Estradiol enhances CIP2A expression by the activation of p70 S6 kinase

Yeon A Choi*, Ja Seung Koo1*, Jeong Su Park, Mi Young Park, Ae Lee Jeong, Ki-Sook Oh and Young Yang

Department of Life Science, Research Center for Women’s Disease, Sookmyung Women’s University, Seoul 140-742, Republic of Korea
1Department of Pathology, Yonsei University College of Medicine, Seoul, South Korea
*(Y A Choi and J S Koo contributed equally to this work)

Abstract

Cancerous inhibitor of PP2A (CIP2A) stimulates the proliferation of various cancer cells, and 17β-estradiol (E2) enhances the proliferation of breast cancer cells. E2 activates epidermal growth factor receptor (EGFR), stimulating the MEK1/2 and PI3K pathways, and CIP2A expression is increased by the MEK1/2-induced transcription factor ETS1. It is possible for E2 to increase CIP2A expression. This study examined whether E2 could increase CIP2A expression and whether CIP2A is highly expressed in estrogen receptor (ER)-positive breast cancer tissues. E2 increased CIP2A expression at the translational level in a c-MYC-independent manner in MCF-7 cells. E2-enhanced proliferation was impaired without CIP2A expression. E2-stimulated EGFR activated the MAPK and PI3K pathways, which converged to activate p70 S6 kinase (S6K). Phosphorylation at all the three phosphorylation sites (S424/T421, T229, and T389) on S6K was required for the phosphorylation of eukaryotic initiation factor 4B (eIF4B), which was responsible for the increase in CIP2A translation. Furthermore, CIP2A expression was higher in ER-positive tissues than in ER-negative tissues. This is the first study, to our knowledge, to demonstrate that CIP2A is a key factor in E2-enhanced proliferation and that estrogen regulates CIP2A expression by non-genomic action through EGFR.

Key Words
- CIP2A
- breast cancer
- ER-positive human breast cancer cells
- estradiol (E2)
- PI3K/AKT
- MAPK pathway
- p70 S6K

Introduction

The most potent and active estrogen in humans is 17β-estradiol (E2), and the biological effects of E2 are mediated through its binding to estrogen receptor (ER)α and ERβ. E2-bound ER acts via genomic and non-genomic mechanisms (Acconcia & Kumar 2006). The genomic actions of E2-bound ER are mediated by its binding to estrogen response elements (EREs) located in the promoters of target genes, leading to transcriptional regulation (Nilsson et al. 2001). For its non-genomic activities, E2-bound ER activates various receptor protein kinases, including epidermal growth factor receptor (EGFR) and insulin-like growth factor receptor (IGFR), in the absence of their ligands (Losel & Wehling 2003, Barletta et al. 2004). Previous studies have indicated that E2-induced EGFR activation plays an important role in breast cancer progression (Song et al. 2010) and that EGFR is actively involved in the function of E2 via its interaction with ER-mediated signaling pathways, leading to MAPK and PI3K activation (Pietras 2003).

The mitogen-activated MAPK pathway and the growth factor-activated EGFR/PI3K pathway converge on mTORC1. One event that occurs downstream of mTORC1 activation is p70 S6 kinase (S6K) activation. The activation of S6K is controlled by multiple phosphorylation events at sites located within the catalytic, linker, and pseudo-substrate domains (Pullen & Thomas 1997). It is known...
that the phosphorylation of T229 in the catalytic domain and that of T389 in the linker domain of S6K are critical for the increase in its kinase activity (Weng et al. 1998). The phosphorylation of the S411, T421, and S424 residues located in the pseudosubstrate domain is involved in the subsequent phosphorylation of T229 and T389 by relieving pseudosubstrate suppression (Pullen & Thomas 1997). However, the precise molecular mechanism of S6K stimulation, in terms of its critical site and the order of phosphorylation events, remains elusive. The activated S6K stimulates the phosphorylation of eukaryotic initiation factor 4B (eIF4B), leading to the enhanced translation of target mRNA (Rogers et al. 2002).

Cancerous inhibitor of PP2A (CIP2A), which is known to enhance the migration and proliferation of tumor cells, is overexpressed in various types of human cancers, including breast (Come et al. 2009, Niemela et al. 2012, Tseng et al. 2012, Laine et al. 2013), prostate (Vaara et al. 2010), lung (Dong et al. 2011), pancreatic (Wang et al. 2013), bladder (Huang et al. 2012a), blood or bone marrow (Lucas et al. 2011, Wang et al. 2011), colon (Teng et al. 2012), ovarian (Bockelman et al. 2011b), cervical (Huang et al. 2010), tongue (Bockelman et al. 2011a), oral (Basile & Czerninski 2010), esophageal squamous (Qu et al. 2012), and head and neck squamous (Ferreira et al. 2004) cancers. CIP2A expression is transcriptionally enhanced by Ets1 (Zhao et al. 2010), and the concurrent binding of Ets1 and Elk1 to the proximal CIP2A promoter is absolutely required for CIP2A expression in cervical, endometrial, and liver carcinoma cell lines (Huang et al. 2012b, Pallai et al. 2012). Although CIP2A expression is upregulated in hormone-related cancers such as breast and prostate cancers, little is known about the effects of specific hormones on CIP2A expression. This study reveals that E2 increases CIP2A expression at the translational level via non-genomic action and that the level of CIP2A is strongly positively correlated with ER-positive breast cancer.

Materials and methods

Reagents and plasmids

The plasmid encoding CIP2A was obtained from Dr Jukka Westermarck at the University of Turku and Abo Akademi University, Finland. ERα expression plasmids were obtained from Dr Myung-Seok Lee at Sookmyung Women’s University. Chemical reagents, including 1,3,5-Tris (4-hydroxyphenyl)-4-propyl-1H-pyrazole (PPT), ICI 182 780, tamoxifen, E2, U0126, LY294002, AKT inhibitor, rapamycin, cycloheximide (CHX), and actinomycin D, were purchased from Sigma–Aldrich. EGF was purchased from PeproTech (Rocky Hill, NJ, USA), and gefitinib was purchased from Santa Cruz Biotechnology. Cells were treated with inhibitors 1 h before E2 treatment. Anti-EGFR neutralizing antibody was purchased from Millipore (Billerica, MA, USA).

