Biochemical and Biophysical Research Communications 457 (2015) 141–147

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

ERβ1 inhibits the migration and invasion of breast cancer cells through upregulation of E-cadherin in a Id1-dependent manner

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ARTICLE INFO

Article history:
Received 2 December 2014
Available online 13 December 2014

Keywords:
ERβ1
E-cadherin
Id1
Migration
Invasion

ABSTRACT

ERβ1 is a member of the nuclear receptor superfamily of ligand-regulated transcription factors. It plays an important role in regulating the progression of breast cancer. However, the mechanisms of ERβ1 in tumorigenesis, metastasis and prognosis are still not fully clear. In this study, we showed that the expression of ERβ1 was positively correlated with E-cadherin expression in breast cancer cell lines. In addition, we found that ERβ1 upregulates E-cadherin expression in breast cancer cell lines. Furthermore, we also found that ERβ1 inhibits the migration and invasion of breast cancer cells and upregulated E-cadherin expression in a Id1-dependent manner. Taken together, our study provides further understanding of the molecular mechanism of ERβ1 in tumor metastasis and suggests the feasibility of developing novel therapeutic approaches to target Id1 to inhibit breast cancer metastasis.

1. Introduction

Breast cancer is the most common malignant tumor among women in the world. Over the last decade, several classic signaling molecules involved in breast tumorigenesis has been revealed and identified as biomarkers and targets of breast cancer therapy, such as estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor-2 (HER2) [1–3]. ERs, exist as ERα and ERβ, are members of the nuclear receptor superfamily of ligand-regulated transcription factors. Similar to other nuclear receptors, the ERs contain three functional domains: the N-terminal domain, the DNA-binding domain (DBD), and the C-terminal or ligand binding domain (LBD) [4]. The DBD is highly conserved between ERα and ERβ and mediates sequence-specific binding of the ERs to estrogen-responsive elements (EREs) in target genes. In contrast, the LBDs of ERα and ERβ moderately conserved showing only 59% amino acid identity [5]. The main endogenous estrogen, 17β-estradiol (E2), binds to both ERα and ERβ with similar affinity. However, the ligand-binding pockets of the two subtypes show minor differences in structure which has allowed the development of selective ER ligands. Importantly, while estrogens via ERα promote proliferation, signaling via ERβ suppresses proliferation and induces apoptosis.

The human ERβ gene is located on chromosome 14q23.2, spanning ~61.2 kb. Multiple ERβ (ERβ1–6) isoforms exist in humans, primates, rats, and mice [6]. The wild-type, full-length human ERβ (also named ERβ1) protein includes 530 amino acids with an estimated molecular mass of 59.2 kDa [7]. ERβ2 was suggested to be a dominant-negative inhibitor of ERα [8]. Additionally, in vitro studies show that ERβ4 and ERβ5 can heterodimerize with ERβ1 and enhance its transactivation in a ligand-dependent manner [9]. Recently, accumulated studies have shown that ERβ act as a tumor suppressor gene [10]. The expression of ERβ has been shown to decrease in breast cancer, prostate cancer, lung cancer, and colorectal cancer [11–14]. Generally higher expression of ERβ was found to be associated with a good prognostic marker or better clinical outcome [15,16]. Published data shown that ERβ1 exerts antiproliferative effects when introduced into ERα positive breast cancer cells [17]. Epithelial to mesenchymal transition (EMT) has been considered as an essential early step in tumor metastasis. EMT is characterized by loss of cellular adhesion, which is mediated by downregulation of adhesion molecules, such as E-cadherin. Recent studies have shown that ERβ1 inhibits EMT and invasion in TNBC cells [18]. However, the mechanisms of ERβ1 in tumorigenesis, development, metastasis and prognosis are still not fully clear.

Recently, our group published a study [19] that presented the Id1 (inhibitor of differentiation/DNA binding) as a novel estrogen

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http://dx.doi.org/10.1016/j.bbrc.2014.12.038
0006-291X/

ERβ1 binding partner. Id1 is a member of the helix-loop-helix (HLH) transcriptional factor family. Id1 lacks the basic domain for DNA binding and function as a dominant inhibitor of the basic HLH transcription factors by forming heterodimers, thus inhibits them from DNA binding and transactivation of their target genes. Recent studies showed that Id1 may function as oncogene [20]. A high level of Id1 expression has been reported in several types of cancers [21]. Id1 has been reported to promote cell proliferation, cell cycle progression, tumor invasion and metastasis through inhibition of tumor suppressor and activation of growth and metastasis promoting pathways [22].

