



## A positive cross-regulation of HER2 and ER- $\alpha$ 36 controls ALDH1 positive breast cancer cells

Lianguo Kang, Yuming Guo<sup>1</sup>, Xintian Zhang, Jun Meng<sup>2</sup>, Zhao-Yi Wang\*

Department of Medical Microbiology and Immunology, Creighton University Medical School, 2500 California Plaza, Omaha, NE 68178, USA

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### ABSTRACT

Accumulating evidence supports the theory that breast cancer arises from a subpopulation of mammary stem/progenitor cell which possesses the ability to self-renew. However, the involvement of estrogen signaling in regulation of breast cancer stem/progenitor cells has not been fully established, mainly because expression and function of ER- $\alpha$  in breast cancer stem cells remains controversial. Previously, our laboratory cloned a variant of ER- $\alpha$ , ER- $\alpha$ 36, and found that ER- $\alpha$ 36-mediated non-genomic estrogen signaling plays an important role in malignant growth of triple-negative breast cancer cells. In this study, we found that ER- $\alpha$ 36 was highly expressed in ER-negative breast cancer SK-BR-3 cells and mediated non-genomic estrogen signaling such as activation of the MAPK/ERK signaling in these cells. Knock-down of ER- $\alpha$ 36 expression in these cells using the shRNA method diminished their responsiveness to estrogen and significantly down-regulated HER2 expression. HER2 signaling activated ER- $\alpha$ 36 transcription through an AP1 site in the ER- $\alpha$ 36 promoter and ER- $\alpha$ 36 physically interacted with HER2. We also found that ER- $\alpha$ 36 is highly expressed in a subset of SK-BR-3 cells that was positive for ALDH1, a breast cancer stem cell marker, and knock-down of ER- $\alpha$ 36 expression reduced the population of ALDH1 positive cells. Our results thus demonstrated that ER- $\alpha$ 36 positively regulates HER2 expression and the population of ALDH1 positive breast cancer cells, and suggested that non-genomic estrogen signaling mediated by ER- $\alpha$ 36 is involved in maintenance and regulation of breast cancer stem cells.

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### 1. Introduction

Tumor-initiating or -stem cells are a subpopulation of tumor cells capable of initiating and driving tumor growth. Accumulating experimental and clinical evidence supports the hypothesis that breast cancer arises from a subpopulation of mammary stem/progenitor cell which possesses the ability to self-renew (reviewed by [1–4]). Al-Hajj et al. were the first to enrich a CD44<sup>+</sup>/CD24<sup>-/low</sup> cell population from human breast cancer that displayed cancer stem cell properties and was capable of forming tumors in immuno-compromised mice with higher efficiency than cells with alternate phenotypes [5]. Later, aldehyde dehydrogenase (ALDH) 1 expression and/or its activity were identified to be a functional marker for breast cancer stem/progenitor cells; fewer ALDH1 positive tumor cells than CD44<sup>+</sup>/CD24<sup>-/low</sup> tumor cells are required

to generate tumors *in vivo* [6]. The breast cancers with ALDH1<sup>high</sup> cancer stem cells are associated with more aggressive phenotypes such as estrogen receptor (ER) negativity, high histological grade, HER2 positivity, as well as poor prognosis [6,7].

Many signaling pathways important for cell growth and survival are involved in maintenance of breast cancer stem/progenitor cells. Recent studies demonstrated that members of human epidermal growth factor receptor (EGFR) such as HER2 play a pivotal role in regulation of human breast cancer stem/progenitor cells; the EGFR/HER2 dual inhibitor, lapatinib, and the HER2 specific monoclonal antibody, trastuzumab, dramatically decrease populations of CD44<sup>+</sup>/CD24<sup>-/low</sup>/ALDH1<sup>high</sup> cells and tumorsphere-forming efficiency. In addition, the population of ALDH1<sup>high</sup> cells was increased by up-regulation of “stemness” genes through HER2 over-expression in breast cancer cells [8–10].

However, the involvement of estrogen signaling, a major signaling pathway in breast cancer development, in regulation of breast cancer stem/progenitor cells has not been fully established. A functional and molecular characterization of mouse mammary “side population” (SP) cells showed that 40% of these cells expressed ER- $\alpha$  [11]. In addition, Clarke et al., reported that ER- $\alpha$  is expressed in a subset of putative normal breast stem/progenitor cells enriched by the SP method and proposed that these ER-positive stem/progenitor cells are directly stimulated by circulating

\* Corresponding author at: Department of Medical Microbiology and Immunology, Creighton University Medical School, Criss III, Room 355, 2500 California Plaza, Omaha, NE 68178, USA. Fax: +1 402 280 3543.

E-mail address: [zywang@creighton.edu](mailto:zywang@creighton.edu) (Z.-Y. Wang).

<sup>1</sup> Current address: Beijing Shenogen Biomedical Co., Ltd., Suite A205, 5 Shangdi Kaituo Road, Haidian District, Beijing 100085, PR China.

<sup>2</sup> Current address: School of Food and Bioengineering, Zhengzhou University of Light Industry, Zhengzhou 450002, PR China.

estrogens [12]. However, Sleeman et al. [13] demonstrated that the ER-expressing luminal epithelial subpopulation contains little *in vivo* stem cell activity; ER expressing cells are distinct from the mammary stem cell population and the effects of estrogen signaling on mammary stem cells are likely to be mediated indirectly [13]. Despite the controversy of receptor expression, mouse mammary stem cells are highly responsive to steroid hormone signaling; ovariectomy markedly diminished mammary stem cell number and outgrowth potential *in vivo* whereas mammary stem cell activity increased in mice treated with estrogen plus progesterone [14]. Estrogen was also found to expand breast cancer stem cells through paracrine FGF/Tbx3 pathway, indicating the indirect effects of estrogen on stem cell activity [15]. However, Simoes et al., recently reported that estrogen treatment reduced the population of stem cells in the normal human mammary gland and in breast cancer cells [16]; overexpression of embryonic stem cell genes such as NANOG, OCT4 and SOX2 reduced ER- $\alpha$  expression and increased the population of breast cancer stem cells as well as properties associated with malignancy, which argues a negative role of estrogen signaling mediated by ER- $\alpha$  in activities of breast cancer stem cells.

