

Mediator of ERBB2-driven Cell Motility (MEMO) Promotes Extranuclear Estrogen Receptor Signaling Involving the Growth Factor Receptors IGF1R and ERBB2*[§]

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Background: Extranuclear ER plays an important role in cancer cell growth regulation through activation of kinase cascades.

Results: MEMO modulates extranuclear functions of ER as well as breast cancer cell growth.

Conclusion: MEMO is a new component of extranuclear ER signalosome and is essential for the regulation of ER-positive breast cancer cell growth.

Significance: MEMO might be an interesting target for ER-positive breast cancer therapy.

In addition to nuclear estrogen receptor (ER) acting as a transcription factor, extranuclear ER also plays an important role in cancer cell growth regulation through activation of kinase cascades. However, the molecular mechanisms by which extranuclear ER exerts its function are still poorly understood. Here, we report that mediator of ERBB2-driven cell motility (MEMO) regulates extranuclear functions of ER. MEMO physically and functionally interacted with ER. Through its interaction with the growth factor receptors IGF1R and ERBB2, MEMO mediated extranuclear functions of ER, including activation of mitogen-activated protein kinase (MAPK) and protein kinase B/AKT, two important growth regulatory protein kinases, and integration of function with nuclear ER. Activation of MAPK and AKT was responsible for MEMO modulation of ER phosphorylation and estrogen-responsive gene expression. Moreover, MEMO increased anchorage-dependent and -independent growth of ER-positive breast cancer cells *in vitro* and was required for estrogen-induced breast tumor growth in nude mice. Together, our studies identified MEMO as a new component of extranuclear ER signalosome and suggest an essential role for MEMO in the regulation of ER-positive breast cancer cell growth.

Estrogen plays a critical role in the development and progression of estrogen-related cancers such as breast cancer (1, 2). Estrogen exerts its biological responses through estrogen receptor (ER)⁴ α and β to regulate networks of gene transcription factors that share structural similarity characterized by several functional domains. The N-terminal estrogen-independent and C-terminal estrogen-dependent activation function domains (AF1 and AF2, respectively) contribute to the transcriptional activity of the two receptors. The DNA binding domain of ER is centrally located. The ligand binding domain, overlapping AF2, shows 58% homology between ER α and ER β . The DNA binding domain is identical between the two receptors except for three amino acids. However, the AF1 domain of ER β has only 28% homology with that of ER α .

Traditionally, ER is thought to be ligand-activated nuclear transcription factors that bind to estrogen-response elements (ERE) of target genes. Upon binding to DNA, ER regulates target gene transcription through recruitment of coactivators and corepressors. Although the majority of ER is in the nucleus mediating both ligand-dependent and ligand-independent gene transcription, evidence from an increasing number of studies clearly shows that a small population of ER is localized at or near the plasma membrane in the presence or absence of estrogen and mediates rapid extranuclear functions of ER (3–7). Extranuclear ER signaling pathway involves insulin-like growth factor 1 receptor (IGF1R) and epidermal growth factor receptor (EGFR), resulting in the activation of many signaling molecules, such as mitogen-activated protein kinase (MAPK), protein kinase B/AKT, and intracellular second messengers (8). MAPK and AKT can phosphorylate ER α , thereby increasing

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⁴ The abbreviations used are: ER, estrogen receptor; MEMO, mediator of ERBB2-driven cell motility; ERE, estrogen-response element; E₂, 17 β -estradiol; IGF1R, insulin-like growth factor 1 receptor; EGFR, EGF receptor.

ER α transcriptional activity (9–14). In cultured cancer cells, membrane-initiated ER α signaling mediates the proliferative effects of estrogen. However, the molecular mechanisms by which extranuclear ER exerts its function are still poorly understood.

The mediator of ERBB2-driven cell motility (MEMO) is a 297-amino acid protein and is not homologous to any known signaling protein (15, 16). The biological function of MEMO is largely unknown. MEMO interacts with ERBB2 and is required for breast cancer cell migration (15, 17). ERBB2 is a member of the epidermal growth factor (EGF, also known as ErbB) family of receptor tyrosine kinases, which also includes EGFR, ErbB3, and ErbB4 (18). MEMO controls ERBB2-regulated microtubule dynamics (19). In this study, we have identified MEMO as a novel ER-interacting protein. Through its interaction with IGF1R and ERBB2, MEMO regulates extranuclear functions of ER α , such as activation of MAPK and AKT pathways and integration of function with nuclear ER α that functions as a transcription factor. Importantly, MEMO is required for estrogen-mediated breast tumor growth.

EXPERIMENTAL PROCEDURES

Plasmids and siRNAs—The estrogen-responsive reporter ERE-Luc and eukaryotic expression vectors for FLAG-tagged ER α and ER β have been described previously (20). The extranuclear ER α mutant ER α (H2+NES) was constructed as reported previously (21). Other mammalian expression vectors encoding FLAG or Myc fusion proteins tagged at the N terminus were constructed by inserting PCR-amplified fragments into pcDNA3 (Invitrogen) or pIRESpuro2 (Clontech). Enhanced green fluorescent protein-tagged MEMO construct was generated by inserting MEMO cDNA into pEGFP-C1 (Clontech). Plasmids encoding GST fusion proteins were made by cloning PCR-amplified sequences into pGEX-KG (Amersham Biosciences). The cDNA target sequences of siRNAs for MEMO were GATGAACACAGTATTGAAA (MEMO siRNA1) and GCAAT-TACTTGAAGAAATA (MEMO siRNA2) and were inserted into pSilencer2.1-U6neo (Ambion) or pSIH-H1-puro (System Biosciences). Expression vectors for siRNA-resistant MEMO containing a silent mutation in the 3'-nucleotide of a codon in the middle of the siRNA-binding site were generated by recombinant PCR. Recombinant lentivirus vectors for ERBB2 siRNA and IGF1R siRNA were used for knockdown of endogenous ERBB2 and IGF1R, respectively, according to the manufacturer's instructions (Santa Cruz Biotechnology).

Yeast Two-hybrid Assay—The bait plasmid pGBKT7-ER α -AF1(1–167) was used to screen a human mammary gland cDNA library fused to the GAL4 activation domain in pACT2 according to the manufacturer's instructions (Clontech). The bait plasmid and the mammary cDNA library were sequentially transformed into AH109 yeast cells as described previously (22).

GST Pulldown Assay—The GST alone and GST fusion proteins were expressed in bacteria and purified according to the manufacturer's instructions (Amersham Biosciences). HEK293T extracts expressing ER α or ER β were incubated with 10 μ g of purified GST derivatives bound to glutathione-Sephar-

ose beads, and the adsorbed proteins were examined as described previously (23).

