfamily, is phosphorylated on multiple serine residues (Castano *et al.*, 1998; Lannigan, 2003). Serine phosphorylation is essential for transcriptional activation in response to β -estradiol (E2) binding and occurs on Ser-118 and Ser-167. Ser-118 is shown to be the major phosphorylation site required for full activation of ER α and to a lesser extent on Ser-104 and Ser-106, all located in the AF-1 domain of the receptor (Lannigan, 2003). General transcription factor IIH (TFIIH) kinase Cdk7 (Chen *et al.*, 2000), mitogen-activated protein kinase (MAPK) (Kato *et al.*, 1995) and glycogen synthetase kinase-3 (Medunjanin *et al.*, 2005) have been reported to phosphorylate Ser-118, and cyclin A-Cdk2 was associated with Ser-104 and -106 phosphorylation (Rogatsky *et al.*, 1999). The molecular mechanisms by which the phosphorylation of ER α is regulated still remain to be clarified. Although protein phosphatase 2A (PP2A) and PP5 have been recently shown to associate with ER α directly and inhibit its transcriptional activity (Lu *et al.*, 2003; Ikeda *et al.*, 2004).

Dual specificity phosphatases (DSPs)/MAPK phosphatases are known to regulate MAPK-mediated signaling pathways, including extracellular signal-regulated kinase (ERK), c-Jun NH₂-terminal kinase (JNK) and p38 MAPK (Theodosiou and Ashworth, 2002). In previous studies, we cloned a distinct class of DSPs with low molecular weight (LMW-DSP1, -DSP2, -DSP4, -DSP6, -DSP10 and -DSP11) (Aoki *et al.*, 2001; Aoyama *et al.*, 2001), which contained a single catalytic domain but lacked a putative common docking site for MAPKs, the cdc25 homology domain. The first LMW-DSP to be cloned was the VH1 protein from *Vaccinia* virus (Guan *et al.*, 1991). A related phosphatase was cloned in mammalian cells and termed VHR, for VH1-related (Ishibashi *et al.*, 1992). Both VH1 and VHR differ from other DSPs in that they are much smaller, only 19 and 21 kDa, respectively. VH1 has been reported to dephosphorylate both MAPK and

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signal transducer and activator of transcription (STAT)1 (Najarro *et al.*, 2001), whereas VHR seems to be specific for ERK and JNK (Denu and Dixon, 1995; Alonso *et al.*, 2001). LMW-DSP-2 dephosphorylated and deactivated p38 MAPK and JNK, but not ERK (Aoyama *et al.*, 2001). LMW-DSP2 is also designated as VHR-related MKPX (VHX) (Alonso *et al.*, 2002) and JNK stimulatory phosphatase-1 (JSP-1) (Shen *et al.*, 2001), JNK pathway-associated phosphatase (JKAP) (Chen *et al.*, 2002). JSP-1 and have been shown to positively regulate JNK activation. These results indicate that LMW-DSP2 may play a regulatory role in the JNK or p38 MAPK pathways. This gene is now designated as *DUSP22*. *DUSP22* has been also demonstrated to belong to a new subgroup of small DSPs anchored at the membranes by an N-terminal myristic acid moiety (Alonso *et al.*, 2004). However, the physiological function of LMW-DSPs has remained unclear as it seems to be less efficient than many other MAPK-specific DSPs. Interestingly, *DUSP22* has been shown to regulate T-cell antigen receptor signaling through ERK2 (Alonso *et al.*, 2002). Recently, we have also demonstrated that *DUSP22* regulates interleukin 6 (IL-6)/leukemia inhibitory factor (LIF)-mediated signaling by dephosphorylating STAT3 (Sekine *et al.*, 2006).

We show here that ER α is a substrate of *DUSP22*, being dephosphorylated at Ser-118 through a direct interaction with *DUSP22*. This suggests an important function of *DUSP22* in the regulation of ER α -dependent transcription in breast cancer cells by negative feedback.

Results

DUSP22 mRNA expression is induced in breast cancer cells in an ER α -dependent manner

Recently, we have demonstrated that *DUSP22*/LMW-DSP-2 regulates IL-6/LIF-mediated signaling by dephosphorylating STAT3 (Sekine *et al.*, 2006). In our previous study, we have shown that E2 suppressed IL-6-induced STAT3 activation and STAT3-mediated gene expression through estrogen receptors in breast cancer cells (Yamamoto *et al.*, 2000). These facts let us to examine whether *DUSP22* expression is induced by E2 breast cancer cells and participates in the E2-mediated suppression of STAT3 activation. Furthermore activation of ER α is also tightly regulated by its phosphorylation status as well as STAT3 (Tora *et al.*, 1989; Castano *et al.*, 1998), indicating the possibility that *DUSP22* may be involved in the regulation of ER α -mediated signaling.

