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Phosphorylation of human estrogen receptor-beta at serine 105 inhibits breast cancer cell migration and invasion

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Abstract

Multiple phosphorylation sites on the human estrogen receptor (hER) α were identified and shown to influence mammary carcinogenesis. In contrast, functional phosphorylation sites of hER β have yet to be experimentally identified and validated. Here, using mass spectrometry, we uncovered three serines (S75, S87, and S105) in the N-terminus of hER β as targets of ERK1/2 and p38 kinases. We raised a specific antibody against phosphorylated S105 (pS105) and demonstrated that this site was endogenously phosphorylated in MDA-MB-231 and BT-474 cells. A phospho-mimetic mutant generated from hER β 1 was found to exhibit higher transactivation activity than hER β 1. Ectopic expression of this mutant inhibited cell migration and invasion, but did not affect cell growth and cell-cycle progression in these cell models. In breast cancer specimens, pS105-hER β immunoreactivity was detected with a higher prevalence and intensity than that of hER β 1. These results underscore the functional importance of the first experimentally identified hER β -phosphorylation site in breast cancer.

Keywords

phosphorylation; estrogen receptor-beta isoforms; phospho-mimetics; breast cancer; invasion; migration; post-translational modification; mass spectrometry; ERK1/2; p38

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1. Introduction

Disruption of hormonal balance has been implicated in breast cancers (BCa), the most common malignancies among women (American Cancer Society, 2010). Estrogen was first discovered as a key factor for the growth of BCa when bilateral oophorectomy was found to result in remission of BCa in a premenopausal woman (Beatson, 1896). In women, a higher incidence of BCa has been linked to higher serum and tissue levels of estrogen (Lamar et al., 2003) and a longer lifetime exposure to estrogen (Paffenbarger, Jr. et al., 1980). Estradiol-17 beta (E2) is believed to trigger nuclear signaling primarily via two estrogen receptors (ERs), ER α and ER β 1 (traditionally referred to as ER β), in human cancers (Leygue et al., 1998; Matthews and Gustafsson, 2003). These two receptors share many of the same ligands but have distinct and diverse cellular functions. In BCa, abnormal growth was found to be driven by ER α (Gaben et al., 2004; Lu and Serrero, 2001) but curtailed by ER β 1 (Koehler et al., 2005; Williams et al., 2008). Thus, ER β 1 appears to function as a tumor suppressor in BCa. This view is further supported by evidence reporting a loss of ER β 1 expression during BCa progression (Leygue et al., 1998).

Post-translational modifications (PTMs) are crucial events in the activation of ERs (Faus and Haendler, 2006; Lannigan, 2003; Le et al., 2011). Phosphorylation is the most extensively studied PTM, partly because of its relatively frequent occurrence and stability (Faus and Haendler, 2006). Studies of human ER α have shown that phosphorylation mediates both ligand- and growth factor-initiated genomic and non-genomic action of the receptor (Lannigan, 2003; Le et al., 2011). Specific phosphorylation sites were identified primarily in the activation function-1 (AF-1) located in the N-terminus of the receptor (Atsriku et al., 2009). Phosphorylation at serine (S) sites, in particular, has been reported to alter protein-protein interaction, subcellular localization, transactivation, and the stability of the human ER α (Lannigan, 2003; Le et al., 2011). Modulation of cancer cell proliferation due to phosphorylation of a specific serine in the ER α has recently been reported (Gburcik and Picard, 2006; Tharakan et al., 2008), and phosphorylation of ER α at various serine sites is currently being evaluated for the classification of BCas (Murphy et al., 2009; Murphy et al., 2006; Skliris et al., 2009).

Analogous information on human ER β 1 is still unavailable because, until now, no phosphorylation sites on the receptor have been experimentally identified and demonstrated to be functional in BCa cells. Our knowledge of human ER β phosphorylation is derived primarily through studies of the mouse ER β (Tremblay et al., 1999; Tremblay et al., 1997). For example, information from the mouse receptor was used to predict and subsequently validated that S87 on the human ER β is a functional phosphorylation site under the regulation of stromal cell-derived factor 1 (or chemokine C-X-C motif ligand 12) in BCa cells (Sauve et al., 2009). Since the number of predicted kinase-specific motifs differs in humans and mice and the AF-1 domain in humans is shorter than that in mice, not all phosphorylation sites in human ER β 1 can be predicted from mouse ER β studies. Therefore, *de novo* identification of phosphorylation sites on human ER β is imperative for filling the data gap concerning the role of this PTM in regulating the function of the human receptor.

