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Research Article

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PES1 promotes breast cancer by differentially regulating ERα and ERβ

Long Cheng,[1,2] Jieping Li,[1,3] Yongjian Han,[1] Jing Lin,[4] Chang Niu,[1] Zhichao Zhou,[1] Bin Yuan,[1] Ke Huang,[5] Jiezhi Li,[1] Kai Jiang,[1] Hao Zhang,[1] Lihua Ding,[1] Xiaojie Xu,[1] and Qinong Ye[1,2]

[1]Department of Medical Molecular Biology, Beijing Institute of Biotechnology, Beijing, People's Republic of China. [2]Institute of Cancer Stem Cell, Cancer Center, Dalian Medical University, Liaoning, People's Republic of China. [3]Department of Clinical Laboratory, Fuzhou General Hospital of Fujian Corps, CAPF, Fuzhou, People's Republic of China. [4]Department of Clinical Laboratory, First Affiliated Hospital, and [5]Department of Obstetrics and Gynecology, Chinese PLA General Hospital, Beijing, People's Republic of China.

The initiation of breast cancer is associated with increased expression of tumor-promoting estrogen receptor α (ERα) protein and decreased expression of tumor-suppressive ERβ protein. However, the mechanism underlying this process is unknown. Here we show that PES1 (also known as Pescadillo), an estrogen-inducible protein that is overexpressed in breast cancer, can regulate the balance between ERα and ERβ. We found that PES1 modulated many estrogen-responsive genes by enhancing the transcriptional activity of ERα while inhibiting transcriptional activity of ERβ. Consistent with this regulation of ERα and ERβ transcriptional activity, PES1 increased the stability of the ERα protein and decreased that of ERβ through the ubiquitin-proteasome pathway, mediated by the carboxyl terminus of Hsc70-interacting protein (CHIP). Moreover, PES1 transformed normal human mammary epithelial cells and was required for estrogen-induced breast tumor growth in nude mice. Further analysis of clinical samples showed that expression of PES1 correlated positively with ERα expression and negatively with ERβ expression and predicted good clinical outcome in breast cancer. Our data demonstrate that PES1 contributes to breast tumor growth through regulating the balance between ERα and ERβ and may be a better target for the development of drugs that selectively regulate ERα and ERβ activities.

Introduction

The association between estrogen and breast cancer was recognized over 100 years ago. Estrogen exerts its function through its 2 nuclear receptors, estrogen receptor α (ERα) and ERβ (1, 2). ER belongs to a superfamily of ligand-activated transcription factors that share structural similarity characterized by several functional domains. N-terminal estrogen-independent and C-terminal estrogen-dependent activation function domains (AF1 and AF2, respectively) contribute to the transcriptional activity of the 2 receptors. The DNA-binding domain of the ERs is centrally located. The ligand-binding domain, overlapping AF2, shows 58% homology between ERα and ERβ. The DNA-binding domain is identical between the 2 receptors, except for 3 amino acids. However, the AF1 domain of ERβ has only 28% homology with that of ERα. The binding of estrogen to ER leads to ER dimerization and its recruitment to the estrogen-responsive elements (EREs) on the promoters of ER target genes, thereby either enhancing or repressing gene activation.

The development of breast cancer is associated with dysregulation of ER expression (3–8). Compared with that in normal breast tissues, the proportion of cells expressing ERα is increased, whereas ERβ expression is reduced, in hormone-dependent breast tumors. The ratio of ERα/ERβ expression is higher in breast tumors than in normal tissues, and ERα and ERβ are antagonistic to each other. ERα mediates the tumor-promoting effects of estrogens, whereas ERβ inhibits breast cancer cell growth. ERβ reduces cell proliferation induced by ERα activation. Although ERα and ERβ have been shown to have a yin-yang relationship in breast tumorigenesis, the molecular mechanism underlying this process remains unclear.

In this study, we show that *PES1* (also known as Pescadillo) plays an essential role in estrogen-induced breast tumor growth through regulation of the yin-yang balance between ERα and ERβ and is the first such gene to be identified to our knowledge. PES1, a breast cancer–associated gene 1 (BRCA1) C-terminal (BRCT) domain-containing protein, is estrogen inducible, and its expression gradually increases during breast cancer development and progression (9–11). Theoretically, in the treatment of patients with ERα-positive breast cancer, in which ERβ is antagonistic to ERα, a drug that decreases transcriptional activity of ERα but increases that of ERβ should be better than the currently used endocrine drugs tamoxifen or fulvestrant, which decrease both ERα and ERβ transactivation (12, 13). We show that, through the ubiquitin-proteasome pathway, PES1 enhances ERα levels but reduces ERβ protein levels, correlating with their respective physiological activities in breast cancer. Thus, PES1 may represent a very promising target for the development of better drugs for breast cancer endocrine therapy.

Results

PES1 differentially regulates transcriptional activity of ERα and ERβ as well as their target genes. To define the exact role of PES1 in breast tumor growth, we investigated whether PES1 regulates estrogen signaling. PES1 overexpression in ERα- and ERβ-positive MCF7 cells (Figure 1A), ERα-positive and ERβ-negative ZR75-1 and T47D cells (Supplemental Figure 1, A and B; supplemental material available online with this article; doi:10.1172/JCI62676DS1), and ERα- and ERβ-negative SKBR3 (Figure 1B) breast cancer cells increased transcription of a luciferase reporter construct containing the ERE in response to the ERα-specific agonist propylpyrazole triol (PPT) but decreased ERE reporter transcription in response to the ERβ-specific agonist diarylpropionitrile (DPN). This effect was PES1 specific because expression of the known

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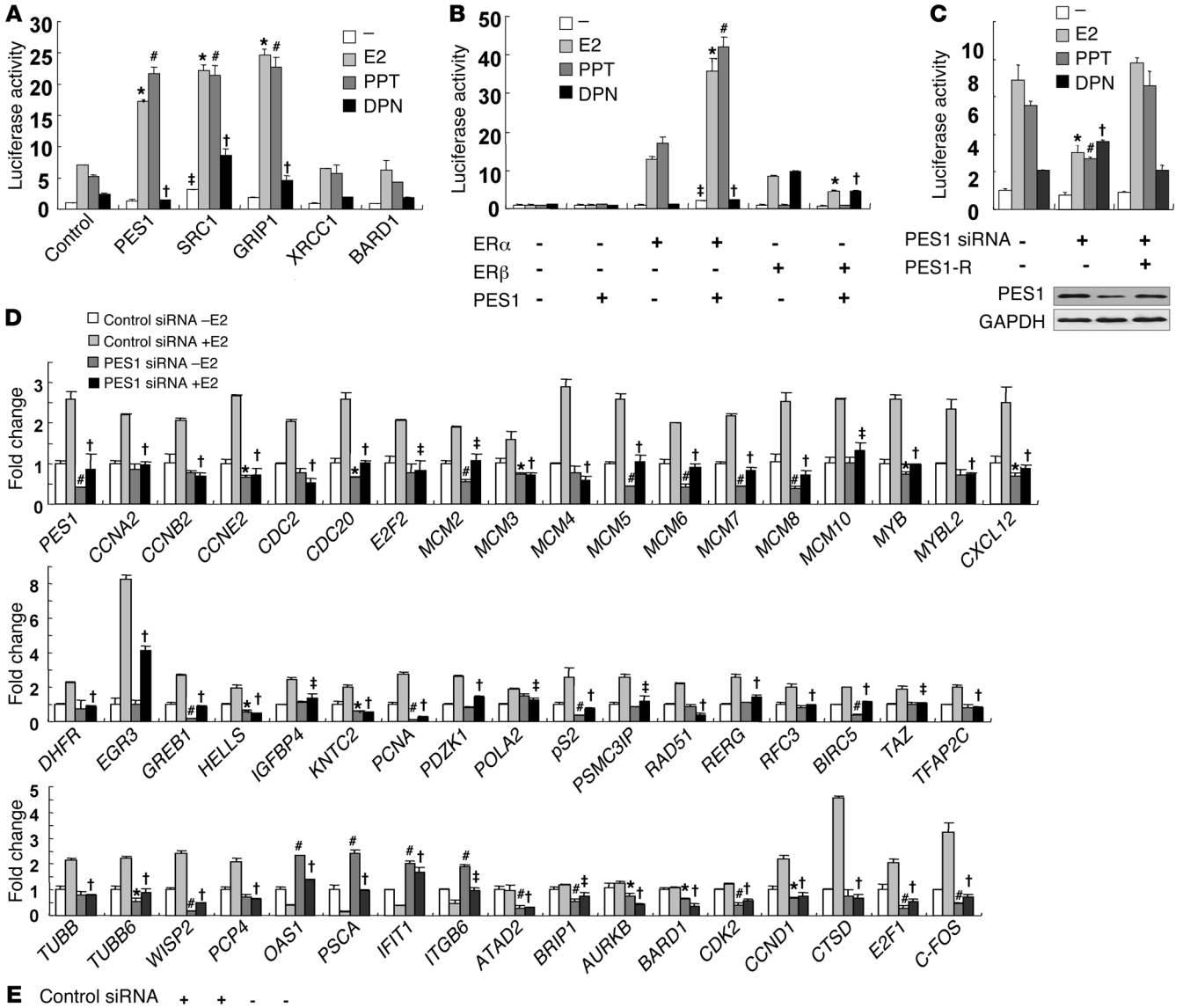