To investigate the involvement of *DUSP22* in ER α -mediated signaling, we first examined whether *DUSP22* expression was regulated by estrogen in human breast cancer cell lines using quantitative real-time polymerase chain reaction (PCR). As shown in Figure 1a and b, a significant induction of *DUSP22* mRNA expression was observed in ER α -positive breast cancer MCF-7 and T47D cell lines. ER α -mediated *cathepsin D* mRNA expression was also observed in these cells. However, any significant induction of *DUSP22* as well as *cathepsin D* mRNA

expression was not observed in the ER α -negative HBL100 cell line (Figure 1c). We attempted to examine the changes of protein level of *DUSP22* by E2 stimulation. Unfortunately, we could not detect alteration of the endogenous protein level of *DUSP22* in the total lysates of these cells, because of the low detection sensitivity of anti-*DUSP22* antibody immunoblotting. These results indicate that expression of *DUSP22* mRNA is induced in breast cancer cells in an ER α -dependent manner.

DUSP22 regulates ER α -mediated transcriptional activation

To examine the effect of *DUSP22* on ER α -induced transcriptional activation, a transient transfection assay was used. The ER α -mediated transcriptional responses were measured by using the reporter construct Vit-luciferase (LUC) in which two copies of an ERE drive expression of the LUC gene (Matsuda *et al.*, 2001b). 293T or HeLa cells were transfected with Vit-LUC together with ER α and treated with E2, and LUC activity was determined. When cells were co-transfected with *DUSP22*, transcriptional activation of Vit-LUC decreased in a dose-dependent manner compared with that of mock transfectants (Figure 2a and b). We further examined whether DSP activity of *DUSP22* was necessary for suppression of ER α activation. Two amino acids in *DUSP22*, namely Asp (D)-57 and Cys (C)-88, have previously been shown to participate in the catalytic mechanism of DSP activity (Aoyama *et al.*, 2001). Wild-type (WT) or catalytically inactive Asp/Ala (D/A) or Cys/Ser (C/S) forms of *DUSP22* together with ER α and Vit-LUC were co-transfected into 293T cells. A decrease of ER α activation was not observed when cells were co-transfected with *DUSP22* D/A or C/S mutant (Figure 2a and b), suggesting that DSP activity of *DUSP22* is essential for deactivation of ER α . To examine the effect of *DUSP22* on the ER α -mediated transcriptional activation under more physiological conditions, we transfected the *DUSP22* WT, D/A or C/S mutant together with Vit-LUC into MCF-7 cells and treated the cells with E2. As shown in Figure 2c, E2-mediated ER α activation was markedly decreased by expression of *DUSP22* WT but not by the D/A or C/S mutant in MCF-7 cells, indicating that *DUSP22* negatively regulates ER α -mediated transcription through its DSP activity. Similarly, we also tested the effects of expression of another DSP, *DUSP19*/LMW-DSP1/SKRP1 (Aoki *et al.*, 2001; Zama *et al.*, 2002), which has been shown to regulate JNK activation (Zama *et al.*, 2002) on E2-induced ER α activation in 293T cells (data not shown). However, we could not observe any significant alteration of ER α activation by expression of *DUSP19*, suggesting that *DUSP22* specifically acts ER α -mediated signaling. Importantly, *DUSP22* has been shown to be modified by the attachment of a myristate to the N-terminal Gly-2 (Alonso *et al.*, 2004). Therefore, N-terminally tagged *DUSP22* constructs lacks myristoylation of Gly-2 (Alonso *et al.*, 2002). We then tested both N-terminal or C-terminal tagged *DUSP22* constructs on ER α -mediated transcription. However, expression of either

Figure 1 Estrogen induced mRNA expression of *DUSP22* in breast cancer cells. Human breast cancer cell lines, MCF-7 (a), T47D (b) and HBL100 (c), were maintained in phenol red-free DMEM containing 10% charcoal-stripped FCS. Cells were treated or untreated with E2 (10^{-7} M) for the indicated periods. Both *DUSP22* and *cathepsin D* expression levels were quantified by RT and quantitative real-time PCR analysis. Data represent the levels of *DUSP22* and *cathepsin D* mRNA normalized to that of a *G3PDH* internal control and are expressed relative to the value at time zero. Data represent the mean of duplicate PCR determinations, which in general varied by <10%. Shown is a representative experiment, which was repeated at least twice with similar results.

N-terminal or C-terminal tagged construct similarly suppressed ER α -mediated transactivation (data not shown), suggesting that myristoylation of DUSP22 has no effect on the DUSP22-mediated suppression of ER α activation.

