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Molecules and Cells

PKA-Mediated Stabilization of FoxH1 Negatively Regulates ERα Activity

Jinah Yum[1,3], Hyung Min Jeong[2,3], Seulki Kim[1], Jin Won Seo[2], Younho Han[2], Kwang-Youl Lee[2,*], and Chang-Yeol Yeo[1,*]

Estrogen receptor α (ERα) mediates the mitogenic effects of estrogen. ERα signaling regulates the normal growth and differentiation of mammary tissue, but uncontrolled ERα activation increases the risk to breast cancer. Estrogen binding induces ligand-dependent ERα activation, thereby facilitating ERα dimerization, promoter binding and coactivator recruitment. ERα can also be activated in a ligand-independent manner by many signaling pathways, including protein kinase A (PKA) signaling. However, in several ERα-positive breast cancer cells, PKA inhibits estrogen-dependent cell growth. FoxH1 represses the transcriptional activities of estrogen receptors and androgen receptors (AR). Interestingly, FoxH1 has been found to inhibit the PKA-induced and ligand-induced activation of AR. In the present study, we examined the effects of PKA activation on the ability of FoxH1 to represses ERα transcriptional activity. We found that PKA increases the protein stability of FoxH1, and that FoxH1 inhibits PKA-induced and estradiol-induced activation of an estrogen response element (ERE). Furthermore, in MCF7 cells, *FoxH1* knockdown increased the PKA-induced and estradiol-induced activation of the ERE. These results suggest that PKA can negatively regulate ERα, at least in part, through FoxH1.

INTRODUCTION

Estrogen receptor α (ERα) is a member of the steroid hormone receptor family that mediates the mitogenic effects of estrogen. ERα signaling regulates the normal growth and differentiation of mammary tissue. However, uncontrolled ERα activation increases the risk of breast cancer (Pike et al., 1993).

Estrogen binding induces the phosphorylation of ERα at specific serine residues (Lannigan, 2003; Nilsson et al., 2001), and ligand-induced ERα phosphorylation facilitates ERα dimerization and binding to the estrogen response elements (Loven et al., 2001). Estrogen also enhances the ERα recruitment of transcription coactivators (Xu and Li, 2003). Additionally, ERα can be activated in the absence of estrogen through many signaling kinases, including AKT, c-Src, cyclin A/CDK2, ERK1/2, p38 MAPK and PKA (Arnold et al., 1995; Cho and Katzenellenbogen, 1993; Feng et al., 2001; Kang et al., 2009; Kato et al., 1995; Lee and Bai, 2002; Rogatsky et al., 1999; Sun et al., 2001). Although the exact mechanisms by which ERα activation occurs are still the subject of debate, it has been proposed that PKA-induced phosphorylation of ERα leads to its activation by inducing conformational changes of ERα toward the agonist, antagonist and coactivators (Michalides et al., 2004; Zwart et al., 2007). In addition, phosphorylation by PKA has been shown to render ERα hypersensitive to estrogen (Cui et al., 2004). However, PKA has been found to inhibit estrogen-dependent cell growth in several ERα-positive breast cancer cell lines, including MCF7 and T47D. In this context, PKA reduced the estrogen binding, dimerization, and promoter binding of ERα and increased the ERα association with Hsp90 (Al-Dhaheri and Rowan, 2007; Chen et al., 1999). The mechanisms by which PKA induces the ligand-independent ERα activation in one context and inhibits the ligand-dependent ERα activation in another context are not clear.

FoxH1 represses the transcriptional activities of estrogen receptors and androgen receptors (AR) (Chen et al., 2005). FoxH1 is a forkhead-box (Fox) transcription factor that plays important roles in mediation of transcriptional regulation of the TGFβ signaling pathway (Schier, 2003; Whitman, 2001). Human *FoxH1* is expressed in several colorectal and prostate cancer cell lines, as well as in many normal adult tissues (Chen et al., 2005; Zhou et al., 1998). Chen et al. reported that FoxH1 interacts with AR to inhibit both its ligand-dependent and ligand-independent activation (Chen et al., 2005). Interestingly, FoxH1 has been found to inhibit the PKA activator forskolin-induced and ligand-induced activation of AR. However, the exact mechanisms by which FoxH1 suppresses the AR and ER activities are not clear.