Previously, we identified and cloned a 36 kDa variant of ER- $\alpha$ , ER- $\alpha$ 36, that is mainly expressed on the plasma membrane and in the cytoplasm, and mediates non-genomic estrogen signaling [17,18]. ER- $\alpha$ 36 lacks both transcription activation function domains AF-1 and AF-2 of the full-length 66 kDa ER- $\alpha$  (ER- $\alpha$ 66), consistent with the fact that ER- $\alpha$ 36 has no intrinsic transcriptional activity [18]. ER- $\alpha$ 36 is generated from a promoter located in the first intron of the ER- $\alpha$ 66 gene [19], indicating that ER- $\alpha$ 36 expression is regulated differently from ER- $\alpha$ 66, consistent with the findings that ER- $\alpha$ 36 is expressed in specimens from ER-negative breast cancer patients and established ER-negative breast cancer cells that lack ER- $\alpha$ 66 expression [18,20,21]. ER- $\alpha$ 36 was found to be over-expressed in triple-negative breast carcinomas [22], and promotes malignant growth of triple-negative breast cancer MDA-MB-231 and MDA-MB-436 cells [23]. Thus, ER- $\alpha$ 36-mediated signaling plays an important role in development and progression of ER-negative breast cancer. However, the molecular mechanisms underlying ER- $\alpha$ 36 action in ER-negative breast cancer still remains largely unknown.

In the present study, we investigated the role of ER- $\alpha$ 36 in ER-negative breast cancer SK-BR-3 cells that express high levels of both ER- $\alpha$ 36 and HER2 and revealed a positive feedback loop between ER- $\alpha$ 36 and HER2 expression. This positive cross-regulation is involved in regulation of ALDH1 positive population of SK-BR-3 cells.

## 2. Materials and methods

### 2.1. Reagents

Polyethylenimine (PEI) and 17 $\beta$ -estradiol (E2) were purchased from Sigma–Aldrich (St. Louis, MO). The dual luciferase assay system was purchased from Promega Corporation (Madison, WI). We developed an affinity-purified rabbit polyclonal anti-ER- $\alpha$ 36 antibody as a custom service from Alpha Diagnostic, Inc. The antibody was raised against a synthetic peptide antigen corresponding to the unique C-terminal 20 amino acids of ER- $\alpha$ 36. The antibody was tested and characterized as described before [18].

Anti-ALDH1 antibody was from BD biosciences (Sparks, MD). Anti-HA mouse monoclonal antibody (mAb), anti-phospho-p44/42 ERK (Thr202/Tyr204) (197G2) mouse mAb, anti-p44/42 ERK (137F5) rabbit mAb, anti-phospho-Akt (Ser473), and anti-Akt antibodies were purchased from Cell Signaling Technology (Boston, MA). The anti-HER2, anti- $\beta$ -actin and the different secondary antibodies were purchased from Santa Cruz Biotechnology (Santa

Cruz, CA). The goat anti-mouse Alexa Fluor 555 and goat anti-rabbit Alexa Fluor 488 antibodies were purchased from Invitrogen (Carlsbad, CA). HER2 inhibitors Lapatinib, AG825, sodium 4-phenylbutyrate (PB) and retinoic acid (RA) were from CalBiochem (San Diego, CA), and the PI3K inhibitor LY294002 was purchased from Tocris Bioscience (Ellisville, MO). The ECL Western Blotting Detection Reagents were from GEHealthcare (Little Chalfont, Buckinghamshire, UK). The “Concert” cytoplasmic RNA purification reagent was purchased from Invitrogen (Carlsbad, CA), and the ProtoScript II RT-PCR kit was obtained from New England Biolabs (Ipswich, MA). Protein A/G plus agarose was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The ALDEFLUOR assay kit was purchased from Stemcell Technologies (Durham, NC).

### 2.2. Cell culture

Breast epithelial cell line MCF10A and breast cancer cell lines MCF7, ZR-75-1, T-47D, H3396, SK-BR-3, MDA-MB-231, MDA-MB-436, and MDA-MB-468 were obtained from American Type Culture Collection (ATCC, Manassas, VA). SK-BR-3, MDA-MB-231, MDA-MB-436, and MDA-MB-468 were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotic–antimycotic. MCF7, ZR-75-1, T-47D, and H3396 were maintained in Improved Minimal Essential Medium (IMEM) from Invitrogen (Carlsbad, CA) supplemented with 10% FBS, 1% non-essential amino-acids, 1% HEPES buffer, 1% antibiotic–antimycotic from Invitrogen (Carlsbad, CA) and 2  $\mu$ g/ml bovine insulin (Sigma, St. Louis, MO). SK-BR-3 cells transfected with the empty expression vector, a control vector expressing shRNA for luciferase or the pER36Sh-1 expression vector were established as described before [23] and were named as SK-BR-3/V, SK-BR-3/L and SK-BR-3/36S, respectively. All cell lines were maintained at 37 °C and 5% CO<sub>2</sub> in a humidified incubator. For E2 treatment, cells were maintained in phenol red-free media with 2.5% charcoal-stripped fetal calf serum (Hyclone, Logan, UT) for three days, and then in serum-free medium for 24 h before experimentation. For ERK activation assays, cells were treated with vehicle (ethanol) and indicated concentrations of E2 for different periods of time.