Reduction of endogenous DUSP22 enhances ER α -mediated transcriptional activation in breast cancer cells
To further explore whether DUSP22 represses ER α -mediated transcriptional activation, we used small

interfering RNA (siRNA) to reduce endogenous expression of *DUSP22* in HeLa or MCF-7 cells. A specific siRNA for *DUSP22* or a control siRNA was transfected into ER α -transfected HeLa or MCF-7 cells. Total RNA isolated from the transfected cells was subjected to quantitative real-time PCR analysis, which confirmed a reduction of *DUSP22* mRNA expression. As shown in Figure 3a and b, approximately 70% reduction in HeLa cells or 60% reduction in MCF-7 cells of the *DUSP22* mRNA expression was observed following *DUSP22* siRNA expression. We then determined the effects of

DUSP22 siRNAs on ER α -induced Vit-LUC activation in these cells. As shown in Figure 3c and d, siRNA-mediated reduced expression of *DUSP22* also resulted in a significant enhancement of ER α -induced Vit-LUC activation in these cells. We next examined the effects of *DUSP22* siRNA on other steroid receptor signalings, such as androgen receptor (AR) and glucocorticoid receptor (GR). The AR-mediated transcriptional responses were measured by using the androgen-dependent reporter construct -285PB-LUC (Matsuda *et al.*, 2001a). HeLa cells were transfected with control or *DUSP22* siRNA together with -285PB-LUC and AR, and treated with dihydrotestosterone (DHT), and LUC activity was determined. Interestingly, siRNA-mediated reduced expression of *DUSP22* resulted in a significant reduction of DHT-induced -285PB-LUC activation in HeLa cells (Figure 3e), suggesting that *DUSP22* may act AR-mediated signaling positively. We also tested the effect of *DUSP22* siRNA on GR-mediated signaling in HeLa cells. The GR-mediated transcriptional responses were measured by using the reporter construct GRE-LUC (Yamamoto *et al.*, 2000). HeLa cells were transfected with control or *DUSP22* siRNA together with GRE-LUC and treated with dexamethasone (DEX), and LUC activity was determined. As shown in Figure 3f, siRNA-mediated reduced expression of *DUSP22* resulted in no alteration of DEX-induced GRE-LUC activation in HeLa cells, indicating that *DUSP22* has no effect on GR-mediated signaling. We further examined the effects of *DUSP22* siRNA on ER α -mediated *cathepsin D* mRNA expression in MCF-7 cells. As shown in Figure 4a, siRNA-mediated reduced expression of

Figure 2 DUSP22 deactivated E2-induced ER α -mediated transcriptional activation. (a) Human embryonic kidney carcinoma cell line, 293T, was maintained in phenol red-free DMEM containing 10% FCS and transfected in a 24-well plate were transfected with ER α (50 ng) and Vit-LUC (100 ng) and/or indicated amounts of empty vector, WT, D/A or C/S of *DUSP22* by the standard calcium precipitation protocol. At 36 h after transfection, the cells were treated with E2 (10^{-9} M) for additional 12 h. The stimulated cells were harvested, and LUC activities were measured. Total cellular protein collected from parallel cultures was analysed for *DUSP22* by Western blot analysis (lower panel). (b) Human cervix carcinoma cell line, HeLa cells in a 24-well plate were transfected with ER α (50 ng) and Vit-LUC (50 ng) and/or indicated amounts of empty vector, WT, D/A or C/S of *DUSP22* using jetPEI. At 36 h after transfection, the cells were treated with E2 (10^{-9} M) for additional 12 h. The cells were harvested and assayed for the LUC activity using the Dual-Luciferase Reporter Assay System. Total cellular protein collected from parallel cultures was analysed for *DUSP22* by Western blot analysis (lower panel). (c) Human breast cancer cell line, MCF-7 cells in a 24-well plate were transfected with Vit-LUC (50 ng) and/or indicated amounts of empty vector, WT, D/A or C/S of *DUSP22* using jetPEI. At 36 h after transfection, the cells were treated with E2 (10^{-7} M) for additional 12 h. The cells were harvested and assayed for the LUC activity using the Dual-Luciferase Reporter Assay System. The results are indicated as fold induction of LUC activity from triplicate experiments, and the error bars represent the s.d. At least three independent experiments were carried out for each assay. Total cellular protein collected from parallel cultures was analysed for *DUSP22* by Western blot analysis (lower panel) * $P < 0.05$.