Figure 1

PES1 differentially regulates transcriptional activity of ERα and ERβ and expression of their target genes. (**A** and **B**) Luciferase reporter assays of ERα and ERβ transcriptional activity in (**A**) MCF7 or (**B**) SKBR3 cells transiently transfected with ERE-LUC and PES1, SRC1, GRIP1, XRCC1, or BARD1 with or without ERα or ERβ and 24-hour treatment with 10 nM E2, 1 nM PPT, or 1 nM DPN. Results shown are mean ± SD of 3 independent experiments. $^{‡}P < 0.01$, $^{*}P < 0.01$, $^{\#}P < 0.01$, $^{†}P < 0.01$ versus empty vector in the (**A**) absence or (**B**) presence of ERα or ERβ with vehicle (–), E2, PPT, and DPN, respectively. (**C**) Luciferase reporter assays in MCF7 cells stably transfected with PES1 siRNA or PES1 siRNA plus siRNA-resistant PES1 (PES1-R) and treated as above. Immunoblot analysis of PES1 expression is shown. Results shown are mean ± SD of 3 independent experiments. $^{*}P < 0.01$, $^{\#}P < 0.01$, $^{†}P < 0.01$ versus control siRNA with E2, PPT, and DPN, respectively. (**D**) Real-time RT-PCR analysis of 47 genes identified by cDNA microarray in our study and 4 genes identified in other studies (*CCND1*, *CTSD*, *E2F1*, and *C-FOS*) in PES1 knockdown MCF7 cells treated or not treated with E2 (+E2 or –E2, respectively) for 24 hours. Data shown are mean ± SD of triplicate measurements that have been repeated 3 times with similar results. $^{*}P < 0.05$, $^{\#}P < 0.01$ versus control siRNA without E2. $^{‡}P < 0.05$, $^{†}P < 0.01$ versus control siRNA with E2. (**E**) Immunoblot analysis of estrogen-responsive gene expression in PES1 knockdown MCF7 cells.

ER cofactors, steroid receptor coactivator-1 (SRC1) or glutamate receptor-interacting protein 1 (GRIP1), did not oppositely regulate ERα and ERβ transactivation, and other BRCT domain–containing proteins, X-ray repair complementing defective repair in Chinese hamster cells 1 (XRCC1) and BRCA1-associated RING domain protein 1 (BARD1), had no effect. As expected, SRC1 and GRIP1 increased the transcriptional activity of both ERα and ERβ (Figure 1A). In contrast, siRNA knockdown of endogenous PES1 reduced transcriptional activity of ERα and enhanced that of ERβ in MCF7 cells (Figure 1C). These effects could be rescued by PES1 reexpression in PES1 knockdown MCF7 cells.

Next, we performed cDNA microarray analysis to monitor gene expression profiles in PES1 knockdown MCF7 cells. In the presence of 17β-estradiol (E2), PES1 regulated the expression of 256 genes, including over 127 previously reported E2-regulated genes (refs. 14–22, Supplemental Figure 2, and Supplemental Tables 1–3). Real-time RT-PCR confirmed the PES1-mediated expression of 47 genes identified in our study and 4 well-known E2-regulated genes (cyclin D1 [*CCND1*], cathepsin D [*CTSD*], E2F transcription factor 1 [*E2F1*], and *C-FOS*) identified in other studies (refs. 14–22 and Figure 1D). The expression of many estrogen-responsive genes known to have important functions in DNA replication (23, 24) and cell cycle regulation (24) was found to be downregulated by PES1 knockdown, including replication factor C (*RFC*), minichromosome maintenance genes, proliferating cell nuclear antigen (*PCNA*), *E2F1*, *E2F2*, *MYB*, *MYC*, cyclin-dependent kinase 1 (*CDC2*), *CCND1*, and survivin (*BIRC5*). Interestingly, some of these genes, such as *MYC*, *CCND1*, *PCNA*, *E2F1*, and *BIRC5*, were reported to be activated by ERα but to be repressed by ERβ (14–22). Consistent with the results of PES1 knockdown, PES1 overexpression increased the transcription of *E2F1*, *CCND1*, cyclin E2 (*CCNE2*), and *CTSD* in the presence and/or absence of E2, with higher magnitude in the presence of E2 (Supplemental Figure 3A). The expression of 10 representative estrogen-responsive genes was further confirmed by immunoblotting (Figure 1E).

PES1 differentially regulates the dimerization and promoter occupancy of ERα and ERβ. Dimerization is a regulatory mechanism of controlling transcription factor activity. Upon dimerization, transcription factors bind to promoter sequences of target genes. ERα and ERβ homodimerization is thought to be critical for ERα and ERβ transcriptional activity, whereas ERα-ERβ heterodimerization facilitates inhibition of ERα transactivation by ERβ (25). In agreement with the findings that ERα and ERβ transcriptional activity was oppositely regulated by PES1, overexpression of PES1 increased ERα homodimerization (Figure 2A) and decreased ERβ homodimerization and ERα-ERβ heterodimerization (Figure 2B). Like ERα and ERβ, PES1 was recruited to the estrogen-responsive *CTSD*, *CCND1*, *E2F1*, and *CCNE2* promoters but not to an unrelated β-actin promoter (Figure 2C). In addition, unlike ERα and ERβ (26, 27), PES1 was not recruited to the distal enhancers of *CTSD* and *CCND1* (Figure 2C). Importantly, consistent with the results of ERα and ERβ transactivation, which was oppositely regulated by PES1, PES1 knockdown decreased ERα promoter occupancy but increased that of ERβ (Figure 2D).

PES1 has been shown to directly interact with the cadmium response element (CdRE) of the heme oxygenase-1 (HO1) promoter (28). We searched potential CdREs of the *CTSD*, *CCND1*, *E2F1*, and *CCNE2* genes and found that *CCND1* and *E2F1* had putative CdREs. The results of EMSA demonstrated that PES1 bound indeed to the CdRE of the HO1 promoter (Figure 2E). However, PES1 did not bind to the putative CdREs of *CCND1* and *E2F1*, suggesting that PES1 may regulate estrogen-responsive gene transcription through EREs.

PES1 oppositely modulates ERα and ERβ protein stability. To investigate how PES1 increases transactivation of ERα but decreases that of ERβ, we examined the effects of PES1 on ERα and ERβ expression. In the absence or presence of E2, PES1 knockdown reduced ERα protein expression but enhanced ERβ protein levels in MCF7 cells (Figure 3A). Reexpression of PES1 in the knockdown cells rescued this effect. PES1 knockdown did not alter *ERα* and *ERβ* mRNA levels in MCF7 cells (Supplemental Figure 3B). Similar trends were obtained in ZR75-1 cells and normal human mammary epithelial cells (HMECs) (Supplemental Figure 3, C–F), suggesting that PES1 regulates ERα and ERβ expression at the posttranscriptional level.

Recent studies show that at least 3 ERβ isoforms, including the wild-type ERβ (ERβ1), ERβ2, and ERβ5, are expressed in breast cancer (29, 30). ERβ is the only fully functional isoform. ERβ2 and ERβ5 can not bind estrogen. All 3 ERβ isoforms can inhibit ERα transcriptional activity (31). Western blot analysis with anti-ERβ or anti-MYC showed that ERβ1/ERβ was indeed expressed in MCF7 cells, because the location of the endogenous band was similar to that of MYC-tagged ERβ1 but not MYC-tagged ERβ2 and ERβ5 (Supplemental Figure 4A). The anti-ERβ used recognized all 3 ERβ isoforms (Supplemental Figure 4A, right panel). In addition, PES1 downregulated ERβ, ERβ2, and ERβ5 (Supplemental Figure 4B).

Since PES1 modulates ERα and ERβ expression at the posttranscriptional level, we first determined the half-life of ERα protein in PES1 knockdown MCF7 cells. In the absence or presence of E2, the half-life of ERα protein in PES1 knockdown cells was reduced from more than 12 hours to approximately 4 hours and from 3 to 2 hours, respectively (Figure 3B). Because ERβ is usually expressed at low levels in breast cancer cell lines, we determined the half-life of ERβ by ERβ overexpression in HEK293T cells. In the absence and presence of E2, PES1 overexpression decreased the half-life of ERβ from approximately 12 to 6 hours and from 9 to 3 hours, respectively (Figure 3C).

Next, we determined effects of PES1 on the protein levels of ERα and ERβ domains. In transfected HEK293T cells, PES1 increased the protein levels of the ERα AF2 domain but reduced the levels of the ERβ AF2 domain (Supplemental Figure 5, A and B). Domain-swapping experiments in which the ERα AF2 domain was exchanged with the ERβ AF2 domain abolished the ability of PES1 to regulate ERα and ERβ protein levels (Supplemental Figure 5C), indicating important roles of the AF2 domains of ERα and ERβ in the regulation of ERα and ERβ protein levels by PES1.

To define the region of PES1 that modulates ERα and ERβ protein levels, we transfected HEK293T cells with a series of PES1 deletion mutants. The 221–322 region of PES1 ($PES1_{\Delta 221–322}$), but not other regions tested, enhanced ERα protein expression, whereas the 311–588 region of PES1 ($PES1_{\Delta 311–415}$) decreased ERβ protein levels (Supplemental Figure 5, D and E). As expected, $PES1_{\Delta 221–322}$ and $PES1_{\Delta 311–415}$ did not change the expression of ERα and ERβ, respectively, but $PES1_{\Delta 221–322}$ reduced ERβ expression, and $PES1_{\Delta 311–415}$ increased ERα expression (Figure 3, D and E). In MCF7 cells, $PES1_{\Delta 221–322}$ decreased ERβ transcriptional activity, and $PES1_{\Delta 311–415}$ increased ERα transcriptional activity, suggesting that the alteration of ERα and ERβ protein levels by $PES1_{\Delta 221–322}$ and $PES1_{\Delta 311–415}$ correlates with their effects on ERα and ERβ transactivation (Figure 3F).