### 2.3. Cell growth assays

Cells were seeded in 35 mm dishes at a density of  $2 \times 10^4$  cells/dish in DMEM supplemented with 10% FBS and were then counted with the ADAM automatic cell counter (Digital Bio., Korea) after different time periods. Three dishes were used for each time point and the experiments were repeated three times.

To test the effects of the anti-ER- $\alpha$ 36 antibody on growth of ALDH<sup>L</sup> and ALDH<sup>H</sup> SK-BR-3 cells, cells sorted after ALDEFLUOR staining were seeded in 35 mm dishes at a density of  $3 \times 10^4$  cells/dish in medium containing 2.5% charcoal–dextran stripped FBS and 5 or 10  $\mu$ g/ml of the anti-ER- $\alpha$ 36 antibody for six days. Cells were then counted with the ADAM automatic cell counter (Digital Bio., Korea). Three dishes were used for each time point and the experiments were repeated three times.

### 2.4. ALDEFLUOR assay

ALDH activity was detected using the ALDEFLUOR assay kit (StemCell Technologies) as recommended by the manufacturer. Briefly, SK-BR-3 cells were suspended in ALDEFLUOR assay buffer containing an ALDH substrate, bodipy-aminoacetaldehyde (BAAA), and incubated for 1 h at 37 °C. A specific inhibitor of ALDH, diethylaminobenzaldehyde (DEAB) was used as a negative control. Flowcytometry cell sorting was performed to separate ALDH<sup>L</sup> and

ALDH<sup>H</sup> SK-BR-3 cells and data was analyzed by FlowJo software (TreeStar, Ashland, OR).

### 2.5. Immunoblot and immunoprecipitation analysis

For immunoprecipitation assays, cells were washed twice with ice-cold PBS and lysed with the lysis buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.4, 0.1% NP-40) supplemented with protease and phosphatase inhibitors (Sigma, St. Louis, MO) for 30 minutes on ice. Cell lysates were then incubated with indicated primary antibodies, or pre-immune serum as a negative control for 1 h at 4 °C. Protein A/G plus agarose was then added and incubated for another 1 h at 4 °C. Precipitates were then extensively washed with the lysis buffer, re-suspended in loading buffer and separated on SDS-PAGE. Western blot analysis was performed as described before [23].

### 2.6. DNA transfection and luciferase assay

HEK293 cells were transfected using PEI transfection reagent with the pER36-736-Luc, pER36-584-Luc, or pER36-513-Luc reporter plasmids as described before [19] and an empty expression vector or the expression vector for HER2 (a kind gift from Dr. Laura Hansen at Creighton University). Cells were co-transfected with a cytomegalovirus-driven *Renilla* luciferase plasmid, pRL-CMV (Promega, Madison, WI) to establish transfection efficiency. Twenty-four hours after transfection, cells were treated with DMSO (vehicle), 10 μM of Lapatinib, or LY294002 for 24 h. Forty-eight hours after transfection, cell extracts were prepared and luciferase activities were determined and normalized using the Dual-Luciferase Assay System (Promega, Madison, WI) and a TD 20/20 Luminometer (Turner BioSystems, Inc., Sunnyvale, CA) as instructed by the manufacturer.

### 2.7. RNA purification and RT-PCR

RNA purification and RT-PCR procedures for ER-α36 and actin were performed as described before [23]. HER2 primers were as follows: forward primer: 5'-AGGGAGTATGTGAATGCC-3'; reverse primer: 5'-GGCCACTGGAATTTTCAC-3'. The procedure of PCR for HER2 was carried out as following: first a denaturing at 94 °C for 3 min, then the remaining PCR was performed at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s (35 cycles). At last, there was a final elongation at 72 °C for 7 min.

### 2.8. DNA mutagenesis

The DNA mutagenesis process was performed as described in [23].

### 2.9. Indirect immunofluorescent staining

Cells were fixed in cold methanol for 5 min, blocked in 10% normal rabbit serum for 20 min, and then incubated with anti-ER-α36 (1:100) or anti-ALDH1 (1:200) antibodies at 4 °C overnight. Secondary antibodies, anti-rabbit Alexa Fluor 488 and anti-mouse Alexa Fluor 555 (1:200) were then added and incubated for 30 min. Sections were then mounted with the mounting medium containing DAPI before inspection under the fluorescent microscope (Nikon, Eclipse E600).

### 2.10. Statistical analysis

Data were summarized as the mean ± standard error (SE) using GraphPad InStat software program. Tukey-Kramer Multiple Comparisons Test was used, and the significance was accepted for *P* values of less than 0.05.

## 3. Results

### 3.1. ER-α36 mediates non-genomic estrogen signaling in ER-negative breast cancer SK-BR-3 cells

ER-α36 is highly expressed in ~40% of ER-negative breast cancer and its expression is significantly correlated with HER2 expression [21]. Recently, we reported a positive feedback loop between EGFR and ER-α36 expression in triple-negative breast cancer MDA-MB-231 and MDA-MB-436 cells; EGFR signaling activates the promoter activity of ER-α36 and ER-α36 stabilizes the steady state levels of EGFR protein [23]. To determine if there exists a similar relationship between HER2 and ER-α36, we first examined HER2 and ER-α36 expression status in eight breast cancer cell lines with normal mammary epithelial MCF10A cells as a control. ER-α36 expression was detected in all of the breast cancer cell lines but not in MCF10A cells (Fig. 1A). Among breast cancer cell lines examined, two cell lines ZR-75-1 and SK-BR-3, also expressed HER2. We used ER-negative breast cancer cell line SK-BR-3 that co-expresses high levels of ER-α36 and HER2 for further study.

