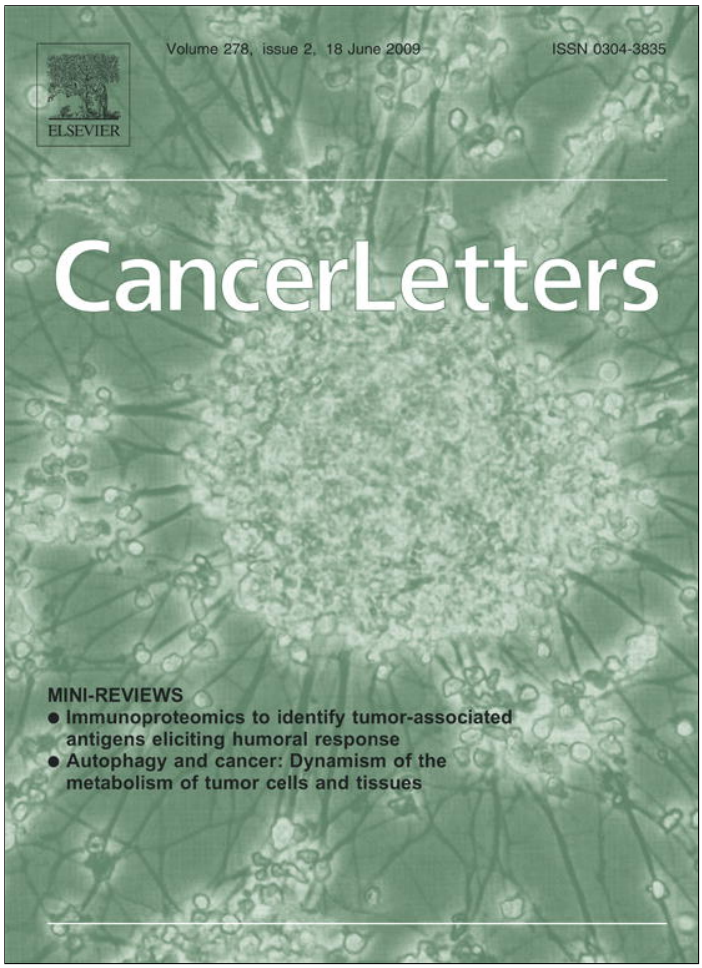

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Identification of a novel estrogen receptor β1 binding partner, inhibitor of differentiation-1, and role of ERβ1 in human breast cancer cells

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ABSTRACT

Estrogen plays an important role in the proliferation and progression of breast cancer. The estrogen signal is mediated by the estrogen receptors (ERα and ERβ). ERα (estrogen receptor α) is an important promoter of growth in breast cancer; however, the role of ERβ (estrogen receptor β) in breast cancer is less clear. In this study, using a yeast two-hybrid screening technique, we identified a novel ERβ1-interacting protein, inhibitor of differentiation-1 (Id1), which is a dominant negative regulator of bHLH transcription factors, and promotes cell proliferation in breast cancer cells. Using mammalian two-hybrid protein–protein interaction assays, we found that the helix–loop–helix domain of the Id1 protein was essential for the physical interaction between ERβ1 and Id1. In addition, we found that 17-β estradiol inhibits ERβ1 binding with Id1. Furthermore, we observed that ERβ1 inhibited cell growth of MDA-MB-231 cells and upregulated p21 expression and that ERβ1 upregulation of p21 is Id1 dependent. Taken together, our study demonstrates a novel ERβ1 binding partner, Id1, and a mechanism by which ERβ1 inhibits breast cancer cell growth through binding with Id1 and upregulating p21 gene expression.

1. Introduction

Estrogen is a key regulator of growth and differentiation in a broad range of target tissues, including the reproductive tract, mammary gland, and the central nervous and skeletal systems [1,2]. Estrogen bind to and activate two estrogen receptors (ERα and ERβ) and exert their effects through a complex array of signaling pathways that mediate genomic and non-genomic events [3,4]. ERs are members of the nuclear receptor superfamily of ligand-regulated transcription factors [5]. ERα, which was originally cloned in the mid-eighties [6,7], is an important regulator of growth and differentiation in the mammary gland and the female reproductive tract. It is also involved in the development of some malignant tumors. ERβ was more recently cloned from the rat prostate in 1996 [8]. The structure of ERα and ERβ diverged early during evolution [9] and differ mostly in the N-terminal A/B and F domains, exhibiting 15% and 18% identity, respectively. However, the ligand binding domain (E domain) is moderately conserved between both receptors showing only 59% amino acid identity [10]. These differences suggest that the two receptors differ in terms of action. ERβ is classified as a type I nuclear receptor, and it forms homodimers and binds to cognate-responsive elements consisting of a palindromic repeat [11]. The human ERβ gene is located on chromosome 14 q23.2 and is ~61.2 kb. Various ERβ mRNA isoforms (ERβ1 ~ 6) have been described in humans, primates, rats, and mice [12,13], and the 530 amino acid form of ERβ1 is considered to be the wild-type, full-length human ERβ [14], and is the only fully functional isoform [11]. ERβ1 tends to form heterodimers with other isoforms

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under the stimulation of estrogens. In contrast, ERβ2, ERβ4, and ERβ5 do not form homodimers and have no innate activities of their own [11].

Recently, ERβ has been considered to act as a tumor suppressor gene [15]. For example, the expression of ERβ has been shown to decrease in various cancers, such as breast cancer [16], prostate cancer [17], lung cancer [18], and colorectal cancer [19]. Accumulated data from protein studies in breast cancer tissues indicate that positive expression of ERβ appears to correlate with a favorable prognosis. Data from a number of tissue culture assays [20], microarray analyses [21,22], and, in particular, ERβ null mice [23], generally support a role of hERβ as a cell proliferation inhibitor. However, until now, the mechanisms responsible for the decreased expression of ERβ in tumors and the biological functions of ERβ are still not fully clear.

Id1 is a member of the helix–loop–helix (HLH) transcriptional factor family. It lacks the basic DNA binding region; therefore, it is considered to act as a dominant negative regulator of basic HLH transcription factors by forming heterodimers [24]. Id1 has multiple functions, including inhibition of differentiation, induction of proliferation, and delaying replicative senescence [25]. Id1 has been reported to promote cell proliferation and cell cycle progression through inactivation of tumor suppressor and activation of growth promoting pathways in mammalian cells [26,27]. Several signaling pathways have been suggested to mediate the function of Id1. For example, Id1 expression promotes cell survival through activation of NF-κB signaling pathway in prostate cancer cells [28]. The Id1-induced invasion ability in breast cancer cells is mediated by inducing the expression of a 120 kDa gelatinase, a member of the type IV collagenase matrix metalloproteinase family [29]. In addition, Id1 is involved in the down-regulation of E-cadherin expression and reorganization of β-catenin and F-actin as well as in transforming growth factor-β-induced EMT in several cell types [30,31].