To this end, in the present study, we identified three serine phosphorylation sites (S75, S87, and S105) localized in the N-terminus of the full-length human ER β 1 using high-accuracy mass spectrometry (MS). Using a newly raised in-house phospho-specific S105 antibody, we showed the PTM to be mediated by E2-induced ERK1/2 activation or osmotic stress-induced p38 activation in BCa cell-line MDA-MB-231 (ER α -negative) and BT-474 (ER α -positive). Use of the phospho-mimetic mutant S105E and the phospho-defective mutant S105A further revealed that pS105 in ER β 1 enhances its ability to inhibit cell migration and invasion in these cancer cell-line models. Immunohistochemistry (IHC) analyses

demonstrated wide-spread S105-phosphorylation (pS105) positivity in BCa specimens. In total, this study identified the first functional phosphorylation site (S105) of the human ER β 1.

2. Materials and Methods

2.1. Breast cancer specimens

Twenty-five formalin-fixed, paraffin-embedded BCa sections were obtained from an archival collection in the Pathology Department at the University of Cincinnati Medical School. All specimens were graded by Dr. J. Wang and his colleagues on the basis of representative hematoxylin and eosin (H&E)-stained sections. The use of the specimens was reviewed and approved by the University's IRB committee.

2.2. In vitro kinase assay

All kinase buffers were prepared according to the manufacturers' instructions. Active recombinant ERK1/2 (#14-439 for ERK1; #14-550 for ERK2), p38 (#14-251), Src (#14-326) and PKA (#14-440) were purchased from Millipore (Billerica, MA). Kinases were incubated with full-length recombinant ER β 1 (Invitrogen, Carlsbad, CA) or myelin basic protein (MBP, positive control, #13-104, Millipore) along with Mg²⁺/adenosine triphosphate (ATP) solution and 5 μ Ci (γ -P³²) ATP (Perkin Elmer, Waltham, MA) at 30°C for 20 min. After termination of *in vitro* phosphorylation reactions, phosphorylated ER β 1 was resolved by SDS/PAGE and detected by autoradiography.

2.3. Phosphorylation-site identification by mass spectrometry

Tryptic digestion was performed as described previously (Lam et al., 2008; Lam et al., 2010). Each digest was analyzed by capillary LC-MS/MS with a Finnigan LTQ-Orbitrap (Thermo Fisher Scientific, MA). Half of the digest was loaded directly onto the 75 μ m $\times$ 100 mm PicoFrit capillary column (New Objective, MA) packed with MAGIC C18 (100 Å, 5 μ m, Michrom Bioresources, CA) at a flow rate of 300 nL/min, and peptides were separated by a gradient comprising 2–60% acetonitrile (ACN)/0.1% formic acid (FA) in 30 min, 60–98% ACN/0.1% FA in 5 min, and held at 98% ACN/0.1% FA for 5 min. The LTQ-Orbitrap was operated in standard data-dependent 'top-three' mode with lock mass function activated (protonated polydimethylcyclsiloxane [Si(CH₃)₂O]₆; m/z 445.120025)] to enable accurate mass measurement (< 2 ppm) of the precursor ions. A survey scan from m/z 300–1600 at 60,000 resolution in the Orbitrap was paralleled by 3 MS/MS scans in the LTQ. Phosphopeptides were detected by neutral loss scanning (–H₃PO₄) of 98, 49, 32.7 amu for 1+, 2+ or 3+ charged precursor ions respectively and consecutive MS³ scans. Multistage activation was also utilized in parallel experiments to confirm the sites of modification. The product ion spectra were searched against the human subset of the International Protein Index (IPI) database (ipi.HUMAN.v3.19) using the SEQUEST search engine in Bioworks 3.3. The database was indexed with fully enzymatic activity and two missed cleavage sites allowed for trypsin. Searching criteria included a mass tolerance of 15 ppm and 1 amu for precursor and fragment ions and variable modifications set on methionine (oxidized methionine: +15.9949), cysteine (carboxyamidomethylated cysteine: +57.0215), and phosphorylated serine/threonine (HPO₃: +79.9663). In addition, phosphorylated serines/threonines were pinpointed in the MS³ by the loss of water (–H₂O, –18). Peptides were filtered according to criteria: XCorr: 1.5 (1+), 2.0 (2+), 2.5(3+); DelCN: > 0.1, SP: >= 300. The MS/MS spectra of the identified phospho-peptides were evaluated manually.