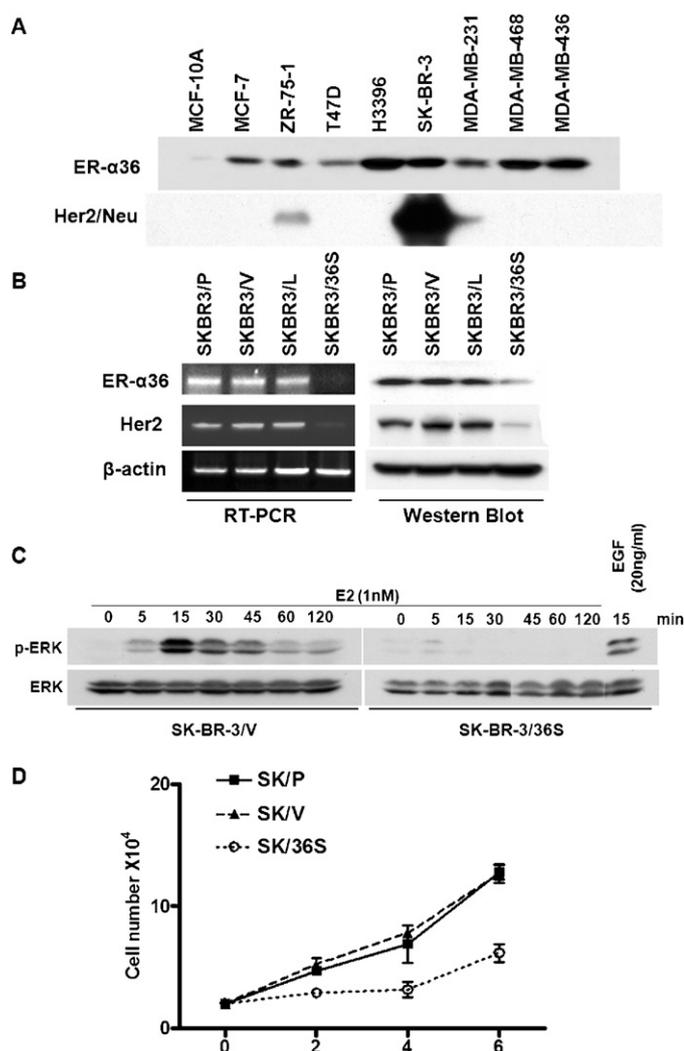
To examine ER-α36 function in SK-BR-3 cells, we knocked down ER-α36 expression using the small hairpin RNA (shRNA) method. SK-BR-3 cells were stably transfected with an shRNA expression vector targeting the 3' UTR of ER-α36 and established a cell line by pooling more than twenty transfectants. SK-BR-3 cells transfected with an empty expression vector or an expression vector for shRNA against firefly luciferase were used as controls. Both Western blot analysis and reverse transcriptase PCR indicated that ER-α36 expression was knocked-down more than 80% in the shRNA-expression vector-transfected cells compared with control cells (Fig. 1B). Intriguingly, HER2 expression was also dramatically down-regulated in cells with knocked-down levels of ER-α36 at both mRNA and protein levels (Fig. 1B), indicating that ER-α36 mediated signaling is involved in positive regulation of HER2 expression.

We then examine whether 17β-estradiol (E2β) induced phosphorylation of the MAPK/ERK1/2, a typical non-genomic estrogen-signaling event, in control cells (SK-BR-3/V) that were transfected by an empty expression vector and cells transfected with a shRNA expression vector specific for ER-α36 that express knocked-down levels of ER-α36 (SK-BR-3/36S). Cells were treated with 1 nM of E2β for different time periods and cell lysates were analyzed with Western blot using a phospho-specific ERK1/2 antibody. Fig. 1C shows that E2β elicited ERK phosphorylation in control SK-BR-3 cells transfected with an empty expression vector but not in cells with ER-α36 expression knocked-down. However, EGF was still able to induce ERK activation in SK-BR-3 cells with knocked-down level of ER-α36 expression (Fig. 1C), indicating there was no defect of the MAPK/ERK signaling in the cells with ER-α36 expression knocked-down. Time course analysis revealed that ERK phosphorylation occurred within 5 min after E2β application, peaked at 15 min, declined at 30 min but failed to return to the basal level at 120 min (Fig. 1C). These results strongly suggested that ER-α36 mediates non-genomic estrogen signaling in ER-negative breast cancer SK-BR-3 cells.

We then examined the growth rate of these cells by counting cell numbers every other days for six days. As shown in Fig. 1D, the growth rate of SK-BR-3/36S was dramatically decreased compared to control and parental cells. Our data thus indicated that signaling pathways mediated by ER-α36 and HER2 are important for proliferation of ER-negative breast cancer SK-BR-3 cells.