In this study, we show that ERβ1 is positively correlated with E-cadherin in breast cancer cell lines. Overexpression ERβ1 leads to upregulation of E-cadherin, and moreover, we found that ERβ1-induced up-regulation of E-cadherin expression is Id1 dependent. We also demonstrate that Id1-dependent upregulation of E-cadherin by ERβ1 inhibits the migration and invasion of breast cancer cells. Taken together, our studies provide further understanding of the molecular mechanism of ERβ1 in tumor metastasis and suggest the feasibility of developing novel therapeutic approaches to target Id1 to inhibit breast cancer metastasis.

2. Materials and methods

2.1. Cell culture

Human breast cancer cell lines T-47D, MDA-MB-231, SKBR3, MCF-7, MDA-MB-361 and human breast epithelial cell line MCF-10A were obtained from American Type Culture Collection and were cultured according to the ATCC recommendations. All cells were maintained in cell culture at 37 °C in a humidified 5% (v/v) CO_2 atmosphere and passaged at 1:3 dilutions.

2.2. Plasmid construction

The human full-length cDNA of ERβ1 and Id1 were amplified by PCR, inserted into the pIRES expression vector (Clontech) with *Cla*I and *Xba*I sites respectively. The primers for ERβ1 full-length cDNA were 5′-CCATCGAT ATGGATATAAAAAACTCACCAT-3′ (forward) and 5′-GCTCTAGA TCACTGAGACTGTGGGTTCTG-3′ (reverse). The primers for Id1 full-length cDNA were 5′-CCATCGATATGAAAGTCGCC AGTGGCAGCA-3′ (forward) and 5′-GCTCTAGACTAGTGGTCGGATC TGGATCTC-3′ (reverse). The promoter of E-cadherin (2.5-kb) was amplified by PCR with the primers 5′-GGGGTACCTGGTGGCCA TGGTGTGG-3′ and 5′-GAAGATCTTCCAAGGGCCCATGGCT-3′, using MDA-MB-231 cell-derived genomic DNA as template. The promoter sequence was then cloned into *Kpn*I and *Bgl*II sites of pGL3-basic luciferase reporter vector (Promega). The identities of all cloned sequences were confirmed by DNA sequencing.

2.3. Real-time RT-PCR

Total RNA was extracted from cultured cells using TRIzol reagent (Invitrogen) according to the manufacturer's protocols, and the first-strand cDNA was synthesized using Primescript RT reagent kit (TaKaRa). For quantitative analysis of ERβ1 and E-cadherin expression, cDNA was subjected to quantitative PCR using SYBR Green qPCR Master Mix (Promega). The PCR primers were: ERβ1 forward, 5′-CGATGCTTTGGTTTGGGTGAT-3′, and reverse, 5′-GCCCTCTTTG CTTTTACT GTC-3′, E-cadherin forward, 5′-ACA-GCCCCGCCTTATGATT-3′, and reverse, 5′-TCG GAA CCGCTT CCTTCA-3′. β-actin was used as an internal control with the pimers5′-ACCCCGTGCTGCTGACCGAG-3′ (forward) and 5′-TCCCG GCCAGCCAGGTCCA-3′ (reverse).

2.4. Western blot analysis

The whole cell proteins were extracted and the protein concentrations were determined by BCA assay (Pierce). Equal amounts of total proteins were separated in 10% SDS–polyacrylamide gels, transferred to polyvinylidene difluoride membranes (Millipore). Membranes were blocked for 1 h with 5% fat-free dry milk in Tris-buffered saline containing 0.05% Tween 20, incubated with anti-ERβ1 antibody (Santa Cruz), anti-E-cadherin antibody (Santa Cruz), or anti-β-actin (Santa Cruz) at 4 °C overnight, then the membranes were washed and incubated with secondary antibody, the signal detection by the enhanced chemiluminescence detection reagents (Pierce).

2.5. Transient transfections and luciferase assays

MDA-MB-231 and MCF-7 cells were transfected with reporter luciferase expression plasmid E-cadherin promoter-Luc, ERβ1 expression plasmid, or ERβ1 siRNA and corresponding control using Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen). After 24 h, cells were harvested and luciferase activities were measured using a luciferase assay system (Promega). The luciferase activity was normalized against pRL-TK *Renilla* luciferase activity. All transfection experiments were performed in triplicate and repeated three times.

2.6. Construction of stable ERβ1- or Id1-overexpression and knockdown MDA-MB-231 cells

Stable transfection and selection of transfected cells was performed as described previously [19]. The expression of ERβ1 and Id1 in clones of ERβ1- or Id1-overexpression and knockdown were examined by western blot.

2.7. Cell migration and invasion assays

Cell migration and invasion were performed with transwell chambers (Millipore) migration and extracellular matrix-coated invasion chambers (Millipore) according to the manufacture's instruction. Briefly, cells were serum deprived for 24 h after transfected for 24 h with ERβ1 expression plasmid, Id1 expression plasmid, or corresponding control. Then 1×10^5 cells were plated into the upper well of a transwell chamber. The bottom well of the chamber contained 500 μL RPMI-1640 medium supplemented with 5% FBS (for migration assay) or 10% FBS (for invasion assay). After 24 h incubation, the nonmigrating/noninvading cells were removed with a cotton swab, and the filters were fixed in 90% ethanol and stained with 0.1% crystal violet for counting. Values for cell migration or invasion were expressed as the average number of cells per microscopic field over five fields per one filter for triplicate experiments.