Coimmunoprecipitation—Cell extracts were prepared, immunoprecipitated, and analyzed as described previously (23). Immunoprecipitation was performed with anti-FLAG M2 affinity gel (Sigma), anti-c-Myc affinity gel (Sigma), anti-ER α (Millipore), anti-MEMO (Abcam), anti-ERBB2 (Santa Cruz Biotechnology), or anti-IGF1R (Santa Cruz Biotechnology).

Cell Culture, Transfection, and Luciferase Reporter Assay—HEK293T embryonic kidney cells and MCF7, ZR75-1, T47D, and SKBR3 breast cancer cells were purchased from American Type Culture Collection (ATCC) and were routinely grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Hyclone). For hormone treatment experiments, cells were grown in medium containing phenol red-free DMEM supplemented with 10% charcoal-stripped FBS. Lipofectamine 2000 reagent was used for transfections according to the manufacturer's protocol (Invitrogen). Lentiviruses were produced by cotransfection of HEK293T cells with recombinant lentivirus vectors and pPACK Packaging Plasmid Mix (System Biosciences) using Megatran reagent (Origene). Lentiviruses were added to the medium of target cells with 8 μ g/ml Polybrene (Sigma). Stable cell lines were selected in 500 μ g/ml G418 or 1 μ g/ml puromycin. Pooled clones or individual clones were screened by standard immunoblot protocols and produced similar results. Luciferase reporter assays were performed as described previously (24).

Real Time RT-PCR—Total RNA was isolated using TRIzol reagent (Invitrogen) and reverse-transcribed using SuperScript II reverse transcriptase (Invitrogen). Real time PCR was performed as described previously with the primers listed in supplemental Table 1A (25).

Chromatin Immunoprecipitation Assay (ChIP)—ChIP assays were performed as described previously (22) using anti-ER α (Millipore). The primers used for ChIP are listed in supplemental Table S1B.

Anchorage-dependent and -independent Growth Assays—Anchorage-dependent cell growth was determined by a crystal violet assay as described previously (22). For anchorage-independent growth (22), 1×10^4 cells were plated on 6-cm plates containing a bottom layer of 0.6% low melting temperature agar in DMEM and a top layer of 0.3% agar in DMEM. Colonies were scored after 3 weeks of growth.

Animal Experiments—Animal studies were approved by the Institutional Animal Care Committee of Beijing Institute of Biotechnology. Two days after implantation of estrogen pellets (E₂, 0.36 mg/pellet, 60-day release) (Innovative Research of America), 1×10^7 tumor cells were injected into the abdominal mammary fat pad of 6-week-old female nude mice. Tumor growth was monitored by caliper measurements. Excised tumors were weighed, and portions were frozen in liquid nitrogen or fixed in 4% paraformaldehyde for further study.

Statistical Analysis—Differences between variables were assessed by two-tailed Student's *t* test or one-way analysis of variance. Statistical calculations were performed using SPSS13.0. A *p* value <0.05 was considered statistically significant.

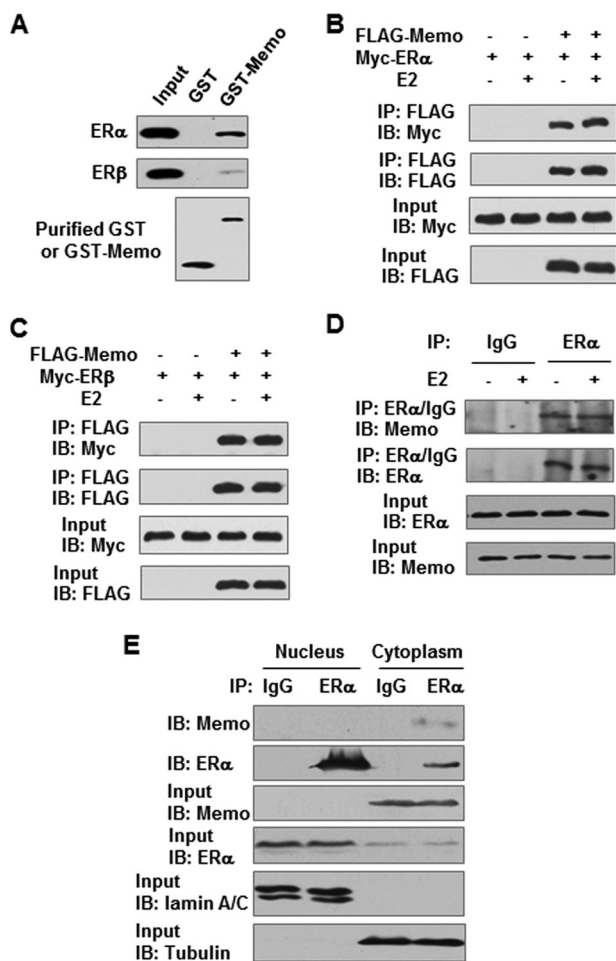


FIGURE 1. MEMO interacts with ER *in vitro* and *in vivo*. A, GST pull-down analysis of purified GST or GST-MEMO fusion protein incubated with lysates of HEK293T cells expressing FLAG-tagged ER α or ER β . Bound proteins were subjected to Western blot with anti-FLAG antibody. B and C, HEK293T cells transiently transfected with FLAG-tagged MEMO and Myc-tagged ER α (B) or ER β (C) in the presence of 10 nM E₂ were immunoprecipitated (IP) with anti-FLAG followed by immunoblotting (IB) with the indicated antibodies. D, coimmunoprecipitation analysis of the endogenous interaction of MEMO with ER α in the presence of 10 nM E₂. Cell lysates from MCF7 cells were immunoprecipitated with antibodies specific for ER α , followed by immunoblotting with the indicated antibodies. IgG, normal serum. E, MCF7 cells were fractionated, and proteins were immunoprecipitated from cytoplasmic and nuclear fractions, with the indicated antibodies or preimmune control serum (IgG). Precipitates were analyzed by immunoblot using the indicated antibodies. Lamin A/C and α -tubulin were used as the nuclear and cytoplasmic marker, respectively.

RESULTS

MEMO Interacts with ER *in Vitro* and *in Vivo*—Yeast two-hybrid screening of a human mammary cDNA library, with the AF1 domain of ER α as bait, identified human MEMO as an ER α -interacting protein (supplemental Fig. 1). Transformation of yeast cells with MEMO together with the controls did not activate the *His*, *Ade*, and *lacZ* reporter genes, indicating the specific interaction of MEMO with ER α in yeast cells. GST pull-down and coimmunoprecipitation assays further demonstrated that MEMO interacted with ER α and ER β *in vitro* and in 293T cells in the absence or presence of 17 β -estradiol (E₂) (Fig. 1, A–C). Importantly, endogenous MEMO associated with ER α in MCF7 breast cancer cells (Fig. 1D). Moreover, subcellular fractionation experiments showed that in MCF7, ZR75-1, and T47D breast cancer cells MEMO resided in the cytoplasm but

not in the nucleus (supplemental Fig. 2). Endogenous cytoplasmic MEMO also interacted with endogenous cytoplasmic ER α in MCF7 cells (Fig. 1E).