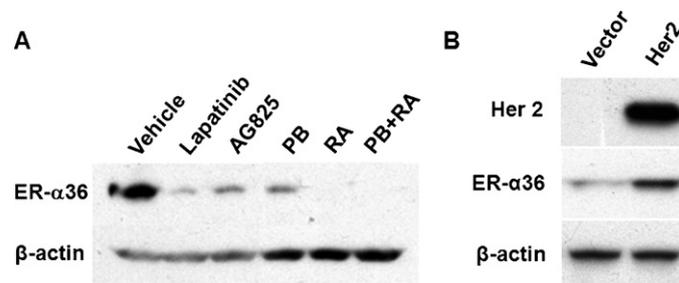
### 3.2. HER2 signaling positively regulates ER-α36 expression

To determine whether HER2 signaling influences ER-α36 expression, we treated SK-BR-3 cells with the HER2 inhibitors



**Fig. 1.** Cross-regulation of ER- $\alpha$ 36 and HER2 expression in ER-negative breast cancer SK-BR-3 cells. (A) Western blot analysis of ER- $\alpha$ 36 and HER2 expression in different breast cancer cell lines and mammary epithelial MCF10A cells. (B) Knock-down of ER- $\alpha$ 36 expression down-regulated HER2 expression. RT-PCR and Western blot analysis of parental SK-BR-3 cells (SK-BR-3/P), SK-BR-3 cells transfected with an empty vector (SK-BR-3/V), with a control vector expressing shRNA for luciferase (SK-BR-3/L) and with the ER- $\alpha$ 36 shRNA (SK-BR-3/36S). (C) SK-BR-3/V and SK-BR-3/36S cells treated with E2 (1 nM) for different time points were assessed with Western blot analysis using phosphorylation specific or non-specific anti-ERK1/2 antibodies. SK-BR-3/36S cells treated with EGF (20 ng/ml) were included as a positive control. (D) SK-BR-3/P, SK-BR-3/V and SK-BR-3/36S cells were counted every other days for six days. Three dishes were used for each time points, the experiments were repeated three times and the data represent the mean  $\pm$  SE.

Lapatinib, AG825, sodium 4-phenylbutyrate (PB), retinoic acid (RA), and PB together with RA. The levels of ER- $\alpha$ 36 expression in these treated cells were analyzed with Western blot. Fig. 2A shows that treatment with the HER2 inhibitors potently down-regulated ER- $\alpha$ 36 expression. To confirm this, human embryonic kidney (HEK) 293 cells that express un-detectable levels of endogenous HER2 and very low levels of ER- $\alpha$ 36 were transiently transfected with a HER2 expression vector. Western blot analysis demonstrated that endogenous ER- $\alpha$ 36 expression was up-regulated in HEK293 cells transfected with the HER2-expression vector but not in cells transfected with an empty expression vector (Fig. 2B). These data thus indicated that ER- $\alpha$ 36 protein concentration is subjected to positive regulation of HER2 signaling.



**Fig. 2.** HER2 signaling positively regulates ER- $\alpha$ 36 expression. (A) HER2 inhibitors down-regulate ER- $\alpha$ 36 expression. SK-BR-3 cells were treated with 10  $\mu$ M of Lapatinib, AG825, sodium 4-phenylbutyrate (PB), retinoic acid (RA) or PB + RA for 48 h. ER- $\alpha$ 36 expression was examined with Western blot analysis. (B) HER2 expression induces endogenous ER- $\alpha$ 36 expression. HEK293 cells were transiently transfected with an empty vector (Vector) or a HER2 expression vector (HER2), and 48 h after transfection, cells were examined for ER- $\alpha$ 36 expression with Western blot analysis. The experiments were repeated three times, and the representative results were shown.

### 3.3. HER2 signaling activates the ER- $\alpha$ 36 promoter activity via an Ap1 site

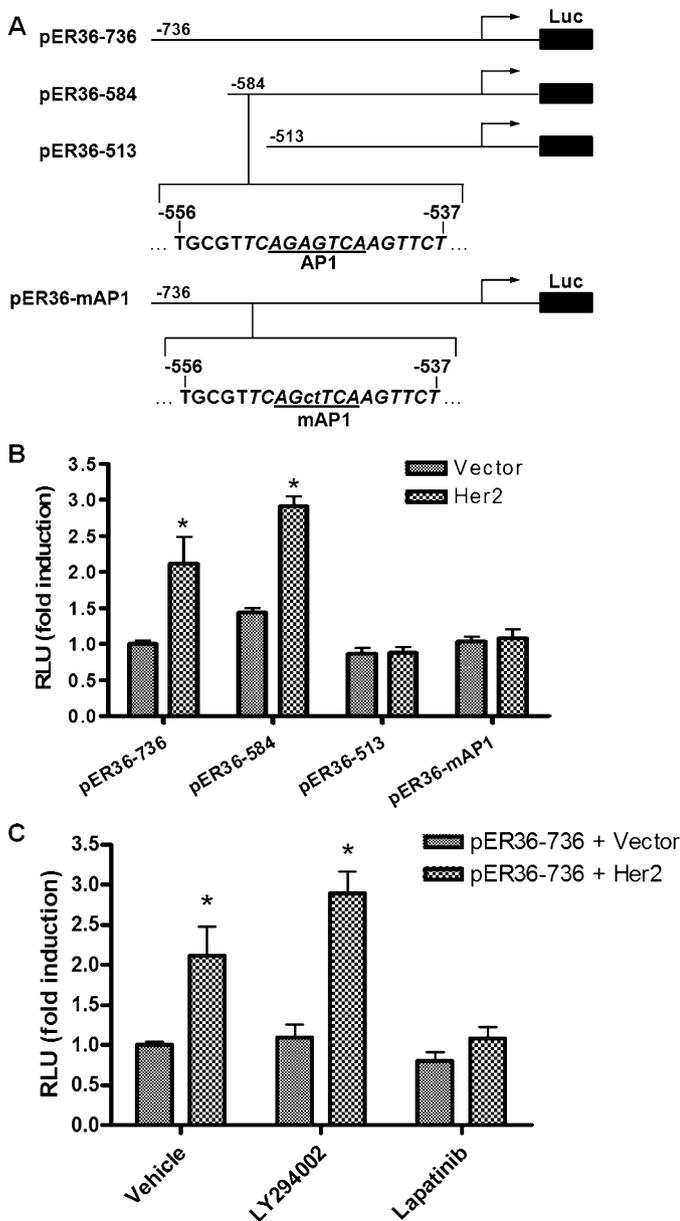
Recently, we reported that EGFR signaling induces the promoter activity of ER- $\alpha$ 36 gene via an Ap-1 binding site located in the 5' flanking sequence of ER- $\alpha$ 36 gene [19]. To examine if HER2 signaling also activates ER- $\alpha$ 36 promoter activity, HEK293 cells were transiently co-transfected with a HER2 expression vector and a luciferase reporter plasmid driven by the ER- $\alpha$ 36 promoter we cloned and characterized before [19]. HER2 co-transfection resulted in a 2- to 3-fold induction of ER- $\alpha$ 36 promoter activity, which was blocked by pre-treatment of the HER2 inhibitor Lapatinib, but not by the PI3K inhibitor LY294002 (Fig. 3C), suggesting the PI3K/AKT signaling is not involved in the activation of ER- $\alpha$ 36 promoter activity. When a series of 5' truncated promoter of ER- $\alpha$ 36 was used, we found that HER2 expression failed to activate the promoter activity of the pER36-513 reporter plasmid (Fig. 3B). Close examination of DNA sequence in the deleted region revealed an AP-1-binding site located at -556 to -537 residues (relative to the transcription initiation site) of the ER- $\alpha$ 36 promoter region (Fig. 3A). Mutation of this AP-1 site abrogated induction of ER- $\alpha$ 36 promoter activity by HER2 (Fig. 3B), indicating that HER2 signaling activates the ER- $\alpha$ 36 promoter activity through an AP-1 dependent signaling pathway.

