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The role of 14-3-3β in transcriptional activation of estrogen receptor α and its involvement in proliferation of breast cancer cells

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ABSTRACT

The estrogen receptor (ER) functions as a transcription factor that mediates the effects of estrogen. ERα, which plays a crucial role in the development and progression of breast cancer, is activated by estrogen binding, leading to receptor phosphorylation, dimerization, and recruitment of co-activators and chaperons to the estrogen-bound receptor complex. The 14-3-3 proteins bind to target proteins via phosphorylation and influence many cellular events by altering their subcellular localization or acting as a chaperone. However, regulation of ERα expression and transactivation by the 14-3-3 proteins has not been reported. We demonstrate that 14-3-3β functions as a positive regulator of ERα through a direct protein–protein interaction in an estrogen-dependent manner. Ectopic expression of 14-3-3β stimulated ERα-mediated transcriptional activity in MCF-7 breast cancer cells. Enhanced ERα transcriptional activity due to 14-3-3β increased the expressions of the endogenous ERα target genes, leading to proliferation of breast cancer cells. We suggest that 14-3-3β has oncogenic potential in breast cancer via binding to ERα and activation of the transcriptional activity of ERα.

1. Introduction

Estrogen-stimulated growth in tumors and normal cells requires the estrogen receptor (ER). ER is a ligand-activated nuclear receptor that regulates the transcription of estrogen-responsive genes important for cell growth, differentiation, and malignant transformation in various target cells [1]. Expression of ER is associated with a large population of breast tumors, and up-regulation of ER is used to select patients who will benefit from hormonal therapy [2]. The effects of estrogen are mediated primarily by direct binding to ER, which homo-dimerizes and interacts with the estrogen response element (ERE) to stimulate the transcription of target genes [3]. ER has two subtypes, α and β. ERα and β are made up of several functional domains. The N-terminal (also known as the A/B region) contains a ligand-independent transactivation domain (AF-1), and is recognized by co-activators and other transcription factors [4]. AF-1 transactivation is also enhanced by the action of second messenger signal pathways, which presumably relieve inhibition by the ligand-binding domain (LBD) [5]. The central zinc finger contains a DNA binding domain (DBD), and the last domain is a C-terminal EF region (also known as the AF-2) that binds the ligand and comprises the ligand binding domain (LBD). When estrogen binds to the LBD of ER, AF-1 is necessary for estrogen action in reproductive targets [6].

The 14-3-3 proteins are a family of highly conserved 30 kDa acidic regulatory proteins expressed in wide range of organisms and tissues [7]. The 14-3-3 proteins play crucial roles in diverse processes, including cell cycle regulation, DNA repair, apoptosis, cell differentiation, and cell adhesion [8]. The mammalian 14-3-3 isoforms β, γ, ε, η, σ, τ, and ζ are encoded by seven individual genes [9]. The 14-3-3 proteins bind to target proteins via phosphorylation, and preferentially recognizes the phosphorylated motifs RSXpSXP and RXXXpSXP, which share a normal region in the consensus Akt phosphorylation elements that are preserved in numerous Akt substrates [9]. Possible modes of action for the 14-3-3 proteins on target proteins include directed conformational change, modification of nuclear/cytoplasmic localization, protection of phosphorylated states, masking the phosphorylated region of a target protein, and scaffolding [10,11].

In this study, we investigated the role of the 14-3-3 proteins on ERα transcriptional activity in breast cancer cells. We found that 14-3-3β interacts with ERα, and regulates the transcriptional activity of ERα in a ligand-dependent manner, leading to enhancement of breast cancer cell proliferation.