2.4. Generation and characterization of the anti-pS105-ER β antibody and its utilization for immunohistochemical (IHC) and immunoblotting studies

Rabbit polyclonal monospecific antibody against the phosphoserine at 105 on human ER β (pS105-ER β) was commercially generated from immunizing New Zealand White rabbits with a KLH-conjugated peptide (CAEPQK*SPW), and then affinity-purified by phosphorylated peptide-conjugated column (affinity-purified package, New England Peptide, Gardner, MA). Its specificity was tested based on our published protocols (Leung et al., 2010). Briefly, pS105-ER β (1:300) was incubated with 10 $\times$ excess (by weight) of S105 peptide or 105 peptide at 4°C overnight. Antibody-peptide complex was centrifuged at 14,000 $\times$ g for 10 min and the supernatant was used for immunohistochemical (IHC) analyses. The antibody was used for of expression of the phosphorylated receptor in formalin-fixed and paraffin-embedded archival BCa sections (n=25), according to our previously published protocol (Leav et al., 2001). Histological evaluation and IHC scoring were performed by Dr. J. Wang, a board-certified clinical pathologist, and his associate in a blinded-manner. The Allred score (Allred et al., 1993; Leung et al., 2010) was used to quantify pS105-ER β positivity. In parallel, the ER β 1-specific antibody, GC17 (Leav et al., 2001), was used to quantify the ER β 1 expression. Pictures were captured using AxioCam MRm camera and Axiovision 4.7 software (Carl Zeiss, Thornwood, NY, USA). For immunoblotting analysis, pS105-ER β (1:1000) was incubated with 200 $\times$ molar excess of S105 peptide or 105 peptide at 4°C overnight. Antibody-peptide complex was centrifuged at 14,000 $\times$ g for 10 min and the supernatant was used for immunoblotting.

2.5. Cell culture and transfection

HEK293 and MDA-MB-231 were purchased from American Type Culture Collection (ATCC, Manassas, VA). BT-474 was a generous gift from Dr. Nira Ben-Jonathan (University of Cincinnati, OH). HEK293 cells was cultured in ATCC-recommended medium supplemented with 10% FBS (recognized as complete medium); MDA-MB-231 was cultured in MEMalpha medium (Invitrogen) supplemented with 10% FBS and 2 mM L-glutamine (recognized as complete medium); BT-474 was cultured in DMEM medium (ATCC) supplemented with 10% FBS. Wild-type (pDEST40-ER β 1), mutants (pDEST40-ER β 1-S105A, pDEST40-ER β 1-S105E), or control vector (pDEST40) was transfected into each cell line by the following methods. For MDA-MB-231 and BT-474 cells, 5 μ g of vector was incubated with 20 μ l of FuGENE HD transfection reagent (Roche Diagnostics, Indianapolis, IN). For HEK293 cells, 3 μ g of vector was mixed with 20 μ l of lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA). After 24 h of transfection, the cells were replated for other assays.

2.6. In cellulo induction of receptor phosphorylation in cell model systems and quantification by Western blot analysis

MDA-MB-231 cells and BT-474 cells cultured in phenol-red and serum-free DMEM for 24 h were treated for 15 min (MBA-MB-231) or 30 min (BT-474) with 10 nM E2, an ERK1/2 inducer or 0.7 M NaCl, a p38 inducer. PD 98059 (25 μ M, EMD chemicals, Gibbstown, NJ) and SB 203580 (10 μ M, EMD chemicals) were used to block ERK1/2- and p38-induced phosphorylation, respectively. These inhibitors were added 30 min before E2 or NaCl treatment. The treated cells were collected and 100 μ g of protein extract was analyzed by immunoblotting experiments. The following antibody dilutions were used: 1:200 for in-house anti-pS105-ER β ; 1:200 for anti-ER β (H-150, Santa Cruz Biotechnology Inc., Santa Cruz, CA), 1:1000 for anti-pERK1/2, anti-ERK1/2, anti-p38 (Cell Signaling Technology, Danvers, MA); 1:5000 for anti- β -actin (Abcam); 1:10,000 for IRD800 dye-conjugated donkey anti-mouse or donkey anti-rabbit antibodies (Rockland Immunochemicals, Gilbertsville, PA). The signals were detected by Odyssey Infrared Imaging System (LI-COR

Biotechnology, Lincoln, NE). β -actin served as loading controls, and phosphorylated and unphosphorylated forms of a kinase were positive controls of the kinase action.