In this study, by using a yeast two-hybrid screening technique, we identified a novel ERβ1-interacting protein, Id1, which interacts with multiple signaling transduction pathways. In addition, we found that the HLH domain of the Id1 protein was responsible for the interaction between ERβ1 and Id1. We also demonstrate that the physical interaction between ERβ1 and Id1 is essential for ERβ1-mediated induction of p21 expression and cell survival in MDA-MB-231 cells. Taken together, our results provide further understanding of the molecular mechanism of inhibition of cell proliferation mediated by ERβ1 proteins.

2. Materials and methods

2.1. Cell culture

Human breast cancer cell lines MDA-MB-231 (purchased from American Type Culture Collection, Manassas, VA, USA), were maintained in modified MEM media supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 20 μg/mL gentamycin. Cell cultures were grown at 37 °C in a humidified atmosphere of 5% CO_2 in a Hereaus CO_2 incubator. Prior to experiments, the modified MEM medium was removed and cells were washed with PBS. Then, a defined media containing DMEM/F12 media supplemented with 1.0 mg/mL human albumin, 5.0 mg/L human transferrin and 5.0 mg/L bovine insulin, was added and cells were maintained for 24 h. During the experiments, cells were grown on defined media. 17-β estradiol (E2) was purchased from Sigma and dissolved in 100% ethanol.

2.2. Yeast two-hybrid screen

The yeast two-hybrid LexA system was provided by Clontech. Experiments were performed according to the manufacturer's instructions. The DNA fragments of the LBD domain encoding 277 amino acids residues 254–530, which covers the extreme C-terminal end of human ERβ1, were cloned by PCR from the full-length ERβ1 cDNA, which had been cloned from MDA-MB-231 cells. The DNA was subcloned into pLexA to construct the bait vector pLexA-LBD by EcoRI and XhoI sites. The pLexA-LBD was then transfected into the yeast host strain EGY48. We found, by repression assays, that the bait protein did not have transcription activation for leu and lacZ reporter genes. Human fetus brain cDNA library plasmids pB42AD (Clontech) were screened in EGY48 yeast containing the bait vectors pLexA-LBD and p8op-lacZ. The clones containing galactose dependent lue+ and lacZ+ were isolated. The library plasmids in positive yeast clones were rescued by means of transforming KC8 *Escherichia coli*, and were sequenced and analyzed by BLASTn.

2.3. Immunofluorescence microscopy

Cells were grown on glass coverslips and fixed with 3.7% paraformaldehyde. Then, the cells were permeated by 0.4% Saponin for 2 h. After blocking in normal mouse and rabbit serum, the cells were incubated with double primary antibodies: mouse monoclonal anti-ERβ1 antibody (1:500; Chemicon International) and rabbit polyclonal anti-Id1 antibody (1:400; Santa Cruz). Double secondary antibodies were FITC-labeled anti-mouse antibody (1:50; Serotec) and Texas red-labeled anti-rabbit antibody (1:400; KPL). The cells were analyzed by confocal laser microscopy (LEICA, TCS-NT 165 123).

2.4. Western blotting and immunoprecipitation

The cell lysates were collected using mammalian protein extraction reagent (Pierce) with protease inhibitors. The protein concentration of each sample was determined by the BCA-200 protein assay kit (Pierce). The proteins were resolved on 12% SDS–polyacrylamide gels and transferred onto nitrocellulose membranes. The membrane was blocked in blocking buffer (5% non-fat milk in TBST), and then incubated with the mouse anti-human ERβ1 monoclonal antibodies (Serotec) or rabbit anti-human Id1 polyclonal antibody (Santa Cruz) at 4 °C overnight. HRP-conjugated goat anti-mouse or goat anti-rabbit IgG was

used as the secondary antibody (Santa Cruz). Chemiluminescent luminol reagent (Santa Cruz) was used according to the manufacturer's instructions. Immunoprecipitation was done according to research applications (Santa Cruz). The MDA-MB-231 cells were washed with PBS and lysed in ice-cold RIPA buffer containing protease inhibitors. 400 μg of cell lysate proteins was incubated in each tube with 14 μL mouse anti-human ERβ1 monoclonal antibody or 14 μL rabbit anti-human Id1 polyclonal antibody for 2 h at 4 °C. Then, 20 μL of protein G-agarose was added and incubated at 4 °C overnight on a rocker platform. After centrifugation, the agarose beads were washed four times with RIPA at 4 °C, and suspended in sample buffer. The precipitates were identified with Western blotting.

2.5. Mammalian two-hybrid protein–protein interaction assays

The system was provided by Clontech. The entire coding sequence of human ERβ1 was subcloned in-frame into pM vector (Clontech) encoding the GAL4 DNA binding domain. The full-length and four types of truncated cDNAs of Id1 were amplified by PCR using templates of human Id1 cDNA (gifts of E. Hara, J. Campisi and M. A. Israel), and subcloned in-frame into the pVP16 vector (Clontech) encoding the VP16 transactivation domain. pG5CAT reporter vectors (0.5 μg) containing a chloramphenicol acetyltransferase (CAT) reporter gene under the control of the GAL4 response element and 2.5 μg of each of the above-mentioned constructed vectors were co-transfected into 2.5×10^5 MDA-MB-231 cells per well in six-well plates with the LF2000 reagent (GIBCO BRL). After 48 h, the cells were harvested and the extracts were assayed for CAT activity using CAT ELISA assay kits (Roche).

In 17-β estradiol interference experiments, co-transfected MDA-MB-231 cells with pG5CAT reporter vectors, pVP16-Id1, and pM-ERβ1 were treated for 6 h in 5, 10 and 20 nM 17-β estradiol. Then, the cells were assayed for CAT activity. The concentration of 17-β estradiol of 10 nM was within the physiologic range, which could saturate the ER. These concentrations have been widely applied in other studies [32,33].

2.6. Stable transfection and selection of ERβ1- and Id1-transfected cells

The pIRES expression vector contain a cytomegalovirus promoter and pIRES-ERβ1 and pIRES-Id1 encode human ERβ1 and Id proteins, respectively. The full-length cDNA of ERβ1 and Id1 were amplified by PCR, inserted into the pIRES plasmids with Clal and Xbal sites, and were confirmed by DNA sequencing. Transfection was done by Lipofectin (Life Technologies) in accordance with the manufacturer's instructions. MDA-MB-231 cells cultured in 6-cm dishes were washed twice and supplemented with 3 mL Opti-MEM reduced serum medium. The pIRES-ERβ1, pIRES-Id1, and pIRES plasmid DNA (2 μg per 6-cm dish) was mixed with Lipofectin before addition to tumor cells. After transfection, stable transfectants were selected by incubating with 500 μg/mL geneticin (G418, Sigma–Aldrich). The surviving colonies were picked ~2 weeks later. Colonies C1 and C2 (transfected with pIRES-ERβ1), clones D1 and D2 (transfected with pIRES-Id1) and clone V (transfected with pIRES, empty vector), were amplified, and ERβ1 and Id1 expression were determined by Western blotting. Positive clones were maintained in culture medium supplemented with 250 μg/mL G418. The cultures of clones C1, C2, D1, D2, and V in 60-mm dishes were lysed for Western blotting.