MEMO Activates MAPK and AKT and Subsequent ER α Phosphorylation—Because MEMO associates with extranuclear ER α , we tested whether MEMO regulates rapid extranuclear functions of ER, such as activation of MAPK/extracellular signal-regulated kinases 1 and 2 (ERK1/2) and AKT (6). As expected, E₂ rapidly increased phosphorylation of ERK1/2 and AKT in MCF7 cells, which was seen from 5 to 30 min after E₂ treatment (Fig. 2A and data not shown). Importantly, overexpression of MEMO enhanced phosphorylation of ERK1/2 and AKT in both the absence and the presence of E₂. In contrast, stable knockdown of MEMO with MEMO siRNAs reduced ERK1/2 and AKT phosphorylation (Fig. 2B).

Although ER β phosphorylation is largely unknown, ER α has been shown to be phosphorylated at serines (Ser) 104, 106, and 118 by MAPK (9–12). ER α Ser-167 can be phosphorylated by AKT (13, 14). Because MEMO can activate MAPK and AKT, we determined whether MEMO increases phosphorylation of ER α . Indeed, overexpression of MEMO in MCF7 cells increased phosphorylation of ER α at serines 104, 106, 118, and 167 (Fig. 2A), whereas stable knockdown of MEMO decreased ER α phosphorylation at these sites (Fig. 2B). Similar results were obtained in ZR75-1 cells (supplemental Fig. 3, A and B). Moreover, ER α is required for MEMO activation of MAPK and AKT because knockdown of endogenous ER α in MCF7 cells abolished the ability of MEMO to regulate MAPK and AKT (Fig. 2C), suggesting the importance of the interaction between MEMO and ER α . Similar results were observed in ER α -negative HEK293T cells. More importantly, MEMO could activate MAPK and AKT through extranuclear ER α because the previously reported extranuclear ER α mutant ER α (H2+NES) was sufficient for MEMO modulation of MAPK and AKT (21) (supplemental Fig. 3C).

To determine whether estrogen antagonists affect MEMO-induced MAPK and AKT activation as well as ER α phosphorylation, the mixed agonist/antagonist 4-OHT and the pure antagonist ICI182,780 were used. Tamoxifen and ICI182,780 treatment in control siRNA-transfected MCF7 cells had similar effects to those in MEMO siRNA-transfected MCF7 cells, *i.e.* tamoxifen and ICI182,780 antagonized estrogen-mediated effects in both control siRNA- and MEMO siRNA-transfected MCF7 cells (Fig. 2D).

MEMO Enhances ER α Transcriptional Activity through Increased ER α Phosphorylation and ER α Recruitment to Estrogen-responsive Promoters—It has been shown that extranuclear ER can indirectly regulate ER transcriptional activity through ER phosphorylation (26). As MEMO can regulate ER phosphorylation, we determined whether MEMO affects ER transcriptional activity. MEMO overexpression in ER α - and ER β -negative SKBR3 breast cancer cells increased transcription of a luciferase reporter construct containing the estrogen-responsive element (ERE) in an ER α - or ER β -dependent manner in the presence or absence of E₂ (Fig. 3A). Moreover, MEMO increased ER transcriptional activity in ER α - and ER β -positive MCF7 cells regardless of E₂, the ER α -specific agonist propylpyrazole triol, and the ER β -specific agonist diarylpropionitrile

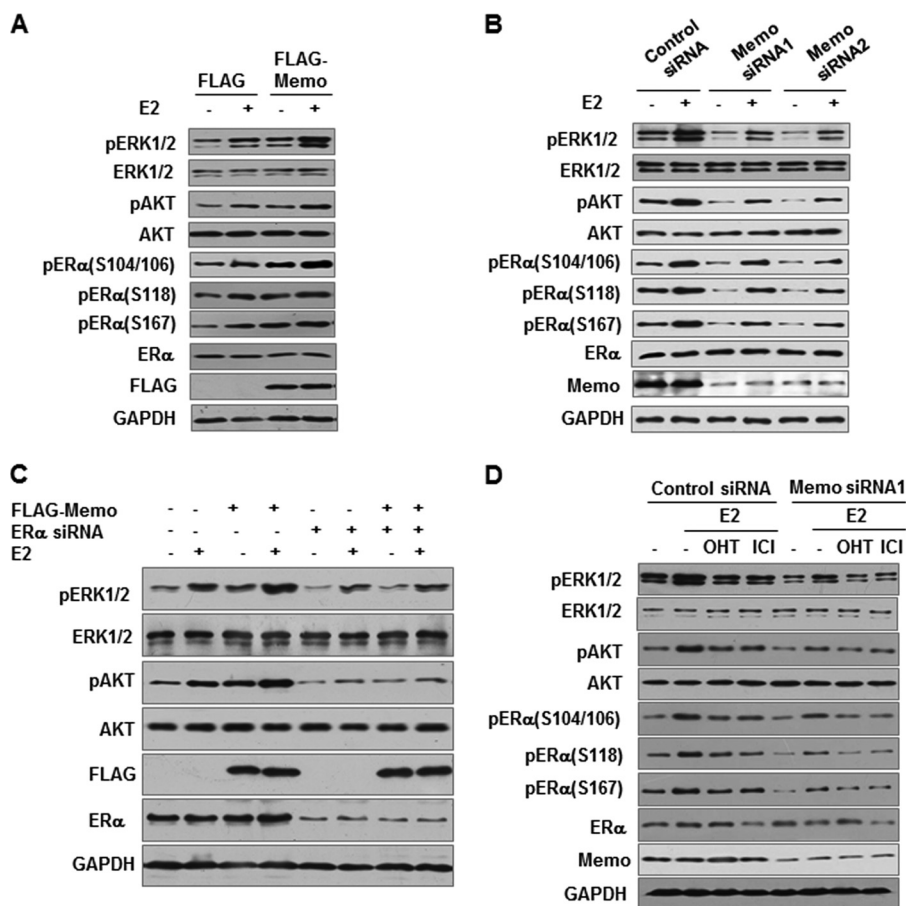


FIGURE 2. MEMO activates ERK1/2 and AKT and subsequent ER α phosphorylation. *A* and *B*, immunoblot analysis of MCF7 cells stably transfected with FLAG-tagged MEMO (*A*) and MEMO siRNA1 or MEMO siRNA2 (*B*) and treated with 10 nM E₂ for 30 min. GAPDH was used as a loading control. *p*, phosphorylation. *C*, immunoblot analysis of FLAG-MEMO-expressing MCF7 cells transfected with ER α siRNA. *D*, immunoblot analysis of MCF7 cells stably transfected with MEMO siRNA1 and treated with 10 nM E₂, 10 nM E₂ plus 100 nM 4-OHT, or 10 nM E₂ plus 10 nM ICI182,780 for 30 min.