DUSP22 resulted in a significant enhancement of E2-induced *cathepsin D* mRNA expression in MCF-7 cells. These results strongly suggest that DUSP22 regulates ER α -mediated transcriptional activation in HeLa and MCF-7 cells. As DUSP22 affects ER α -mediated transcriptional activation, it seemed plausible that DUSP22 might alter ER α -mediated transcription by influencing the ability of ER α to bind to DNA. We then examined the effects of DUSP22 siRNA on the ER α DNA-binding activity to ERE. To this end, we performed DNA

pulldown assays using immobilized ER α Consensus Oligonucleotide. As shown in Figure 4b, after E2 stimulation, the ER α -DNA complex formation was observed in MCF-7 cells. Furthermore, this complex formation was enhanced by siRNA-mediated reduced expression of DUSP22, suggesting that DUSP22 negatively regulates ER α -DNA complex formation.

DUSP22 physically interacts with ER α

One of the mechanisms that would be consistent with the data described above is a direct interaction with ER α by DUSP22, which would trigger its deactivation. First this possibility was tested in co-immunoprecipitation experiments. Expression vectors encoding FLAG-tagged ER α and Myc-tagged DUSP22 or its inactive mutant D/A or C/S were transiently transfected into 293T cells. The transfected 293T cells were lysed and subjected to immunoprecipitation with anti-FLAG antibody. Immunoprecipitates were then used in Western blot analysis with anti-Myc antibody. As shown in Figure 5a, ER α interacted with DUSP22 WT and C/S. However, DUSP22 D/A showed a very weak binding potential to ER α , suggesting that the DUSP22-binding site is in close proximity to Asp-57. These results also indicate that the interaction between ER α and DUSP22 is not required for its DSP activity.

Figure 3 Reduction of endogenous DUSP22 enhances ER α -mediated transcriptional activation in breast cancer cells. (a and b) HeLa (a) or MCF-7 (b) cells in a 24-well plate were transfected with control siRNA or siRNA targeting DUSP22 (25 pmol) using Lipofectamine 2000 (Invitrogen). DUSP22 expression levels were quantified by RT and quantitative real-time PCR. Data represent the levels of DUSP22 mRNA normalized to that of a *G3PDH* internal control and are expressed relative to the value at time zero. Data represent the mean of duplicate PCR determinations, which in general varied by <10%. Shown is a representative experiment, which was repeated at least twice with similar results. (c) siRNA-transfected HeLa cells were then transfected with ER α (50 ng) and Vit-LUC (50 ng) using jetPEI (PolyPlus-transfection). At 36 h after transfection, the cells were treated with E2 (10^{-9} M) for additional 12 h. The cells were then harvested, and LUC activities were measured. The results are indicated as fold induction of LUC activity from triplicate experiments, and the error bars represent the s.d. * P <0.05 (d) siRNA-transfected MCF-7 cells in a 24-well plate were then transfected with Vit-LUC (50 ng) using jetPEI (PolyPlus-transfection). At 36 h after transfection, the cells were treated with E2 (10^{-7} M) for additional 12 h. The cells were then harvested, and LUC activities were measured. The results are indicated as fold induction of LUC activity from triplicate experiments, and the error bars represent the s.d. * P <0.05 (e) siRNA-transfected HeLa cells were then transfected with AR (10 ng) and -285PB-LUC (0.2 μ g) using jetPEI (PolyPlus-transfection). At 36 h after transfection, the cells were treated with DHT (10^{-8} M) for additional 12 h. The cells were then harvested, and LUC activities were measured. The results are indicated as fold induction of LUC activity from triplicate experiments, and the error bars represent the s.d. * P <0.05 (f) siRNA-transfected HeLa cells were then transfected GRE-LUC (0.2 μ g) using jetPEI (PolyPlus-transfection). At 36 h after transfection, the cells were treated with DEX (10^{-7} M) for additional 12 h. The cells were then harvested, and LUC activities were measured. The results are indicated as fold induction of LUC activity from triplicate experiments, and the error bars represent the s.d. ** P >0.1.

Figure 4 Reduction of endogenous DUSP22 enhances E2-induced gene expression and E2-induced DNA-binding activity of ER α in breast cancer cells. **(a)** siRNA-transfected MCF-7 cells in a 24-well plate were treated with E2 (10^{-7} M) for the indicated periods. *Cathepsin D* expression levels were quantified by RT and quantitative real-time PCR analysis. Data represent the levels of *cathepsin D* mRNA normalized to that of a *G3PDH* internal control and are expressed relative to the value at time zero. Data represent the mean of duplicate PCR determinations, which in general varied by <10%. Shown is a representative experiment, which was repeated at least twice with similar results. **(b)** siRNA-transfected MCF-7 cells in a 6-well plate were treated with E2 (10^{-7} M) for the indicated periods. Pull-down assays were carried out using the immobilized ER α Consensus Oligonucleotide-Sepharose. Specifically bound proteins were eluted and subjected to Western blot analysis using anti-ER α antibody (upper panel). Densitometric quantification of the above results was also shown (middle panel). Relative intensity of DNA-bound ER α was normalized to total ER α of the same sample. An aliquot (1%) of the input samples was analysed for ER α by Western blot analysis (lower panel).