2. Materials and methods

2.1. Cells and reagents

MCF-7 and T47D human breast cancer cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin

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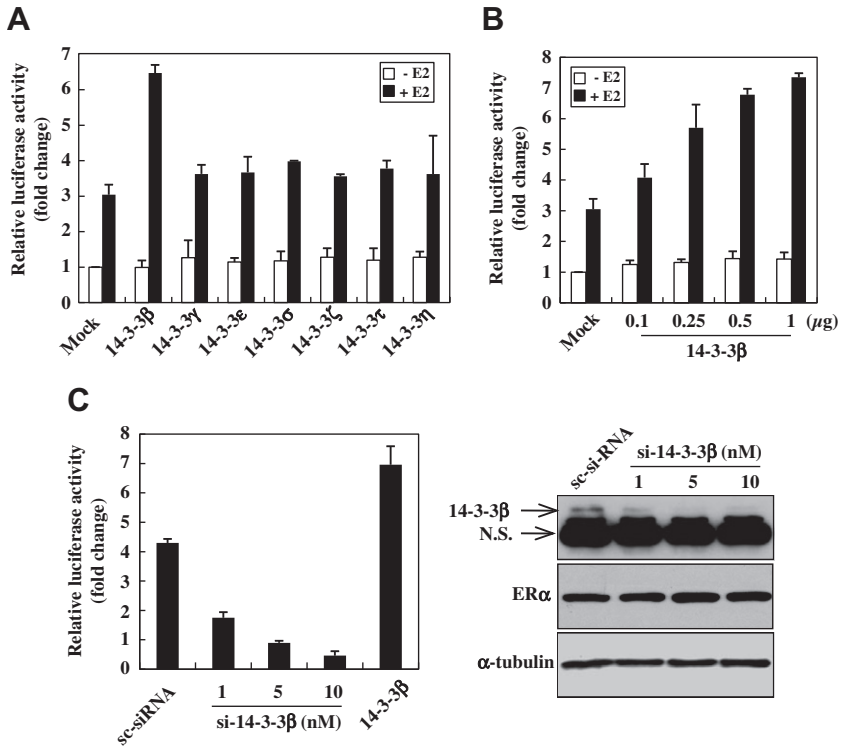

Fig. 1. 14-3-3β enhances the estrogen-induced transcriptional activity of ERα. (A and B) MCF-7 cells were transfected with various 14-3-3 isoforms (A) or the indicated amounts of 14-3-3β (B), and the pMMTV–luciferase reporter construct. After 24 h of transfection, cells were treated with or without 100 nM E2 for 12 h in 10% charcoal-stripped medium and harvested for the luciferase assay. (C) MCF-7 cells were transfected with the indicated amounts of si-14-3-3β, and the pMMTV–luciferase reporter construct. Scrambled siRNA (sc-siRNA, 10 nM) was used as a negative control. After 24 h of transfection, cells were treated with 100 nM E2 for 12 h in 10% charcoal-stripped medium and harvested for the luciferase assay (left panel). The luciferase activity was normalized by β-galactosidase activity, and the experiments were performed in triplicate. Data are expressed as the mean ± SD and are presented as the relative luciferase activity. The expression of 14-3-3β and ERα was determined by immunoblotting (right panel). N.S. indicates non-specific bands.

(100 μg/ml). HEK 293T cells were grown in DMEM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). Lipofectamine 2000 reagent was obtained from Invitrogen (Carlsbad, CA). Anti-ERα, anti-14-3-3β, anti-HA, anti-cyclin D_1, anti-α-tubulin, anti-GST antibodies and Protein A/G agarose were from Santa Cruz Biotechnology (Santa Cruz, CA). Charcoal and 17-β-estradiol (E2) were obtained from Sigma (St. Louis, MO).

2.2. Transient transfection and luciferase reporter assay

The transcriptional activity of ERα was measured by a luciferase reporter assay using the pMMTV-Luc reporter plasmids. MCF-7 cells were seeded into 6-well plate at a density of 5×10^5 cells/well. Cells at 70–80% confluence were co-transfected with 0.2 μg pMMTV-Luc and 0.2 μg pSV-β-galatosidase for 24 h. Transfected cells were incubated with 10% RPMI 1640 containing charcoal-stripped FBS and stimulated with 100 nM E2 for 24 h. Luciferase activity was assayed according to the manufacture's protocol (Promega) using Luminometer 20/20^n (Turner BioSystems, Sunnyvale, CA).