2.7. Stable transfection and selection of ERβ1- and Id1-silencing cells

RNAi was performed using the pSilencer 3.1H1 hygro vector (Ambion) to direct the expression of the relevant hairpin double-stranded sequence. The selection of small interfering RNA (siRNA) target sites was dependent on siRNA Design (Ambion). A sequence of 19 nucleotides in Id1 was found and compared with the human genome database using BLAST (http://www.ncbi.nlm.nih.gov/BLAST). The hairpin siRNA template oligonucleotides, 5′-G A T C G A C G T G C T T C G C G G G T G C A A T T C A A G A C G T T G C A C C C G C G A A G C A C G T T T T T T T T A-′3 And 5′-A G C T T A A A A A A A A C G T G C T T C G C G G G T G C A A C G T C T T G A A T T G C A C C C G C G A A G C A C G T C-′3 for ERβ1, and 5′-G A T C C G C T C G G A A T C C G A A G T T G G A T T C A A G A G A T C C A A C T T C G G A T T C C G A G T T T T T T T G G A A A-3′ and 5′-A G C T T T T C C A A A A A A A C T C G G A A T C C G A A G T T G G A T C T C T T G A A T C C A A C T T C G G A T T C C G A G C G-3′ for Id-1, were chemically synthesized, annealed, and cloned into the pSilencer 3.1H1 hygro vector between the BamHl and Hindlll site. They were referred to as pSilence-ERβ1 and pSilencer-Id1, respectively. The pNegative vector (negative control of the pSilencer 3.1H1 hygro vector that expresses a hairpin siRNA with limited homology to any known sequences in the human, mouse and rat genomes) was also transfected as a control. Stable transfections were obtained by sustained selection of hygromycin B (Stratagene) at a concentration of 200 μg/mL. The positive reconstructs were validated by sequencing. MDA-MB-231 cells were transfected with expression plasmids of pSilence-ERβ1 (clone si-ERβ1-1 and si-ERβ1-2), pSilencer-Id1 (clone si-Id1-1 and si-Id1-2) or pNegative vector (clone si-V) by FuGene6 reagent (Roche) according to the manufacturer's instructions. Positive clones were maintained in culture medium supplemented with 200 μg/mL hygromycin B. ERβ1 and Id1 expression in clones si-ERβ1-1, si-Id1-1 and si-V were examined by Western blotting. The expression of $p21^{CIP1/WAF1}$, $p^{16INK4a}$, p53, MMP9 and E-cadherin in clones of si-ERβ1-1, C2, si-Id1-1 and D1 were examined by Western blotting.

2.8. Growth rate analysis

The above cell clones were seeded in 24-well plates at a density of $1 \times 10^4 \sim 2 \times 10^4$ cells per well. At each time-point, cells were collected by trypsinization and centrifugation. The cells were counted with a hemocytometer. All samples were prepared in quadruplicate and the entire experiment was repeated twice.

2.9. $p21^{WAF1/CIP1}$ luciferase reporter gene assay

The human $p21^{CIP1/WAF1}$ promoter construct, WWP-luc, was a gift of Dr. Bert Vogelstein (Johns Hopkins University) [34]. The 2.4-kb genomic fragment pair containing the $p21^{CIP1/WAF1}$ cDNA start site was subcloned into the SacI and HindIII sites of the luciferase reporter vector, pGL3-basic (Promega), to create wild-type pGL3-p21P-luc (p21-Luc). The reporter gene constructs were transiently transfected in MDA-MB-231 cells and some cell clones (1.0 μg/1.0 × 10^6 cells) by using the FuGENE 6 reagent. Twenty-four hours after transfection, cells were supplemented with fresh medium. The cells were rinsed with PBS and then harvested using 1× passive lysis buffer. Luciferase activity of the cell lysate was then determined using a dual-luciferase reporter assay kit (Promega). The activity was expressed as relative luciferase units (RLU) per mg protein. pGL3-control vector, vacant vector pGL3-Basic, and pRL vector were purchased from Promega and used as control reporter plasmid.

2.10. Statistical analysis

Data are presented as means ± SD of three independent experiments. Student's *t-test* was used to determine the statistical difference between experimental and control groups. Differences were considered statistically significant at a level of $P < 0.05$.

3. Results

3.1. Identification of an ERβ1-interacting protein, inhibitor of differentiation-1 (Id1)

To identify novel ERβ1-interacting proteins, we used the LBD domain cDNA of human ERβ1 as bait in a yeast two-hybrid system (LexA) to screen a human fetus brain cDNA library. Eight positive clones were selected (data not shown). Sequencing analysis and database comparison revealed that three positive clones had high sequence similarity with *Homo sapiens* inhibitor of DNA binding 1, dominant negative helix–loop–helix protein (Id1) (GenBank™ accession number NM_002165). The three positive clones have respective sequence identities of 96% (647/674), 97% (681/702) and 98% (576/588).

To confirm the *in vivo* interaction between ERβ1 and Id-1, we screened some human breast cancer cell lines (T47D, SK-BK-3, MCF-7 and MDA-MB-231) by western blotting for both ERβ1 and Id-1 positive cells in Fig. 1. MDA-MB-231 cells, with relatively high levels of endogenous ERβ1 and Id-1 [35,24], were used in the immunoprecipitation experiments. We also investigated the sub-cellular distribution of ERβ1 and Id1 with immunofluorescence. As shown in Fig. 2A, both ERβ1 and Id1 were distributed and co-localized in the cytoplasm of MDA-MB-231 cells. The immunoprecipitation from the lysates of MDA-MB-231 cells were carried out with anti-ERβ1 and anti-Id1 antibodies. A single band at about 19 kDa was recognized by the anti-Id1 antibody in both precipitates analyzed by western blotting. On the other hand, ERβ1 protein, with a molecular weight about 60 kDa, was identified in both co-precipitates (Fig. 2B). These data strongly support that ERβ1 associates with Id1 in human breast cancer cells.

To identify the binding domain for Id-1 binding to ERβ1, we constructed a full-length Id1 vector and four vectors expressing truncated forms of Id1 (△Id1*a* 142–155 aa; △Id1*b* 110–155 aa; △Id1*c* 69–155 aa; △Id1*d* 1–109 aa, Fig. 3A). We used mammalian two-hybrid protein–protein interaction assays to further investigate the interaction *in vivo* and delineate the binding activities. We found that ERβ1 or Id1 alone could not stimulate the expression of the CAT report gene, whereas the co-expression of ERβ1 and Id1 did stimulate expression of the CAT report gene. Furthermore, the full-length Id1, △Id1*a*, and △Id1*b*, containing

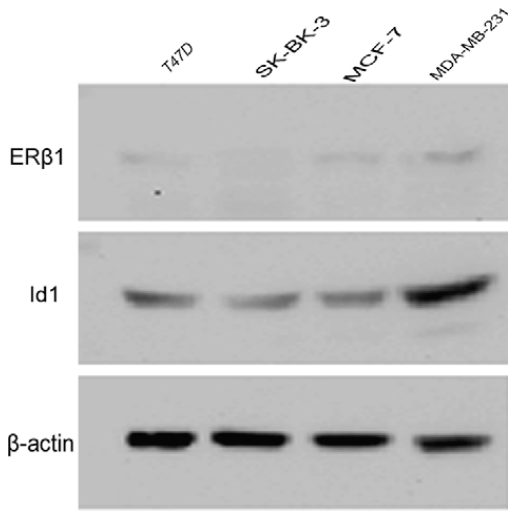

Fig. 1. The expression pattern of ERβ1 and Id1 expression in different breast cancer cell lines. The expression of ERβ1 and Id1 genes in different breast cancer cell lines (T47D, SK-BK-3, MCF-7, and MDA-MB-231 cells) were examined by Western blotting. The β-actin was used to confirm equal protein loading. Each lane was loaded with up to 30 μg protein.