To further confirm that endogenous DUSP22 interacts with ER α *in vivo*, co-immunoprecipitation experiments were performed using cell extracts obtained from T47D cells, in which both proteins could be detected using specific antibodies. As shown in Figure 5b, anti-DUSP22 immunoprecipitates from cell extracts of T47D cells contained ER α protein. This result suggests that endogenous DUSP22 interacts and forms a complex with ER α in T47D cells.

To further delineate the domains in ER α that mediate protein–protein interactions between ER α and DUSP22, co-immunoprecipitation experiments were performed with a series of mutant ER α proteins (Figure 5c). As shown in Figure 5d, the AF-1 domain of ER α (12–175) did not interact with DUSP22. Both the DNA-binding (176–395) and the AF-2 domain of ER α (396–595) interacted with DUSP22, although the DNA-binding domain of ER α (176–395) showed a weak interaction with DUSP22. These data suggest that the C-terminal region containing the DNA-binding and AF-2 domain is required for ER α to interact with DUSP22.

DUSP22 dephosphorylates ER α Ser-118

It is well known that phosphorylation of ER α modulates the activity of both AF-1 and AF-2 (Tora *et al.*, 1989; Castano *et al.*, 1998). Among the sites of potential phosphorylation, Ser-118 in the ER α AF-1 domain has been particularly intensively studied with regard to the state of its phosphorylation and consecutive potentiation of ER α activity. Thus, the effect of DUSP22 on ER α Ser-118 phosphorylation was examined. First, we examined whether E2 stimulation induces ER α Ser-118 phosphorylation and complex formation between ER α and DUSP22 in 293T cells. Expression vectors for ER α together with or without DUSP22 WT were transfected into 293T cells and treated with E2. The transfected 293T cells were lysed and subjected to immunoprecipitation with anti-FLAG antibody. Immunoprecipitates were then used in Western blot analysis with anti-Myc antibody or anti-phospho-ER α Ser-118. As shown in Figure 6a, ER α strongly interacted with DUSP22 WT in the presence of E2, whereas E2-induced phosphorylation of ER α Ser-118 decreased by expression of DUSP22 WT. We further confirmed the effect of DUSP22 on E2-induced ER α Ser-118 phosphorylation. As shown in Figure 6b, E2-induced ER α Ser-118 phosphorylation decreased by expression of DUSP22 in a dose-dependent manner. DUSP22 WT, D/A or C/S mutant was also co-transfected with FLAG-tagged ER α into 293T cells and treated with E2. Upon co-expression of LMW-DUSP22 WT, E2-induced ER α Ser-118 phosphorylation remarkably decreased, compared with mock, DUSP22 D/A and C/S transfectants (Figure 6c). Comparable expression of DUSP22 WT, D/A and C/S mutant was confirmed by immunoblotting with anti-Myc antibody. These results indicate that DUSP22 dephosphorylates ER α Ser-118 and negatively regulates E2-induced ER α transcriptional activation. We next examined the effect of DUSP22 siRNA on E2-induced phosphorylation of ER α Ser-118 in MCF-7 cells. As shown in Figure 7a, DUSP22 siRNA treatment significantly enhanced E2-induced phosphorylation of ER α Ser-118. These results indicate that endogenous DUSP22 dephosphorylates ER α and negatively regulates E2-mediated ER α transcriptional activation in MCF-7 cells. To further confirm the dephosphorylation action of DUSP22 on ER α , recombinant glutathione-S-transferase (GST) fusion proteins containing full-length DUSP22 WT were expressed in *Escherichia coli* and purified. GST-DUSP22 WT

Figure 5 DUSP22 and ER α physically interact *in vivo*. (a) 293T cells (1×10^7 cells) were transfected with FLAG-tagged ER α (7.5 μ g) together with or without WT, D/A or C/S of Myc-tagged DUSP22 (10 μ g). At 48 h after transfection, the cells were lysed, and immunoprecipitated with anti-FLAG antibody and immunoblotted with anti-Myc (upper panel) or anti-FLAG antibody (middle panel). Total cell lysates (1%) were blotted with anti-Myc (bottom panel). (b) Human breast cancer T47D cells (2×10^7) were lysed, and immunoprecipitated with control IgG or anti-DUSP22 antibody and immunoblotted with anti-ER α antibody (upper panels) or anti-DUSP22 antibody (lower panels). (c) Domain structure of ER α and mutant fragments is schematically shown. (d) 293 T cells (1×10^7) were transfected with a series of Myc-tagged ER α mutants (10 μ g) and DUSP22 (10 μ g). At 48 h after transfection, cells were lysed and immunoprecipitated with anti-FLAG antibody, and immunoblotted with anti-Myc antibody (upper panel) or anti-FLAG antibody (middle panel). Total cell lysates (1%) were blotted with anti-Myc antibody (lower panel).