Cell culture and transfection

The human breast cancer cell lines MDA-MB-231 (<33 passages, ATCC No. HTB-26), MCF-7 (<30 passages, ATCC No. HTB-22), T47D (<20 passages, ATCC No. HTB-133), and SK-BR-3 (<18 passages, ATCC No. HTB-30) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The ATCC authenticated the MDA-MB-231, MCF-7, T47D and SK-BR-3 cell lines by short tandem repeat (STR) profiling. MDA-MB-231 and MCF-7 cells were cultured in DMEM (HyClone, Logan, UT, USA), and T47D and SK-BR-3 cells were cultured in RPMI (HyClone). All the cells were supplemented with 10% fetal bovine serum (HyClone) at 37 °C in a humidified incubator under 5% CO2. MCF-7 cells were incubated in phenol red-free DMEM (WelGENE, Daegu, Korea) supplemented with 10% charcoal-stripped FCS (Sigma) and non-essential amino acids (Sigma) for 1 day before treatment with E2. For transfection studies, the cells were transfected using Lipofectamine LTX (Invitrogen) following the manufacturer’s protocol.

RNA interference assay

Targeted siRNA oligonucleotides were purchased from Bioneer (Seoul, Korea). The following sequences were used for the construction of the siRNAs: siCIP2A1 forward: 5'-GACAGUGUCGUACAGACUCUCUU-3' and siCIP2A1 reverse: 5'-AGAGUGGUACACUGACAGUUCG-3'; siCIP2A2 forward: 5'-CACCAGUUGUUCUUGUAU-3' and siCIP2A2 reverse: 5'-AUCAACAGACAUUGUGGUG-3'; siMYC1 forward: 5'-GACAGUGUCAGUCCUGA-3' and siMYC1 reverse: 5'-UCAGGACUCUGACAGUC-3'; siMYC2 forward: 5'-CGUAGGAAGUAAGGAAGA-3' and siMYC2 reverse: 5'-UUCUUACUUCUUUCUACG-3'; and GFP forward: 5'-GUUCAGGGUCCGGCAG-3' and GFP reverse: 5'-CUGCCGGACACGGCUGAAC-3'. The cells were transfected with 20 nM of siRNA using Lipofectamine LTX (Invitrogen).

Proliferation and cell viability assays

For the proliferation assay, the cells were transfected with the CIP2A expression plasmid, CIP2A siRNAs,
and MYC siRNAs. The transfected cells were seeded in 12-well plates at a density of $3 \times 10^5$ cells/well and treated with the indicated concentrations of E2. The number of viable cells was determined by counting the cells 24 h after E2 treatment. The dead cells were identified using Trypan Blue staining. For the cell viability assay, the cells were transfected with the CIP2A expression plasmid, CIP2A siRNAs, and MYC siRNAs. The transfected cells were seeded in 12-well plates at a density of $3 \times 10^4$ cells/well and treated with E2. CellTiter-Blue reagent (Promega) was added to the plates 20 µl/well 24 h after E2 treatment and the plates were gently shaken for 10 s. The plates were incubated under the standard cell-culture conditions for 1–2 h, and the fluorescence was measured at 560/590 nm using a VICTOR 3 multilabel plate counter (Perkin Elmer, Waltham, MA, USA).

**RT-PCR**

Total cellular RNA was extracted using the RNeasy Plus Kit (Takara, Shiga, Japan). An RNA sample of 3 µg was reverse-transcribed with 0.5 µg of random primers using reverse transcriptase (Fermentas, Ontario, Canada) at 42 °C for 1 h. The following primers were used for amplification: CIP2A forward: 5’-GGGAATTCCCT-GATTTCCCTTCA-3’ and CIP2A reverse: 5’-CCCTCGAG-CTAGAGTCTTACCTCAT-3’ and β-actin forward: 5’-GTGGGGCGCCCCCAGGCACCA-3’ and β-actin reverse: 5’-CTCTTTAATGTCACGCACGAT-3’. PCRs were carried out using 30 cycles of amplification. Each amplification cycle consisted of 30 s of denaturation at 94 °C, 30 s of annealing at 55 °C, and 30 s of extension at 72 °C. The reactions were carried out using a PTC-100 instrument (MJ Research, Inc., Waltham, MA, USA). The PCR mixtures were run on a 1% agarose gel and photographed.

**Immunoblot analysis and antibodies**

Total cell lysates were prepared 24 h after transfection with the CIP2A expression plasmid, mixed with 5 × SDS sample buffer, and sonicated for 15 s. The sonicated samples were heated at 95 °C for 5 min and separated electrophoretically on a 12% SDS–polyacrylamide gel. Subsequently, the proteins were transferred onto a 0.45 µm nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK) for 2 h. The membrane was incubated with antibodies specific for CIP2A, MYC, ERα (Santa Cruz), phospho-AKT, AKT, phospho-mTOR, mTOR, phospho-ERK, ERK, phospho-S6K (T389, T421/S424), S6K, phospho-eIF4B, eIF4B (Cell Signaling Technology, Inc., Danvers, MA, USA), and phospho-S6K T229 (R&D Systems, Minneapolis, MN, USA). The membrane was then incubated with anti-rabbit or anti-mouse IgG antibody conjugated to HRP (Assay Designs, Ann Arbor, MI, USA) at room temperature for 2 h. The proteins were visualized using an enhanced chemiluminescent substrate (Thermo Fisher Scientific, Logan, UT, USA) and analyzed using a LAS3000 luminescent image analyzer (Fuji Film, Tokyo, Japan).