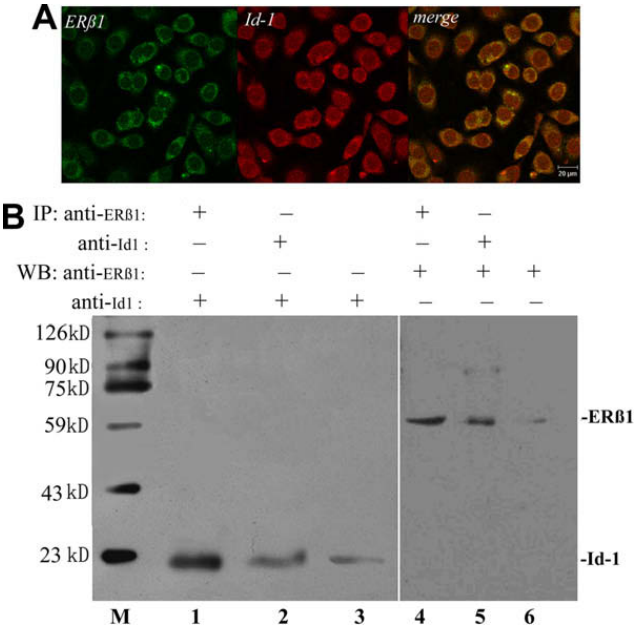

Fig. 2. Co-localization and co-immunoprecipitation analysis of endogenous ERβ1 and Id1 in MDA-MB-231 cells. (A) Co-localization of ERβ1 and Id1 in MDA-MB-231 cells. Each image represents the same field visualized by confocal laser microscopy. ERβ1 and Id1 were stained with FITC in green and Texas Red, respectively. Composite image shows co-localization of ERβ1 and Id1 proteins in yellow particles at cytoplasm. (B) Co-immunoprecipitation of MDA-MB-231 cell lysates. Lane 1 shows Id1 protein that was identified from the ERβ1 antibody co-precipitates. ERβ1 proteins in these co-precipitates are shown in lane 4. Lane 5 shows ERβ1 proteins that were detected from Id1 antibody co-precipitates, Id1 proteins in the same co-precipitates are shown in lane 2. Lanes 3 and 6 are Id1 and ERβ1 proteins, respectively, from MDA-MB-231 cell lysates. Lanes 3 and 6 were loaded with up to 30 μg protein. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the HLH domain, bound to the ERβ1 and stimulated the expression of the CAT gene, increasing the activity by 2–3 fold compared with △Id1c or △Id1d binding to ERβ1 (Fig. 3B). These results suggest that the HLH

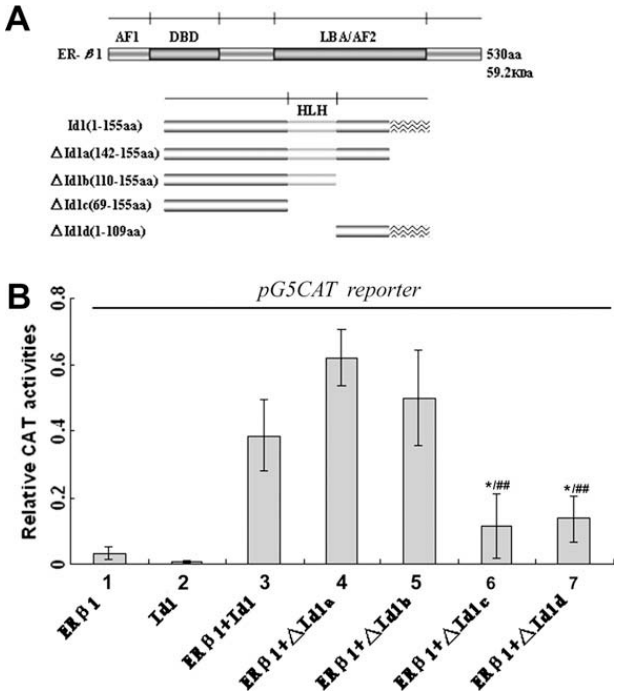

Fig. 3. Binding activities of ERβ1 and Id1 in MDA-MB-231 cell lines. Binding activities of ERβ1 and Id1, or four truncated forms of Id1 in the MDA-MB-231 cell line. (A) The constructed fusion proteins in mammalian two-hybrid protein–protein interaction system were used to compare the binding activities between the full-length ERβ1 and wild-type Id1, or truncated forms of Id1. (B) The reporter plasmid pG5CAT expressing CAT and the pM-GAL-DBD construct of ERβ1 were co-transfected into MDA-MB-231 cells with either a pVP16-AD construct of Id1 or four types of truncated Id1. In the presence of pG5CAT, two groups of control cells were transfected with only pM-ERβ1 and pVP16-Id1 constructs. CAT activity was measured at an absorbance of 415 nm. Error bars represent standard deviations ($n = 3$, three independent experiments in duplicate; $^{*}p < 0.05$, column 6 or 7 *vs.* column 3; $^{\#\#}p < 0.01$, column 6 or 7 *vs.* column 4 or 5).

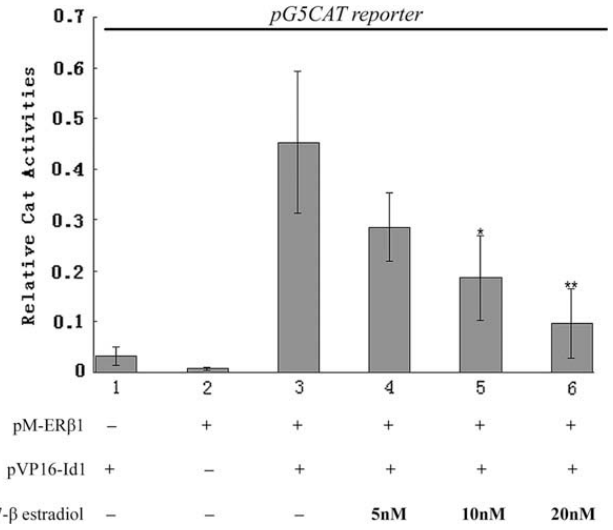

Fig. 4. Effects of 17-β estradiol on ERβ1 binding with Id1. The constructed fusion proteins in a mammalian two-hybrid protein–protein interaction system were used to determine the effect of 17-β estradiol on ERβ1 binding with Id1. In the presence of pG5CAT, two groups of control cells (columns 1 and 2) were transfected with only pVP16-Id1 or pM-ERβ1 constructs; in the other groups, cells were co-transfected with pM-ERβ1 and pVP16-Id1 constructs. At 48 h after transfection, and after exposure to 17-β estradiol for 6 h, the CAT activity of columns 4, 5 and 6 (5, 10 or 20 nM 17-β estradiol, respectively), were examined by CAT ELISA assays. The CAT activity was lower in the cells treated with 17-β estradiol compared with the untreated cells (column 3) ($^{*}p < 0.05$, $^{**}p < 0.01$), and was dose-dependent. Error bars represent standard deviations ($n = 3$, three independent experiments in duplicate).

domain (Id1, △Id1*a*, and △Id1*b*) is an important factor that regulates the expression of the CAT reporter gene, and that the helix–loop–helix domain of Id1 is essential for the physical interaction between ERβ1 with Id1.