2.7. Site-directed mutagenesis

Site-directed mutagenesis was used to prepare a phospho-defective mutant S105A (ER β 1-S105A), in which serine was changed to an alanine, and a phospho-mimetic mutant S105E (ER β 1-S105E), in which serine was replaced with a glutamate. The QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used with pENTR-ER β 1 as the DNA template and complementary pairs of primers with S105A or S105E mutation: ER β 1-S105A-F: 5'-gtg tgc gga acc tca aaa ggc tcc ctg gtg tga agc-3'; ER β 1-S105A-R: 5'-gct tca cac cag gga gcc ttg tga ggt tcc gca tac -3'; ER β 1-S105E-F: 5'-ctg tat gcg gaa cct caa aag gag ccc tgg tgt gaa gca aga tc-3'; ER β 1-S105E-R: 5'-gat ctt gct tca cac cag ggc tcc ttg tga ggt tcc gca tac ag-3'. The sequence identities of mutants were verified by sequencing (Macrogen, Seoul, Korea). The mutant vectors were subcloned into a mammalian expression vector, pcDNA-DEST40, by Gateway LR Clonase II recombination reaction (Invitrogen, Carlsbad, CA). The resulting vectors were designated as pDEST40-ER β 1-S105A and pDEST40-ER β 1-S105E, respectively.

2.8. ERE Reporter assay

ER β 1 transactivation activity was performed according to our previously published method (Leung et al., 2006). Cells were transfected with 300 ng of control vector or ER β 1 plasmids, together with 150 ng of ERE-luciferase reporter plasmid and 100 ng of β -galactosidase plasmid. E2 (1nM) or MPP dihydrochloride (Tocris Bioscience, Ellisville, MO, 10 μ M) was added after 24 h, and luciferase activity was measured after another 24 h. Control was added with 0.01% DMSO. Transactivation activity was performed with Bright Glo Luciferase assay kit (Promega) by using Wallac 1420 Vector2 Multilabel counter (PerkinElmer), and normalized by β -galactosidase activity (β -galactosidase enzyme assay kit; Promega). Results were presented as 'relative light units'.

2.9. Cell viability assay and cell-cycle analysis

ER β 1, S105A, or S105E vectors transfected cells were allowed to grow for 3 days in the complete medium and cell viability was assessed by CellTiter 96 Aqueous Non-Radioactive Cell Proliferation MTS assay (Promega) according to the manufacturer's recommended protocol. Cell cycle analysis was carried out by transfecting cells with plasmids and harvesting them after 72 h. Cells were fixed, stained with PI, and subjected to flow cytometry analysis of DNA content as described previously (Chan et al., 2010).

2.10. Cell migration and invasion assays

For cell migration, MDA-MB-231 and BT-474 cells were transfected with ER β 1-, ER β 1-S105A-, and ER β 1-S105E-transfected cells as mentioned before. A scratch was made on a confluent layer of cells in the complete medium. Distance of migration was calculated by half of the difference between the width of the wound 0 h and 48 h after scratching. Cell growth was monitored by MTS assay stated in section 2.9 above. For cell invasion, transfected cells were seeded in serum-free medium in the upper chamber and cells invaded to the complete medium in the lower chamber were fixed, stained, and counted after 24 h as described previously (Leung et al., 2010).

2.11. Statistical Analysis

One-way ANOVA was used to compare means among experimental groups.