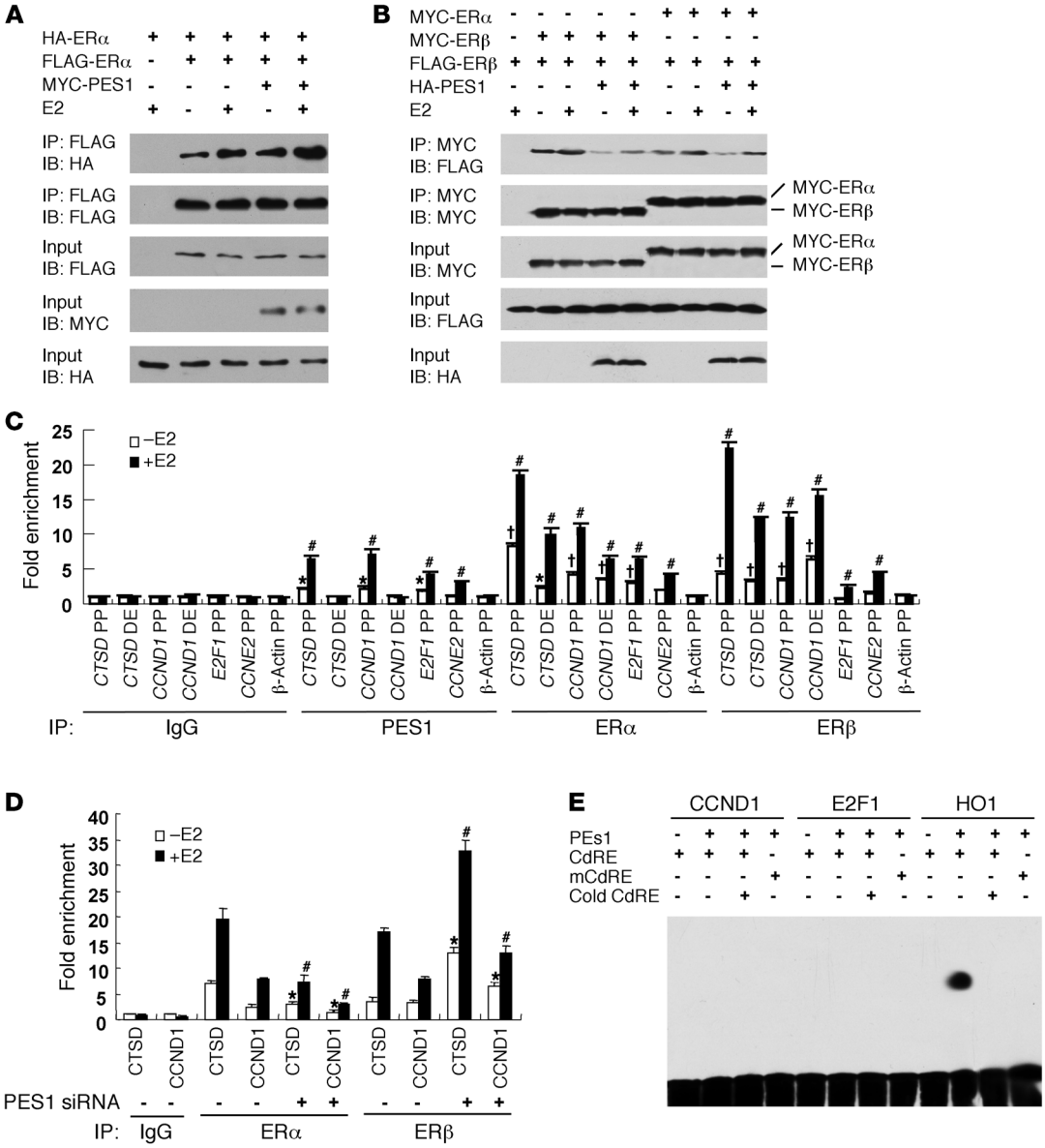

Figure 2

PES1 differentially modulates the dimerization and promoter occupancy of ERα and ERβ. (**A** and **B**) Coimmunoprecipitation analysis of ERα and ERβ homodimerization and ERα-ERβ heterodimerization. HEK293T cells transiently transfected with the indicated HA-, FLAG-, and MYC-tagged constructs were immunoprecipitated with (**A**) anti-FLAG or (**B**) anti-MYC antibodies, followed by immunoblotting as indicated. (**C**) ChIP analysis of the occupancy of PES1, ERα, or ERβ on the indicated estrogen-responsive proximal promoters (PPs) or distal enhancers (DEs) in MCF7 cells treated with 10 nM E2 for 1 hour. The β-actin promoter was included as a negative control. IgG, normal serum. Data shown are mean ± SD of triplicate measurements that have been repeated 3 times with similar results. $^{*}P < 0.05$, $^{\dagger}P < 0.01$ versus respective IgG without E2. $^{\#}P < 0.01$ versus respective IgG with E2. (**D**) ChIP analysis of the occupancy of ERα or ERβ on CTSD and CCND1 promoters in MCF7 cells stably transfected with control siRNA or PES1 siRNA and treated as in **C**. Data shown are mean ± SD of triplicate measurements that have been repeated 3 times with similar results. $^{*}P < 0.01$ versus respective control siRNA without E2. $^{\#}P < 0.01$ versus respective control siRNA with E2. (**E**) EMSA using the in vitro–translated PES1 and the biotin-labeled CdRE or mutated CdRE (mCdRE) probe. Cold probe was used for competition experiments.

The E3 ubiquitin ligase CHIP is important for PES1 modulation of ERα and ERβ protein stability. The finding that PES1 regulates ERα and ERβ protein stability suggests that the ubiquitin-proteasome pathway may be involved in this process. Indeed, addition of the proteasome inhibitor MG132 or lactacystin blocked PES1 knockdown–mediated ERα degradation and PES1 overexpression–mediated ERβ degradation (Figure 4, A and B, and data not shown). Overexpression of PES1 or $PES1_{\Delta 311-415}$, which increases ERα protein levels, reduced ERα ubiquitination, whereas expression of $PES1_{\Delta 211-322}$, which does not increase ERα protein levels, did not (Supplemental Figure 6A). Likewise, overexpression of PES1 or $PES1_{\Delta 211-322}$, which decreases ERβ protein levels, increased ERβ