3.2. 17-β Estradiol regulates ERβ1 binding with Id1 in MDA-MB-231 cells

To investigate the role of 17-β estradiol (E2) in regulation of ERβ1 binding with Id1, we used the mammalian two-hybrid protein–protein interaction system. The constructs used for reporter assays are illustrated in Fig. 3B. The co-transfected MDA-MB-231 cells were treated for 6 h with 5, 10 or 20 nM 17-β estradiol, respectively. We found that pM-ERβ1 or pVP16-Id1 alone could not stimulate the expression of the CAT report gene (Fig. 4, columns 1 and 2), whereas co-transfection of pM-ERβ1 and pVP16-Id1 did stimulate expression of the CAT report gene (Fig. 4, column 3). When treated with 10 or 20 nM 17-β estradiol, CAT activity was inhibited (Fig. 4, columns 5 and 6). These data show that 17-β estradiol can inhibit the interaction between ERβ1 with Id1, and the ability is concentration dependent (Fig. 4, columns 3, 4, 5, and 6). These results demonstrate that 17-β estradiol may be an inhibitor of ERβ1 binding with Id1.

3.3. ERβ1 inhibits MDA-MB-231 cell growth

To study the biological function of ERβ1 on MDA-MB-231, we established stable ERβ1-overexpressing MDA-MB-231 cells (clone C1, C2) and stable ERβ1-silencing MDA-MB-231 cells (clone si-ERβ1-1, si-ERβ1-2). Growth assay curves showed that ERβ1 gene expression decreased the growth of MDA-MB-231 cells (Fig. 5A, C, and E). We also prepared Id1-overexpressing MDA-MB-231 cells (clone D1, D2) and Id1-silencing cells (si-Id1-1, si-Id1-2). Our results showed that Id1 gene expression results in increased growth of MDA-MB-231 cells, which was consistent with previous studies (Fig. 5B, D, and F). Meanwhile, our results suggested that the ERβ1-overexpressing or -silencing did not regulate the expression of Id1 gene in MDA-MB-231 cells (Fig. 5E lane 3 and Fig. 5F lane3), and the Id1-overexpressing or -silencing did not regulate the expression of ERβ1 gene (Fig. 5E lane4 and Fig. 5F lane 4).

To investigate the mechanisms involved in ERβ1-induced suppression of cell growth and in Id-1-induced proliferation of MDA-MB-231 cells, we examined the expression levels of Id-1-regulated genes on cell growth ($p21^{CIP1/WAF1}$ and $p16^{INK4a}$), apoptosis (p53) and invasion (MMP9 and E-cadherin) in clones C2, D1, si-ERβ1-1, and si-Id1-1, compared with that of the controls (Fig. 5G). The $p21^{WAF1/CIP1}$ expression was reduced in si-ERβ1-1 cells (Fig. 5G, lane 2 *vs.* lane 1) and Id1-expressing D1 cells (Fig. 5G, lane 5 *vs.* lane 1). Expression of $p21^{WAF1/CIP1}$ was increased in ERβ1-expressing C2 cells (Fig. 5G, lane 3 *vs.* lane 1) and si-Id1-2 cells (Fig. 5G, lane 4 *vs.* lane 1). Similarly, $p^{16INK4a}$ expression was affected in a similar pattern to $p21^{WAF1/CIP1}$ (Fig. 5G), albeit more weakly. The expression of p53 and MMP9 was high in nearly all clones, and not obviously in response to the changes of ERβ1 and Id1; E-cadherin was weakly expressed in all clones (Fig. 5G). These results indicated that the $p21^{WAF1/CIP1}$ and $p^{16INK4a}$ proteins are upregulated by ERβ1, and downregulated by Id1. Response patterns of $p21^{WAF1/CIP1}$ and $p^{16INK4a}$ may contribute to better understanding of the mechanism by which ERβ1 inhibits MDA-MB-231 cell growth. Indeed, we had hypothesized that interactions between ERβ1 and Id-1 may play a role in mediating cell proliferation in breast cancer.

3.4. ERβ1-induced up-regulation of $p21^{WAF1/CIP1}$ expression is Id-1 dependent

Because up-regulation of $p21^{WAF1/CIP1}$ expression by ERβ1 was confirmed by western blotting (Fig. 5G), we examined whether the effect of ERβ1 on $p21^{WAF1/CIP1}$ expression was mediated by Id-1. A reporter assay with p21 promoter-Luc was used; the clones of Id1-expressing D2 and Id1-silencing si-Id1 cells were co-transfected with increasing amounts of ectopic ERβ1 (pIRES-ERβ1). In the si-Id1 clone, activity of the p21 promoter-Luc remained unchanged and was independent of the increasing expression of ERβ1 (Fig. 6A, columns 2, 3, 4, and 5). In contrast, in the