3. Results

3.1. Identification of S75, S87, and S105 as ERK1/2- and p38-susceptible phosphorylation sites of human ER β 1

In *in vitro* assays, in the presence of phosphate donor ATP, recombinant human ER β 1 was shown to be phosphorylated by ERK1/2 (phosphorylation results are similar for ERK1 and ERK2, thus ERK1 was presented and used in subsequent experiments) and p38, and weakly phosphorylated by PKA and Src (Fig. 1A). MS analysis and site mapping (sequence coverage of ER β 1: 44%) following *in vitro* phosphorylation of the recombinant human ER β 1 by the various kinases revealed S75 (QTTpSPNVLWPTPGHLSPLVVHR), S87 (QTTSPNVLWPTPGHLpSPLVVHR), and S105 (QLSHLYAEPQKpSPWCEAR; Fig. 1B) as common ERK1 and p38 phosphorylation sites. All three sites are in the N-terminus of the receptor. Accurate mass measurement of the phosphopeptide containing S105 demonstrated a mass deviation of 1.5 ppm of the theoretical mass (Fig. 1B, upper panel). In addition, MS² exhibits a continuous *b*- and *y*- ion series covering the pS105. Because MS² analyses of phosphopeptides are often accompanied by a loss of phospho-group (neutral loss), leading to the limited fragmentation, we used a consecutive neutral loss-triggered MS³ to confirm the identity of the peptide (Fig. 1B, lower panel). To our satisfaction, database searching of both MS² and MS³ spectra revealed the same match (QLSHLYAEPQKpSPWCEAR). MS² (with or without multistage activation) gave enough fragmentation to identify both S75 and S87 as phosphorylation sites, thus a MS³ analysis was deemed unnecessary. All peptide masses measured were within 10 ppm of the theoretical masses. Owing to the fact that both PKA and Src did not effectively phosphorylate ER β , the present study did not follow up with these two kinases.

3.2. Antibody validation and induction of pS105 in ER β by ERK1/2 and p38 signaling

We successfully raised a specific antibody to pS105-ER β . We validated its specificity by immunohistochemistry (IHC, Fig. 2A) and immunoblotting (IB, Fig. 2B), in which the positive signal was abrogated by phosphorylated peptide but not unphosphorylated peptide against serine 105 site of human ER β . Immunoblotting result further showed that recombinant ER β was not able to be phosphorylated in the absence of the substrate ATP, despite the presence of an active recombinant ERK1 (Fig. 2B). The availability of this new antibody allows us to study the function of S105.

To confirm that S105 is a target site for ERK1/2 and p38, we treated a recombinant ER β 1 with the respective kinase and observed a single band with the correct size only when the phosphate donor ATP was present (Fig. 2C). To test whether pS105 occurs *in cellulo* (Fig. 2D), we stimulated MDA-MB-231 (ER α -negative) and BT-474 (ER α -positive) cells, both express endogenous ER β , with E2 to activate ERK1/2 (Henson and Gibson, 2006; Kelly and Levin, 2001) or with 0.7 M NaCl to activate p38 (Meriin et al., 1999). Both E2 and NaCl did not change the protein level of basal ER β but increased the level of phosphorylated ER β at S105 (pS105-ER β) in both cell lines. Such phosphorylation events were abolished by a MEK1 inhibitor, PD 98059 (Fig. 2D, upper panel), or by a p38 inhibitor, SB 203580 (Fig. 2D, lower panel). Collectively, these data indicate that S105 on human ER β is a target of ERK1/2 and p38.

3.3. Phospho-mimetic ER β 1-S105E enhanced ligand-dependent transactivation

To determine if pS105 affects the transactivation of ER β 1, we first compared transcriptional activities of the wild-type receptor and its phospho-mimetic (ER β 1-S105E) or phospho-defective (ER β 1-S105A) mutants in the HEK293 cell background using an ERE-luciferase reporter. In the presence of E2, ER β 1-S105E exhibited significantly higher transactivation activity than did ER β 1 and ER β 1-S105A (Fig. 3A). ER β 1-S105E inhibited ER α .

transactivation in a dose-dependent manner (Supplementary Fig. S1). In the absence of ligand, the mutants exhibited transactivation activities that were very low but similar to those of the wild-type ER β 1 (Fig. 3A). In a BCa-cell background, the phospho-mimetic mutant ER β 1-S105E showed an enhanced E2-induced transactivation in both MDA-MB-231 (ER α -negative) and BT-474 (ER α -positive) cells when compared with wild-type ER β 1 (Fig. 3B and C). Expectedly, the phospho-defective mutant ER β 1-S105A induced a significantly weaker transactivation when compared with that of wild-type ER β 1 following E2-stimulation (Fig. 3B). Similar result was obtained when the cells were treated with DPN (Diarylpropionitrile, an ER β -specific agonist), except that DPN did not trigger the transactivation in the control vector-transfected cells when compared with the results treated with E2 (Supplementary Fig. S2). The E2-induced transactivation in control vector-transfected BT-474 cells was due to endogenous ER α , which can be blocked by MPP dihydrochloride (an ER α -specific antagonist, data not shown). Blockade of ER α caused cell death, so it was not applied to subsequent cell studies. These data indicate that pS105 can augment the ligand-induced transactivation activity of ER β 1 in both ER α -positive or negative BCa cell background and inhibit the transactivation of ER α .