(Fig. 3*B* and supplemental Fig. 4*C*), suggesting that MEMO regulates ER transcriptional activity in a ligand-independent manner. Similar results were observed in ER α -positive ZR75-1 and T47D cells (supplemental Fig. 4, *B* and *C*). Consistent with the results of MEMO overexpression, knockdown of endogenous MEMO in MCF7 cells decreased ERE-Luc reporter activity independently of ligands, including EGF and IGF, which have been reported to stimulate ER transcriptional activity through ER phosphorylation (Fig. 3*C*) (10). Importantly, MEMO increased wild-type ER α -mediated ERE-LUC reporter transcription much greater than the ERE-LUC reporter transcription mediated by mutant ER α in which serines 104, 106, 118, and 167 were mutated to alanine (Fig. 3*D*). These data suggest that phosphorylation of ER α at these sites is critical for the enhancement of ER α transcriptional activity by MEMO.

To investigate whether MEMO affects promoter occupancy of ER α , we performed ChIP experiments for the estrogen-responsive pS2, *c-Fos*, and CCND1 promoters. As expected, ER α was recruited to the pS2, *c-Fos*, and CCND1 promoters but not to a region ~2-kb upstream of the pS2, *c-Fos*, or CCND1 promoters (Fig. 3*E*). Importantly, MEMO knockdown decreased ER α recruitment to the *c-Fos* and CCND1 promoters, but not the pS2 promoter, indicating that MEMO selectively regulates ER α recruitment to estrogen-responsive promoters.

MEMO Increases ER α Target Gene Expression through Activation of MAPK and AKT and Subsequent ER α Phosphorylation—To validate the reporter assays in which MEMO increases ER α transcriptional activity, we performed real time RT-PCR analysis using MCF7 cells stably transfected with MEMO siRNA or control siRNA. The results showed that, in the presence or absence of E₂, MEMO knockdown reduced the transcription of eight previously reported E₂-regulated genes (Fig. 4*A*), including *c-Fos* (cellular FBJ osteosarcoma oncogene), *c-Jun* (cellular *ju-nana*), cathepsin D, CCND1/cyclin D1, *c-myc* (cellular myelocytomatosis virus oncogene), STC2 (stanniocalcin 2), PCP4 (Purkinje cell protein 4), and CA2 (carbonic anhydrase 2), many of which play key roles in cell proliferation regulation (27–30). Consistent with the results of ChIP experiments, MEMO did not alter pS2/TFF1 (trefoil factor 1) mRNA expression (Fig. 4*A*). In addition, MEMO did not regulate ER mRNA expression. Importantly, stable MEMO expression in MCF7 cells increased the protein levels of *c-Fos*, *c-Jun*, cathepsin D, cyclin D1, and *c-Myc*, but not pS2, in an E₂-independent manner (Fig. 4*B*). In contrast, siRNA knockdown of endogenous MEMO in MCF7 cells reduced the protein expression of *c-Fos*, *c-Jun*, cathepsin D, cyclin D1, and *c-Myc*, but not pS2 (Fig. 4*C*), suggesting that MEMO selectively regulates estrogen-responsive gene expression. Similar results were obtained in

MEMO Modulation of Extranuclear ER

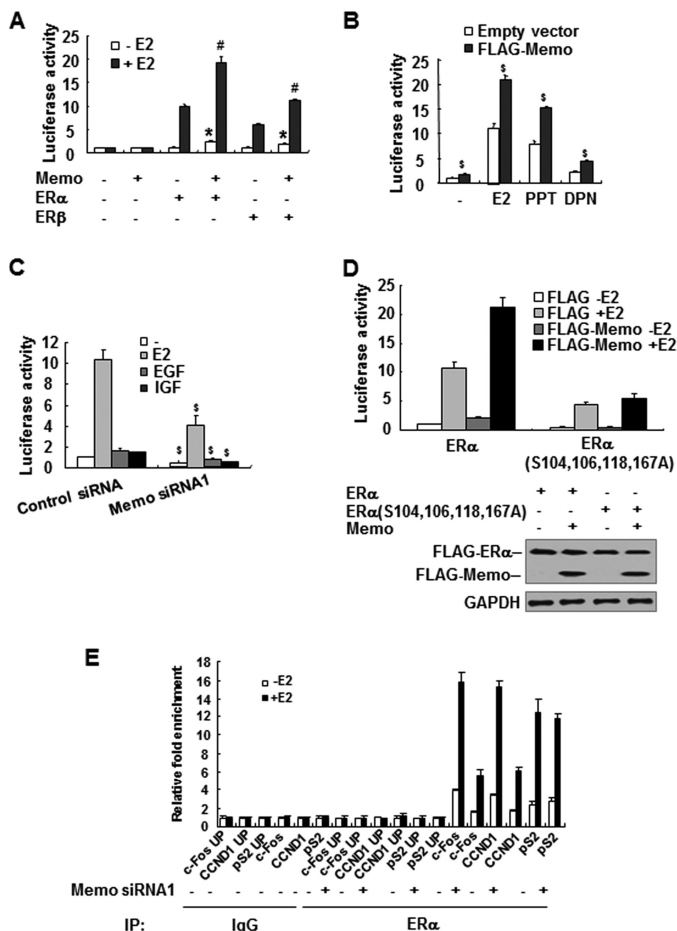


FIGURE 3. MEMO increases ER transcriptional activity as well as ER recruitment to estrogen-responsive promoters. *A* and *B*, luciferase reporter assays of ER α and ER β transcriptional activity in SKBR3 (*A*) or MCF7 (*B*) cells transfected with ERE-LUC and FLAG-tagged MEMO with or without ER α or ER β and treated with 10 nM E₂, 1 nM propylpyrazole triol, or 1 nM diarylpropionitrile for 24 h. The results shown are means \pm S.D. of three independent experiments. *, $p < 0.01$ versus ER α or ER β in the absence of E₂. #, $p < 0.01$ versus ER α or ER β in the presence of E₂. \$, $p < 0.01$ versus corresponding to empty vector. *C*, luciferase reporter assays of ER transcriptional activity in MEMO knockdown MCF7 cells (Fig. 2*B*) transfected with ERE-LUC and treated with 10 nM E₂, 100 ng/ml EGF, or 100 ng/ml IGF1. The results shown are means \pm S.D. of three independent experiments. \$, $p < 0.01$ versus corresponding control siRNA. *D*, luciferase reporter assays of ER α transcriptional activity in SKBR3 cells transfected with ERE-LUC and FLAG-tagged MEMO with wild-type ER α or ER α (S104A,S106A,S118A,S167A). The results shown are means \pm S.D. of three independent experiments. \$, $p < 0.01$ versus corresponding control siRNA. *Bottom*, immunoblot analysis with anti-FLAG in the presence of E₂. *E*, ChIP analysis of the occupancy of ER α on the indicated estrogen-responsive promoters in MCF7 cells stably transfected with control siRNA or MEMO siRNA1 and treated with 10 nM E₂ for 1 h. UP, upstream of the indicated promoters. IgG, normal serum; IP, immunoprecipitation.