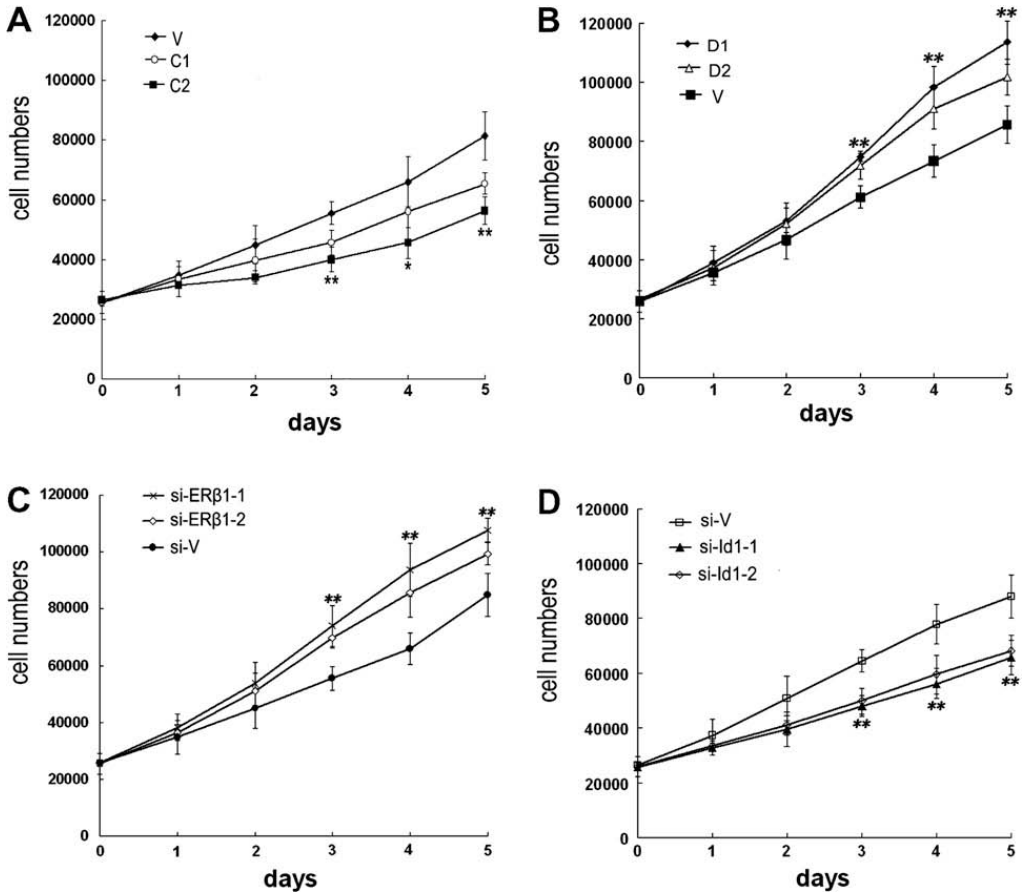

Fig. 5. Effects of ERβ1 and Id1 expression or suppression on cell growth in MDA-MB-231 cells. Cells were cultured in defined media for 24 h and subjected to various assays. Blots shown are representative of at least three independent experiments. Clone V, stable transfectant of MDA-MB-231 cells expressing empty pIRES plasmid; clones C1 and C2, stable clones of MDA-MB-231 cells expressing pIRES-ERβ1; clones D1 and D2, stable clones of MDA-MB-231 cells expressing pIRES-Id1; clone si-V, stable clones of expressing pNegative vector; clones si-ERβ1-1 and si-ERβ1-2, stable clones of knockdown ERβ1; clones si-Id1-1 and si-Id1-2, stable clones of knockdown Id1. The data were analyzed by Student's *t*-test and asterisks indicate significant differences. Error bars represent standard deviations ($n = 3$, three independent experiments in duplicate). (A) Overall cell growth of clones C1, C2, and V were examined by cell count. Clones C1 or C2 compared with clone V ($^{**}p < 0.01$, $^{*}p < 0.05$). (B) Growth curves of clones D1, D2, and V. Clones D1 or D2 compared with clone V ($^{**}p < 0.01$). (C) Growth curves of clones si-ERβ1-1, si-ERβ1-2 and si-V, clones si-ERβ1-1 or si-ERβ1-2 compared with clone si-V ($^{**}p < 0.01$). (D) Growth curves of clones si-Id1-1, si-Id1-2, and si-V, clones si-Id1-1 or si-Id1-2 compared with clone si-V ($^{**}p < 0.01$). In (E) with clones V, C2, D1, and (F) clone si-V, si-ERβ1-1 and si-Id1-1, the expression of ERβ1 and Id1 genes was examined by western blotting. β-actin was used to confirm equal protein loading. (G) The expression of p21, p16, p53, MMP9, and E-cadherin genes in MDA-MB-231 cells and clones si-ERβ1-1, C2, si-Id1-1, and D1 were examined by western blotting. The data shown are relative to β-actin in the same lane. Each lane was loaded with up to 30 μg protein.

Id1-expressing clone D2 cells, the increase in p21 promoter-Luc activity was dependent on the increased expression of ERβ1 (Fig. 6A, columns 7, 8, 9, and 10). These results indicate that ERβ1-mediated up-regulation of p21 expression is Id1 dependent. In si-ERβ1-1 and C2 clones, the decreased activity of p21 promoter-Luc activity was dependent on the increased Id1 expression (Fig. 6B, columns 2, 3, 4, 5, 7, 8, 9, and 10). In si-ERβ1-1 cells, Id1 significantly inhibited the activity of p21 promoter-Luc (Fig. 6B, columns 2, 3, 4, 5) and, in ERβ1-expressing cells, the ability of Id1 to inhibit the activity of p21 promoter-Luc was reduced. These results indicate that the Id1-mediated inhibition of p21 promoter-Luc activity is independent of ERβ1, but ERβ1 reduced the ability of Id1 to inhibit p21 expression. Taken together, $p21^{WAF1/CIP1}$ may contribute to ERβ1-mediated inhibition of MDA-MB-231 cell growth, a role which is Id1 dependent; in other words, ERβ1 inhibits MDA-MB-231 cell growth by upregulating the expression of $p21^{WAF1/CIP1}$, through binding with Id1, and removing the Id1 restraint on $p21^{WAF1/CIP1}$ expression.

4. Discussion

In this study, we used the LBD domain cDNA of human ERβ1 as a bait to screen a human fetus brain cDNA library in yeast two-hybrid system, and identified a novel ERβ1-interacting protein, Id1. We also found that the HLH domain of Id1 is essential for its binding to the LBD domain of ERβ1. In addition, we demonstrated that interaction between ERβ1 and Id1 was inhibited by 17-β estradiol. Our results also suggest that ERβ1 up-regulation of $p21^{WAF1/CIP1}$ gene expression is Id1 dependent, which decrease cell growth not through regulating the expression of Id1 gene directly, but binding with Id1, whilst removing Id1 inactivation of $p21^{WAF1/CIP1}$ expression. Our results demonstrate that Id1 is a downstream signal of ERβ1 and we provide a novel mechanism by which ERβ1 suppresses growth of breast cancer cells, and thus that ERβ1 is a tumor suppressor.

Estrogens are considered to be major driving forces in breast tumorigenesis and breast cancer progression [36,37]. Current evidence suggests that estrogen action is primarily mediated through ERα and ERβ [38]. ERs regulate gene expression through distinct DNA response elements.