In this study, we examined the effects of PKA activation on the ability of FoxH1 to repress ERα transcriptional activity. Because FoxH1 inhibits PKA-induced AR activation and PKA can regulate the activities and stabilities of its target proteins, we evaluated PKA to determine if it negatively regulated ERα through FoxH1. We found that PKA activation increased the protein stability of FoxH1, while FoxH1 inhibited the forskolin-induced and estradiol-induced activation of an estrogen response element (ERE). Addi-

[1]Department of Life Science and Division of Life and Pharmaceutical Sciences, Ewha Womans University, Seoul 120-750, Korea, [2]College of Pharmacy and Research Institute of Drug Development, Chonnam National University, Gwangju 500-757, Korea, [3]These authors contributed equally to this work.
*Correspondence: kwanglee@chonnam.ac.kr (KYL); cyeo@ewha.ac.kr (CYY)

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tionally, PKA inhibition relieves the FoxH1-mediated repression of ERα. Furthermore, *FoxH1* knockdown was found to lead to increased PKA-induced and estradiol-induced activation of the ERE in MCF7 cells. Taken together, these results suggest that PKA can negatively regulate ERα, at least in part, through FoxH1. Our results may provide a basis for understanding how PKA inhibits estrogen-dependent cell growth.

MATERIALS AND METHODS

Cell lines

HEK 293T human embryonic kidney cells and MCF7 human breast cancer cells were maintained in DMEM supplemented with 10% FBS and antibiotics.

Plasmids, siRNAs and transient transfection

The following plasmids were used for transient transfection: ERE-Luc (a luciferase reporter containing an estrogen response element), pCMV-β-Gal, pCMV-GFP, pCS4+ERα (human estrogen receptor α), pCS4+HA-FoxH1 (HA-tagged human FoxH1) and pCS4 + HA-Ub (HA-tagged human ubiquitin). Human *FoxH1* ON-TARGET*plus* siRNA pool and ON-TARGET *plus* Non-Targeting siRNA pool were purchased from Dharmacon. Plasmids and siRNAs were transfected using Effectene transfection reagent (Qiagen) according to the manufacturer's protocols.

Western blot analyses

293T cells were lysed in ice-cold lysis buffer [25 mM HEPES (pH 7.5), 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 25 mM NaF, 1 mM EDTA, 1 mM Na_3VO_4, 250 μM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin]. Cell lysates were cleared by centrifugation and subjected to SDS-PAGE. Proteins were transferred to PVDF membranes and then visualized using the appropriate primary antibodies, horseradish peroxidase-conjugated secondary antibodies and ECL reagent.

Luciferase assays

293T cells and MCF7 cells were transfected with combinations of plasmids and siRNAs. After 24 h, the 293T cells were incubated in fresh growth media and the MCF7 cells were incubated in DMEM supplemented with 5% dextran-coated charcoal-stripped FBS. Cells were then treated with combinations of Forskolin, H89 and estradiol for 12 h. The luciferase activities were then measured using a Luciferase Reporter Assay Kit (Promega) and normalized using the corresponding β-Gal activities for transfection efficiency. All experiments were performed in triplicate and repeated three times.

RT-PCR

Total RNA from MCF7 cells was isolated using TRIZOL reagent, after which cDNA was synthesized using a Superscript III First Strand Synthesis Kit (Invitrogen). The cDNA was then amplified by PCR using the following primers: FoxH1 forward 5′-CAC AGA GGC CTC TCA GAA G-3′, FoxH1 reverse 5′-CTG GAA AGA CTC CAT TCG-3′, GADPH forward 5′-ACC ACA GTC CAT GCC ATC AC-3′, GADPH reverse 5′-TCC ACC ACC CTG TTG CTG TA-3′.