2.8. Statistical analysis

Data are expressed as mean ± standard deviation (SD) of three independent experiments. The statistical differences between experimental and control groups. were determined by Student's *t* test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. ERβ1 expression is positively correlated with E-cadherin expression in breast cancer cells

To investigate the relationship between the ERβ1 and E-cadherin, the expression of ERβ1 and E-cadherin in six human breast

cancer cell lines were examined by real time RT-PCR (Fig. 1A) and western blot (Fig. 1B). Then, we investigated the correlation between ERβ1 and E-cadherin. As shown in Fig. 1C and D, a positive correlation was observed between ERβ1 and E-cadherin both at mRNA (R squared = 0.93, $P < 0.01$) and protein (R squared = 0.75, $P < 0.01$) levels.

3.2. ERβ1 upregulates E-cadherin expression in breast cancer cell lines

To investigate whether ERβ1 is involved in the regulation of E-cadherin, MDA-MB-231 cell were transfected with a pIRES-ERβ1 plasmid in order to overexpress ERβ1, then the real-time RT-PCR and western blot assays were performed. As shown in Fig. 2, overexpression of ERβ1 increased the expression of E-cadherin at both mRNA (Fig. 2A) and protein levels (Fig. 2B). Three siRNAs against ERβ1 mRNA were synthesized, and the one (siERβ1–2) with the best silence effect was selected to be used in the subsequent experiments (Fig. 2C). Fig. 2D and E showed that the upregulation of E-cadherin induced by ERβ1 was dramatically attenuated when the expression of ERβ1 was knocked down by siERβ1–2. To further inquire whether E-cadherin was subjected to ERβ1 regulation, MDA-MB-231 cells were cotransfected with pro-E-cadherin-Luc (contain the promoter region of E-cadherin) and pIRES-ERβ1 or ERβ1 siRNA, then the report assays were performed. The results showed that the E-cadherin promoter-Luc activity was increased significantly when overexpression of ERβ1 and was decreased when cotransfected with ERβ1 siRNA (Fig. 2F). To further confirm ERβ1 upregulates E-cadherin expression, we analyzed the expression of E-cadherin in other two breast cancer cell lines T-47D and MCF-7 (Supplemental Figs. 1 and 2). The above results indicated that ERβ1upregulates the expression of E-cadherin at transcriptional level.

3.3. ERβ1-induced up-regulation of E-cadherin expression is Id1 dependent

Recently, our group published a study that presented the Id1 (inhibitor of differentiation-1) as a novel estrogen ERβ1 binding partner [19]. Therefore, we hypothesized that ERβ1-induced upregulation of E-cadherin may be Id1 dependent. In order to verify this assumption, we examined whether the effect of ERβ1 on E-cadherin expression was mediated by Id1. We established stable ERβ1 or Id1-overexpression MDA-MB-231 cells and stable ERβ1 or Id1-knockdown MDA-MB-231 cells, and the expression of ERβ1 (Fig. 3A) and Id1 (Fig. 3B) in the stable cells were examined by western blot. As shown in Fig. 3, after transfected with pIRES-ERβ1 in Id1-knockdown MDA-MB-231 cells or Id1-overexpression MDA-MB-231 cells, the expression of E-cadherin was increased in Id1-knockdown cells and decreased Id1-overexpression cells (Fig. 3C). Whereas, transfected with pIRES-Id1 in ERβ1-knockdown cells or ERβ1-overexpression cells resulted in a reduction of E-cadherin expression in ERβ1-knockdown cells and a raise of E-cadherin expression in ERβ1-overexpression cells (Fig. 3D). Furthermore, a reporter assay with E-cadherin promoter-Luc was used, the Id1-knockdown or Id1-overexpression cells were cotransfected with increasing amounts pIRES-ERβ1. In the Id1-knockdown cells, the activity of E-cadherin promoter-Luc was independent of the increasing expression of ERβ1. In contrast, in the Id1-overexpression cells, the increase in E-cadherin promoter-Luc was dependent on the increasing expression of ERβ1 (Fig. 3E). Also, we cotransfected with increasing amount pIRES-Id1 and E-cadherin promoter-Luc in ERβ1-knockdown or ERβ1-overexpression cells. In ERβ1-knockdown cells, the activity of E-cadherin promoter-Luc was significantly inhibited by the increasing expression of Id1, however, in ERβ1-overexpression cells, the activity of E-cadherin