2.3. GST pull-down assay

HEK 293T cells were transfected with HA-ERα and mGST-14-3-3β. Cells were washed with phosphate-buffered saline (PBS), lysed in RIPA buffer, and centrifuged at 16,000g for 10 min at 4 °C. Cell lysates were incubated with Glutathione–Sepharose 4B beads for 6 h at 4 °C. The beads were then washed with cold RIPA buffer, resuspended in sample buffer, and separated by SDS–PAGE followed by Western blotting. The experimental procedures for immunoprecipitation is as described [12].

2.4. Semi-quantitative RT-PCR

Total RNA was isolated using Trizol (Invitrogen) according to the manufacturer's protocol. The cDNA was synthesized with 2 μg of total RNA using SuperScript reverse transcriptase (Bioneer, Daejeon, South Korea). PCR was performed using specific primers (ERα sense 5′-CGACGCCAGGA-3′; antisense 5′-CTCTCATGTC-3′, pS2 sense 5′-TGCTGTTTCG-3′; antisense 5′-CTGCAGAAGT-3′, cyclin D_1 sense 5′-GGATGCTGGA-3′; antisense 5′-GAGAGGAAGC-3′, PR sense 5′-CCAGCCAGAG-3′; antisense 5′-TTCAGACATC-3′. GAPDH was amplified as an internal control.

2.5. Colony forming assay

All proliferation assays were based on MTT method. After transfection of the 14-3-3β plasmid and si-14-3-3β, cells were seeded in 96-well plate at a density of 1×10^3 cells/well. Cells were treated with E2 for 72 h. The absorbance was measured at 595 nm. For colony forming assay, cells were seeded in 6-well plate at a density of 1×10^4 cells/well and allowed to attach for 24 h. Cells were then treated with or without 100 nM E2. After 10 days, colonies were fixed with fixing solution (methanol:acetic acid = 3:1) for 10 min at room temperature and stained with 0.01% crystal violet solution. Plates were washed with PBS and were photographed.

2.6. Statistics analysis

Data are presented as the mean ± SD. Statistical evaluation was carried out by the Student's *t*-test. Data were considered statistically significant when $p < 0.05$. All statistical analysis was performed by the computer program Prism (GraphPad Software, La Jolla, CA).