RESULTS

PKA activation increases the FoxH1 protein levels

We evaluated PKA activation to determine if it affected the protein levels of FoxH1. Briefly, 293T cells were transfected with human FoxH1 and treated with increasing amounts of the PKA activator, forskolin. The protein levels of FoxH1 were then examined by Western blotting. Forskolin increased the FoxH1 protein levels in a dose-dependent manner (Fig. 1A). Next, we evaluated the effects of forskolin on FoxH1 protein levels to determine if they could be reversed by inhibition of the PKA activity using the PKA inhibitor, H89. The forskolin-induced increase of FoxH1 protein levels was abolished by co-treatment with H89 (Fig. 1B, left panel). Taken together, these results suggest that PKA activation increases the protein levels of FoxH1.

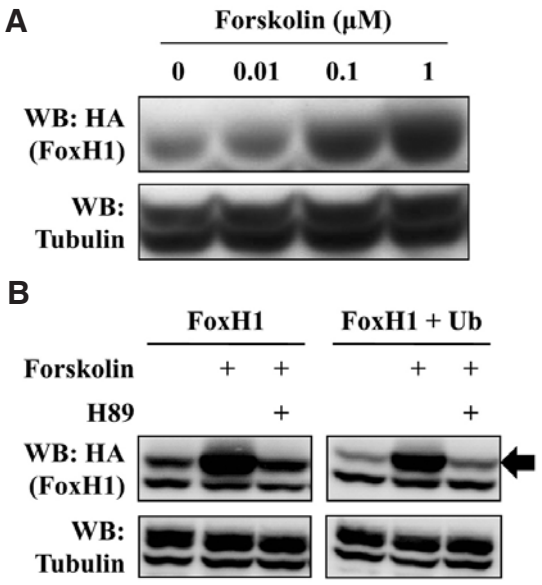

Fig. 1. PKA activation increases the FoxH1 protein levels. (A) 293T cells were transfected with HA-tagged human FoxH1 expression plasmid (0.5 μg). Cells were then treated with increasing amounts of the PKA activator, forskolin (0.01 to 1 μM final concentration) or with vehicle (DMSO) alone. (B) 293T cells were transfected with HA-Ubiquitin (0.25 μg) and/or HA-FoxH1 (0.5 μg) expression plasmids. Cells were then treated with the indicated combinations of forskolin (1 μM), the PKA inhibitor, H89 (1 μM), or DMSO alone. The levels of FoxH1 protein were then examined by anti-HA Western blotting [WB: HA (FoxH1)]. The arrow indicates HA-tagged FoxH1. Tubulin was used as a loading control [WB: Tubulin].

PKA may increase the FoxH1 protein levels by regulating the transcription, translation or protein stability of FoxH1. Because FoxH1 was expressed from a heterologous CMV promoter in our assay and the CMV promoter-derived FoxH1 transcripts contain heterologous untranslated regions, it is not likely that PKA increased the transcription or translation of FoxH1 under the conditions used for this study. Accordingly, we evaluated PKA to determine if it modulates the protein degradation of FoxH1. To accomplish this, 293T cells were transfected with FoxH1 alone or with ubiquitin, and the FoxH1 protein levels were then examined. Ubiquitin co-expression led to decreased FoxH1 protein levels (Fig. 1B, left versus right panels). These results suggest that PKA may modulate the ubiquitin-mediated protein degradation of FoxH1.

PKA increases the FoxH1 protein stability

Next, we evaluated PKA activation to determine if it affected the protein stability of FoxH1. To examine the patterns of FoxH1 protein turnover, FoxH1 transfected cells were treated with a translation inhibitor, cycloheximide, for increasing amounts of