### 3.4. ER- $\alpha$ 36 physically interacts with HER2

To elucidate the molecular mechanism by which ER- $\alpha$ 36 functions to mediate non-genomic estrogen signaling in SK-BR-3 cells, we examined whether ER- $\alpha$ 36 interacts with HER2. SK-BR-3 cells were transiently transfected with an expression vector for HA-tagged ER- $\alpha$ 36 and co-immunoprecipitation/Western blot assays were performed with cell lysates from transfected cells. Fig. 4 shows that HER2 and ER- $\alpha$ 36 co-existed in the immunoprecipitates of the anti-HER2 and anti-HA antibodies. Thus, the presence of HER2 and ER- $\alpha$ 36 in the same protein complex suggests an interaction between them and also suggests that ER- $\alpha$ 36 may function through HER2.

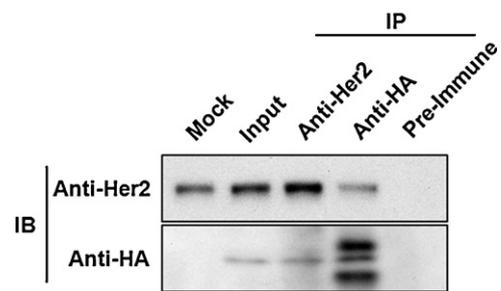
### 3.5. ER- $\alpha$ 36 positively regulates ALDH1 expressing breast cancer cells

Previously, it was reported that the HER2 signaling pathway is involved in the positive regulation of ALDH1 positive breast cancer cells [8–10]. We decided to examine whether ER- $\alpha$ 36 is also involved in regulation of ALDH positive breast cancer cells. First, we



**Fig. 3.** HER2 signaling activates ER- $\alpha$ 36 promoter activity. (A) Schematic structures of luciferase reporter plasmid driven by different 5' truncated promoters of ER- $\alpha$ 36. The -736, -584, and -513 indicate residues upstream of the transcription initiation site, respectively. An AP1 binding site is also indicated that was mutated in the pER36-mAP1 plasmid. (B) The luciferase activities in HEK293 cells transfected with different reporter plasmids together with an empty expression vector or an expression vector for HER2. Columns: means of four independent experiments; bars, SE. \* $p < 0.05$ , for cells transfected with the HER2 expression vector vs an empty expression vector. (C) HEK293 cells were transfected with the pER36-736 reporter plasmid with the empty expression vector or the HER2 expression vector, and then treated with DMSO vehicle, 10  $\mu$ M of LY294002 or Lapatinib for 24 h. The luciferase activities were then normalized and analyzed. Results shown in graph are means from four experiments; bars, SE. \* $p < 0.05$  for cells treated with vehicle vs different inhibitors.

enriched ALDH1 positive cells from SK-BR-3 cells with flowcytometry cell sorting after stained with the ALDEFLOUR kit. Western blot analysis revealed that the ALDH1 positive/high (ALDH1<sup>high</sup>) SK-BR-3 cells expressed higher levels of ER- $\alpha$ 36 and exhibited higher levels of phosphorylated AKT compared to ALDH1 negative/low (ALDH1<sup>low</sup>) cells, while no significant changes of HER2 expression were observed (Fig. 5A). We also performed the immunofluorescence staining analysis with anti-ALDH1 and ER- $\alpha$ 36 antibodies in SK-BR-3/V and SK-BR-3/36S cells. Fig. 5C shows that ER- $\alpha$ 36 was



**Fig. 4.** ER- $\alpha$ 36 physically interacts with HER2. Co-immunoprecipitation and Western blot analysis of ER- $\alpha$ 36 and HER2 in SK-BR-3 cells. Cells transiently transfected with an expression vector of HA-tagged ER- $\alpha$ 36 were lysed and the cell lysates were immunoprecipitated with anti-HER2 or anti-HA antibodies, or with pre-immune rabbit serum as a negative control. The immunoprecipitates were then separated by SDS-PAGE and probed with anti-HER2 and anti-HA antibodies.