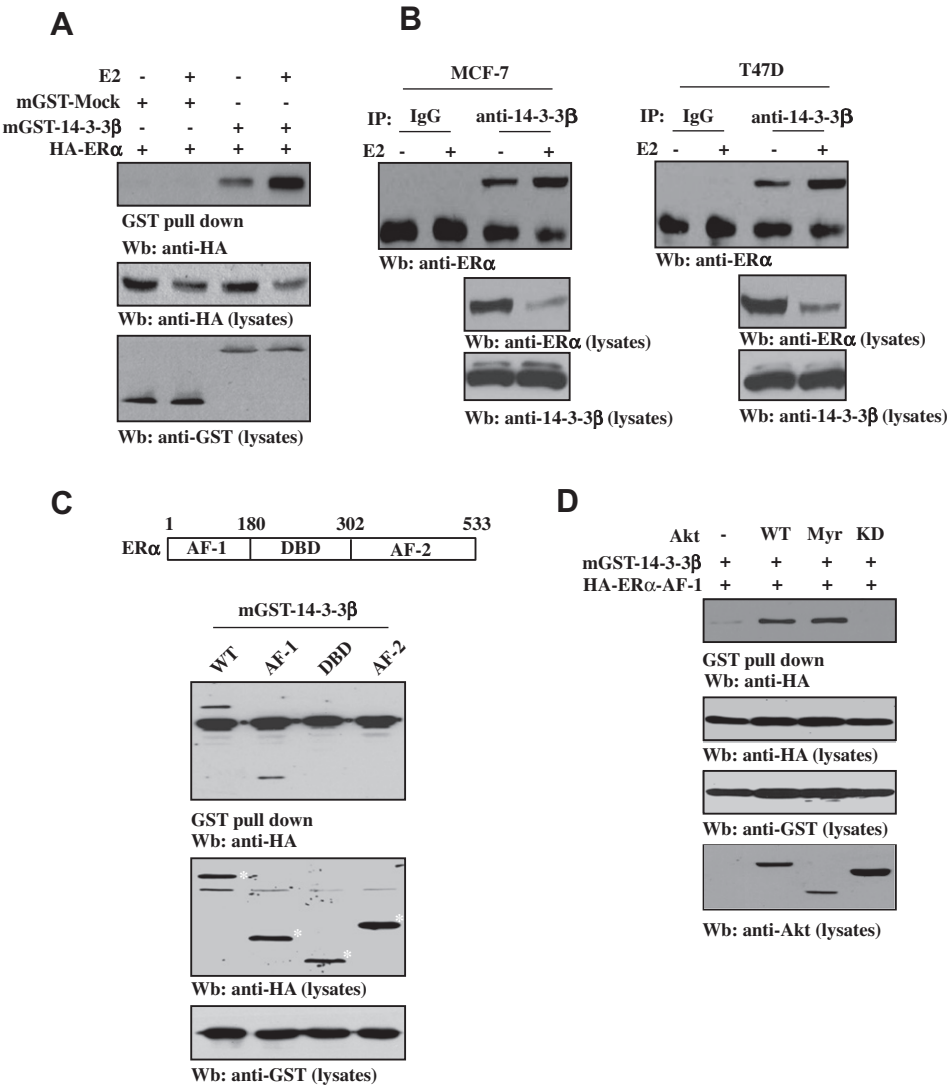

Fig. 2. 14-3-3β interacts with ERα. (A) HEK 293T cells were co-transfected with mGST-Mock or mGST-14-3-3β and HA-ERα. Transfected 14-3-3β proteins were pulled down using glutathione beads. Proteins bound to the beads were analyzed by 12% SDS–PAGE followed by immunoblotting using anti-HA antibody. (B) MCF-7 and T47D cells were treated with or without 100 nM E2 for 24 h and subjected to immunoprecipitation using anti-ERα antibody. IgG was used as a negative control. (C) After transfection with mGST-14-3-3β and the HA-ERα deletion fragments into HEK293T cells, cells were lysed and the cell lysates were pulled down using glutathione beads. Proteins bound to the beads were analyzed by 12% SDS–PAGE followed by immunoblotting using anti-HA antibody. (D) HEK293T cells were co-transfected with HA-ERα-AF-1, mGST-14-3-3β and the wild type Akt (WT), myristolyated Akt (Myr), or kinase dead Akt (KD). Transfected 14-3-3β proteins were pulled down using glutathione beads. Proteins bound to the beads were analyzed by 12% SDS–PAGE followed by immunoblotting using anti-HA antibody.

3. Results

3.1. 14-3-3β enhances the estrogen-induced transcriptional activity of ERα

To investigate the effects of various 14-3-3 isoforms on the transcriptional activity of ERα, we performed a luciferase assay using an ERE-containing MMTV-promoter reporter gene. Among various 14-3-3 isoforms, 14-3-3β increased the transcriptional activity of ERα by 2-fold in the presence of E2; however, other isoforms did not affect ERα transactivation (Fig. 1A). As shown in Fig. 1B, 14-3-3β increased ligand-induced transactivation of ERα in a dose-dependent manner in MCF-7 breast cancer cells. We also examined the effects of siRNA for 14-3-3β (si-14-3-3β) on the transcriptional activity of ERα. Results showed that the ligand-dependent transcriptional activation of ERα was inhibited by si-14-3-3β in a dose-dependent manner (Fig. 1C, left panel). si-14-3-3β effectively reduced the expression of 14-3-3β, whereas the ERα protein expression was not affected by si-14-3-3β (Fig. 1C, right panel). These results indicate that 14-3-3β enhances the estrogen-induced transcriptional activity of ERα.