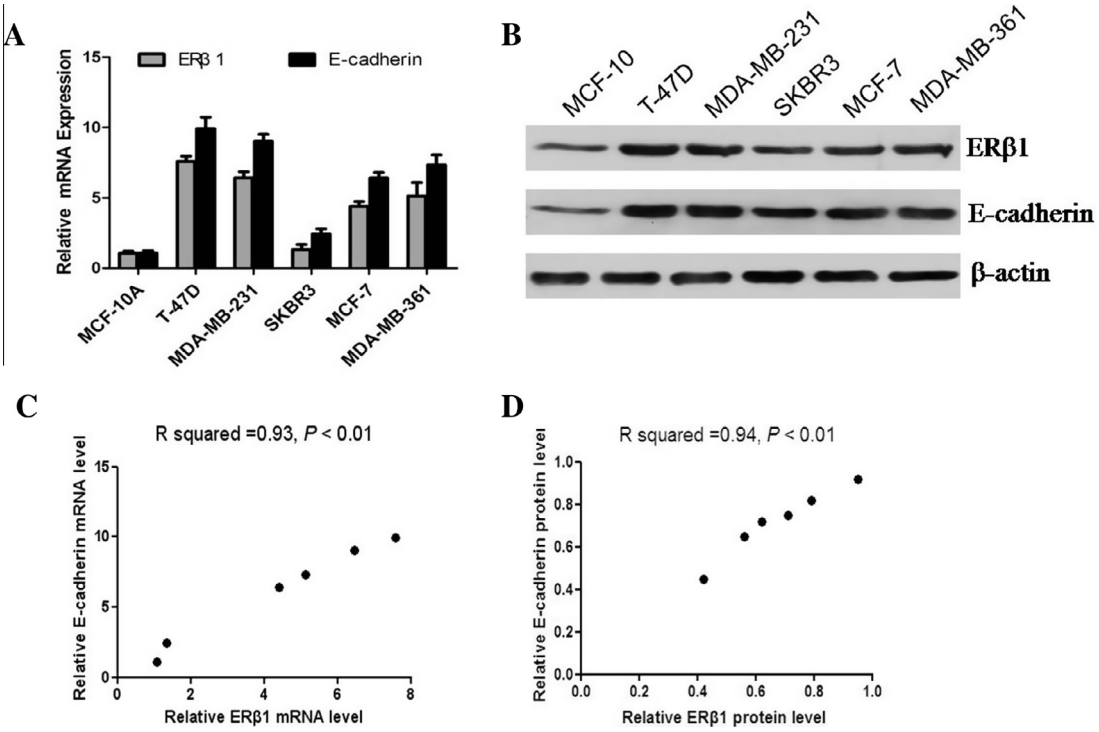

Fig. 1. ERβ1 expression is positively correlated with E-cadherin expression in breast cancer cells. Real time RT-PCR (A) and western blot (B) analysis of ERβ1 and E-cadherin expression in MCF-10A, T-47D, MDA-MB-231, SKBR3, MCF-7, MDA-MB-361 cell lines. Correlation between the ERβ1 and E-cadherin in breast cancer cells at mRNA (C) and protein (D) levels. The mRNA levels were determined by real time RT-PCR and normalized with the β-actin mRNA level. The protein levels were determined by western blot analysis and normalized with the β-actin protein level.