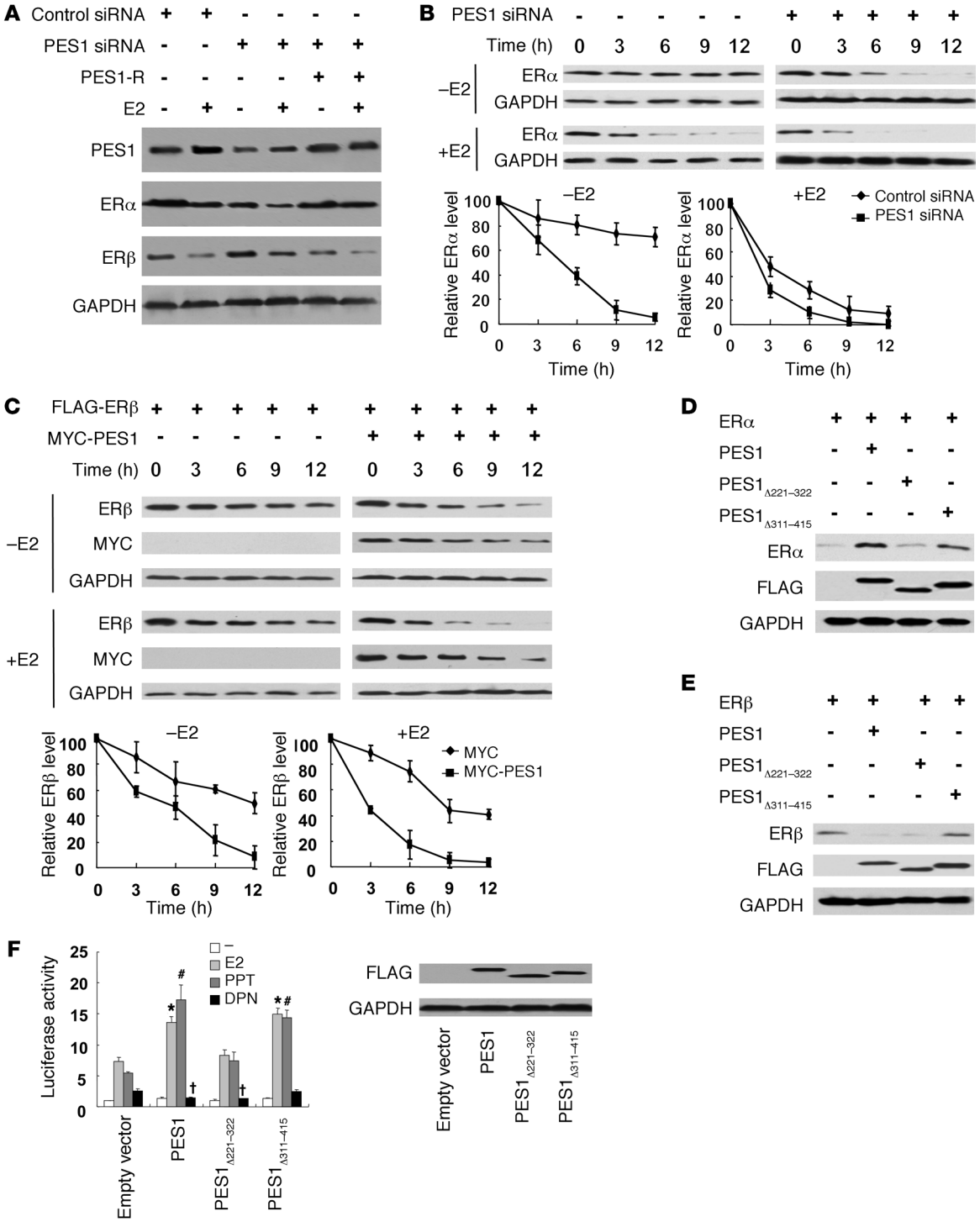

Figure 3

Modulation of ERα and ERβ stability by PES1 correlates with their respective transcriptional activity. (**A**) Immunoblot analysis of MCF7 cells stably transfected with PES1 siRNA or PES1 siRNA plus siRNA-resistant PES1 and treated with E2 for 24 hours. (**B**) Immunoblot analysis of ERα in MCF7 cells stably transfected with control siRNA or PES1 siRNA at the indicated times after exposure to the protein synthesis inhibitor cycloheximide (20 mg/ml) in the absence or presence of 10 nM E2. Graphs show quantification of immunoblot data. (**C**) Immunoblot analysis of ERβ in HEK293T cells transiently transfected with FLAG-tagged ERβ (FLAG-ERβ) and MYC-tagged PES1 (MYC-PES1) and treated as in **B**. (**B** and **C**) Data shown are mean ± SD of 3 independent experiments. (**D** and **E**) Immunoblot showing (**D**) ERα and (**E**) ERβ protein levels in HEK293T cells transiently transfected with ERα or ERβ and FLAG-PES1, FLAG-PES1$_{\Delta 221–322}$, or FLAG-PES1$_{\Delta 311–415}$. (**F**) Luciferase reporter assays of ERα and ERβ transcriptional activity in MCF-7 cells transiently transfected with ERE-LUC and FLAG-tagged PES1, PES1$_{\Delta 221–322}$, or PES1$_{\Delta 311–415}$ and treated with 10 nM E2, 1 nM PPT, or 1 nM DPN for 24 hours. Results shown are mean ± SD of 3 independent experiments. $^{*}P < 0.01$, $^{\#}P < 0.01$, $^{\dagger}P < 0.01$ versus empty vector with E2, PPT, and DPN, respectively. Immunoblot analysis of FLAG-tagged PES1, PES1$_{\Delta 221–322}$, or PES1$_{\Delta 311–415}$ in the presence of 10 nM E2 is shown.

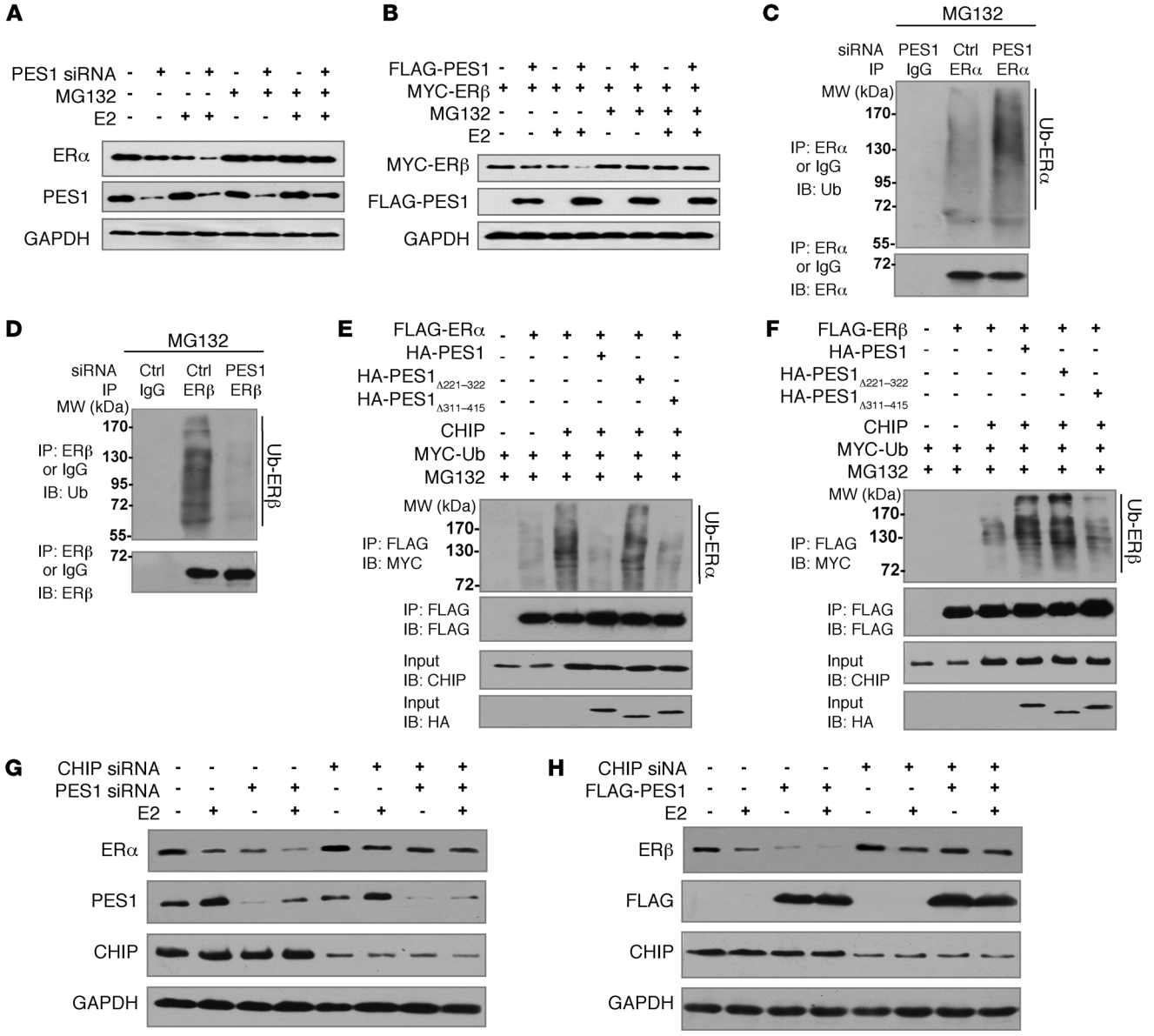

Figure 4

PES1 oppositely regulates ERα and ERβ stability through the CHIP-mediated ubiquitin-proteasome pathway. (**A**) Immunoblot analysis of MCF7 cells stably transfected with PES1 siRNA or control siRNA and treated with 10 nM E2 or 10 nM E2 plus the proteasome inhibitor MG132 (10 μM). (**B**) Immunoblot analysis of HEK293T cells transiently transfected with FLAG-PES1 and MYC-ERβ and treated as in **A**. (**C** and **D**) Ubiquitination of endogenous ERα and ERβ. Cell lysates from MCF-7 cells stably transfected with PES1 siRNA were immunoprecipitated with antibodies specific for (**C**) ERα or (**D**) ERβ, followed by immunoblotting with the indicated antibodies. Ub, ubiquitin. (**E** and **F**) Effects of PES1 on CHIP-mediated ERα and ERβ ubiquitination. Coimmunoprecipitation was performed in HEK293T cells transiently transfected with the indicated constructs. (**G** and **H**) Effects of PES1 on CHIP-mediated degradation of ERα and ERβ. Western blot analysis of MCF-7 cells transfected with (**G**) CHIP siRNA and PES1 siRNA or with (**H**) CHIP siRNA and FLAG-PES1 and treated with 10 nM E2 for 24 hours.

ubiquitination, whereas PES1$_{\Delta 311–415}$, which does not decrease ERβ protein levels, did not (Supplemental Figure 6B). Importantly, PES1 knockdown increased the ubiquitination of endogenous ERα but decreased the ubiquitination of endogenous ERβ in MCF7 cells (Figure 4, C and D).

ERα and ERβ are substrates of carboxyl terminus of Hsc70-interacting protein (CHIP) (32–34), an E3 ubiquitin ligase. Intriguingly, PES1 or PES1$_{\Delta 311–415}$, but not PES1$_{\Delta 211–322}$, reduced CHIP-mediated ERα ubiquitination (Figure 4E). Likewise, PES1 or PES1$_{\Delta 211–322}$, but not PES1$_{\Delta 311–415}$, increased CHIP-mediated ERβ ubiquitination (Figure 4F). CHIP knockdown greatly inhibited the ability of PES1 to regulate ERα and ERβ ubiquitination (Supplemental Figure 6, C and D). Furthermore, consistent with the ubiquitination results, CHIP knockdown almost abolished the effects of PES1 on ERα and ERβ degradation (Figure 4, G and H). These data suggest that CHIP plays a key role in the modulation of ERα and ERβ protein levels by PES1.