3.2. 14-3-3β interacts with ERα

Since Akt phosphorylates ERα on the S167 residue, and Akt substrates often form a complex with 14-3-3 isoforms [13], we investigated whether 14-3-3β binds to ERα. Results from a GST pull-down assay showed that 14-3-3β interacted with ERα in the absence of E2, and the interaction between 14-3-3β and ERα was increased by E2 treatment (Fig. 2A). To determine whether endogenous 14-3-3β binds to ERα, we performed immunoprecipitation in MCF-7 and T47D cells. As shown in Fig. 2B, ERα co-immunoprecipitated with 14-3-3β, and the interaction was increased in the presence of E2 in both cell lines. To determine which domain of ERα is involved in interaction with 14-3-3β, we generated various deletion mutants of ERα, and performed a

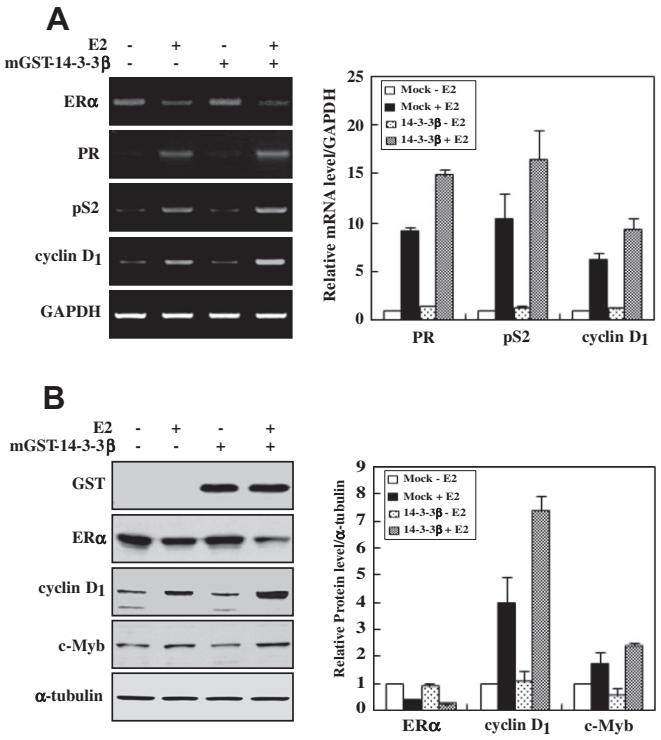

Fig. 3. Enhancement of ERα transcriptional activity by 14-3-3β leads to induction of ERα-mediated target gene expression. (A) MCF-7 cells were transfected with 14-3-3β in 10% charcoal-stripped medium for 24 h. Cells were then treated with 100 nM E2 for 24 h. Total RNA was extracted from the cells, the mRNA expression levels of ERα, PR, pS2 and cyclin D_1 were determined by RT-PCR analysis. The analysis was repeated three times, and GAPDH was regarded as a loading control. (B) MCF-7 cells were transfected with 14-3-3β in 10% charcoal-stripped medium for 24 h. Cells were then treated with 100 nM E2 for 24 h. The protein levels of ERα, cyclin D_1 and c-Myb were detected by Western blotting. Tubulin was used as an internal control.

GST pull-down assay. Results showed that 14-3-3β bound to the AF-1 domain of ERα; however, the DBD and AF-2 domains did not bind to 14-3-3β (Fig. 2C). We next examined whether Akt phosphorylation is involved in interaction between 14-3-3β and the AF-1 domain of ERα. HEK293 cells were co-transfected with various Akt mutants, AF-1, and 14-3-3β. Results showed that 14-3-3β bounds to AF-1 in the presence of the wild-type Akt and the dominant active myristoylated Akt, whereas 14-3-3β did not bind to AF-1 in cells transfected with the dominant negative kinase-dead (KD) Akt (Fig. 2D). These results indicate that 14-3-3β interacts with ERα through the AF-1 domain of ERα in a ligand-dependent manner and Akt phosphorylation is critical in this event.