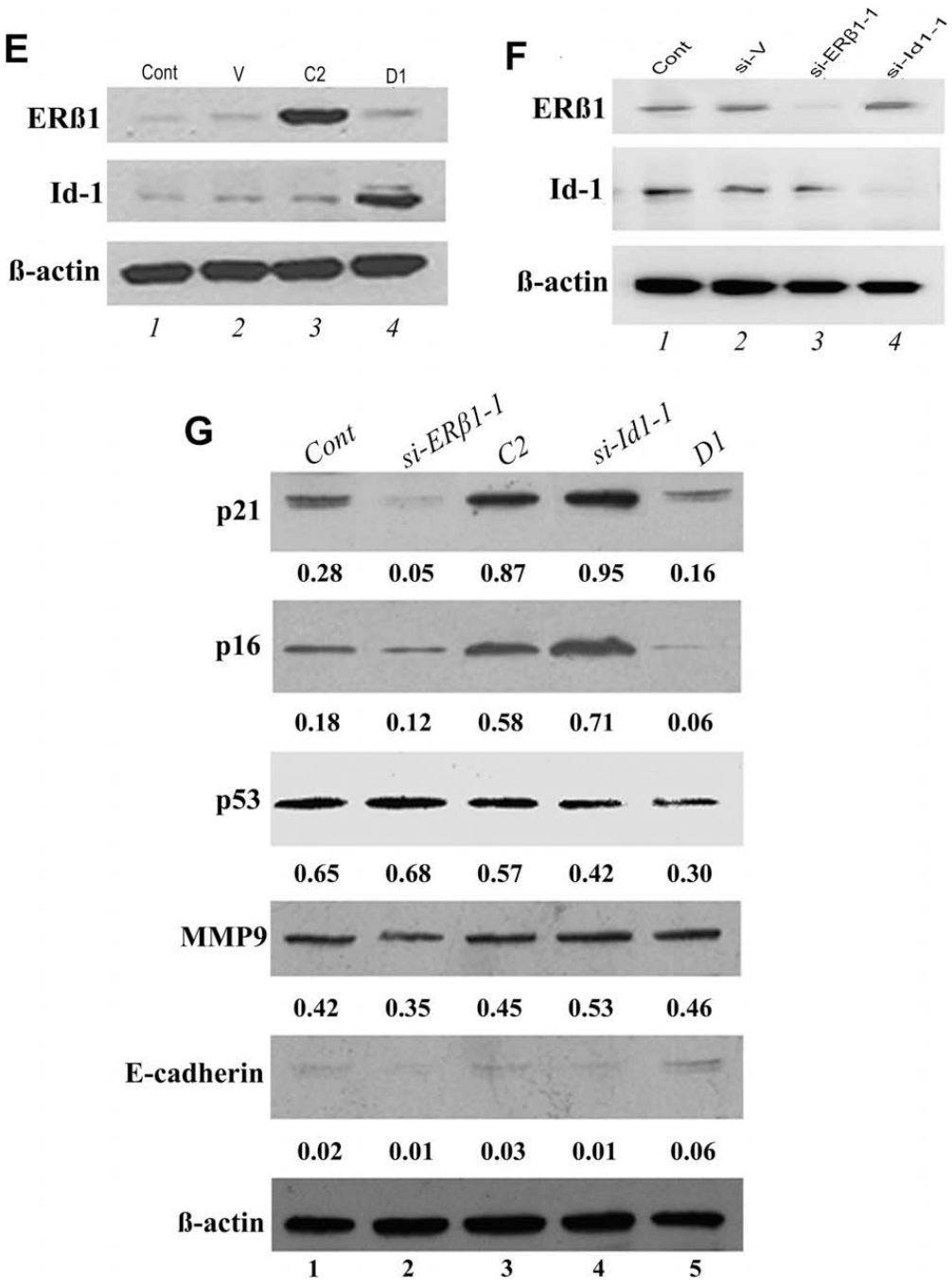

Fig. 5 (*continued*)

The classic mechanism of estrogen signaling is through an estrogen response element (ERE). Estrogen signaling also occurs through alternative mechanisms where liganded ERs are tethered to DNA via association with other transcription factor complexes, including Fos/Jun or SP-1. Previous studies suggest that expression of ERβ declines during breast tumorigenesis [39,16]. This down-regulation of ERβ in breast tumors, compared with normal breast tissue, further supports the putative role of ERβ as a tumor suppressor [40], although the role and mechanism of ERβ in human breast cancer remains unclear [41]. In five out of seven clinic studies, increasing levels of ERβ were associated with better disease outcomes [42], and better disease-free survival [43,44]. In this study, we found that expressing ERβ1 inhibited cell growth of MDA-MB-231 cells (Fig. 5A), and silencing ERβ1 accelerated the proliferation of cells (Fig. 5C), which is consistent with prior studies [45]. Furthermore, we found a novel ERβ1-interacting protein, Id1, a dominant negative helix–loop–helix protein, using the yeast two-hybrid system, and characterized interactions between ERβ1 and Id1 (Fig. 2B). Id1 primarily acts as a dominant inhibitor of basic HLH proteins by forming non-functional Id-bHLH heterodimers. Because most of the bHLH proteins positively activate genes in cell differentiation, Id proteins are thought to be negative regulators of differentiation [46,47]. Increased Id1 expression has been