3.4. Activation of ER β 1-S105E inhibited cell migration and invasion but not cell growth/cycle in MDA-MB-231 and BT-474 cells

To elucidate the functional roles of S105 in ER β , we transfected MDA-MB-231 and BT-474 cells with wild-type ER β 1, ER β 1-S105E, or ER β 1-S105A plasmid. Ectopic expression of the wild-type ER β 1 and its mutants in MDA-MB-231 and BT-474 cells did not alter cell growth (Fig. 4A) or cell-cycle progression (Fig. 4B). Ectopic expression of ER β 1 inhibited cell invasion in MDA-MB-231 but not BT-474 cells, whereas transgenic expression of phospho-mimetic mutant ER β 1-S105E induced a drastic suppression of both cell migration and cell invasion in both cell lines (Fig. 4C and D, $p < 0.01$ compared with the control vector-transfected cells). Transgenic expression of ER β 1-S105A did not inhibit migration or invasion when compared with the control vector-transfected cells.

3.5. Detection of endogenous Serine-105 phosphorylation of ER β in human BCa specimens

Since S105 resides in the N-terminus of ER β , our anti-pS105 antibody recognizes wild-type ER β 1 as well as its isoforms, which only differ from ER β 1 at their C-termini (Leung et al., 2006). To gain insight of the potential role of pS105 in BCa when compared with ER β 1 that has been widely shown to be anti-proliferative (Warner and Gustafsson, 2010), we performed IHC with GC17 (an ER β 1-specific antibody) and our pS105-antibody.

In 25 BCa specimens, pS105 signal was detected in benign duct (myoepithelial and luminal secretory cells, Fig. 5A), benign lobule (Fig. 5B), ductal carcinoma *in situ* (DCIS, Fig. 5C) and invasive cancer (Fig. 5D). Nuclear staining was stronger than cytoplasmic staining in both benign luminal epithelial ($p = 0.1$) and cancer cells ($p < 0.01$, Table 1A). Occasional negative staining was detected regardless of the clinical classifications (Fig. 5A–D, red dotted arrows). Nuclear and cytoplasmic pS105-staining in cancer (Grade I/II and III) and their adjacent benign glands showed similar positivity (Table 1A). Importantly, pS105-staining was not correlated with ER α positivity or TNM staging (data not shown). Unlike the wide-spread pS105 positivity in invasive BCas (Fig. 5D), both nuclear and cytoplasmic ER β 1 signals were significantly diminished in invasive BCas as compared with signals in benign glands regardless of cancer grade, ER α positivity, and TNM staging (Table 1B, $p < 0.01$, Fig. 5I and J).

Collectively, the prevalence of pS105-ER β staining was higher in the nucleus than in the cytosol and did not mirror the ER β 1 positivity in BCa specimens.

4. Discussion

Protein phosphorylation is one of the most common PTMs in mammalian cells, playing crucial roles in regulating the functions of the target proteins and their downstream cellular processes (Lannigan, 2003). Such phosphorylation can occur at serine, threonine, and tyrosine residues, with serine phosphorylation being the most common modification. However, because of the low stoichiometry of phosphorylation and the transient nature of this PTM *in vivo* (Jorgensen and Linding, 2008; Mayya and Han, 2009), localizing phosphorylation sites in a protein still presents great challenges. In the past, progress has been hindered by the limited availability of high throughput, accurate methods. The recent advent of high-resolution tandem MS has allowed rapid developments in the field. The mapping of the human ER β has not been reported until now. In this study, we have taken advantage of this new technology and successfully uncovered three phosphorylation sites (S75, S87, and S105) in ERK1/2- or p38-treated recombinant human ER β 1 protein (wild-type). Worthy of mention is that all three sites are in the N-terminus of the protein and likely regulate the function of the AF-1 domain, a relatively understudied region of the receptor. However, since the substrate specificity of a kinase is generally broader *in vitro* than *in vivo* (Lannigan, 2003), the existence of these phosphorylated sites in tissue samples and in cell models must be further validated. We thus have developed and validated a highly specific antibody to pS105 that can be used for IHC and IB analyses to uncover the physiological roles of pS105.