3.3. Enhancement of ERα transcriptional activity by 14-3-3β leads to induction of ERα-mediated gene expression

Since 14-3-3β binds to ERα and regulates the transcriptional activity of ERα, we investigated whether the increased ERα transcriptional activity due to 14-3-3β affects expressions of ERα target genes, including cyclin D_1, pS2, c-Myb and PR. E2 decreased the mRNA expression of ERα as previously known (Fig. 3A). The E2-stimulated mRNA expressions of PR, pS2, and cyclin D_1 were increased in cells transfected with 14-3-3β, compared to cells transfected with a mock vector (Fig. 3A). We also examined the effect of 14-3-3β on the protein expressions of ERα target genes using Western blot analysis. As shown in Fig. 3B, overexpression of 14-3-3β remarkably increased the protein expression levels of cyclin D_1 and c-Myb in response to estrogen in MCF-7 cells. These results indicate that 14-3-3β increases the transcriptional activity of ERα in response to estrogen, leading to enhancement of target gene expression in MCF-7 cells.

3.4. 14-3-3β increases estrogen-induced proliferation of breast cancer cells

ERα is a major regulator of cellular growth in estrogen-dependent breast cancer cells [3,14]. Since 14-3-3β regulates ERα transcriptional activity, we investigated whether 14-3-3β plays a role in proliferation of breast cancer cells. MCF-7 cells transfected with 14-3-3β exhibited increased proliferation in response to E2, compared to cells transfected with a mock vector (Fig. 4A). We also examined the effect of si-14-3-3β on proliferation of breast cancer cells. Results from a MTT assay showed that estrogen-induced proliferation of MCF7 cells was inhibited by si-14-3-3β (Fig. 4A). We confirmed these results using a colony forming assay. MCF-7 cells transfected with 14-3-3β and si-14-3-3β were cultured with or without E2 for 10 days. Results showed that 14-3-3β increased colony formation of E2 treated cells approximately 2.2-fold (Fig. 4B). However, si-14-3-3β decreased estrogen-induced proliferation of MCF7 cells by 4.5-fold (Fig. 4B). These results indicate that 14-3-3β increases the transcriptional activity of ERα and expressions of ERα target genes, leading to enhancement of estrogen-induced proliferation of breast cancer cells.

4. Discussion

The association of the 14-3-3 proteins with client proteins occurs through defined high affinity peptide motifs, two of which (RSXpSXP or RXXXpSXP) are highly conserved and recognized by all 14-3-3 isoforms [7]. In most cases, binding occurs only if a specific serine within the motif is phosphorylated by Akt [8]. Recent reports demonstrate that the 14-3-3 proteins bind to their target proteins and regulate their functions [15]. However, there is less evidence that they regulate gene transcription through interactions with transcription factors [16]. The current study was designed to investigate the novel functions of the 14-3-3 proteins and to explore the molecular mechanism underlying their function. We found that (1) 14-3-3β interacts with ERα and the interaction is Akt-dependent, (2) 14-3-3β regulates the transcriptional activity of ERα in a ligand-dependent manner, (3) 14-3-3β increases expressions of ERα target genes, and (4) 14-3-3β increases breast cancer cell proliferation.

Results from a binding assay showed that 14-3-3β binds to the N-terminal AF-1 domain of ERα. Our results are supported by a report that binding of estrogen to ERα induces the phosphorylation of ERα by Akt [17]. ERα that is activated by the PI3k/Akt signal pathway contains a site that shares common residues in the concord 14-3-3 binding motifs, indicating that 14-3-3β binds to Akt phosphorylation sites of ERα. We also identified a positive regulatory effect of 14-3-3β on estrogen-induced ERα transcriptional activity. In E2-treated MCF-7 cells, 14-3-3β increased the ERE-containing MMTV promoter activity and expressions of ERα target genes, leading to an increase in breast cancer cell proliferation. These results are consistent with a current report that regulation of ERα transcriptional activity is critical in the development and progression of breast cancer. Once ERα is activated by estrogen, ERα-induced proteins, including pS2, PR, and cyclin D_1, initiate independent signal pathways that converge at or before cyclin E_1/Cdk2 activation, resulting in proliferation of breast cancer cells [18]. We propose that 14-3-3β plays a critical role in promoting the concerted actions of an ERα-mediated multifunction via regulation of estrogen-stimulated transcriptional activation of ERα in breast cancer cells.