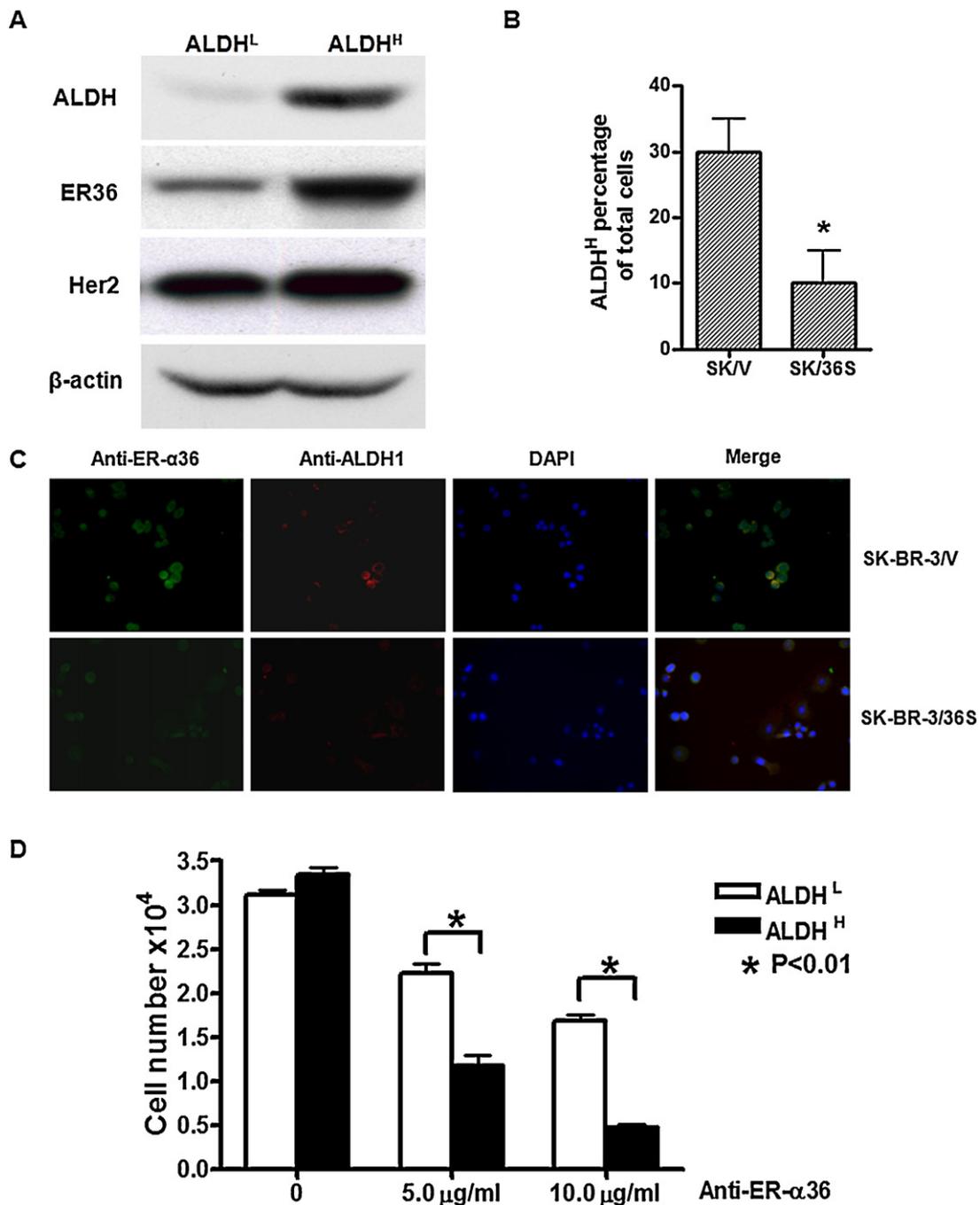
highly expressed in SK-BR-3/V cells that were also ALDH1 positive. The numbers of ALDH1<sup>high</sup> cells were significantly decreased in SK-BR-3/36S cells compared to SK-BR-3/V cells (Fig. 5B), suggesting that ER- $\alpha$ 36 is involved in maintenance of ALDH1<sup>high</sup> breast cancer cells.

To further confirm the role of ER- $\alpha$ 36-mediated non-genomic estrogen signaling in ALDH1<sup>high</sup> breast cancer cells, we tested the effects of a specific anti-ER- $\alpha$ 36 antibody on ALDH1<sup>high</sup> cell population. Recently, we demonstrated that the anti-ER- $\alpha$ 36 antibody blocked ER- $\alpha$ 36-mediated non-genomic estrogen signaling such as activation of the MAPK/ERK signaling [24]. We first enriched ALDH1<sup>high</sup> cells from SK-BR-3 cells using the ALDEFLOUR kit and flowcytometry, and treated the ALDH1<sup>high</sup> and ALDH1<sup>low</sup> cells with different concentrations of the ER- $\alpha$ 36 antibody for six days. We found that treatment of these cells with anti ER- $\alpha$ 36 antibody significantly inhibited the growth of ALDH1<sup>high</sup> cells (Fig. 5D), indicating that ER- $\alpha$ 36-mediated non-genomic estrogen signaling plays an important role in maintenance and proliferation of ALDH1<sup>high</sup> breast cancer cells.