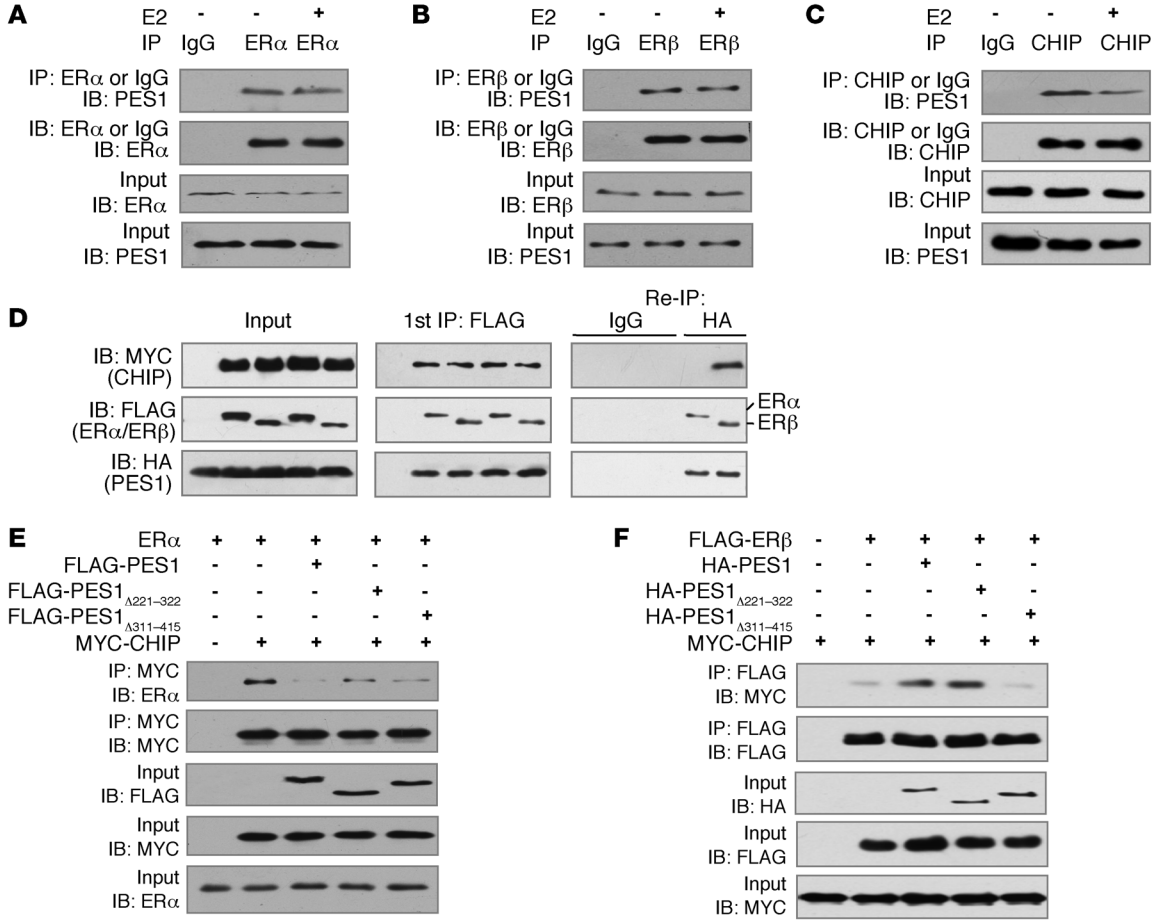

Figure 5

PES1 and CHIP formed a complex with ERβ but not with ERα. (**A**–**C**) Coimmunoprecipitation analysis of the endogenous interaction of PES1 with (**A**) ERα, (**B**) ERβ, or (**C**) CHIP. Cell lysates from MCF-7 cells were immunoprecipitated with antibodies specific for (**A**) ERα, (**B**) ERβ, or (**C**) CHIP, followed by immunoblotting with the indicated antibodies. (**D**) PES1, ERβ, and CHIP formed a complex. HEK293T cells transfected with the indicated plasmids were immunoprecipitated with anti-FLAG (1st IP). The immune complexes were eluted with FLAG peptide and reimmunoprecipitated (Re-IP) with anti-HA or normal IgG, followed by immunoblotting with the indicated antibodies. (**E** and **F**) Effects of PES1 on the interaction of CHIP with (**E**) ERα and (**F**) ERβ. Coimmunoprecipitations were carried out in HEK293T cells transiently transfected with the indicated constructs.

PES1 forms a complex with CHIP and ERβ but not with CHIP and ERα. Based on our findings that PES1 regulates CHIP-dependent ERα and ERβ degradation, we tested whether PES1 physically interacts with ER and CHIP. Indeed, GST pull-down and coimmunoprecipitation experiments showed that exogenous PES1 protein associated with exogenous ERα and ERβ proteins as well as exogenous CHIP protein, both in vitro and in vivo (Supplemental Figure 7, A and B, and Supplemental Figure 8, A and B). Importantly, in the absence or presence of E2, endogenous ERα, ERβ, or CHIP from MCF7 cell lysates specifically coimmunoprecipitated with endogenous PES1 (Figure 5, A–C). Moreover, confocal immunofluorescence analysis of MCF7 cells revealed that PES1 also colocalized with ERα and ERβ in the absence or presence of E2 (Supplemental Figure 7, C and D). These data strongly indicate that PES1 interacts with ERα, ERβ, and CHIP.

To map interaction regions of ERα and ERβ in PES1, we performed coimmunoprecipitation experiments using transfected HEK293T cells. $PES1_{1–110}$, $PES1_{111–220}$, $PES1_{221–322}$, and $PES1_{311–588}$ interacted with ERα and ERβ, whereas $PES1_{415–588}$ did not (Supplemental Figure 7, E and F). On the other hand, coimmunoprecipitation assays showed that PES1 interacted with the AF1 and AF2 domains, but not the DNA-binding domain, of ERα but only with the AF2 domain of ERβ (Supplemental Figure 7, G and H).

To define the region of CHIP that interacts with ERα, ERβ, and PES1, we used CHIP deletion mutants in coimmunoprecipitation assays. ERβ and PES1 interacted with both $CHIP_{1–126}$, containing the tetratricopeptide repeat domain, and $CHIP_{127–304}$, containing the U-box domain, but ERα interacted only with $CHIP_{1–126}$ (Supplemental Figure 8, C–E). Importantly, PES1 and CHIP formed a complex with ERβ but not with ERα, possibly because ERβ has more interaction regions in CHIP than ERα (Figure 5D). Based on these observations, we tested whether PES1 affects the interaction of CHIP with ERα and ERβ. Consistent with the effects of PES1 on CHIP-mediated ERα and ERβ degradation, PES1 or $PES1_{Δ311–415}$ reduced the interaction between CHIP and ERα, whereas $PES1_{Δ211–322}$ did not (Figure 5E and Supplemental Figure 9A). Likewise, PES1 or $PES1_{Δ211–322}$ increased the interaction between CHIP and ERβ,

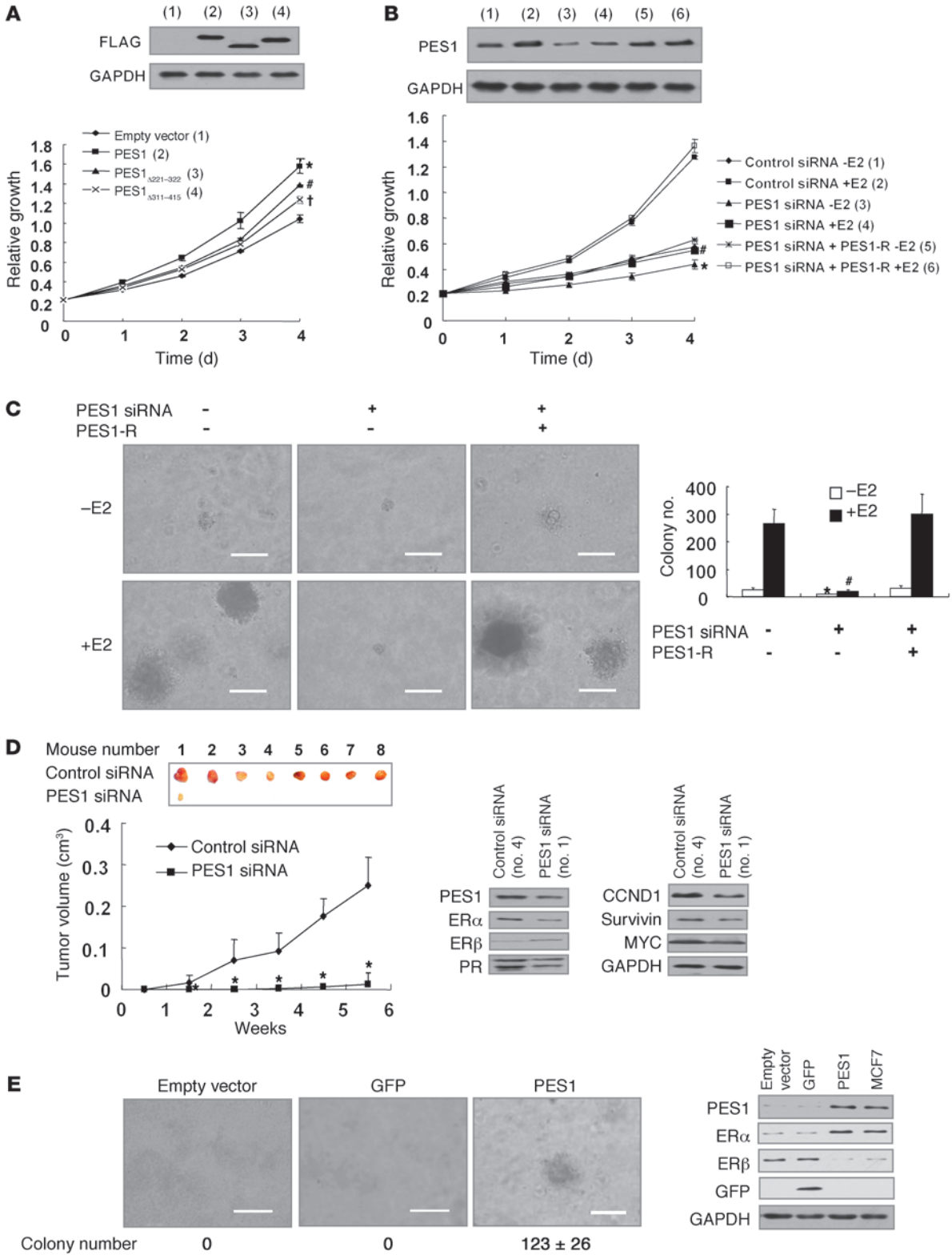
A
(1) (2) (3) (4)
FLAG
GAPDH
Empty vector (1)
PES1 (2)
PES1Δ221–322 (3)
PES1Δ311–415 (4)
Relative growth
Time (d)
B
(1) (2) (3) (4) (5) (6)
PES1
GAPDH
Control siRNA -E2 (1)
Control siRNA +E2 (2)
PES1 siRNA -E2 (3)
PES1 siRNA +E2 (4)
PES1 siRNA + PES1-R -E2 (5)
PES1 siRNA + PES1-R +E2 (6)
Relative growth
Time (d)
C
PES1 siRNA
PES1-R
–E2
+E2
Colony no.
–E2
+E2
D
Mouse number 1 2 3 4 5 6 7 8
Control siRNA
PES1 siRNA
Tumor volume (cm3)
Weeks
Control siRNA (no. 4)
PES1 siRNA (no. 1)
PES1
ERα
ERβ
PR
CCND1
Survivin
MYC
GAPDH
E
Empty vector
GFP
PES1
Colony number
0
0
123 ± 26
Empty vector
GFP
PES1
MCF7
PES1
ERα
ERβ
GFP
GAPDH