**Immunofluorescent staining**

MCF-7 cells were seeded onto 0.1% gelatin-coated glass coverslips in a 12-well plate. After 24 h, the cells were fixed with 70% methanol for 90 s, washed with PBS, and subsequently permeabilized with 0.1% Triton X-100 at room temperature for 10 min. After the removal of the permeabilizing solution and washing with PBS, the cells were blocked with 0.1% BSA at room temperature for 30 min and immunostained with a primary antibody (1:300 anti-CIP2A) at 4 °C overnight. The secondary antibody used was goat anti-mouse IgG F(ab’2)-TRITC (Santa Cruz Biotechnology). The nuclei were stained with Hoechst dye (5 mg/ml).

**ELISA**

ELISA was carried out using the RayBio Human EGF ELISA Kit (ELH-EGF-001; RayBiotech, Inc., Norcross, GA, USA). MCF-7 cells were transfected with siCIP2A, cultured in a phenol-red-free medium, and then treated with E2. The medium was harvested and diluted for use in ELISA at 24 h after E2 treatment.

**Human breast cancer tissue**

Patients who were diagnosed with invasive ductal carcinoma not otherwise specified and underwent surgical excision at Severance Hospital between January 2006 and December 2006 were included in the study. The Institutional Review Board of Yonsei University Severance Hospital approved this study. All 258 patients, none of whom received preoperative hormonal therapy or neoadjuvant chemotherapy, were included in this study. All hematoxylin and eosin (H&E)-stained slides for each case were retrospectively reviewed by a breast pathologist (J S Koo). The histological grade was assessed using the Nottingham grading system (Elston & Ellis 1991). Tumor staging was based on the 7th American Joint Committee on Cancer (AJCC) criteria. The clinicopathological

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parameters evaluated for each breast cancer case included patient age at initial diagnosis, lymph node metastasis, tumor recurrence, distant metastasis, and patient survival.

**Immunohistochemistry**

Immunohistochemical staining for all the cases was carried out on formalin-fixed, paraffin-embedded tissue sections using the indicated antibodies (Table 1). Briefly, 5 μm-thick sections were obtained with a microtome, transferred onto positively charged slides, and dried at 62 °C for 30 min. After incubation with primary antibodies, immunodetection was carried out with biotinylated anti-mouse immunoglobulin and then with peroxidase-labeled streptavidin using a labeled streptavidin biotin kit with the 3,3′-diaminobenzidine chromogen as substrate. The primary antibody incubation step was omitted in the negative control. Slides were counterstained with Harris hematoxylin.

**Interpretation of immunohistochemical staining results**

All immunohistochemical markers were assessed by light microscopy. ER- or progesterone receptor (PR)-positive tumors were defined as having 1% or more positively stained nuclei (Hammond et al. 2010). HER2 staining was analyzed according to the American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) guidelines using the following categories: 0, no immunostaining; 1+, weak incomplete membranous staining, <10% of tumor cells; 2+, complete membranous staining, either uniform or weak in at least 10% of tumor cells; and 3+, uniform, intense membranous staining in at least 30% of tumor cells (Wolff et al. 2007). HER2 immunostaining was recorded as positive when strong (3+) membranous staining was observed, whereas staining categories 0 to 1+ were recorded as negative. Samples with 2+ HER2 (ERBB2) expression were evaluated for HER2 amplification by FISH (Spielman et al. 2004).

CIP2A immunohistochemical staining was evaluated based on the percentage of stained cells and the immunostaining intensity (graded 0 (negative), 1 (weak), 2 (moderate), or 3 (strong)). The proportion of stained cells and the staining intensity were multiplied to obtain the H-score (range: 0–300). CIP2A expression was divided into low expression and high expression categories with the median H-score as the cut-off (median of CIP2A: 30). Ki-67 labeling indices (LI) were scored by counting the number of positively stained nuclei and expressed as a percentage of total tumor cells.

**FISH**

FISH was carried out using the PathVysion HER2 DNA Probe Kit (Vysis, Downers Grove, IL, USA) according to the manufacturer’s instructions. Invasive tumors were first examined on hematoxylin and eosin-stained slides to confirm their histology. The HER2 gene copy number in the cells was evaluated using an epifluorescence microscope (Olympus, Tokyo, Japan) according to the ASCO/CAP guidelines (Wolff et al. 2007). At least 60 tumor cell nuclei in three separate regions were investigated for HER2 and chromosome 17 signals. An absolute HER2 gene copy number <4 or a HER2 gene:chromosome 17 copy number ratio (HER2:Chr17 ratio) <1.8 was considered HER2-negative. An absolute HER2 gene copy number between 4 and 6 or a HER2:Chr17 ratio between 1.8 and 2.2 was considered HER2-equivocal. An absolute HER2 gene copy number >6 or a HER2:Chr17 ratio >2.2 was considered HER2-positive.

**Human breast cancer molecular subtype classification**

Breast cancer molecular subtypes were classified according to the immunohistochemistry results for ER, PR, HER2, and Ki-67 and the FISH results for HER2 as follows (Goldhirsch et al. 2011): luminal A subtype: ER- or PR-positive and HER2-negative and Ki-67 LI <14%; luminal B subtype: ER- or PR-positive and HER2-negative and Ki-67 LI ≥14%; HER2-positive: ER- or PR-positive and HER2 overexpressed or/and amplified; HER2-overexpression subtype: ER- and PR-negative and HER2 overexpressed or/and amplified; and triple-negative breast cancer (TNBC): ER-, PR-, and HER2-negative.