exhibited catalytic activity against an artificial substrate, *p*-nitrophenylphosphate (Aoyama *et al.*, 2001, data not shown). 293T cells that had been co-transfected with ER α were treated with E₂, and the Ser-118 phosphorylated ER α was immunoprecipitated. The indicated amounts of the recombinant GST or GST-DUSP22 WT fusion proteins were added to the immune complexes and incubated. As shown in Figure 7b, the Ser-118 phosphorylation level of ER α was clearly reduced by GST-DUSP22 WT. Incubation of the immune complexes with empty GST resulted in no reduction in Ser-118 phosphorylation levels of ER α .

Discussion

Estrogen receptors are transcription factors that regulate gene expression by both estrogen-dependent and estrogen-independent pathways (Mangelsdorf *et al.*, 1995). Kinase-mediated ER phosphorylation contributes to transcriptional activation of the ER by both these pathways (Tora *et al.*, 1989; Couse and Korach, 1999). However, the roles of phosphatases in the regulation of ER-mediated signaling pathway are less well defined. PP2A associates with ER α inhibiting phosphorylation of Ser-118, a site important for

Figure 6 DUSP22 dephosphorylated ER α on Ser-118. **(a)** 293T cells (1×10^7 cells) were transfected with FLAG-tagged ER α ($7.5 \mu\text{g}$) together with or without DUSP22 WT ($10 \mu\text{g}$). At 36 h after transfection, the cells were treated with or without E2 (10^{-7}M) for 30 min. The cells were then lysed and immunoprecipitated with anti-FLAG antibody, and immunoblotted with anti-FLAG antibody (upper panel), anti-ER α antibody (middle panel) or anti-pER α Ser-118 antibody (lower panel). Total cell lysates (1%) were blotted with anti-Myc antibody (bottom panel). **(b)** 293T cells (1×10^7) were transfected with FLAG-tagged ER α ($7.5 \mu\text{g}$) and/or DUSP22 WT (5 – $15 \mu\text{g}$). At 36 h after transfection, the cells were treated or untreated with E2 (10^{-7}M) for 30 min. The cells were then lysed and immunoprecipitated with anti-FLAG antibody, and immunoblotted with anti-pER α Ser-118 antibody (upper panel) or anti-FLAG antibody (middle panel). Total cell lysates (1%) were blotted with anti-Myc antibody (lower panel). **(c)** 293T cells (1×10^7) were transfected with FLAG-tagged ER α ($10 \mu\text{g}$) and/or Myc-tagged DUSP22 WT, D/A or C/S ($10 \mu\text{g}$). At 36 h after transfection, the cells were treated or untreated with E2 (10^{-7}M) for 30 min. The cells were then lysed and immunoprecipitated with anti-FLAG antibody, and immunoblotted with anti-pER α Ser-118 antibody (upper panel) or anti-FLAG antibody (middle panel). Total cell lysates (1%) were blotted with anti-Myc antibody (lower panel).

ligand-independent activation of ER α (Lu *et al.*, 2003). The Ser/Thr phosphatase PP5 binds to and suppresses ER-mediated transcriptional activation (Ikeda *et al.*, 2004). Although the phosphatase Cdc25B stimulates ER α activity, the stimulation is independent of the phosphatase activity of Cdc25B (Ma *et al.*, 2001). Protein phosphatase magnesium-dependent 1d also stimulates ER α activity through an indirect interaction (Proia *et al.*, 2006). We here show the first example of an estrogen-induced DSP whose activity is required for optimal regulation of ER α -mediated transcription. Our study shows not only that overexpression of DUSP22 reduces ER α activity. Reduction of endogenous DUSP22 expression in MCF-7 breast cancer cells enhances endogenous ER α transcriptional activity by influencing DNA-binding activity of ER α . Furthermore, DUSP22 directly interacts with ER α *in vivo* and dephosphorylates ER α Ser-118. These results strongly indicate that DUSP22 plays an important in the ER α -mediated transcription through a negative feedback loop in breast cancer cells.