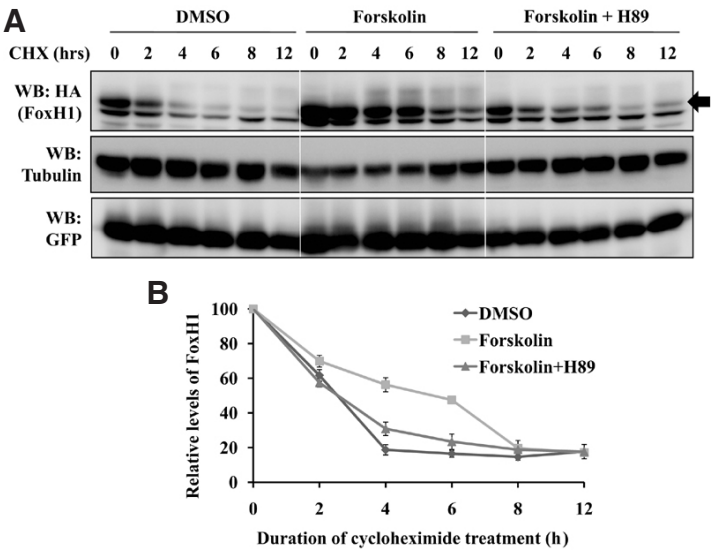

Fig. 2. PKA increases the protein stability of FoxH1. (A) 293T cells were transfected with HA-FoxH1 (0.5 μg) and pCMV-GFP (0.1 μg). Cells were then treated with the indicated combinations of forskolin (1 μM), H89 (1 μM) or DMSO alone. After 12 h, cells were treated with a translation inhibitor, cycloheximide (CHX, 40 mg/ml), for the indicated times (h). The levels of FoxH1 protein were then examined by anti-HA Western blotting [WB: HA (FoxH1)]. The arrow indicates HA-tagged FoxH1. The levels of GFP were compared to evaluate the transfection efficiency [WB: GFP], and tubulin was used as a loading control [WB: Tubulin]. (B) The levels of FoxH1 in panel A were determined by densitometry. The levels of FoxH1 protein in CHX untreated cells (0 h) were considered to be 100%. The experiment was repeated three times and a representative result is shown.

time in the presence of forskolin or H89. Treatment with cycloheximide led to the gradual degradation of FoxH1 (Fig. 2). However, co-treatment with forskolin significantly prolonged the half-life of FoxH1, whereas H89 abolished the forskolin-induced increase of the FoxH1 protein half-life. Taken together, these results suggest that PKA activation increases the FoxH1 protein levels by modulating the protein stability of FoxH1.

PKA inhibition abolishes the repression of ERα transcriptional activity by FoxH1

FoxH1 has been shown to repress the ligand-induced transactivation of androgen receptors and estrogen receptors (Chen et al., 2005). Therefore, we examined the effects of PKA activation or inhibition on the ability of FoxH1 to repress ER transcriptional activity. Briefly, 293T cells were transfected with a luciferase reporter containing an estrogen response element (ERE) along with combinations of ERα and FoxH1. The cells were then treated with combinations of estradiol, forskolin and H89. ERα, estradiol and forskolin induced the activation of ERE, whereas FoxH1 reduced the ERα-, estradiol- and forskolin-induced activation of ERE (Fig. 3). Interestingly, inhibition of PKA abolished the suppression of ERE activation by FoxH1. These results suggest that PKA modulates ERα, at least in part, through FoxH1.

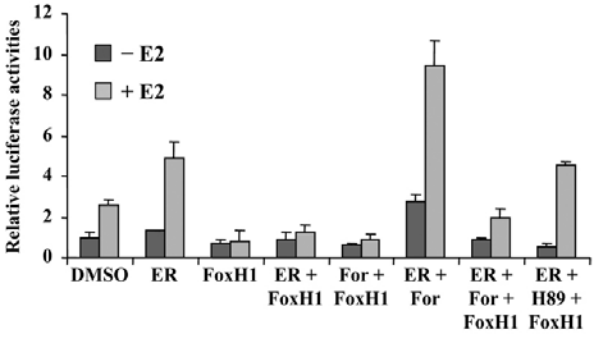

Fig. 3. PKA inhibition abolishes the suppression of ER transcriptional activity by FoxH1. 293T cells were transfected with pCMV-β-Gal (0.05 μg), ERE-Luc (0.1 μg), and the indicated combinations of ERα (0.25 μg) and FoxH1 (0.25 μg) expression plasmids. Cells were then treated with the indicated combinations of forskolin (For, 1 μM), H89 (1 μM), estradiol (E2, 10 nM) or DMSO alone. Luciferase activities were normalized to the corresponding β-galactosidase activities. The experiment was performed in triplicate and the average relative luciferase activities ± S.D. are shown.