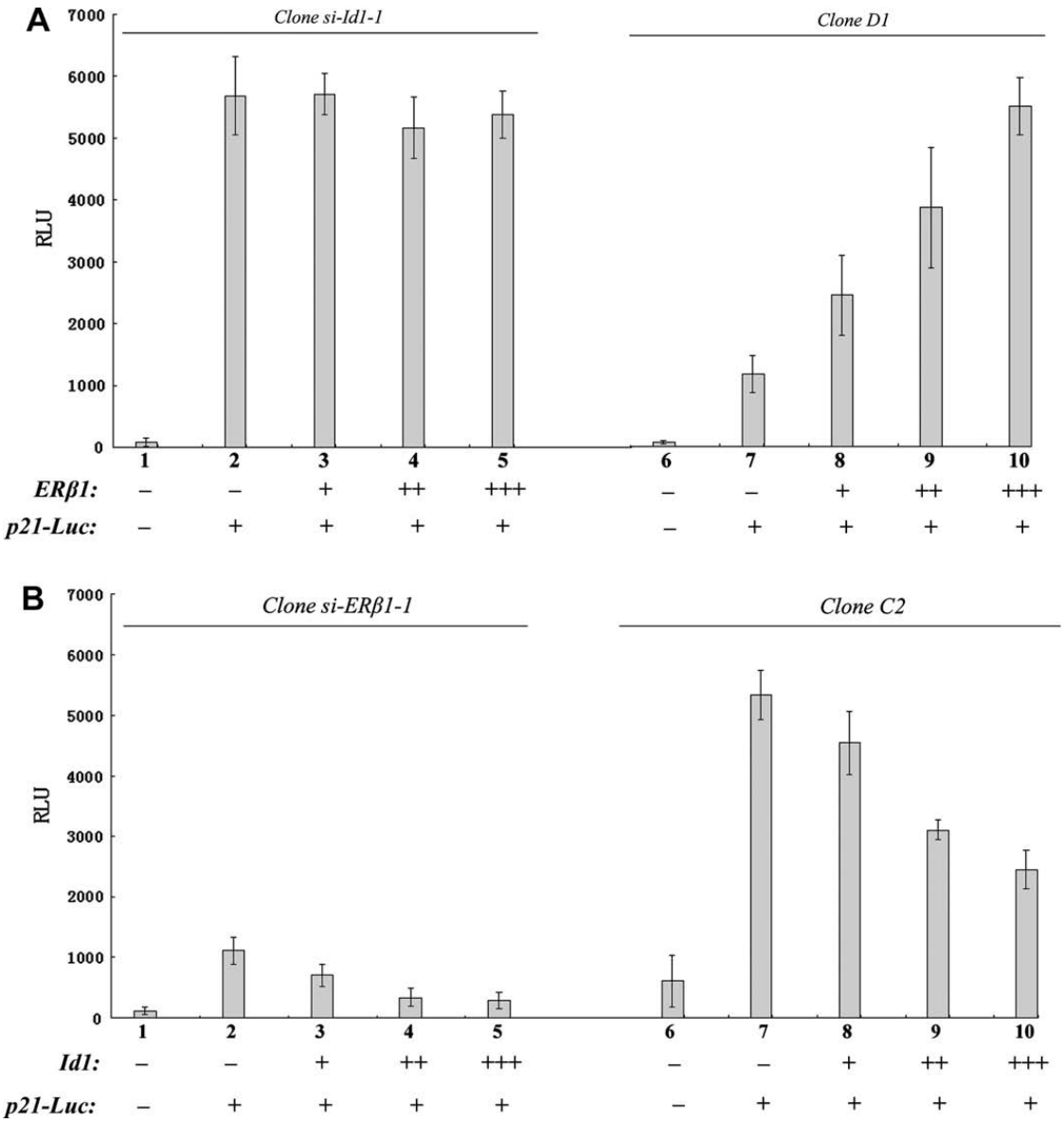

Fig. 6. ERβ1 promotion of $p^{21CIP1/WAF1}$ expression is Id1 dependent. The effect of ERβ1 and Id-1 on expression of $p21^{CIP1/WAF1}$ was investigated by a reporter gene assay using the $p21^{CIP1/WAF1}$ promoter reporter gene (p21-Luc). Error bars indicate standard deviation ($n = 3$, three independent experiments in duplicate). +, ++, and +++ correspond to 0.5, 1.0, and 1.5 μg DNA. Reporter activities are presented as RLU. (A) In clone si-Id1-1 cells (Id1 knockdown; columns 1–5), and clone D1 cells (Id1 expression; columns 6–10), p21-Luc, and pIRES-ERβ1, respectively, were co-transfected with increasing amounts of ectopic ERβ1. (B) In clone si-ERβ1-1 cells (ERβ1 knockdown; columns 1–5), and clone C2 cells (ERβ1 expression; columns 6–10), p21-Luc and pIRES-Id1, respectively, were co-transfected with increasing amounts of ectopic Id1.

found in several types of primary tumors including breast [48]. ETS-domain transcription factors [49], mouse Id associated-1 (MIDA1) [50] and paired box (PAX) transcription factors that interact with Id proteins in the context of the cell cycle and growth control [51]. Moreover, we identified that ERβ1 is another novel non-bHLH partner that interacts with Id1 (Figs. 2–4).

It was recently reported that ectopic expression of Id1 resulted in increased aggressiveness and metastasis in breast cancer cells [52], and up-regulation of Id-1 has also been correlated with increased tumor stage in several human cancers [53]. In this study, we found that upregulating Id1 increased cell growth of MDA-MB-231 cells (Fig. 5B), and downregulating Id1 decreased cell growth (Fig. 5D). In addition, we found that the HLH domain of Id1 was essential for the physical interaction between Id1 and ERβ1 (Fig. 3). Furthermore, we found that 17-β estradiol (E2) interfere with the interaction between ERβ1 and Id1 (Fig. 4). Early studies showed that enforced expression of Id genes in immortalized fibroblast cell lines causes disruption of cytoskeletal organization and loss of adhesion [54]. Subsequently, Id genes have been shown to function as either cooperating oncogenes or as dominant oncogenes in various contexts. Id1 genes and their well-documented oncogenic ability to promote cell proliferation are in accord with the tumor suppressor properties of some bHLH proteins, whose activities are antagonized by Id proteins. For example, overexpression of bHLH proteins such as

E47 in cell lines typically leads to suppression of growth by inducing cell cycle arrest at the G1 phase [55]. This probably occurs, at least in part, through transcriptional activation of the gene encoding the p21, CDK2 inhibitor [56]. ERβ is involved in genistein-induced expression of p21 in PC-3 cells [45], and Id-1 through a molecular mechanism, regulating p53 and NF-κB pathways, and modulates the Bax and Bcl-2 genes, thus providing a survival advantage under exogenous stress in MCF-7 breast cancer cells [57]. A series of genes involved in tumor invasion, for example, E-cadherin and MMP9, are responsible for Id1 gene silencing [58].

To investigate the mechanisms involved in ERβ1-induced suppression of cell growth in human breast cancer cells, the effect of Id gene silencing on representative genes involved in apoptosis (p53), proliferation ($p21^{CIP1/WAF1}$, $p16^{INK4a}$), and tumor invasion (E-cadherin and MMP9) was investigated by western blotting of ERβ1-expressing, Id1-expressing, ERβ1-silencing, and Id1-silencing clones and compared with that in control cells.

Our results show that the $p21^{CIP1/WAF1}$ and $p16^{INK4a}$ genes respond to increased expression of Id1 and ERβ1, but not p53, E-cadherin or MMP9 (Fig. 5G). $p21^{CIP1/WAF1}$ and $p16^{INK4a}$ are also the most frequently reporter genes to respond to Id1 induction in advanced cancers [59,60]. These results provide further evidence for a novel molecular pathway by which ERβ1 regulates cell growth through Id-1 inactivation of $p21^{CIP1/WAF1}$. It is possible that high levels of Id1 in many human breast cancer cells may lead to reduced $p21^{CIP1/WAF1}$ and $p16^{INK4a}$ expression, which provides a survival advantage for cancer progression. In this study, we found that the ERβ1-induced p21-Luc reporter gene expression is Id1 dependent, which not through regulating the expression of Id1 gene (Fig. 5E lane 3 and Fig. 5F lane 3), might through binding with Id1, implying that the role of ERβ1 is to maintain the $p21^{CIP1/WAF1}$ gene at a higher level, and overcoming the suppressive activity of Id1. Clinical studies also shown that Id1 overexpression was found to be related with higher microvessel densities in the ER-negative breast cancer [61]. The influence of Id1 on clinical outcome seems much stronger in patients with negative estrogen receptor status compared to those with positive status, implying that ER is associated with biological function of Id1 [62].

These results suggest that the interaction between ERβ1 and Id1 is essential for ERβ1-induced $p21^{CIP1/WAF1}$ expression (Fig. 6A). Because Id1-induced expression of the p21-Luc reporter gene is effective irrespective of ERβ1 expression, but that Id1-induced inhibition of p21-Luc is suppressed by ERβ1 (Fig. 6B), and because both ERβ1 and Id1 are expressed in the same human breast cancer cells, it is tempting to speculate that the relationship between ERβ1, Id1, and 17-β estradiol plays an important role in human breast cancer. Especially, the 17-β estradiol might inhibit the function of ERβ1-Id1 pathway (Fig. 4).

In summary, our study demonstrates the role of the ERβ1 binding partner, Id1, and suggests a novel molecular mechanism, which mediates the function of ERβ1 in suppressing breast cancer cells through binding with Id1, and overcoming Id1 inactivation of $p21^{CIP1/WAF1}$ expression.

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