ZR75-1 cells (supplemental Fig. 4, *D* and *E*). Moreover, 4-OHT and ICI182,780 antagonized the estrogen-mediated effect on the target gene expression in both control siRNA- and MEMO siRNA-transfected MCF7 cells (Fig. 4*C*).

To investigate whether activation of MAPK and AKT is responsible for MEMO modulation of ER α phosphorylation and target gene expression, PD98059 and LY294002, which are MAPK and PI3K/AKT inhibitors, respectively, were used to treat MCF7 cells stably transfected with either MEMO or empty vector. As expected, the MAPK and AKT inhibitors decreased phosphorylation of ERK1/2 and AKT as well as phosphorylation of ER α at serines 104, 106, 118, and 167 (Fig. 4*D*).

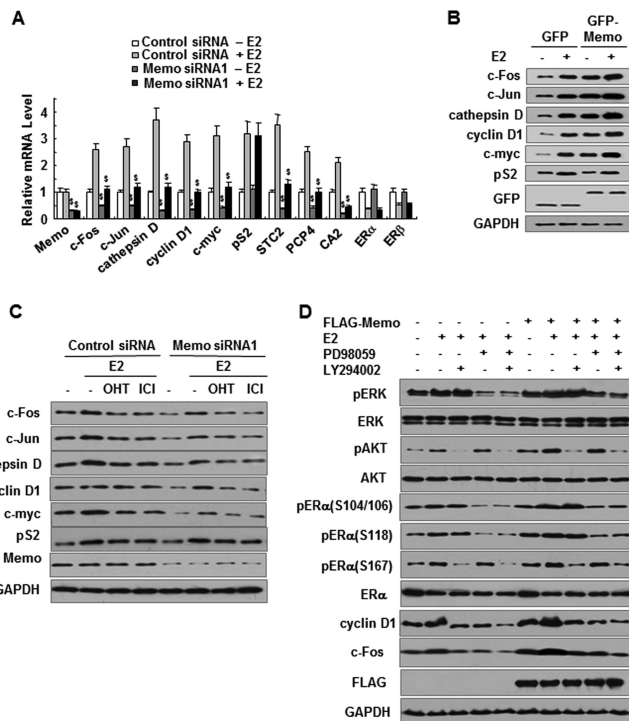


FIGURE 4. MEMO enhances ER α phosphorylation as well as the expression of estrogen-responsive genes through activation of ERK1/2 and AKT. *A*, real time RT-PCR analysis of estrogen-responsive genes in MEMO knockdown MCF7 cells treated with 10 nM E₂ for 24 h. *B* and *C*, immunoblot analysis of estrogen-responsive gene expression in MCF7 cells stably transfected with GFP-tagged MEMO (*B*) or MEMO siRNA1 (*C*) in the presence of 10 nM E₂, 10 nM E₂ plus 100 nM 4-OHT, or 10 nM E₂ plus 10 nM ICI182,780 for 24 h. *D*, MEMO stably expressed MCF7 cells were treated with E₂ or E₂ plus PD98059 or E₂ plus LY294002 for 24 h. Cell lysates were blotted with the indicated antibodies.

Such inhibition impaired the effect of MEMO on the expression of the estrogen-responsive proteins cyclin D1 and c-Fos. These data suggest that MEMO increases ER α phosphorylation and estrogen-responsive gene expression at least in part through activated MAPK and AKT.

MEMO and ER α Interaction Is Required for Activation of MAPK and AKT as Well as Estrogen-responsive Gene Expression—To further determine whether the interaction of MEMO and ER α is necessary for activation of MAPK and AKT as well as estrogen-responsive gene expression, we first used deletion analysis to map the interaction domains of ER α in coimmunoprecipitation assays. The AF1 and AF2 domains of ER α interacted with MEMO, whereas the ER α DNA binding domain did not (Fig. 5*A*). Next, we determined which MEMO protein region mediates interaction with ER α by GST pull-down assay. GST-MEMO(75–146) and GST-MEMO(140–211) bound ER α , whereas GST-MEMO(1–81), GST-MEMO(205–297), or GST alone did not (Fig. 5*B*). The MEMO(75–146) and the MEMO(140–211) interacted with ER α more weakly than full-length MEMO, suggesting that both regions of MEMO are required for maximal interaction with ER α . The AF1 and AF2 domains of ER α also interacted with MEMO(75–146) and MEMO(140–211), and MEMO(140–211) had higher binding affinity to the AF1 and AF2 of ER α than MEMO(75–146) (Fig. 5*C*). Further domain mapping showed that deletion of the amino acid region 161–204 of MEMO greatly attenuated the

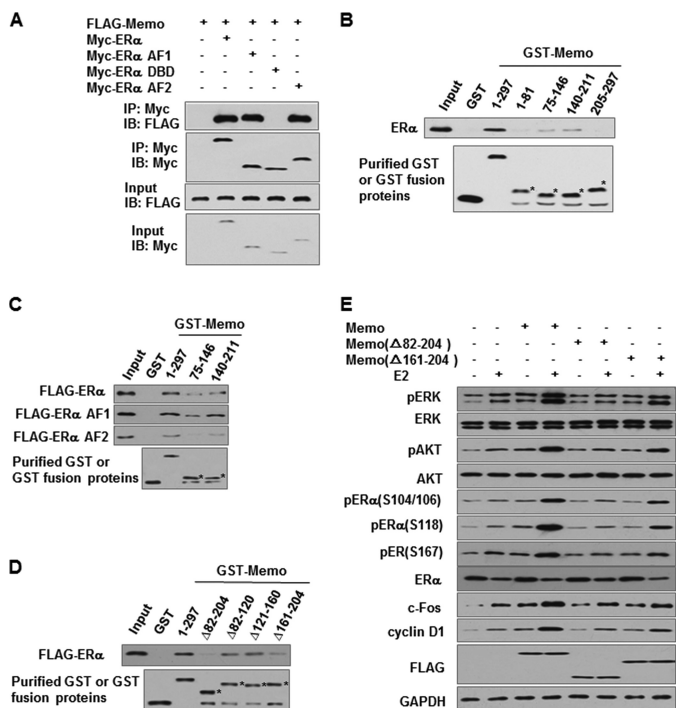


FIGURE 5. Interaction of MEMO with ER α is required for MEMO modulation of phosphorylation of ERK1/2, AKT, and ER α as well as the expression of estrogen-responsive genes. *A*, mapping of the MEMO interaction regions in ER α . HEK293T cells transfected with FLAG-tagged MEMO and Myc-tagged ER α , ER α AF1, ER α DNA binding domain, or ER α AF2 were immunoprecipitated (IP) with anti-Myc followed by immunoblotting (IB) with the indicated antibodies. *B–D*, mapping of the regions of MEMO that interact with ER α . Purified GST or GST-MEMO deletion mutant proteins were incubated with lysates of HEK293T cells expressing FLAG-tagged ER α (*B–D*), ER α AF1, or ER α AF2 (*C*). Bound proteins were subjected to Western blot with anti-FLAG. Asterisks indicate the positions of the expected purified proteins. *E*, immunoblot analysis of activation of ERK1/2 and AKT, ER α phosphorylation, and estrogen-responsive gene expression in MCF7 cells transfected with FLAG-tagged MEMO, MEMO(Δ 82–204), or MEMO(Δ 161–204) and treated with 10 nM E₂ for 24 h.