DUSP22 is abundantly expressed in testis (Aoki *et al.*, 2001; Aoyama *et al.*, 2001). Recently, we have shown that DUSP22 regulates IL-6-/LIF-mediated transcription by dephosphorylating STAT3 (Sekine *et al.*, 2006). In mammals, STAT3 is expressed in developing acrosomes of round spermatids in the adult testis (Murphy *et al.*, 2005). In testes, the addition of LIF to cocultures enhances the *in vitro* survival of both primary Sertoli cells and proliferating gonocytes without affecting cell proliferation (Jenab and Morris, 1998). IL-6 and interferon- γ (IFN- γ) activate STAT3 and induce *c-fos* gene expression in Sertoli cells (Jenab and Morris, 1997). These findings indicate that DUSP22 modulates STAT3-mediated signaling in testis. Estrogen is also thought to have a regulatory role in the testis, because estrogen biosynthesis occurs in testicular cells and the absence of ER α caused adverse effects on spermatogenesis and steroidogenesis (Couse *et al.*, 2001; Carreau *et al.*, 2002). Moreover, several chemicals that are present in the environment, designated xenoestrogens, because they have the ability to bind and activate ER α , are known to affect testicular gene expression (Safe, 2004). We have previously demonstrated that estrogen suppresses IL-6-/LIF-mediated transcription through a direct interaction between ER α and STAT3 (Yamamoto *et al.*, 2000). The ER α -dependent protein inhibitor of activated STAT3 (PIAS3) induction is also involved in ER α -dependent repression of STAT3 transcriptional activation (Wang *et al.*, 2001). Our present study suggests that ER α -dependent DUSP22 induction may participate in ER α -mediated repression of STAT3 transcriptional activation. Therefore, estrogens regulate spermatogenesis and steroidogenesis in testis by modulating STAT3 activity via a direct interaction with ER α and DUSP22 induction. Furthermore, their activities are also regulated by DUSP22 through a direct interaction with ER α . In this study, we also proposed the possibility that DUSP22 may positively regulate AR-mediated signaling. This finding may support an important role in spermatogenesis and steroidogenesis

Figure 7 Reduction of endogenous DUSP22 enhances E2-induced phosphorylation of ER α Ser-118 and *in vitro* dephosphorylation of ER α Ser-118 by DUSP22. **(a)** siRNA-transfected MCF-7 cells in a 6-well plate were treated with E2 (10^{-7} M) for the indicated periods. The cells were then lysed and immunoprecipitated with anti-ER α antibody, and immunoblotted with anti-pER α Ser-118 (upper panel) or anti-ER α antibody (middle panel). Densitometric quantification of the above results was also shown (lower panel). Relative intensity of pER α was normalized to total ER α of the same sample. **(b)** 293T cells (1×10^7) were transfected with Myc-tagged ER α (7.5 μ g). At 48 h after transfection, cells were then lysed, immunoprecipitated with anti-Myc antibody and subjected to an *in vitro* dephosphorylation assay by incubation with the indicated GST or GST-DUSP22 WT (15–30 μ g). After termination of the incubation, proteins were separated by SDS-PAGE and analysed with anti-pER α Ser-118 antibody (upper panel). The same blot was reprobed with anti-Myc antibody after stripping.

in testis by modulating STAT3 activity. As androgens has been also shown to modulate STAT3 activation (Matsuda *et al.*, 2001a).

DUSP22 has been originally shown to regulate JNK activation (Aoyama *et al.*, 2001; Shen *et al.*, 2001; Chen *et al.*, 2002). E2 is also reported to induce apoptosis through JNK activation in ER-positive MCF-7 cells (Altioek *et al.*, 2006). Although we could not detect any activation of JNK using anti-phospho-JNK antibody, our siRNA experiments revealed that siRNA-mediated reduced expression of DUSP22 enhanced E2-induced cell growth in MCF-7 cells (data not shown). This result suggests that DUSP22 may also regulate JNK signaling pathway in breast cancer cells.

In summary, we demonstrated that DUSP22 is an important regulator of ER α function and thereby may

have critical roles in spermatogenesis and the progression of diseases, such as breast cancer or prostate disease. More detailed understanding of the interaction between ER α and DUSP22 is therefore important as this new information may provide new therapeutic approaches for these and other pathological conditions.

Materials and methods

Reagents and antibodies

E2, DHT and DEX was purchased from Wako Chemicals (Osaka, Japan). Expression vectors, ER α (HEGO), AR, Vit-LUC, -285PB-LUC, GRE-LUC were kindly provided by Dr P Chambon (Institut de Chimie Biologique, Strasbourg, France) Dr F Saatcioglu (University of Oslo, Oslo, Norway).

Epitope-tagged ER α and its mutants were described previously (Matsuda *et al.*, 2001b; Yamamoto *et al.*, 2002). Epitope-tagged WT DUSP22 (DUSP22 WT) and its D/A or C/S mutant were described previously (Aoki *et al.*, 2001; Aoyama *et al.*, 2001). Anti-Myc, anti-GST, anti-ER α antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-FLAG M2 monoclonal antibody and rabbit polyclonal anti-FLAG antibody were purchased from Sigma (St Louis, MO, USA). Anti-phospho-ER α (pER α) Ser118 was purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-DUSP22 antibody was described previously (Aoyama *et al.*, 2001).