Figure 6
PES1 transforms normal HMECs and is required for estrogen-induced breast carcinogenesis. (**A**) Anchorage-dependent growth assays in MCF-7 cells transiently transfected with FLAG-tagged PES1, $PES1_{\Delta 221–322}$, or $PES1_{\Delta 311–415}$. The transfection efficiency is approximately 30%. Cell viability was assessed at the indicated times. *$P < 0.01$ versus empty vector on day 4. #$P < 0.05$, †$P < 0.01$ versus PES1 on day 4. Immunoblot analysis with anti-FLAG is shown. (**B**) Anchorage-dependent growth assays in MCF-7 cells stably transfected with PES1 siRNA or PES1 siRNA plus siRNA-resistant PES1. Cells were treated with 10 nM E2 and analyzed as in **A**. *$P < 0.01$ versus control siRNA without E2 on day 4. #$P < 0.01$ versus control siRNA with E2 on day 4. Immunoblot analysis with anti-PES1 is shown. (**C**) Anchorage-independent growth assays in MCF-7 cells stably transfected as in **B**. Scale bar: 50 μm. (**A**–**C**) Data are shown as mean ± SD of 3 independent experiments. *$P < 0.01$ versus control siRNA without E2. #$P < 0.01$ versus control siRNA with E2. (**D**) Volume of xenograft tumors derived from MCF-7 cells expressing control siRNA or PES1 siRNA. Data are shown as mean ± SD ($n = 8$ for control siRNA; $n = 1$ for PES1 siRNA due to absence of visible tumors in the other 7 mice). *$P < 0.01$ versus control siRNA. Representative tumor tissues were subjected to immunoblot analysis with indicated antibodies. (**E**) Anchorage-independent growth assays in HMECs infected with recombinant lentivirus carrying GFP or PES1. Scale bar: 50 μm. Immunoblotting with the indicated antibodies is shown.

whereas $PES1_{\Delta 311–415}$ did not (Figure 5F and Supplemental Figure 9B). These effects are specific for CHIP, because PES1 did not change the interaction of ERα and ERβ with E6-associated protein, another E3 ubiquitin ligase for ERα and ERβ (refs. 35, 36, and Supplemental Figure 9, C and D).

PES1 is required for estrogen-mediated breast tumor growth. Next, we determined the effect of PES1 on breast cancer cell growth. In assays of anchorage-dependent growth, MCF7 cells transfected with PES1 grew faster than those transfected with $PES1_{\Delta 211–322}$, $PES1_{\Delta 311–415}$, or empty vector, and MCF7 cells transfected with $PES1_{\Delta 211–322}$ or $PES1_{\Delta 311–415}$ grew faster than those transfected with empty vector (Figure 6A). In contrast, PES1 knockdown almost completely abolished E2-mediated growth stimulation of MCF7 cells (Figure 6B), and this phenotype was rescued by PES1 reexpression. Similar results were observed in ZR75-1 and T47D cells (Supplemental Figure 10, A and B). PES1 knockdown also greatly inhibited anchorage-independent growth of MCF7, ZR75-1, and T47D cells (Figure 6C and Supplemental Figure 10, C and D), and again, the observed effects were rescued by PES1 reexpression in MCF7 cells (Figure 6C). Furthermore, all mice inoculated with MCF7 or ZR75-1 cells expressing control siRNA developed tumors in the presence of E2 but not in the absence of E2 (Figure 6D, Supplemental Figure 10E, and data not shown), suggesting that both MCF7 and ZR75-1 cell lines are estrogen dependent. In contrast, in mice inoculated with MCF7 or ZR75-1 cells expressing PES1 siRNA, only 1 or 3, respectively, out of 8 mice developed tumors in the presence of E2, and these showed late latency and a much smaller tumor size (Figure 6D and Supplemental Figure 10E). The tumors in mice inoculated with MCF7 cells expressing PES1 siRNA had reduced protein levels of ERα, progesterone receptor (PR), MYC, CCND1, and survivin, and increased levels of ERβ (Figure 6D). With the exception of those concerning ERβ, similar effects were observed in ERβ-negative ZR75-1 cells (Supplemental Figure 10E).

Using growth in soft agar as an index of transformation, we examined the effect of PES1 on transformation of HMECs. HMECs with elevated PES1 expression levels, equivalent to those of MCF7 cells, could grow in soft agar, whereas HMECs expressing green fluorescent protein or empty vector could not (Figure 6E). PES1 overexpression increased expression of ERα but decreased that of ERβ.

Correlation of PES1 with ERα and ERβ in patients with breast cancer. The implication of ERα in breast cancer has been widely investigated with high-quality ERα antibodies. Thus, we examined the specificity of anti-ERβ and anti-PES1 antibodies. We confirmed the specificity of the antibodies by immunoblotting of lysates from MCF7 cells transfected with ERβ siRNA or PES1 siRNA (Supplemental Figure 11A), immunofluorescence analysis of ERβ or PES1 knockdown MCF7 cells (Supplemental Figure 11B), and immunohistochemical staining of breast cancer samples incubated with anti-ERβ or anti-PES1 antibodies preincubated with their respective antigens (Supplemental Figure 11C). Intriguingly, consistent with our findings that PES1 upregulated ERα and downregulated ERβs (ERβ, ERβ2, and ERβ5), immunohistochemical staining of 116 breast cancer tissues and 92 normal tissues adjacent to breast cancer, using antibodies with confirmed specificity, showed that PES1 expression correlated positively with ERα expression ($P < 10^{-4}$) but negatively with expression of ERβs ($P < 10^{-4}$) (Figure 7, A and B). These data strongly suggest important pathological roles of PES1 in breast cancer.

PES1 predicts clinical outcome of tamoxifen therapy. To determine the clinical relevance of PES1 modulation of ER, we analyzed the survival follow-up information of 65 subjects. PES1 overexpression was significantly associated with better disease-free survival ($P = 0.001$) and overall survival ($P = 0.002$) in patients with breast cancer who received tamoxifen treatment (Figure 7, C and D). To verify the effects of PES1 on tamoxifen sensitivity, we performed animal experiments. Overexpression of PES1 in ZR75-1 cells increased ERα expression and caused increased sensitivity to tamoxifen (Supplemental Figure 10F).

Discussion

An increasing number of studies show that estrogen signaling depends principally on the balance between ERα and ERβ expression (3, 4). The disturbance of such balance and, especially, a predominance of proliferative ERα protein over antiproliferative ERβ protein may cause cancer in estrogen-responsive organs. Thus, elucidating the regulation of the balance between ERα and ERβ expression may not only provide novel mechanistic insights into estrogen-induced tumorigenesis but also improve current endocrine therapy for estrogen-related cancers. Our work reveals for what we believe to be the first time that PES1 plays an essential role in estrogen-induced breast tumor growth through regulation of the yin-yang balance between ERα and ERβ (Figure 7E). First, we demonstrated that PES1 enhances transcriptional activity of ERα and reduces that of ERβ and modulates many estrogen-responsive genes. Second, consistent with this regulation of ERα and ERβ transcriptional activity, PES1 increased the stability of the ERα protein and decreased that of ERβ through CHIP-mediated ubiquitin-proteasome pathway. Third, PES1 can transform normal HMECs through increased ERα protein and decreased ERβ protein and is required for estrogen-induced breast tumor growth in nude mice. Fourth, expression of PES1 correlates positively with ERα expression and negatively with ERβ expression in patients with breast cancer. Since