interaction of MEMO with ER α (Fig. 5*D*). Importantly, the MEMO(Δ 82–204) deletion mutant, which lacks two ER α -binding sites, or the MEMO(Δ 161–204) deletion mutant, which greatly reduces the interaction of MEMO with ER α , completely or almost abolished the ability of MEMO to activate MAPK and AKT, ER α phosphorylation, and estrogen-responsive gene expression (Fig. 5*E*). Combined with the previous results (Fig. 2*C*), these data suggest that interaction of MEMO with ER α is required for MEMO modulation of ER α function.

MEMO Activation of MAPK and AKT Requires IGF1R and ErbBs—Phosphorylation of MAPK and AKT plays a critical role in transducing signals from membrane ER to nuclear ER (6). ER has no intrinsic kinase domain and thus is not capable of phosphorylating other proteins. Like ER, MEMO also did not have intrinsic kinase activity by an *in vitro* kinase assay (data not shown). It has been reported that ER initiates E₂ rapid non-genomic signals by forming a protein complex with many signaling molecules, including IGF1R and EGFR/ErbB1, which have kinase activities (31). The formation of the protein complex leads to the activation of MAPK and AKT pathways. MEMO has been shown to interact with ERBB2, another EGFR family member (15). Thus, we hypothesized that MEMO may activate MAPK and AKT through its interaction with IGF1R

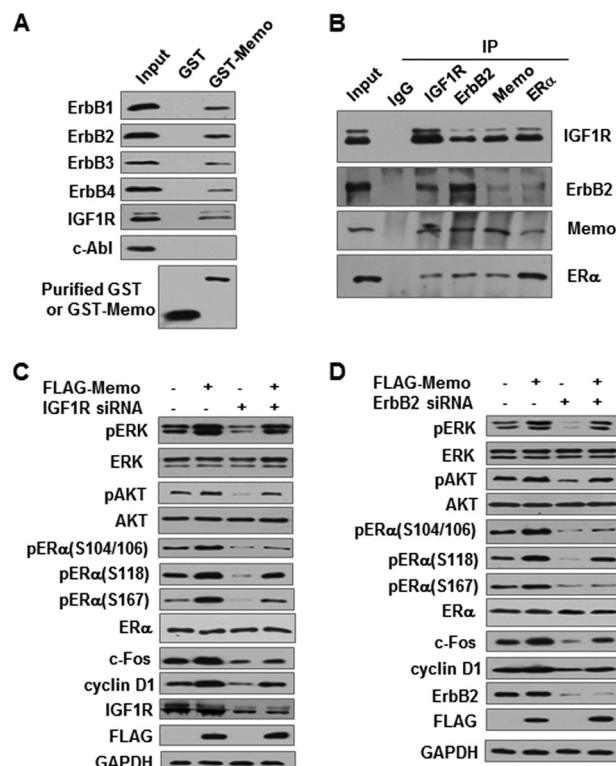


FIGURE 6. MEMO activates ERK1/2 and AKT and increases ER α phosphorylation through its interaction with IGF1R and ERBB2. *A*, GST pull-down analysis of purified GST or GST-MEMO fusion protein incubated with MCF7 whole cell lysates. Bound proteins were subjected to Western blot with the indicated antibodies. *B*, identification of MEMO, ER α , ERBB2, and IGF1R within the same complex by reciprocal coimmunoprecipitation assays. MCF7 cell lysates were immunoprecipitated with each of the antibodies or normal IgG as the control. All the immunoprecipitates were detected by immunoblotting with the indicated antibodies. *C* and *D*, immunoblot analysis of phosphorylation of ERK1/2, AKT, and ER α as well as the expression of estrogen-responsive genes in MEMO-expressing MCF7 cells infected with lentiviral particles carrying IGF1R siRNA (*C*) or ERBB2 siRNA (*D*).

and the members of the EGFR family. Indeed, GST pull-down experiments showed that MEMO interacted not only with IGF1R but also with the EGFR family members ErbB1–4 (Fig. 6*A*). As a control, MEMO did not interact with the nonreceptor tyrosine kinase *c-Abl* (cellular Abelson leukemia oncogene). These results suggested that MEMO, ER α , and IGF1R or ErbBs might coexist in the same functional complex. To verify this, we performed reciprocal coimmunoprecipitation assays with each of the antibodies against these proteins. MEMO, ER α , IGF1R, and ERBB2 could form a protein complex in MCF7 cells (Fig. 6*B*).

Next, we investigated whether MEMO activates MAPK and AKT through IGF1R and ERBB2. Knockdown of endogenous IGF1R or ERBB2 in MCF7 cells greatly attenuated MEMO activation of ERK1/2 and AKT, leading to reduced phosphorylation of ER α at serines 104, 106, 118, and 167 as well as reduced expression of the estrogen-responsive proteins *c-Fos* and cyclin D1 (Fig. 6, *C* and *D*). These results suggest that MEMO activates MAPK and AKT and subsequent ER targets through IGF1R and ERBB2.

MEMO Is Required for Estrogen-mediated Breast Tumor Growth—Next, we determined the effect of MEMO on breast cancer cell growth. In assays of anchorage-dependent growth,