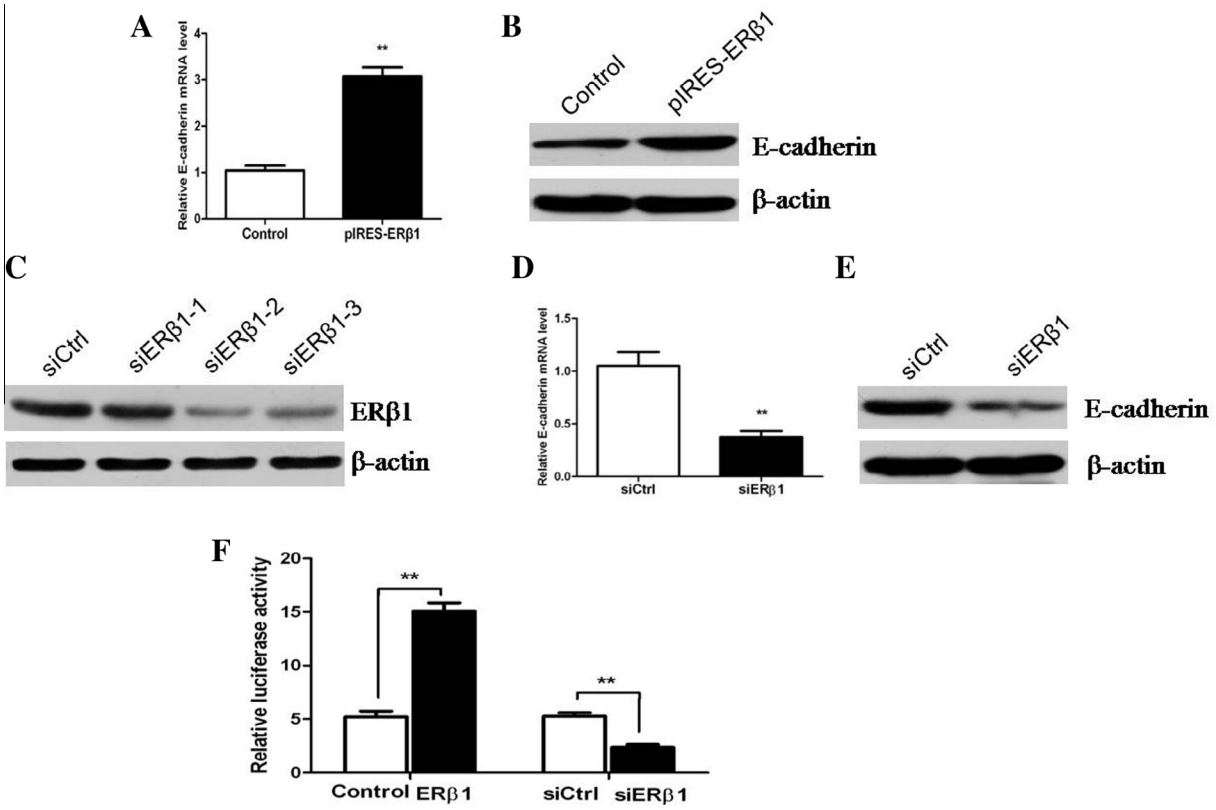

Fig. 2. ERβ1 upregulates E-cadherin expression in breast cancer cell lines. Real time RT-PCR (A) and western blot (B) analysis of E-cadherin mRNA and protein after transfection with pIRES-ERβ1(ERβ1expression plasmid) or control (empty vector pIRES) for 48 h in MDA-MB-231 cells. (C) Three non-overlapping siRNAs to inhibit ERβ1 expression were transfected into MDA-MB-231 cells, the ERβ1 expression was detected by western blot. (D and E) MDA-MB-231 cells were transfected with control siRNA or ERβ1 siRNA (10 nM each), then, real time RT-PCR (D) and western blot (E) analysis of E-cadherin mRNA and protein expression. (F) MDA-MB-231 cells were cotransfected with the reporter plasmid 0.5 μg pGL/pro-E-cadherin and 0.5 μg pIRES-ERβ1, or 10 nM ERβ1 siRNA, or control. After 24 h, the cell extracts were prepared and the luciferase assays were performed. The luciferase activity was normalized against pRL-TK *Renilla* luciferase activity. Data are mean ± SD of three independent experiments. $^{**}P < 0.01$ *vs.* control.

promoter-Luc was restored. Moreover, the activity of E-cadherin promoter-Luc was decreased by the increasing expression of Id1in ERβ1-overexpression cells (Fig. 3F). The same findings were reproduced in two additional breast cancer cell lines T-47D and MCF-7 (Supplemental Figs. 3 and 4). These results indicate that ERβ1 upregulate the expression of E-cadherin through binding with Id1, and reducing the Id1 suppression on E-cadherin expression.

3.4. Id1-dependent upregulation of E-cadherin by ERβ1 inhibits the migration and invasion of breast cancer cells

To study the effect of Id1-dependent upregulation of E-cadherin on the cell migration and invasion, transwell migration and invasion assays were carried out. As shown in Fig. 4, the migration and invasion of Id1-knockdown cells and ERβ1-overexpression cells were decreased, however, the migration and invasion of Id1-overexpression and ERβ1-knockdown cells were increased. Moreover, overexpression of ERβ1 attenuated the Id1-promoted migration (Fig. 4A) and invasion (Fig. 4C), and overexpression of Id1 impaired the ERβ1-inhibited migration (Fig. 4B) and invasion (Fig. 4D). Furthermore, the inhibition of cell migration and invasion mediated by Id1-dependent upregulation of E-cadherin was also found in T-47 and MCF-7 breast cancer cell lines (Supplemental Figs. 5 and 6). These results indicate that Id1-dependent upregulation of E-cadherin by ERβ1 was associated with the decreased migration and invasion of breast cancer cells.

4. Discussion

Estrogen is essential for growth and development of the mammary glands and has been associated with the promotion and growth of breast cancer. Estrogen exerts most of its effects through two nuclear estrogen receptors (ERs), ERα and ERβ. The full-length human ERβ (also named ERβ1) protein includes 530 amino acids is the most widely expressed ER in normal mammary tissue and is expressed in both luminal and myoepithelial cells. Previous reports have shown that total ERβ levels decline during breast tumorigenesis [11]. Generally higher expression of ERβ was found to be associated with a good prognostic marker or better clinical outcome. Recent studies have shown that ERβ act as a tumor suppressor gene [9,23], however, the mechanisms of ERβ1 in tumorigenesis, development, metastasis and prognosis remain unclear.