**Table 1** Clone, dilution, and source of antibodies used in the human breast cancer samples

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Dilution</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIP2A</td>
<td>Polyclonal</td>
<td>1:100</td>
<td>Abcam, Cambridge, UK</td>
</tr>
<tr>
<td>Tumor phenotype related</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ER</td>
<td>SP1</td>
<td>1:100</td>
<td>Thermo Scientific, San José, CA, USA</td>
</tr>
<tr>
<td>PR</td>
<td>PgR</td>
<td>1:50</td>
<td>Dako, Glostrup, Denmark</td>
</tr>
<tr>
<td>HER2</td>
<td>Polyclonal</td>
<td>1:1500</td>
<td>Dako</td>
</tr>
<tr>
<td>Ki-67</td>
<td>MIB-1</td>
<td>1:150</td>
<td>Dako</td>
</tr>
</tbody>
</table>

ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.
Statistical analysis

Data were analyzed using SPSS for Windows, Version 12.0 (SPSS, Inc.). Student’s t-test and χ² test were used to evaluate continuous and categorical variables respectively. In analyses data with multiple comparisons, a corrected P value with application of Bonferroni multiple comparison procedure was used. The significance level was set at 0.05.

Results

E₂ stimulates cell proliferation by increasing CIP2A expression

CIP2A is highly expressed in breast cancer tissues and stimulates the growth of breast cancer cells. E₂ is also a well-known growth-stimulating hormone that acts on breast cancer cells. Thus, we examined whether E₂ is able to stimulate CIP2A expression in the ER-positive breast cancer cell line MCF-7. ER-positive MCF-7 cells and ER-negative MDA-MB-231 cells were incubated in a steroid-free medium overnight, followed by E₂ treatment for 24 h. CIP2A expression was increased in a dose-dependent manner in ER-positive MCF-7 cells but not in ER-negative MDA-MB-231 cells. Because CIP2A and MYC regulate each other’s expression in a reciprocal fashion, MYC expression was also examined. Similar to CIP2A expression, MYC expression was also increased by E₂ in MCF-7 cells but not in MDA-MB-231 cells (Fig. 1A). To understand the kinetics of E₂-induced CIP2A expression, MCF-7 cells were treated with E₂ for the indicated time periods. CIP2A expression was increased 24 h after treatment, whereas MYC expression was enhanced beginning at 3 h (Fig. 1B). This result indicates that E₂ may not

Figure 1

E₂ stimulates MCF-7 cell proliferation by increasing CIP2A expression. The cells were incubated in phenol-red-free DMEM for 24 h and subsequently treated with the indicated concentrations of E₂. The blots were re-probed with anti-β-actin antibody as a loading control. (A) The indicated cell lines were treated with E₂ for 24 h. CIP2A and MYC levels were measured using an immunoblot assay. (B) MCF-7 cells were treated with 100 nM E₂ for the indicated time periods. (C) MCF-7 cells were transfected with 20 nM siRNAs specific for GFP, CIP2A, and MYC, and CIP2A and MYC levels were measured in these cells using an immunoblot assay. (D) MCF-7 cells were transfected with 20 nM siRNAs specific for GFP, CIP2A, and MYC and subsequently replated 24 h later. After incubation with phenol red-free DMEM for 24 h, the cells were treated with 100 nM E₂ for 24 h. The viable cells were counted after Trypan Blue staining. (E) MCF-7 cells were treated as described in (D) and the degree of cell proliferation was measured using the CellTiter-Blue assay. All the experiments were repeated three times, and representative results are shown. The immunoblot data were quantified using the GelQuant software and plotted (A–C lower panels). *P < 0.01 and **P < 0.001 indicate statistically significant differences.
directly increase the transcription or translation of CIP2A and that MYC may mediate E2-induced CIP2A expression. To test this possibility, MCF-7 cells were transfected with siMYC before treatment with E2. E2 was able to induce CIP2A expression similarly in siGFP control and siMYC cells (Fig. 1C), indicating that MYC is not involved in E2-induced CIP2A expression. Next, because E2 can enhance the proliferation of ER-positive cells, we investigated whether E2 could enhance the proliferation of MCF-7 cells in the absence of CIP2A. MCF-7 cells transfected with two different siCIP2As or siMYCs were treated with E2, and the efficiency of depletion by two different siCIP2As or siMYCs is shown in Fig. 1C. Cell number was measured 24 h after treatment. CIP2A depletion did not increase proliferation in the presence of E2, whereas E2 increased cell proliferation in MYC-depleted cells (Fig. 1D). To further confirm this fact, MCF-7 cells were treated under the same condition used for cells shown in Fig. 1D, and the degree of cell proliferation was measured using the CellTiter-Blue assay (Fig. 1E). The CellTiter-Blue assay yielded results similar to those shown in Fig. 1D. These results indicate that E2 stimulates cell proliferation through CIP2A induction via a MYC-independent pathway in MCF-7 cells.

**E2 enhances CIP2A expression at the translational level**

It is well known that E2-bound ERα enhances the transcription of its target gene. Thus, the level of CIP2A gene transcription was examined by RT-PCR in MCF-7 cells after E2 treatment. Surprisingly, CIP2A mRNA levels were not affected by E2 treatment (Fig. 2A). When MCF-7 cells were treated with E2 in the presence of CHX to block the translation of CIP2A mRNA, E2 failed to increase CIP2A protein levels (Fig. 2B). CIP2A mRNA levels were not affected by CHX treatment (Fig. 2B, lower panel). However, actinomycin D-treated cells exhibited an E2-induced increase in CIP2A expression (Fig. 2C), although CIP2A mRNA levels were reduced by actinomycin D treatment (Fig. 2C, lower panel). These findings imply that E2 enhances CIP2A production via a translational mechanism. To determine whether the localization of CIP2A is altered after E2 treatment, immunofluorescent staining was carried out after E2 treatment. CIP2A was mainly localized in the cytoplasm (Fig. 2D).