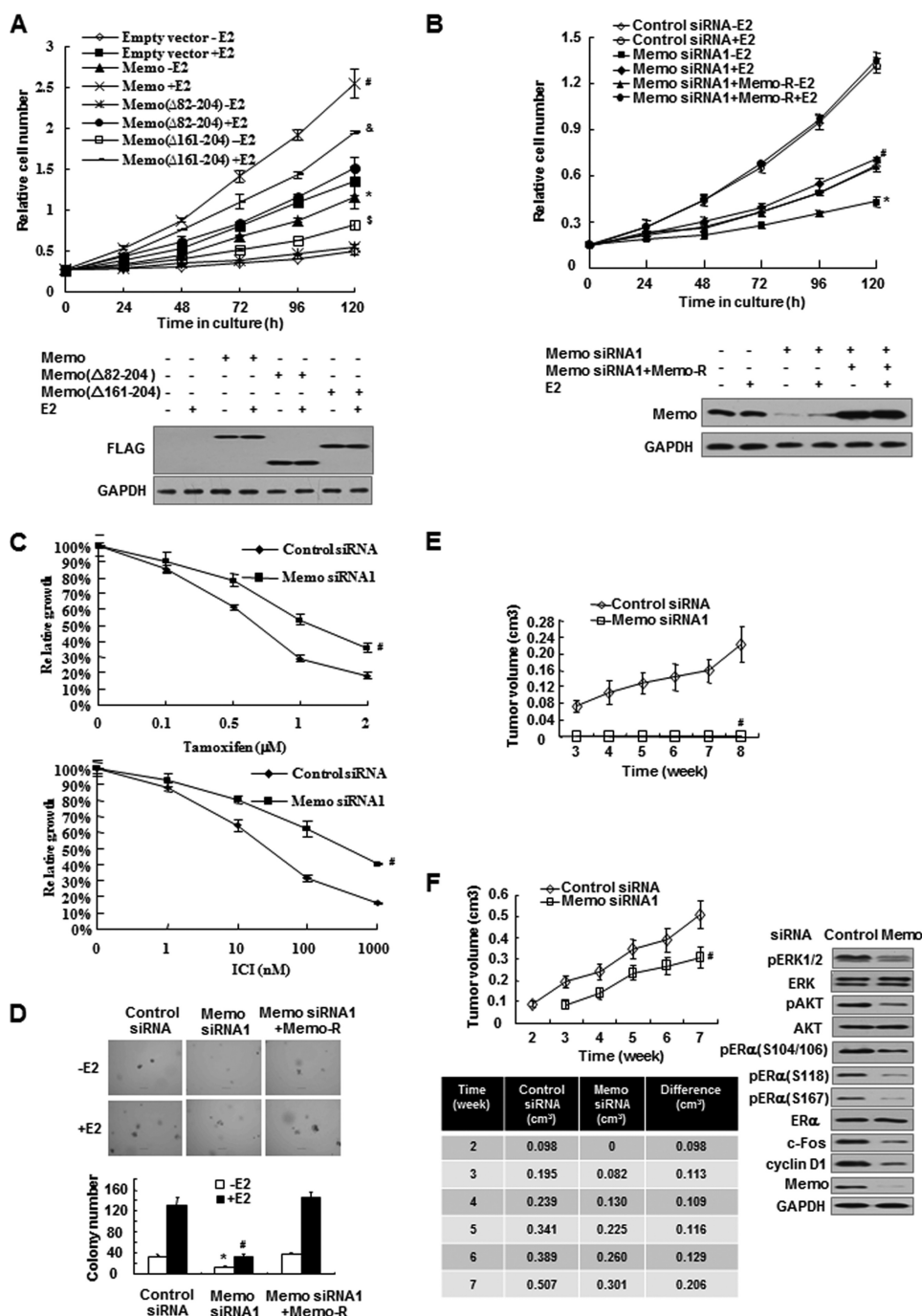


FIGURE 7. MEMO is required for ER α -positive breast tumor growth. *A*, anchorage-dependent growth assays in MCF7 cells transfected with FLAG-tagged MEMO, MEMO(Δ 82-204), or MEMO(Δ 161-204) with or without 10 nM E₂ treatment. Cell viability was assessed at the indicated times. Data are presented as means \pm S.D. of three independent experiments. *Bottom*, immunoblot analysis with anti-FLAG. *B*, anchorage-dependent growth assays in MCF7 cells transfected with MEMO siRNA1 or MEMO siRNA1 plus siRNA-resistant MEMO. Cells were treated and analyzed as in *A*. *Bottom*, immunoblot analysis with anti-MEMO. *C*, MCF7 cells stably transfected with control siRNA or MEMO siRNA1 were treated with different doses of 4-OHT or ICI182,780. Cell viability was assessed 5 days after treatment. *D*, anchorage-independent growth assays in MCF7 cells transfected as in *B*. Scale bar, 50 μ m. *E* and *F*, volume of xenograft tumors derived from MCF7 (*E*) or ZR75-1 (*F*) cells expressing control siRNA or MEMO siRNA1. Detailed tumor volumes were listed in the table (*F*). Data are presented as mean \pm S.D. ($n = 10$). Representative tumor tissues were subjected to immunoblot analysis with the indicated antibodies (*F*, right panel). *, $p < 0.01$ versus empty vector or control siRNA without E₂. #, $p < 0.01$ versus empty vector or control siRNA with E₂. \$, $p < 0.05$ versus empty vector or control siRNA without E₂. &, $p < 0.05$ versus empty vector or control siRNA with E₂.

MCF7 cells stably transfected with MEMO grew faster than those transfected with empty vector both in the presence and in the absence of E₂ (Fig. 7A). The above-mentioned deletion mutants, MEMO(Δ 82-204) and MEMO(Δ 161-204), completely or almost abolished the ability of MEMO to increase the

growth of MCF7 cells. In contrast, MEMO knockdown greatly reduced E₂-mediated growth stimulation of MCF7 cells (Fig. 7B), and this phenotype was rescued by MEMO re-expression. Similar results were observed in ZR75-1 cells (supplemental Fig. 5, A and B). As determined by anchorage-dependent

growth assays, knockdown of MEMO reduced the sensitivity of MCF7 cells to the estrogen antagonists 4-OHT and ICI182,780 (Fig. 7C). MEMO knockdown also greatly inhibited anchorage-independent growth of MCF7 and ZR75-1 cells in the presence or absence of E_2 (Fig. 7D and supplemental Fig. 5, C and D), and again, the observed effects were rescued by MEMO re-expression in MCF7 cells (Fig. 7D). Furthermore, all mice inoculated with MCF7 or ZR75-1 cells expressing control siRNA developed tumors in the presence of E_2 but not in the absence of E_2 (Fig. 7, E and F, and data not shown), suggesting that both MCF7 and ZR75-1 cell lines are estrogen-dependent. In contrast, none of the mice inoculated with MCF7 cells expressing MEMO siRNA developed tumors in the presence of E_2 (Fig. 7E), and mice inoculated with ZR75-1 cells expressing MEMO siRNA showed late latency and a much smaller tumor size (Fig. 7F). The tumors in mice inoculated with ZR75-1 cells expressing MEMO siRNA had reduced phosphorylation of ERK1/2, AKT, ER α (Ser-104 and Ser-106), ER α (Ser-118), and ER α (Ser-167), as well as expression of c-Fos and cyclin D1 (Fig. 7F).

DISCUSSION

In this study, we have identified MEMO as a new component of extranuclear ER α signaling. MEMO integrates extranuclear and nuclear ER α actions. Importantly, MEMO plays an essential role in ER α -positive breast cancer cell growth through regulation of many growth-related genes. Thus, the findings provide novel mechanistic insights into the growth of ER α -positive breast cancer cells, the number of which increases during breast cancer development.