E-cadherin, a single-span transmembrane glycoprotein of five repeats and cytoplasmic domain, is expressed primarily in epithelial cells. E-cadherin serves as an invasion or metastasis suppressor. Over-expression of E-cadherin has been shown to reduce the progression and invasiveness of tumors, as well as the formation of metastases [24]. E-cadherin is a tumor suppressor protein that used as a prognostic marker for breast cancer [25]. Several tumor suppressor proteins have also been shown to regulate the expression of E-cadherin in breast cancer, such as Retinoblastoma (Rb) [26]. However, it is not clear whether E-cadherin is regulated by ERβ1. In this study, we found that ERβ1 expression is positively correlated with E-cadherin expression in breast cancer cells and ERβ1 upregulates E-cadherin expression in breast cancer cell lines.

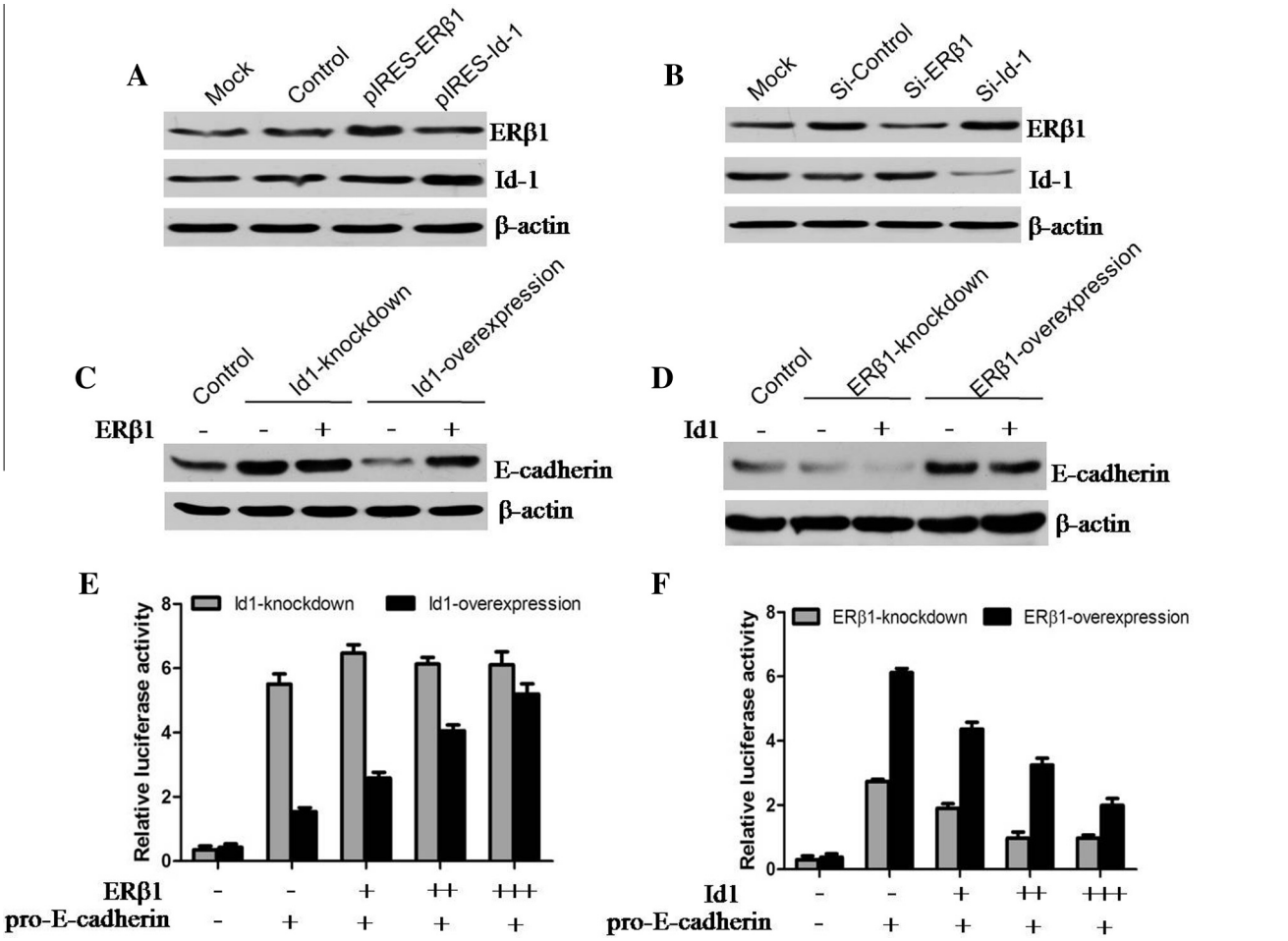

Fig. 3. ERβ1-induced up-regulation of E-cadherin expression is Id1 dependent. (A and B) Western blot detected the expression of ERβ1 and Id1 in the stable ERβ1 or Id1-overexpression MDA-MB-231 cells (A) and ERβ1 or Id1-knockdown MDA-MB-231 cells (B). (C) Id1-knockdown MDA-MB-231 cells or Id1-overexpression MDA-MB-231 cells were transfected with pIRES-ERβ1 respectively. After 48 h, the E-cadherin protein was examined by western blot. (D) Western blot analysis of E-cadherin protein after transfection with pIRES-Id1 in ERβ1-knockdown cells or ERβ1-overexpression cells. (E) Id1-knockdown or Id1-overexpression cells were cotransfected with E-cadherin promoter-Luc and increasing amounts pIRES-ERβ1(+, ++, and +++ correspond to 0.2, 0.5, and 1 μg DNA) for 24 h, then the luciferase assays were performed. (F) E-cadherin promoter-Luc and increasing amounts pIRES-Id1 were cotransfected into ERβ1-knockdown or ERβ1-overexpression cells for 24 h, then the luciferase assays were performed. The luciferase activity was normalized against pRL-TK *Renilla* luciferase activity. Data are mean ± SD of three independent experiments.

These results indicated that ERβ1 can regulate the expression of E-cadherin, however, the molecular mechanism of ERβ1-induced upregulation of E-cadherin expression is not clear.