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**Figure 2**

E2 enhances CIP2A expression at the translational level. (A) MCF-7 cells were treated with 100 nM E2 for the indicated time periods. CIP2A mRNA levels were measured using RT-PCR. (B) MCF-7 cells were treated with 50 µM cycloheximide (CHX) 1 h before E2 treatment, and CIP2A levels were measured using an immunoblot assay (upper panel) and CIP2A mRNA levels were measured using RT-PCR (lower panel). (C) MCF-7 cells were treated with 1 µg/ml actinomycin D 1 h before E2 treatment, and CIP2A levels were measured using an immunoblot assay (upper panel) and CIP2A mRNA levels were measured using RT-PCR (lower panel). (D) The cellular localization of CIP2A was determined by immunofluorescent staining 24 h after E2 treatment. All the experiments were repeated three times, and representative results are shown. The immunoblot data were quantified using the GelQuant software and plotted (B and C lower panels). *P < 0.01 and **P < 0.001 indicate statistically significant differences.
E2 increases CIP2A expression via ERα

It is well known that E₂ functions by binding to two intracellular ERs, ERα and ERβ, or by binding to the ER-independent membrane receptor GPR30. To determine whether ER is responsible for the increase in CIP2A expression, MCF-7 cells were treated with tamoxifen, which binds to the ER but does not activate it. Tamoxifen blocked E₂-induced CIP2A expression at a concentration of 4 μM (Fig. 3A). To further confirm this finding, MCF-7 cells were treated with the high-affinity ERα antagonist ICI 182 780. Treatment with ICI 182 780 inhibited E₂-induced CIP2A expression in a dose-dependent manner (Fig. 3B). Because ICI 182 780 can also act as an agonist of the high-affinity membrane ER GPR30, it is unlikely that E₂ induces CIP2A expression through GPR30. To confirm this assumption, ER-negative and GPR30-positive SK-BR-3 cells were treated with E₂. As expected, E₂-treated SK-BR-3 cells exhibited no induction of CIP2A expression (Fig. 3C), indicating that E₂ enhanced CIP2A expression through ERα. Next, ERα-depleted MCF-7 cells were treated with E₂. E₂-induced CIP2A expression was impaired in ERα-depleted cells (Fig. 3D). It was found that PPT, an ERα-specific agonist, alone could induce CIP2A expression in the absence of E₂ in MCF-7 cells (Fig. 3E). These findings indicate that CIP2A expression is regulated through an ERα-dependent pathway.

Figure 3

E₂ enhances CIP2A expression via ERα. (A) MCF-7 cells were incubated in phenol-red-free medium for 24 h and subsequently treated with or without tamoxifen (μM) at the indicated concentrations for 1 h before stimulation with 100 nM of E₂. CIP2A levels were measured using an immunoblot assay. (B) MCF-7 cells were treated with or without the ERα antagonist ICI 182 780 (nM) at the indicated concentrations for 1 h before treatment with E₂. CIP2A levels were measured using an immunoblot analysis. (C) MCF-7 and SK-BR-3 cells were treated with the indicated concentrations of E₂ (nM) for 24 h, and CIP2A levels were subsequently measured using an immunoblot analysis. (D) MCF-7 cells were transfected with 20 nM siRNAs specific for GFP and ERα and subsequently treated with 100 nM E₂. CIP2A and ERα levels were measured using an immunoblot assay. (E) MCF-7 cells were treated with the ERα agonist PPT (Nilsson et al. 2001) for 24 h, and CIP2A levels were measured using an immunoblot analysis. All the experiments were repeated three times, and representative results are shown. The immunoblot data were quantified using the GelQuant software and plotted (lower panels). *P<0.01 and **P<0.001 indicate statistically significant differences.
E2 increases CIP2A protein levels through a MAPK-dependent pathway

The function of E2 involves both direct and indirect interactions between ERα and components of growth-regulating signal pathways, including the PI3K/AKT and MAPK pathways (Meyer et al. 2009). Therefore, we examined whether the PI3K/AKT pathway is associated with E2-induced CIP2A expression. The cells were treated with LY294002, an inhibitor of PI3K, 1 h before E2 treatment. When E2-induced AKT phosphorylation was completely abolished by LY294002, the elevation of CIP2A protein levels by E2 treatment was impaired (Fig. 4A). To determine whether AKT activation is involved in CIP2A expression, the cells were treated with AKT inhibitor 1 h before E2 treatment. The E2-induced increase in CIP2A expression was inhibited by AKT inhibitor (Fig. 4B). Because mTOR is a well-known downstream target of the PI3K/AKT pathway, we examined whether mTOR is associated with E2-enhanced CIP2A expression. As expected, the mTOR inhibitor rapamycin completely inhibited the E2-dependent increase in CIP2A expression (Fig. 4C). Next, we examined the effect of the MAPK pathway on the E2-dependent increase in CIP2A expression. When MCF-7 cells were treated with U0126, an inhibitor of MEK1/2, 1 h before E2 treatment, the E2-dependent increase in CIP2A protein levels was impaired (Fig. 4D). To further confirm this finding, we used another ER-positive cell line T47D. The cells were treated with E2 in the presence of inhibitors. E2-induced CIP2A expression was also inhibited (Supplementary Figure 1, see section on supplementary data given at the end of this article). These results indicate that E2 increases CIP2A expression by stimulating the MAPK pathway as well as the PI3K/AKT pathway. Because both the PI3K/AKT and MAPK pathways converge on mTOR, we examined whether mTOR could be inhibited by the inhibition of the MAPK pathway.

Figure 4
E2 increases CIP2A levels through the MAPK and PI3K pathways. (A) MCF-7 cells were pre-treated with the PI3K inhibitor LY294002 (10 μM) for 1 h before treatment with E2. (B and F) MCF-7 cells were pre-treated with the AKT inhibitor (2 μM) for 1 h before treatment with E2. (C and E) MCF-7 cells were pre-treated with the mTOR inhibitor rapamycin (100 nM) for 1 h before treatment with E2. (D and G) MCF-7 cells were pre-treated with the MEK inhibitor U0126 (10 μM) for 1 h before treatment with E2. After pretreatment with various inhibitors, the cells were treated with E2 for 24 h without medium exchange. The levels of the indicated proteins were measured using an immunoblot analysis.
Unexpectedly, however, the inhibition of MAPK did not affect mTOR activation (Fig. 4D), suggesting that MAPK may activate a downstream signal component of mTOR.