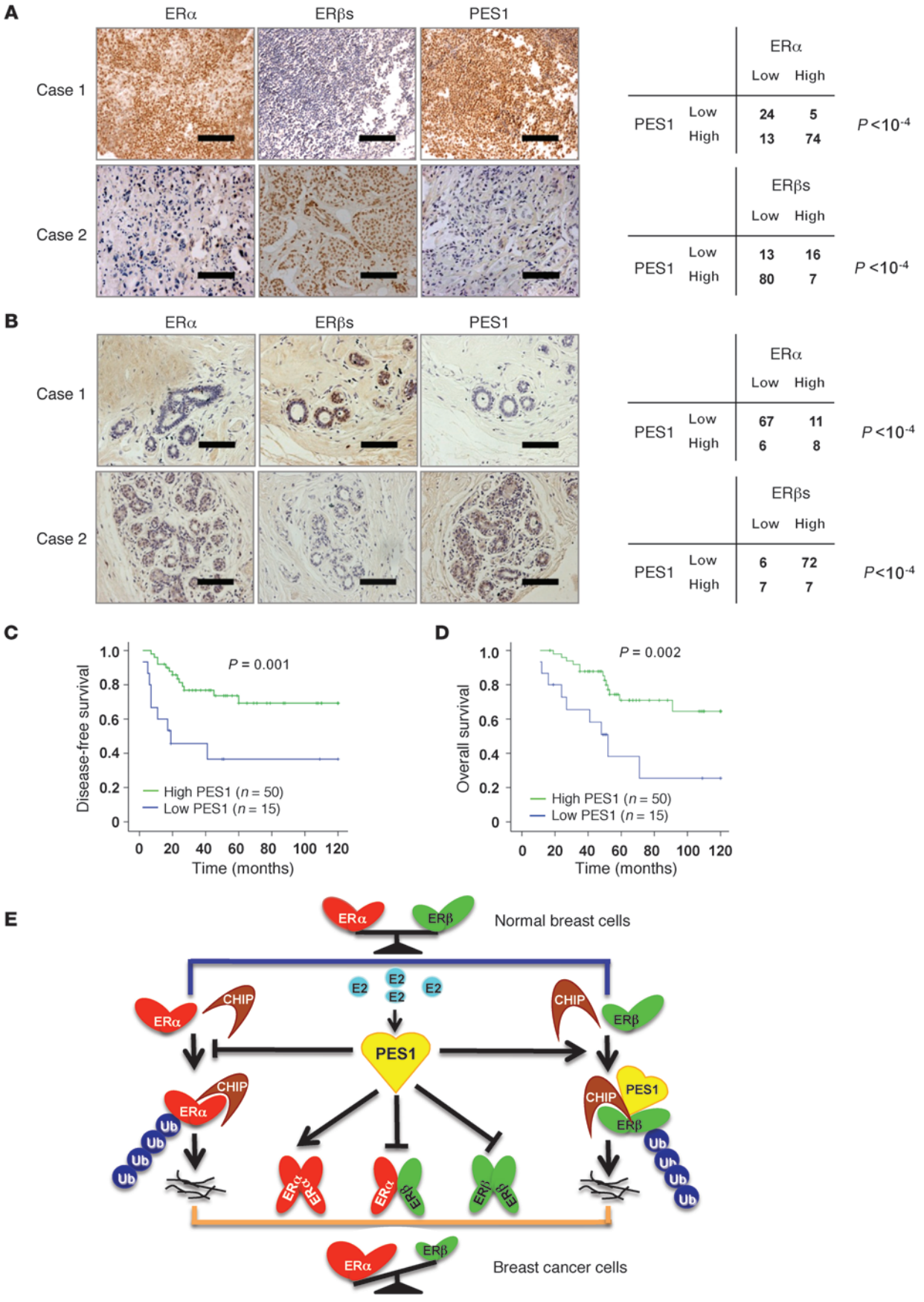
A
ERα
ERβs
PES1
Case 1
Case 2
ERα
Low High
PES1 Low 24 5
High 13 74
P <10-4
ERβs
Low High
PES1 Low 13 16
High 80 7
P <10-4
B
ERα
ERβs
PES1
Case 1
Case 2
ERα
Low High
PES1 Low 67 11
High 6 8
P <10-4
ERβs
Low High
PES1 Low 6 72
High 7 7
P<10-4
C
Disease-free survival
P = 0.001
High PES1 (n = 50)
Low PES1 (n = 15)
Time (months)
D
Overall survival
P = 0.002
High PES1 (n = 50)
Low PES1 (n = 15)
Time (months)
E
ERα
ERβ
Normal breast cells
E2
CHIP
PES1
Ub
Breast cancer cells

Figure 7
Association of PES1 with ERα and ERβ in breast cancer. (**A** and **B**) Expression of ERα, ERβ, and PES1 in (**A**) human breast cancer tissues and (**B**) normal tissues adjacent to breast cancer. Representative immunohistochemical staining of PES1, ERα, and ERβ is shown at top. Original magnification, ×20. Scale bar: 100 μm. A summary of (**A**) 116 breast cancer tissues or (**B**) 92 normal breast tissues is shown below, with tissues categorized by low and high expression of PES1 and ERα or ERβ. Case 1 and case 2 refer to 2 representative samples. The *P* value was generated using the χ^2 test. (**C** and **D**) Kaplan-Meier estimate of (**C**) disease-free survival and (**D**) overall survival in 65 patients with breast cancer who received tamoxifen treatment. Marks on graph lines represent censored samples. High PES1 and low PES1 refer to samples with high and low levels of PES1 expression, respectively. (**E**) Proposed model for PES1 modulation of the balance between ERα and ERβ. The estrogen-inducible protein PES1 blocks interaction of ERα with CHIP through its interaction with CHIP and instead forms a complex with ERβ and CHIP, leading to reduced degradation of ERα and increased degradation of ERβ by CHIP. In turn, PES1 enhances transcriptional activity of ERα but reduces that of ERβ through increased ERα homodimerization and decreased ERβ homodimerization and ERα-ERβ heterodimerization. Disruption of the balance between ERα and ERβ by PES1 contributes to breast tumorigenesis.

dysregulation of the balance between ERα and ERβ has also been reported to be associated with other cancers, such as colon cancer and thyroid cancer (37, 38), our data suggest that PES1 may not only act as a determinant of breast tumorigenesis but also play an important role in the development of other cancers. PES1 may be a useful target for hormone-related cancer therapy.

Estrogen stimulates breast cancer cell growth through ERα, and use of antiestrogens blocks this stimulating response (3, 39, 40). As approximately 70% to 80% of all breast cancers are ERα positive at the time of diagnosis, ERα expression has considerable implications for cancer biology and therapy. Tamoxifen is an antiestrogen drug that was developed over 30 years ago and has been widely used in the treatment of all stages of ERα-positive breast cancer (39, 40). Tamoxifen binds and blocks ERα on the surface of cells, preventing estrogens from binding and activating the cell. Due to the discovery of a second form of the ER, ERβ, in 1996, the role of tamoxifen in breast cancer endocrine therapy appears to be complicated. Tamoxifen also binds ERβ and inhibits ERβ transcriptional activity. However, many lines of evidence demonstrate that ERβ has an antiproliferative function when reintroduced into ERα-positive breast cancer cells (3, 5–7). In many ways, ERβ seems to oppose the action of ERα. The same problem remains for another endocrine drug, fulvestrant (41, 42). It is indicated for the treatment of ERα-positive metastatic breast cancer in postmenopausal women after treatment with other antiestrogens. Fulvestrant binds to ER and prevents structural changes that are necessary for ER to initiate gene transcription. On the other hand, fulvestrant degrades ER, which also reduces gene transcription. Like tamoxifen, fulvestrant also inhibits both ERα and ERβ transcriptional activity. Ideally, in the treatment of patients with ERα-positive breast cancer, in which ERβ is antagonistic to ERα, a drug that reduces transcriptional activity of ERα but enhances that of ERβ should be better than the currently used endocrine drugs tamoxifen or fulvestrant. We demonstrate that PES1 increases ERα but decreases ERβ protein levels, correlating with their respective physiological activities in breast cancer. Thus, PES1 represents a very promising target for the development of better drugs for endocrine cancer therapy.

Estrogen has been shown to stimulate the expression of PES1 protein (9–11). Our cDNA microarray analysis indicated that PES1 regulates the expression of 256 genes in MCF7 breast cancer cells, including over 127 previously reported estrogen-responsive genes (14–22). Moreover, PES1 knockdown almost abolishes estrogen-mediated anchorage-dependent and -independent growth of breast cancer cells, and most of mice inoculated with PES1 knockdown breast cancer cells do not develop tumors, even in the presence of estrogen. These results suggest that PES1 is a key mediator of estrogen signaling and that a positive feedback loop of estrogen/PES1 promotes malignant growth of breast cancer cells.

PES1 has been demonstrated to play important roles in embryonic development (43), ribosome biogenesis (44–46), cell cycle regulation (47), and chromosome stability (45), although molecular mechanisms underlying these processes remain largely unknown. We show that PES1 modulates many estrogen-responsive genes that are known to have important functions in DNA replication, cell cycle regulation, and DNA repair. For example, PES1 can regulate the expression of a large number of cell cycle–related genes, including cyclin A2 (*CCNA2*), cyclin B2 (*CCNB2*), *CCND1*, *CCNE2*, cyclin-dependent kinase 1 (*CDK1*), *CDK2*, *E2F1*, *E2F2*, cell-division cycle gene 20 (*CDC20*), *PCNA*, *MYC*, *MYB*, and minichromosome maintenance genes (*MCM2*–*MCM8* and *MCM10*). In normal human cells, cellular division is an ordered, tightly regulated process, involving multiple cell cycle checkpoints that ensure genomic integrity. Altered regulation of the cell cycle is a hallmark of human cancers (48). Cyclins and their associated CDKs are the central machinery that governs cell cycle progression. Overexpression of cyclin D1, the major regulatory subunit for CDK4, is common in human cancers of epithelial cell origin (49). Approximately 50% of human breast cancers express abnormally high levels of cyclin D1, which is maintained throughout subsequent stages of breast cancer progression, from in situ carcinoma to invasive carcinoma. Both cyclin D1 and CDK2 are required for mammary tumorigenesis induced by the ErbB-2 oncogene (50). Like cyclin D1, MYC and MYB are also overexpressed in breast tumors (51, 52). Overexpression of MYC contributes to breast cancer development and progression and is associated with poor clinical outcome. MYC regulates cell cycle at the G_1/S transition through activation of downstream targets such as cyclin E/CDK2. In addition, MYC promotes cell cycle progression through activation of cyclin D1, CDK4, E2F1, and E2F2. The expression of MYB protein was shown to be important for estrogen-stimulated proliferation of breast cancer cells (52). MYB controls G_2/M cell cycle transition by direct regulation of cyclin B1 expression. Like cyclin D1, MYC, and MYB, E2F1 is also an estrogen-inducible protein (53). E2F1 is necessary for estrogen regulation of breast cancer cell proliferation. Interestingly, a large number of genes involved in the control of the cell cycle contain regulatory binding sites for E2F1 (54) (e.g., *CCNA2*, cyclin D3, *CCNE2*, *CDK1*, *MYC*, and *PCNA* as well as *E2F1* itself). DNA replication takes place in the S phase of the cell cycle. The highly orchestrated process of DNA replication ensures the accurate inheritance of genetic information from one cell generation to the next. The MCM proteins are essential for the process of DNA replication (55, 56). Loss of MCM function results in DNA damage and genome instability. The fact that PES1 can regulate many key molecules of cell cycle, DNA replication, and DNA repair suggests the importance of PES1 in breast tumorigenesis and as a therapeutic target. It will be interesting to investigate how PES1 regulates these molecules.