FoxH1 knockdown enhances the ligand- and PKA-induced activation of ERα

To conduct a functional knockdown of the endogenous *FoxH1*, we first examined the expression of *FoxH1* in MCF7, MDA-MB231 and T47D human breast cancer cells. *FoxH1* is expressed in all three cell lines (data not shown). We then examined the efficiency and specificity of *FoxH1* siRNA in MCF7 cells. *FoxH1* siRNA, but not the control siRNA, led to a significant reduction in the *FoxH1* transcript levels (Fig. 4A).

Using *FoxH1* siRNA, we examined the effects of endogenous *FoxH1* on ERα transcriptional activity. MCF7 cells were transfected with ERE-Luc and siRNA, and the transfected cells were then treated with combinations of estradiol, forskolin and H89. *FoxH1* knockdown led to a significant increase in the forskolin and/or estradiol-induced activation of ERE (Fig. 4B). Additionally, *FoxH1* knockdown led to increased ERE activation in non-stimulated or PKA inhibited cells. These results suggest that FoxH1 represses the ligand-induced or PKA-induced activation of ERα.

DISCUSSION

We examined the effects of PKA on the ability of FoxH1 to repress ERα transcriptional activity. We found evidence of a novel regulatory mechanism in which PKA inhibits ERα activation, at least in part, through FoxH1. Specifically, we found that the PKA activator, forskolin, increased the protein stability of FoxH1. Additionally, H89 was found to relieve the repression of ERα transcriptional activity induced by FoxH1. Finally, *FoxH1* knockdown led to further increases in the estradiol and forskolin-induced activation of an estrogen response element.

PKA activates ERα in the absence of estrogen, while it inhibits estrogen-dependent cell growth in certain contexts. For the ligand-independent activation of ERα, PKA phosphorylates ERα at serine 305 (Cui et al., 2004; Michalides et al., 2004; Zwart et al., 2007). This change reduced acetylation at lysine 303 of ERα, rendering it hypersensitive to estrogen, while it induced the conformational change that enabled an anti-estrogen tamoxifen to bind with an ERα activator. To inhibit the