The downstream targets of mTOR are S6K and 4EBP1 (Sarbassov et al. 2005); therefore, we examined S6K and 4EBP1 phosphorylation after mTOR activation by E2. E2-stimulated mTOR phosphorylated S6K but not 4EBP1 (Fig. 4E). To understand how E2-mediated signaling pathway converges on S6K, we analyzed three S6K phosphorylation sites because S6K must be phosphorylated at T389 by mTOR, at T229 by PDK1, and at T421/S424 ERK to be activated. Rapamycin treatment completely blocked phosphorylation at all the three sites (Fig. 4E). Because AKT functions as an upstream regulator of mTOR, the level of S6K phosphorylation was also examined after treatment with an AKT inhibitor. As with the mTOR inhibitor, phosphorylation at all the three sites was inhibited by the AKT inhibitor (Fig. 4F). These results indicate that PI3K/AKT-mediated mTOR activation is responsible for the phosphorylation of S6K at all the three sites during E2 treatment. Next, we examined the effect of the MAPK pathway on S6K phosphorylation because both the PI3K/AKT and MAPK pathways are involved in the increase in CIP2A expression. The phosphorylation levels of T229 and T389 were not altered, whereas the phosphorylation of T421/S424 was completely blocked, as expected (Fig. 4G). Collectively, eIF4B, a downstream target of S6K, was not phosphorylated when all the three phosphorylation sites of S6K were inhibited (Fig. 4E and F) or only T421/S424 was inhibited (Fig. 4G). These findings indicate that all the three phosphorylation sites in S6K must be phosphorylated to activate the downstream target eIF4B.

**E2-bound ERα enhances CIP2A expression through EGFR**

Because E2 can activate the PI3K/AKT and MAPK pathways through EGFR activation (Levin 2003), it is possible that E2 stimulates EGF secretion. To explore this possibility, we examined EGF levels 24 h after E2 treatment using a human EGF ELISA kit. EGF secretion was not increased by E2 in the presence or absence of CIP2A (Fig. 5A). This indicates that E2-induced CIP2A expression is not mediated by extracellular EGF. However, EGF increased CIP2A expression and elf4B phosphorylation in a dose-dependent manner (Fig. 5B), and this EGF-induced increase in CIP2A expression was inhibited by treatment with AKT inhibitor, rapamycin, or U0126 (Supplementary Figure 2, see section on supplementary data given at the end of this article). These data indicate that E2 is able to activate intracellular EGFR kinase in the absence of extracellular EGF. To further confirm this finding, the cells were treated with the EGFR tyrosine kinase inhibitor gefitinib, which blocks EGFR kinase activity, before E2 treatment. Gefitinib completely inhibited the E2-dependent increase in CIP2A expression (Fig. 5C). To further confirm this finding, we used lapatinib as another intracellular EGFR/HER2 inhibitor. Lapatinib treatment...
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E2-bound ER stimulates EGFR kinase activity to induce CIP2A expression (Supplementary Figure 3). This observation provides strong evidence that E2-induced CIP2A expression. However, E2 did not increase CIP2A expression at the translational level in normal mammary tissues, E2 does not cause ER-positive cells to proliferate. Instead, E2 stimulates ER-positive cells to produce and release paracrine growth factors to induce CIP2A expression in the absence of extracellular EGF.

Table 2  Patient clinicopathological characteristics according to breast cancer phenotype

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Total n = 258 (%)</th>
<th>Luminal A n = 127 (%)</th>
<th>Luminal B n = 63 (%)</th>
<th>HER2 n = 18 (%)</th>
<th>TNBC n = 50 (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years, mean ± s.d.)</td>
<td>51.2 ± 11.1</td>
<td>51.9 ± 11.5</td>
<td>48.1 ± 10.0</td>
<td>54.8 ± 8.2</td>
<td>51.9 ± 11.8</td>
<td>0.063*</td>
</tr>
<tr>
<td>Histological grade</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>I</td>
<td>59 (22.9)</td>
<td>48 (37.8)</td>
<td>8 (12.7)</td>
<td>1 (5.6)</td>
<td>2 (4.0)</td>
<td>0.162†</td>
</tr>
<tr>
<td>II/III</td>
<td>199 (77.1)</td>
<td>79 (62.2)</td>
<td>55 (87.3)</td>
<td>17 (94.4)</td>
<td>48 (96.0)</td>
<td></td>
</tr>
<tr>
<td>Tumor stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>162 (62.8)</td>
<td>85 (66.9)</td>
<td>39 (61.9)</td>
<td>13 (72.2)</td>
<td>25 (50.0)</td>
<td>0.467†</td>
</tr>
<tr>
<td>T2/T3</td>
<td>96 (37.2)</td>
<td>42 (33.1)</td>
<td>24 (38.1)</td>
<td>5 (27.8)</td>
<td>25 (50.0)</td>
<td></td>
</tr>
<tr>
<td>Nodal status</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No metastasis</td>
<td>166 (64.3)</td>
<td>77 (60.6)</td>
<td>40 (63.5)</td>
<td>13 (72.2)</td>
<td>36 (72.0)</td>
<td></td>
</tr>
<tr>
<td>Metastasis</td>
<td>92 (35.7)</td>
<td>50 (39.4)</td>
<td>23 (36.5)</td>
<td>5 (27.8)</td>
<td>14 (28.0)</td>
<td></td>
</tr>
<tr>
<td>Estrogen receptor status</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Negative</td>
<td>73 (28.3)</td>
<td>2 (1.6)</td>
<td>3 (4.8)</td>
<td>18 (100.0)</td>
<td>50 (100.0)</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>185 (71.7)</td>
<td>125 (98.4)</td>
<td>60 (95.2)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
<tr>
<td>Progesterone receptor status</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Negative</td>
<td>94 (36.4)</td>
<td>13 (10.2)</td>
<td>13 (20.6)</td>
<td>18 (100.0)</td>
<td>50 (100.0)</td>
<td></td>
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<tr>
<td>Positive</td>
<td>164 (63.6)</td>
<td>114 (89.8)</td>
<td>50 (79.4)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
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</tr>
<tr>
<td>HER2 status</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Negative</td>
<td>208 (80.6)</td>
<td>127 (100.0)</td>
<td>31 (49.2)</td>
<td>0 (0.0)</td>
<td>50 (100.0)</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>50 (19.4)</td>
<td>0 (0.0)</td>
<td>32 (50.8)</td>
<td>18 (100.0)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
<tr>
<td>Ki-67 LI (% , mean ± s.d.)</td>
<td>15.5 ± 17.5</td>
<td>4.6 ± 3.6</td>
<td>18.0 ± 12.7</td>
<td>19.8 ± 13.3</td>
<td>38.5 ± 21.4</td>
<td>&lt;0.001**</td>
</tr>
</tbody>
</table>