#### 4. Discussion

Previously, we observed a significant correlation between ER- $\alpha$ 36 and HER2 expression in breast cancer patients [21]. Recently, we reported that co-expression of ER- $\alpha$ 36 and HER2 were detected 10 out of 19 cases of ER-negative apocrine breast cancer [22]. Here, we used an ER-negative breast cancer cell line SK-BR-3 as a model to study the underlying mechanisms of the correlation between ER- $\alpha$ 36 and HER2 expression.

Approximately 20–25% of breast cancers have an amplification of the HER2 gene or overexpression of its protein product [25]. Overexpression of this receptor in breast cancer is associated with increased disease recurrence and worse prognosis [26,27]. However, the molecular mechanisms by which breast cancer cells gain HER2 overexpression are largely unknown. Previous studies have demonstrated an interaction between the ER- $\alpha$ 66 and HER2 signal transduction [28,29]. For example, 17 $\beta$ -estradiol is able to reduce HER2 expression in an ER- $\alpha$ 66-dependent manner [30], which may provide an explanation to the infrequent co-expression of these two receptors in breast cancer [31–33]. In contrast, a significant positive correlation between HER2 overexpression and ER- $\beta$  expression has been reported [34,35]. It was reported that forced expression of ER- $\beta$ 1 in ER-positive breast cancer MCF7 cells induced HER2 expression [36]. Here, we revealed another mechanism for HER2 overexpression in breast cancer cells. We found that HER2 and ER- $\alpha$ 36 positively regulate each other's expression in SK-BR-3 cells; HER2 signaling activates the promoter



**Fig. 5.** ER-α36 positively regulates the population of ALDH<sup>high</sup> SK-BR-3 cells. (A) Western blot analysis of cell lysates of SK-BR-3 cells with ALDH1 low/negative expression (ALDH<sup>L</sup>) and ALDH1 high expression (ALDH<sup>H</sup>). (B) Decrease of ALDH1 positive cells in SK-BR-3 cells with ER-α36 expression knocked-down. The numbers of ALDH1 positive cell in 100 counted cells were scored. The results shown are means of four independent experiments; bars, SE. \**p* < 0.05 for SK-BR-3/V vs SK-BR-3/36S cells. (C) Immunofluorescence staining of SK-BR-3/V and SK-BR-3/36S cells. Cells were fixed and stained by anti-ALDH1 (red) and anti-ER-α36 (green) antibodies and photographed. The representative photos were shown with 10× amplification. (D) ALDH<sup>L</sup> and ALDH<sup>H</sup> SK-BR-3 cells were treated with 0, 5 or 10 μg/ml of anti-ER-α36 antibody for 6 days. The survived cell numbers were then counted. Results shown in graph are means of three independent experiments; bars, SE. \**p* < 0.01 for cells treated with control vs cells treated with the antibody. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

activity of ER-α36 and ER-α36 activates HER2 transcription. This positive cross-regulation may provide a molecular explanation to the observations that ER-α36 and HER2 expression is significantly correlated in primary breast cancer [21,22]. These findings are similar to our recent report of a positive feedback loop between ER-α36 and EGFR expression that promotes malignant growth of triple-negative breast cancer cells [23]. Thus, the interplay between growth factor receptors and ER-α36 may play an important role in development and progression of subsets of breast cancer that highly express ER-α36.

In the present study, we also demonstrated ER-α36 physically interacted with HER2 in co-immunoprecipitation assay, which provided a molecular mechanism by which ER-α36 mediates non-genomic estrogen signaling in ER-negative breast cancer SK-BR-3 cells. Consistent with this, we recently found that ER-α36 mediates non-genomic estrogen signaling pathway in triple-negative breast cancer MDA-MB-231 and MDA-MB-436 cells via interaction with the EGFR/Shc/Src complex [23]. Our results thus suggested that growth factor receptors play integral roles in ER-α36-mediated non-genomic estrogen signaling.

The involvement of estrogen signaling in regulation of breast cancer stem/progenitor cells has not been fully established, mainly because expression and function of ER- $\alpha$ 66 in breast cancer stem/progenitor cells remains controversial. Thus, our findings demonstrated that ER- $\alpha$ 36 is highly expressed in ALDH1<sup>high</sup> SK-BR-3 cells and knock-down of ER- $\alpha$ 36 expression reduced ALDH1<sup>high</sup> cell population are noteworthy. Previously, the importance of HER2 signaling in maintenance of ALDH1<sup>high</sup> breast cancer stem cells has been reported [8–10]. Currently, it is not clear whether ER- $\alpha$ 36-mediated estrogen signaling is directly involved in positive regulation of ALDH1<sup>high</sup> breast cancer stem cells or indirectly through activation of HER2 expression. However, the finding that the ER- $\alpha$ 36 specific antibody significantly reduced the population of ALDH1<sup>high</sup> cells in SK-BR-3 cell line suggested that the non-genomic estrogen signaling mediated by ER- $\alpha$ 36 is involved in positive regulation of breast cancer stem cells. It is also likely that ER- $\alpha$ 36-mediated non-genomic estrogen pathway is involved in the estrogen effects observed in normal mammary stem cells before [14].

In summary, we have shown that ER- $\alpha$ 36 positively regulates HER2 expression and the population of ALDH1<sup>high</sup> breast cancer cells, suggesting that non-genomic estrogen signaling mediated by ER- $\alpha$ 36 contributes to development and progression of ER-negative breast cancer that express ER- $\alpha$ 36. Thus, ER- $\alpha$ 36 is a novel player in non-genomic estrogen signaling that may play important roles in maintenance and regulation of normal mammary stem cells as well as breast cancer stem cells.

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