Several membrane and cytoplasmic adaptor proteins, including caveolins (32, 33), striatin (34), p130Cas (35), the adaptor protein Shc (36), hematopoietic PBX-interacting protein (37, 38), and modulator of nongenomic action of estrogen receptor/proline-, glutamic acid-, and leucine-rich protein (MNAR/PELP1) (39), have been shown to interact with membrane/cytoplasmic ER α . E_2 increases the interaction of ER α with these proteins. However, MEMO interacts with ER α and regulates ER α transcriptional activity in an E_2 -independent manner. It has been reported that stimulation of numerous growth factor receptors leads to a ligand-independent increase in ER α transcription, presumably by ER α phosphorylation (10). Indeed, MEMO forms a complex with the growth factor receptors IGF1R and ERBB2, resulting in the phosphorylation of the ligand-independent AF1 domain of ER α . The fact that E_2 -independent activation of ER α by MEMO suggests that MEMO may play roles in the development and progression of both E_2 -independent and -dependent cancers. Because two to three proteins are usually studied for protein-protein interaction, whether there is more than one extranuclear ER α -containing complex remains to be investigated.

Extranuclear ER α initiates rapid action response by binding to estrogen. Although we grew cells with phenol red-free DMEM supplemented with 10% charcoal-stripped FBS, there may be residual estrogens in the culture media, which activate rapid estrogen response, followed by ER α phosphorylation and transcriptional activation. In addition, the charcoal-stripped FBS also contained many growth factors, which activate growth factor signaling and may cross-talk with estrogen signaling (40).

The fact that knockdown of MEMO can inhibit basal ER α transcriptional activity and MEMO regulates ER α transcriptional activity in a ligand-independent manner suggests that a small amount of estrogen may be sufficient for MEMO modulation of ER α transcriptional activation.

It has been shown that the ligand-independent activity of ER α is a result of ER α phosphorylation at multiple sites, including serines 104, 106, 118, 167, and 305 (41). Phosphorylation of ER α at these sites increases ER α transcriptional activity. However, the clinical significance of ER α phosphorylation at these sites is complicated. It has been reported that phosphorylation at some sites in ER α is associated with a better clinical outcome, whereas phosphorylation at other sites is associated with a poorer clinical outcome most often in patients treated with tamoxifen (42). For instance, ER α phosphorylation at serine 118 or 167 is associated with a better clinical outcome in patients treated with tamoxifen, whereas elevated phosphorylation at serine 305 predicts tamoxifen resistance. The clinical significance of ER α phosphorylation at serines 104 and 106 is unknown. Our study showed that MEMO increased ER α phosphorylation at serines 104, 106, 118, and 167, and tamoxifen antagonized such effect. Moreover, knockdown of MEMO induced tamoxifen resistance in cultured breast cancer cells. It will be interesting to determine whether MEMO is a predictive marker in breast cancer patients treated with tamoxifen.

Overexpression of ERBB2 is associated with aggressive breast tumors that are more likely to metastasize (18). MEMO was shown to be required for ERBB2-driven breast cancer cell migration (15). Whether MEMO has other biological functions remains unknown. We demonstrate that MEMO plays an essential role in ER α -positive breast cancer cell growth. MEMO not only activates MAPK and AKT, two important growth regulatory protein kinases (43), but also enhances the expression of many growth-related estrogen-responsive proteins, including c-Fos (44), c-Jun (45), cyclin D1 (46), and c-myc (47). c-Fos and c-Jun belong to the activating protein-1 (AP-1) transcription factor that plays a critical role in tumorigenesis and progression. They regulate the expression of AP-1 target genes by forming heterodimer (c-Jun/c-Fos) or homodimer (c-Jun/c-Jun) and by binding to the AP-1 site within gene promoters. Both c-Fos and c-Jun are overexpressed in human breast cancer. c-Fos knockdown with antisense cDNA suppresses breast cancer cell growth. c-Jun overexpression in MCF7 cells produces a tumorigenesis-, invasive-, and hormone-resistant phenotype. Overexpression of cyclin D1 is common in human cancers of epithelial cell origin. Cyclin D1 is required for mammary tumorigenesis induced by ERBB2. Like cyclin D1, c-Myc is also overexpressed in breast tumors. Overexpression of c-Myc contributes to breast cancer development and progression and is associated with poor clinical outcome. The fact that MEMO can regulate many key oncogenes suggests the importance of MEMO in breast cancer development and progression and as a therapeutic target.

MCF7 and ZR75-1 are two well characterized ER α -positive breast cancer cell lines. In our study, ZR75-1 cells are easier to form tumors in the presence of estrogen in nude mice than MCF7 cells. This may be due to differential gene expression profiling between the two cell lines. It has been reported that

ZR75-1 cells selectively use genes for energy, protein synthesis, and sugar metabolism and other pathways differently from MCF7 cells (48). Such characteristics may impart more aggressiveness to ZR75-1 than MCF7. The difference in gene expression between MCF7 and ZR75-1 cells may also affect the ability of MEMO to regulate breast cancer cell growth in nude mice. None of the mice inoculated with MEMO siRNA-expressing MCF7 cells developed tumors in the presence of E₂ and mice inoculated with MEMO siRNA-expressing ZR75-1 cells revealed late latency and a much smaller tumor size. Although MEMO siRNA-expressing ZR75-1 tumors grew with similar kinetics to control tumors 2–6 weeks after inoculation, significant different kinetics seems to be observed 7 weeks after inoculation. This trend might be more obvious if tumors had grown for more than 7 weeks.

Overexpression of ERBB2 occurs in ~30% of breast cancer and is associated with poor clinical outcome such as shorter survival and shorter time to relapse (18). ERBB2 is therefore an attractive target for drug development. Accordingly, humanized monoclonal antibodies against ERBB2 (Herceptin[®], trastuzumab) have been developed and used in the treatment of patients with breast cancer overexpressing ERBB2. However, not all patients whose tumors overexpress ERBB2 respond to trastuzumab treatment (49). The mechanisms underlying trastuzumab resistance include the inability or reduced capacity of trastuzumab binding to ERBB2 and activation of downstream signaling pathways such as PI3K/AKT signaling. In some breast cancer patients, trastuzumab is unable to interfere with the ERBB2 heterodimers by EGFR or ErbB3. Interaction of ERBB2 with other proteins such as mucin-4 sterically hinders ERBB2 from binding to trastuzumab (49). Activation of PI3K/AKT signaling has been considered as the major determinant of trastuzumab resistance. Trastuzumab-mediated growth inhibition was lost in breast cancer cells that overexpressed both IGF1R and ERBB2. This may be due to cross-talk between IGF1R and ERBB2 signaling (50). IGF1R and ERBB2 interact with each other and synergistically stimulate PI3K/AKT signaling. The findings that MEMO, ER α , IGF1R, and ERBB2 form a complex and MEMO activates the AKT pathway raise the possibility that MEMO may be involved in trastuzumab resistance. Whether MEMO prevents ERBB2 from binding to trastuzumab remains to be elucidated. The essential role of MEMO in ER α -positive breast tumor growth might make it an interesting target for breast cancer therapy.

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