TNBC, triple-negative breast cancer. *P value was calculated using Student’s t-test. †P value was calculated using χ² test. ‡P value was corrected using Bonferroni multiple comparison procedure.

Discussion

Breast tumors are typical hormone-dependent tumors, and nearly 70% of breast cancers are ER-positive. In normal mammary tissues, E2 does not cause ER-positive cells to proliferate. Instead, E2 stimulates ER-positive cells to produce and release paracrine growth factors to induce the proliferation of neighboring cells (Mallepell et al. 2006). In contrast, in primary breast cancers, ERα mediates cancer cell proliferation via paracrine and/or autocrine mechanisms. ERα can activate multiple pathways, such as the ERK and AKT pathways, in a non-genomic manner (Razandi et al. 2003, Pedram et al. 2006), and EGFR is required for ERα-activated signal transduction (Razandi et al. 2003). Additionally, the inhibition of the ERK and PI3K/AKT pathways can prevent the estrogen-induced increase in the S-phase cell population in MCF-7 cells (Lobenhofer et al. 2000). In this study, E2 treatment increased CIP2A expression at the translational level by activating ERK and PI3K/AKT in MCF-7 cells, and the intracellular EGFR kinase inhibitor gefitinib blocked E2-induced CIP2A expression. However, E2 did not stimulate the secretion of EGF in MCF-7 cells. Thus, E2...
enhances the proliferation of cells expressing ER through enhanced CIP2A expression and cannot aid the proliferation of neighboring ER-negative and EGFR-positive tumor cells through the EGFR pathway.

E2-bound ERα is known to induce proliferation in ER-positive breast cancer cells. Although the underlying mechanism is not fully known, one potential mechanism involves ERα binding to the MYC promoter to transcriptionally enhance MYC gene expression early in the cell cycle, leading to an increase in the proliferation of breast cancer cells (McEwan et al. 2012). It is known that MYC stimulates the expression of CIP2A (and vice versa) (Bockelman et al. 2012). Thus, it is possible that E2-induced MYC expression increases CIP2A expression. However, E2 increased CIP2A expression even in MYC-depleted cells, which is possible because E2 increases CIP2A expression at the translational level and does not require a MYC-mediated increase in transcription. To our knowledge, this is the first study to explore the translational regulation of CIP2A expression.

mTOR integrates signals from Ras/MAPK and PI3K/AKT activation, which are hallmarks of many cancers (Carracedo et al. 2008). However, mTOR inhibition therapy was not successful as expected because the inhibition of mTORC1 leads to the activation of AKT and ERK by upregulating growth factor receptors CIP2A staining in the four breast cancer subtypes is shown as a graph. Molecular subtypes used are as follows: (A) luminal A (n = 127); (B) luminal B (n = 63); (H) HER2-positive (n = 18); (T) triple negative (n = 50). The box plots show the median (thick lines) and the lower and upper quartiles (boxes). Raw immunohistochemical results for CIP2A: C, negative intensity; D, weak intensity; E, moderate intensity; F, strong intensity. (Shah et al. 2004, Zhang et al. 2007) or by releasing PI3K from mTORC1-negative feedback loop inhibition (Carracedo et al. 2008). However, a higher dose of rapamycin inhibits AKT and ERK phosphorylation mainly via mTORC2 (Chen et al. 2010). In this study, treatment with a high dose of rapamycin suppressed ERK activation. Thus, it is conceivable that a high dose of rapamycin inhibits ERK-induced T421/S424 phosphorylation of S6K. The blockage of S6K phosphorylation at T421/S424 suppressed CIP2A translation. Thus, E2-stimulated mTORC2 activation may be involved in CIP2A translation via ERK activation; this hypothesis is supported by a recent study indicating that E2 activates mTORC2 in an ERα-dependent manner (Kumari Kanchan et al. 2012). The activated S6K stimulates an increase in the translation of its target mRNA (Radimerski et al. 2002). The phosphorylation of three sites on S6K (T389, T229, and T421/S424) is required for its activation. Two models have been proposed to explain the activation of S6K. In the conventional model, T421/S424 sites are phosphorylated before T389 phosphorylation by mTORC1 and subsequent T229 phosphorylation by PKD1. In the alternative model, phosphorylation at S371 by an unknown kinase is the first step before the subsequent phosphorylation of T389, T229, and T421/S424 (Magnuson et al. 2012). In this study, S6K phosphorylated at T389 and T229 but not at T421/S424.
failed to phosphorylate eIF4B, showing that T421/S424 phosphorylation of S6K by ERK is indispensable for the activation of S6K via T389 and T229 phosphorylation. This implies that T389 and T229 can be phosphorylated without T421/S424 phosphorylation and that the alternative model is preferable over the conventional model in this study.