Methods

Plasmids and siRNAs. The estrogen-responsive reporter construct ERE-Luc and eukaryotic expression vectors for FLAG-tagged ERα and ERβ have been described previously (57–59). Other mammalian expression vectors encoding FLAG-, MYC-, or HA-fusion proteins tagged at the amino terminus were constructed by inserting PCR-amplified fragments into pcDNA3 (Invitrogen) or pIRESpuro2 (Clontech). Plasmids encoding GST fusion proteins were generated by cloning PCR-amplified sequences into pGEX-KG (Amersham Pharmacia Biotech). The cDNA target sequences of siRNAs for PES1 and CHIP were ACACAAGAAGAAGGTTAAC and GCACGACAAGTACATGGCGGA, respectively, and were inserted into pSilencer2.1-U6neo (Ambion) and pSIH-H1-puro (System Biosciences). Expression vectors for siRNA-resistant PES1 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 PES1 or GFP were made by inserting PCR-amplified fragments into pCDH-EF1-MCS-T2A-puro (System Biosciences).

Cell culture, transfection, and luciferase reporter assay. HEK293T embryonic kidney cells and MCF7, ZR75-1, T47D, and SKBR3 breast cancer cells were routinely cultured in DMEM (Invitrogen) containing 10% FBS (Hyclone). Normal HMECs (Invitrogen) were cultured in HMEC medium (Invitrogen). For hormone treatment experiments, cells were cultured in medium containing phenol red–free DMEM supplemented with 10% charcoal/dextran-treated FBS (Hyclone). Lipofectamine 2000 reagent was used for transfections following 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 collected 48 hours after transfection and added to the medium of target cells with 8 μg/ml polybrene (Sigma-Aldrich). Stable cell lines were selected in 500 μg/ml G418 or 1 μg/ml puromycin for approximately 2 months. Pooled clones or individual clones were screened by standard immunoblot protocols and produced similar results. PES1 knockdown stable cell lines grew very slowly and could only be passaged several times. Luciferase reporter assays were performed as described previously (59).

cDNA microarray analysis. cDNA generated from RNA was labeled with Cy3 (PES1 siRNA) and Cy5 (control siRNA), mixed, and hybridized to human oligo chip 35 k v2.0 containing 35,000 human gene elements (CapitalBio). The chip was scanned by LuxScan 10K/A (CapitalBio), and data were analyzed using MAS 3.0 Software (CapitalBio). All results were given as the gene expression ratio (ratio of the intensity of Cy3 to that of Cy5). Genes with more than or equal to 2-fold intensity change were considered of interest and subjected to further investigation by gene ontology analysis and pathway analysis based on the Kyoto Encyclopedia of Genes and Genomes database.

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 with the primers listed in Supplemental Table 4A as described previously (57).

ChIP assay. ChIP assays were performed as described previously (57) using anti-ERα (Millipore), anti-ERβ (Novus Biologicals), and anti-PES1 (Bethyl Laboratories). The primers used for ChIP are listed in Supplemental Table 4B.

Cycloheximide chase assay. Transfected cells were cultured in phenol red–free DMEM medium supplemented with 10% charcoal-stripped FBS for 3 days. Cells were treated with 20 μg/ml cycloheximide for different time periods in the presence or absence of 10 nM E2. Cell lysates were analyzed by immunoblotting with antibodies specific for ERα (Sigma-Aldrich), FLAG (Sigma-Aldrich), and GAPDH (Santa Cruz Biotechnology Inc.).

Ubiquitination assay. Transfected cells were treated with the proteasome inhibitor MG132 (10 μM) for 4 hours. The cells were lysed in RIPA buffer and immunoprecipitated with anti-FLAG (Sigma-Aldrich), anti-ERα (Millipore), or anti-ERβ (Novus Biologicals) antibodies as described previously (60). The immunocomplexes were subjected to immunoblot analysis with antibodies specific for MYC or ubiquitin (both from Santa Cruz Biotechnology Inc.).

GST pull-down assay. GST fusion proteins were expressed in pGEX-KG and purified according to the manufacturer's instructions (Amersham Pharmacia Biotech). HEK293T extracts expressing ERα, ERβ, or CHIP were mixed with 10 μg of GST derivatives bound to glutathione-Sepharose beads, and the adsorbed proteins were analyzed as previously described (61).

Coimmunoprecipitation. Cell extracts were prepared, immunoprecipitated, and analyzed as previously described (61). Immunoprecipitation was performed with anti-FLAG M2 Affinity Gel (Sigma-Aldrich), anti-MYC Affinity Gel (Sigma-Aldrich), anti-ERα (Millipore), anti-ERβ (Novus Biologicals), or anti-CHIP (Santa Cruz Biotechnology Inc.).

Immunofluorescence. Immunofluorescence was performed as previously described (57). Briefly, cells grown on glass coverslips were fixed, permeabilized, and blocked in normal goat serum. The coverslips were then incubated with rabbit anti-PES1 (Bethyl Laboratories) and mouse anti-ERα (Santa Cruz Biotechnology Inc.) or mouse anti-PES1 (Santa Cruz Biotechnology Inc.) and rabbit anti-ERβ (Millipore), followed by incubation with corresponding secondary antibodies. Nuclei were counterstained with DAPI. Confocal images were collected using a Radiance2100 confocal microscope (Bio-Rad).

EMSA. The probes for EMSA were labeled with the Biotin 3′-End DNA Labeling Kit (Pierce) as instructed by the manufacturer. The sequences of the labeled oligonucleotides for the CdRE of the *HO-1* gene (28), the putative CdRE of the *CCND1* gene, and the putative CdRE of the *E2F1* gene were 5′-AATTCGGCGGATTTTGCTAGATTTTGCG-3′ (CdRE-*HO-1*), 5′-AGTTTCATATTTGCTAGATATCAGTGTTTG-3′ (CdRE-*CCND1*), and 5′-TTTGAACCTGATGCTAGATCTTTTTATTTT-3′ (CdRE-*E2F1*), respectively (the core sequence is underlined). The mutated sequences were 5′-AATTCGGCGGATTTTGCTGAATTTTGCG-3′ (mCdRE-*HO-1*), 5′-AGTTTCATATTTGCTGAATATCAGTGTTTG-3′ (mCdRE-*CCND1*), and 5′-*z*TTTGAACCTGATGCTGAATCTTTTTATTTT-3′ (mCdRE-*E2F1*). EMSA was performed using in vitro–translated protein or the same amount of unprogrammed lysate (Promega) with LightShift Chemiluminescent EMSA Kits (Pierce). For competition experiments, a 100-fold molar excess of unlabeled CdRE was mixed with the biotin-labeled probe. The resulting protein-DNA complexes were analyzed by electrophoresis on a polyacrylamide gel, followed by chemiluminescent detection.

Anchorage-dependent and -independent growth assays. Anchorage-dependent cell proliferation was analyzed by a crystal violet assay as described previously (59). For anchorage-independent growth (57), 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. 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. When tumors reached the volume of approximately 100 mm^3, we randomly allocated the mice to groups in which they received placebo or tamoxifen pellets (Innovative Research of America). 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.

Immunohistochemistry. Immunohistochemical staining was performed as described previously (59) using rabbit anti-ERα (Millipore), rabbit anti-ERβ (Millipore), and rabbit anti-PES1 (Bethyl Laboratories) as primary antibodies.

Statistics. Differences among variables were assessed by χ^2 analysis or 2-tailed Student's *t* test. Estimation of disease-free and overall survival was performed using the Kaplan-Meier method, and differences between sur-

vival curves were determined with the log-rank test. Statistical calculations were performed using SPSS 13.0. *P* values of less than 0.05 were considered statistically significant.

Study approval. Animal studies were approved by the Institutional Animal Care Committee of Beijing Institute of Biotechnology. Breast cancer samples were obtained from Chinese PLA General Hospital, with the informed consent of patients and with institutional approval for experiments from Chinese PLA General Hospital and Beijing Institute of Biotechnology.

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Address correspondence to: Qinong Ye, Department of Medical Molecular Biology, Beijing Institute of Biotechnology, 27 Tai-Ping Lu Rd., Beijing 100850, China. Phone: 8610.6818.0809; Fax: 8610.6824.8045; E-mail: yeqn66@yahoo.com.

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