The classic mechanism of ERα and ERβ regulate gene transcription is direct binding to estrogen-regulated elements (ERE). Besides this direct binding regulation mechanism, recent studies have shown that ERα and ERβ can regulate gene transcription indirectly by protein–protein interaction with transcription factor activator protein-1 (AP-1) or Sp1 [27,28]. Recently, our group published a study [19] that presented the Id1 as a novel estrogen ERβ1 binding partner. The Id (inhibitor of DNA binding) genes were originally identified in murine myoblasts, where they prevented myogenic basic helix-loop-helix (bHLH) transcription factors from binding muscle-specific regulatory elements. Id protein family consists of four members (Id1 to Id-4). As well as other Id proteins, Id1 contains a well-conserved HLH domain that binds to the basic HLH (bHLH) proteins. Id1 proteins dimerize with bHLH proteins through HLH domains, and inhibits bHLH proteins bind to DNA to activate the transcription of target genes containing E-boxes (CANNTG) in their promoters. Thus, Id proteins are dominant-negative regulators of bHLH function. Early studies have shown that Id1 control the differentiation of muscle, neurons, mammary, and B and T-cells. Recently, Id1 has been implicated as an oncogene which may play role in the tumorigenesis of many cancers which include breast, colon, and prostate cancer. Recent reports show that a number of signaling pathways involved in tumor progression can be activated or inhibited by overexpression of Id1. For example, overexpression of Id1 was found to induce serum-independent cell growth through inactivation of the p16/RB tumor suppressor pathway [29]. In addition, Id1 expression was found to protect the cells from chemotherapeutic drug-induced apoptosis through activation of the Raf-1/MAPK and JNK pathways. Moreover, overexpression of Id1 was found to induce MT1-MMP and inhibit E-cadherin protein expression, leading to invasion of breast cancer [30] cells. In this study, we found that overexpression ERβ1 decreased MDA-MB-231 cells migration and invasion and upregulation Id1 increased cell migration and invasion. To investigate the mechanisms involved in ERβ1-induced suppression of cell migration and invasion in human breast cancer, we detected the expression of E-cadherin of ERβ1-expressing, Id1-expressing, ERβ1-silencing, and Id1-silencing cells and compared with that in control cells. Our results show that ERβ1-induced E-cadherin expression is Id1 dependent, which not through regulating the expression of Id1. These results provide a new evidence for a novel molecular pathway by which ERβ1 maintain the E-cadherin at a higher level through binding with Id1 and overcoming the suppressive activity