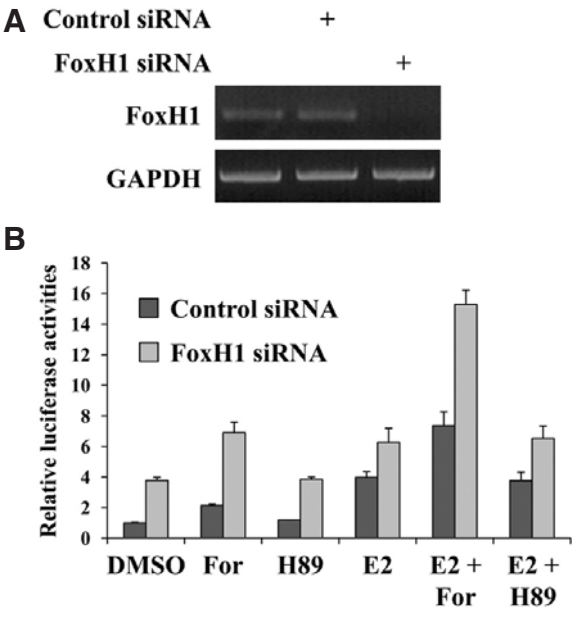

Fig. 4. *FoxH1* knockdown enhances the ligand- and PKA-induced increases in ER transcriptional activity. (A) FoxH1 siRNA reduces the levels of *FoxH1* mRNA. MCF7 cells were transfected with FoxH1 siRNA or control siRNA. The levels of *FoxH1* mRNA were examined by RT-PCR. GAPDH is used as a loading control. (B) MCF7 cells were transfected with pCMV-β-Gal (0.05 μg), ERE-Luc (0.1 μg), and FoxH1 or control siRNA (100 nM final concentration). Cells were then treated with the indicated combinations of forskolin (For, 1 μM), H89 (1 μM), estradiol (E2, 10 nM) or DMSO. Luciferase activities were normalized with the corresponding β-galactosidase activities. The experiment was performed in triplicate and the average relative luciferase activities ± S.D. are shown.

ligand-dependent ERα activation, PKA also alters the phosphorylation profile of ERα. Specifically, PKA increases phosphorylation at serines 236 and 305 and decreases phosphorylation at serine 118 (Al-Dhaheri and Rowan, 2007; Chen et al., 1999). These changes lead to a reduction in ligand binding, the dimerization and DNA binding of ERα, and increased Hsp90 association with ERα. The results of the present study suggest that PKA modulates the activity of ERα indirectly through post-translational modification of ERα regulators, as well as through the direct modification of ERα.

The phosphorylation and ligand-independent activation of ERα by PKA may occur immediately after PKA activation, whereas the FoxH1-mediated inhibition of ERα by PKA may occur relatively late. This is because the accumulation of FoxH1 to a level sufficient to inhibit ERα would require time. Based on this assumption, a short-term activation of PKA may increase ER transcriptional activity, whereas a sustained activation of PKA may decrease ER transcriptional activity due to the accumulation of FoxH1 protein. However, further investigation is needed to determine if short-term activation and sustained activation of PKA have different effects on ERα activation, and to determine if pre-treatment with forskolin reduces the effect of FoxH1 knockdown.

FoxH1 physically interacts with AR. Chen et al. reported that FoxH1 interacts with the activation function 1 (AF-1) domain of AR and colocalizes with AR (Chen et al., 2005). The AF-1 domain contributes to the ligand-independent transcriptional activity of AR, and this domain is conserved in steroid hormone receptors including ERα (McKenna et al., 1999). The interaction between FoxH1 and AR was inhibited by coexpression of ANT-1 (Chen et al., 2005). ANT-1 enhances AR transactivation through direct interaction with the AF-1 domain of AR, suggesting that the interaction between FoxH1 and AR was inhibited by ANT-1 as a result of competition between FoxH1 and ANT-1 for binding to the AF-1 domain. These results suggest that FoxH1 represses AR transcriptional activity through a physical interaction with AR. Although FoxH1 can function as a DNA-binding transcription factor, the transcriptional activity of FoxH1 is not required for the inhibition of AR. A FoxH1 mutant defective in DNA binding ($FoxH1_{H83R}$) inhibited AR to a similar extent as wild type FoxH1 (Chen et al., 2005). This observation further supports the notion that FoxH1 inhibits AR through a physical interaction. Based on the structural and mechanistic similarities between AR and ERα, FoxH1 is likely to inhibit ERα activity by binding to the AF-1 domain of ERα.

FoxH1 plays important roles in mediation of the transcriptional regulation of the TGFβ signaling pathway (Schier, 2003; Whitman, 2001). Human *FoxH1* is expressed in most adult tissues and several cancer cell lines (Chen et al., 2005; Zhou et al., 1998). *FoxH1* is also expressed in embryos from the early stages of development. To date, most studies of FoxH1 functions have been focused on its role during embryogenesis, while little is known about its roles in adult tissues (Chen et al., 2005; 1997; Kofron et al., 2004; Roessler et al., 2008; Watanabe and Whitman, 1999). We found evidence that TGFβ signaling also increases the FoxH1 protein levels (data not shown). Because the TGFβ signaling pathway controls a wide range of biological processes, including cell proliferation and tumorigenesis (Massagué and Gomis, 2006), further studies are needed to understand the roles that FoxH1 plays in adult tissues.

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