In this study, we showed for the first time, to our knowledge, that CIP2A expression is higher in the tumor tissue of ER-positive patients than in that of ER-negative patients and that luminal A types and luminal B types exhibit higher CIP2A expression than triple-negative types. Additionally, we showed that E2 increases CIP2A expression through ERα, and the high expression of CIP2A in ER-positive patients clearly supports the correlation between ER and CIP2A expression. On the other hand, it has been reported that ER-positive cell lines exhibit higher PP2CB expression and activity than ER-negative cell lines (Gopalakrishna et al. 1999). Because CIP2A inhibits tumor suppressor PP2CB activity, it is possible that E2-enhanced CIP2A expression overcomes the tumor suppressor PP2CB activity in ER-positive breast cancer tissues. Thus, it will be worth determining whether breast cancer tissues exhibiting high levels of CIP2A and ER expression have low PP2CB activity.

While preparing this manuscript, it was reported that CIP2A expression is significantly related to histological grade, lymph node metastasis, distant metastasis, and triple-negative status in breast cancer (Yu et al. 2013). However, high CIP2A expression was found to be associated with ER positivity, PR positivity, luminal subtype, and lower Ki-67 LI in this study, but not with histological grade and lymph node metastasis. There are possible explanations for this inconsistency. Of the patients enrolled in the study of Yu et al. 35% were CIP2A-positive, but 77% of patients were positive in this study. This big difference could arise from different CIP2A antibody use. Yu et al. did not mention the source of the CIP2A antibody. In addition, Yu et al. divided the CIP2A-positive group into two levels, but we divided it into three levels according to CIP2A intensity. In this study, three levels of CIP2A expression were divided into low and high expression categories according to the median H-score as the cut-off (median for CIP2A: 30), whereas two levels of CIP2A expression were divided into CIP2A-positive or CIP2A-negative in the study of Yu et al. It means that our classification of the CIP2A-positive group is more elaborate. In other words, a tissue classified as CIP2A-positive by Yu et al. could be grouped with the CIP2A low-expression sample.

Table 3  Correlations between clinicopathological factors and CIP2A expression

<table>
<thead>
<tr>
<th>Parameters</th>
<th>CIP2A expression</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low n=137 (%)</td>
<td>High n=121 (%)</td>
</tr>
<tr>
<td>Age (years, mean±s.d.)</td>
<td>51.8±11.0</td>
<td>50.5±11.2</td>
</tr>
<tr>
<td>Histological grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>24 (17.5)</td>
<td>35 (28.9)</td>
</tr>
<tr>
<td>II/III</td>
<td>113 (82.5)</td>
<td>86 (71.1)</td>
</tr>
<tr>
<td>Tumor stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>81 (59.1)</td>
<td>81 (66.9)</td>
</tr>
<tr>
<td>T2/T3</td>
<td>56 (40.9)</td>
<td>40 (33.1)</td>
</tr>
<tr>
<td>Lymph node status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No metastasis</td>
<td>93 (67.9)</td>
<td>73 (60.3)</td>
</tr>
<tr>
<td>Metastasis</td>
<td>44 (32.1)</td>
<td>48 (39.7)</td>
</tr>
<tr>
<td>Estrogen receptor status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>51 (37.2)</td>
<td>22 (18.2)</td>
</tr>
<tr>
<td>Positive</td>
<td>86 (62.8)</td>
<td>99 (81.8)</td>
</tr>
<tr>
<td>Progesterone receptor status</td>
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<td></td>
</tr>
<tr>
<td>Negative</td>
<td>61 (44.5)</td>
<td>33 (27.3)</td>
</tr>
<tr>
<td>Positive</td>
<td>76 (55.5)</td>
<td>88 (72.7)</td>
</tr>
<tr>
<td>HER2 status</td>
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<tr>
<td>Negative</td>
<td>115 (83.9)</td>
<td>93 (76.9)</td>
</tr>
<tr>
<td>Positive</td>
<td>22 (16.1)</td>
<td>28 (23.1)</td>
</tr>
<tr>
<td>Molecular subtype</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Luminal A</td>
<td>59 (43.1)</td>
<td>68 (56.2)</td>
</tr>
<tr>
<td>Luminal B</td>
<td>29 (21.2)</td>
<td>34 (28.1)</td>
</tr>
<tr>
<td>HER2</td>
<td>11 (8.0)</td>
<td>7 (5.8)</td>
</tr>
<tr>
<td>TNBC</td>
<td>38 (27.7)</td>
<td>12 (9.9)</td>
</tr>
<tr>
<td>Ki-67 LI (%, mean±s.d.)</td>
<td>19.1±19.6</td>
<td>11.5±13.9</td>
</tr>
</tbody>
</table>

TNBC, triple-negative breast cancer. *P value was calculated using Student’s t-test. †P value was calculated using χ² test. ‡P value was corrected using Bonferroni multiple comparison procedure.
This could be another cause for the differences. On the other hand, ER-positive tissues have been widely reported as exhibiting low levels of Ki-67 (Urruticoechea et al. 2005). Since CIP2A expression is high in ER-positive tissues, it is possible that the CIP2A high-expression category exhibits low Ki-67 LI, although CIP2A expression increases the proliferation of tumor cells. Although we do not know the exact relationship between Ki-67 and CIP2A, further study on Ki-67 regulation by E2-bound ER could give us an answer.

In summary, E2 enhances CIP2A expression at the translational level through the intracellular activation of EGFR. Both the ERK and mTOR signaling pathways converge on S6K, leading to eIF4B phosphorylation. Three sites on S6K must be phosphorylated for its complete activation. A summary of the signal transduction pathway of E2-induced CIP2A expression is shown in Supplementary Figure 4, see section on supplementary data given at the end of this article.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-13-0453.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
Y A Choi carried out the molecular studies and drafted the manuscript. J S Koo carried out immunohistochemistry and statistical analysis. J S Park and M Y Park acquired the data. A L Jeong and K S Oh carried out the molecular studies and helped to design the study. Y Yang participated in the design of the study and wrote the manuscript. All authors read and approved the final manuscript.

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