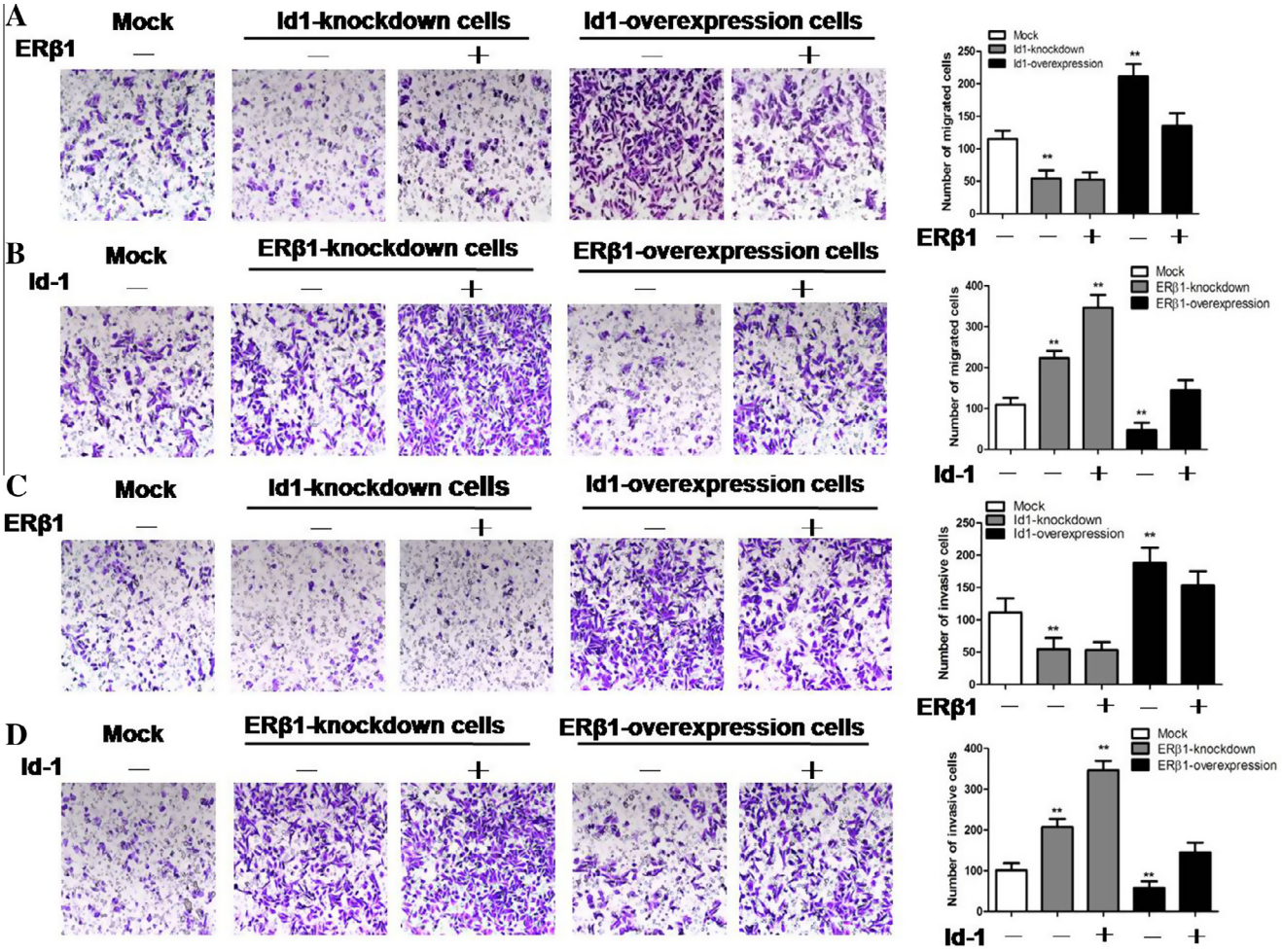

Fig. 4. Id1-dependent upregulation of E-cadherin by ERβ1 inhibits the migration and invasion of breast cancer cells. (A and C) After transfected with pIRES or pIRES-ERβ1 for 24 h, the cells were seeded in transwell filters (A) or ECM gel-coated transwell culture chambers (C) and incubated for 24 h, then the transwell migration assay (A) or invasion assay (C) were conducted. (B and D) Cells were transfected with pIRES or pIRES-Id1 for 24 h, then the cells were seeded in transwell filter, and after 24 h incubation, the cell migration (B) and invasion (D) were determined by transwell assay. Transwell assays were performed in triplicates with mean ± SD, and representative images are displayed. $^{**}P < 0.01$.

of Id1. Clinical studies also found that much higher levels Id1 in patients with negative estrogen receptor status compared to those with positive status, suggesting that there a closely correlated between ER and the biological function of Id1 [31].

In conclusion, our study showed that the expression of ERβ1 was positively correlated with E-cadherin expression in breast cancer cells and ERβ1 upregulates E-cadherin expression in breast cancer cell lines. We also found that ERβ1 inhibits the migration and invasion of breast cancer cells and upregulated E-cadherin expression in a Id1-dependent manner. This study provides a novel molecular mechanism of ERβ1 in tumor metastasis and suggests the feasibility of developing novel therapeutic approaches to target Id1 to inhibit breast cancer metastasis.

Acknowledgment

This work was supported by National Nature Science Foundation of China (No. 81102031).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.12.038.

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