Cell culture, transfection and LUC assays

Human breast cancer cell lines, MCF-7, T47D and HBL100, were kindly provided from Cell Resource Center for Biomedical Research (Tohoku University, Sendai, Japan) maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). Before stimulation, the cells were cultured for 24 h in phenol red-free DMEM containing 10% charcoal-stripped FCS followed by treatment with E2 (Matsuda *et al.*, 2001b). Human cervix carcinoma cell line, HeLa, and MCF-7 were maintained in DMEM containing 10% FCS (Matsuda *et al.*, 2001b; Sekine *et al.*, 2006) and transfected with Vit-LUC together with or without ER α using jetPEI (PolyPlus-transfection, Strasbourg, France) according to the manufacturer's instructions. Before stimulation, the cells were cultured for 24 h in phenol red-free DMEM containing 10% charcoal-stripped FCS followed by treatment with E2. The cells were harvested and assayed for the LUC activity using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Human embryonic kidney carcinoma cell line, 293T, was maintained in DMEM containing 10% FCS and transfected by the standard calcium precipitation protocol (Sekine *et al.*, 2004). The cells were harvested 36 h after transfection and lysed in 50 μ l of Reporter Lysis Buffer (Promega) 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 for each assay.

Transfection of siRNA

siRNAs targeting DUSP22 and control siRNA used in this study was described previously (Sekine *et al.*, 2006). HeLa or MCF-7 cells were plated on a 24-well plate at 2×10^4 cells/well, and then incubated with an siRNA-Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) mixture at 37°C for 4 h, followed by addition of fresh medium containing 10% charcoal-stripped FCS. The cells were then transfected with or without Vit-LUC, -285PB-LUC or GRE-LUC together with or without ER α or AR using jetPEI as described the above. Twenty-four hours after transfection, the cells were treated with the indicated concentration of steroid hormone for the indicated periods.

Immunoprecipitation and immunoblotting

The immunoprecipitation and Western blotting assays were performed as described previously (Sekine *et al.*, 2005). The cells were harvested and lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, containing 1% NP-40, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride and 10 μ g/ml each of aprotinin, pepstatin and leupeptin). The immunoprecipitates from cell lysates were resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and

transferred to polyvinylidene difluoride transfer membrane (PerkinElmer, Boston, MA, USA). The filters were then immunoblotted with each antibody. Immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (Millipore, Bedford, MA, USA).

DNA-binding assay

Cell extracts from cells treated with or without E2 (10^{-7} M) for the indicated period were prepared as described (Sekine *et al.*, 2006). To measure ER α DNA binding, cell extracts were treated with the immobilized ER Consensus Oligonucleotide (Santa Cruz Biotechnology)-Sepharose conjugate in binding buffer (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, 0.5 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol, 2 μ g/ml poly(dI-dC)) and rotated for 2 h at 4°C (Meyer *et al.*, 2003). Samples were centrifuged, and the pellets were washed three times with binding buffer. Proteins were eluted from the beads by boiling in sample buffer, and SDS-PAGE was performed as described above.

RNA isolation and quantitative real-time PCR

MCF-7, T47D, HBL100 or siRNA-transfected HeLa cells were treated with or without the indicated concentration of E2 for the indicated time (Sekine *et al.*, 2006). Total RNAs were prepared by using Iso-Gen (Nippon Gene, Tokyo, Japan) and used in reverse transcription (RT)-PCR. RT-PCR was performed using RT-PCR high-Plus-Kit (TOYOBO, Tokyo, Japan). Quantitative real-time PCR analyses of DUSP22, cathepsin D, as well as the control G3PDH mRNA transcripts were carried out using the assay-on-demandTM gene-specific fluorescently labeled TaqMan MGB probe in an ABI Prism 7000 sequence detection system (Applied Biosystems, Tokyo, Japan).

In vitro dephosphorylation assay

The GST fusion protein containing DUSP22 WT (GST-DUSP22) were purified on glutathione-Sepharose beads and eluted with neutralized glutathione as described previously (Aoyama *et al.*, 2001). Enzymatic activity of the GST-DUSP22 fusion protein was also determined using *p*-nitrophenylphosphate as described (Aoki *et al.*, 1996).

Statistical Analysis

Statistical analysis was performed using Student's *t*-test, and $P < 0.05$ is considered significant.

Abbreviations

DUSP, dual specificity phosphatase; ER, estrogen receptor; E2, β -estradiol; GST, glutathione-S-transferase; LMW-DSP, low molecular weight-dual specificity phosphatase; LUC, luciferase; PCR, polymerase chain reaction; RT, reverse transcription; siRNA, small interfering RNA; STAT, signal transducer and activator of transcription.

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