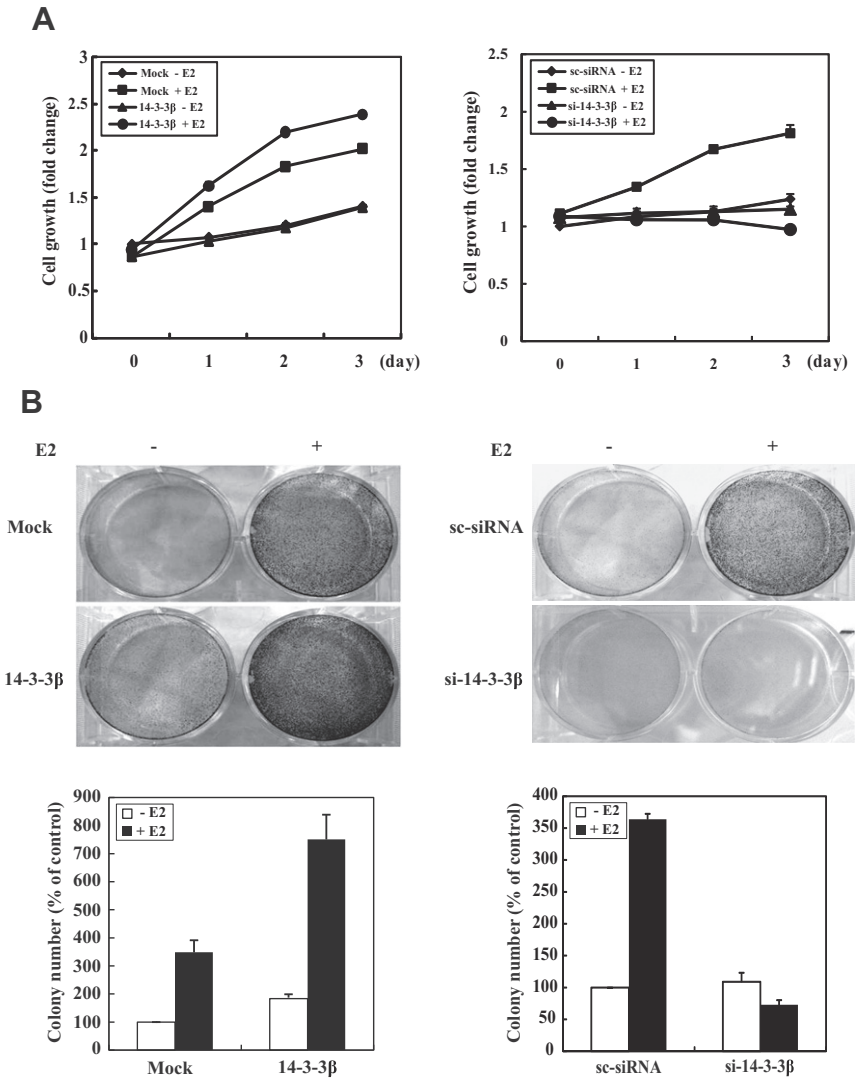

Fig. 4. 14-3-3β increases estrogen-induced proliferation of breast cancer cells. (A) MCF-7 cells were transfected with 14-3-3β and si-14-3-3β. Cells were then treated with 100 nM E2 in 10% charcoal-stripped medium for the indicated time periods and the cell viability was examined by a MTT assay. (B) MCF-7 cells were transfected with 14-3-3β and si-14-3-3β, and treated with or without 100 nM E2 for 10 days. Colonies were fixed with fixing solution (methanol:acetic acid = 3:1) for 10 min at room temperature and stained with 0.01% crystal violet solution. Plates were washed with PBS and were photographed.

Acknowledgments

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