Most nuclear receptors are known to be regulated by unique kinase systems, hence dictating the molecular functions of the receptors (Faus and Haendler, 2006). In this study, we found that both ERK1/2 and p38 can phosphorylate S105 of ER β *in vitro* and *in cellulo* in ER α -negative (MDA-MB-231) and ER α -positive (BT-474) BCa cells. Using S105 phosphomimetic (ER β 1-S105E) and -defective (ER β 1-S105A) mutants of ER β 1 and the specific ER β agonist DPN, we further demonstrated that phosphorylation of ER β at this site may promote the receptor's transactivation activity in the cancer cells. Of interest, Tremblay and co-workers (1999) found that phosphorylation of the analogous site (S124) in the mouse ER β was also ERK1/2-sensitive and associated with increased transactivation of the receptor. Thus, phosphorylation of S105/S124 in ER β by ERK1/2 appears to be a conserved feature in humans and mice. Tremblay and his colleagues further demonstrated that the ERK1/2-mediated phosphorylation of the mouse ER β increased recruitment of a co-activator SRC1 and a co-mediator CBP to the transcriptional complex (Tremblay and Giguere, 2001; Tremblay et al., 1999). However, p38 showed opposite effects on ER β phosphorylation and receptor transactivation activity in humans and mice. First, in mice, p38 represses ER β transactivation via ErbB2/ErbB3 pathway (St-Laurent et al., 2005), whereas in humans, p38 stimulates ER β -mediated transcription (Driggers et al., 2001; Frigo et al., 2006), acting through phosphorylation of a co-activator, glucocorticoid receptor-interacting protein 1 (Frigo et al., 2006). Second, no p38-mediated phosphorylation site has been identified in the mouse or human ER β , prior to this study. Therefore, it remains to be determined if the phosphorylation of S124 of the mouse receptor is a direct target of p38. This is in stark contrast to findings from our study of the human ER β in MDA-MB-231 and BT-474 BCa cell lines clearly demonstrating that S105 is a *bona fide* phosphorylation target of p38. Here, by comparing human and mouse data, we discovered conserved and divergent roles of phosphorylation of an analogous site (human S105/mouse S124) that may be involved in regulating the functions of ER β in the two species. Furthermore, it is not surprising to find that ERK1/2 and p38 are specific inducers of pS105 because both MAP kinases are known to regulate proliferation, survival, apoptosis, and invasion in most cancers (Atanaskova et al., 2002; Frigo et al., 2006). What remain to be deciphered are the pathophysiological conditions that would trigger pS105 in ER β through either or both signaling cascades.

Information on how ER β phosphorylation influences cellular functions, especially in cancers, is sparse. Our major objective in this study was to understand the pathophysiological role pS105 in BCa. Most published reports have demonstrated that ER β participates in growth inhibition of benign and malignant breast epithelial cells (Cheng et al., 2004; Lazennec et al., 2001; Martinetti et al., 2005; Pravettoni et al., 2007) and/or induces apoptosis (Cheng et al., 2004; Hodges-Gallagher et al., 2008; Huang et al., 2006), but no study relates these functions to ER β phosphorylation. In addition, only a few studies on ER β have focused on BCa progression (Lazennec et al., 2001; Lindberg et al., 2010). Thus, an important finding of our study is the observation that transient expression of the phosphomimetic mutant ER β 1-S105E in MDA-MB-231 or BT-474 cells induced significant inhibition of cell migration and invasion without affecting cell proliferation or cell cycle progression. The data suggest that phosphorylation of ER β at one or more specific serine sites (including S105) confers a previously overlooked property to the receptor, i.e. one that is related to the blockade of BCa invasion. Our findings are in concordance with a recent report that ER β increased BCa cell adhesion to different extracellular matrix proteins (mostly laminin) and inhibited cell migration. The clinical relevance of these observations warrants further investigation.

Our results show pS105 immunopositivity was widely distributed in BCa specimens in both nuclear and cytoplasmic compartment. However, it did not mirror the ER β 1 positivity in BCa specimens, which in general diminished in high grade cancer (Leygue et al., 1998; Shaw et al., 2002; Zhao et al., 2003). A disparity in pS105 positivity and ER β 1 immunostaining is expected because S105 is in the N-terminus of the receptor, a region that is common among all reported isoforms (Leung et al., 2006). Thus, a pS105 antibody should recognize any isoforms whose S105 is susceptible to similar kinase regulation. Our data therefore support the hypothesis that other ER β isoforms can be phosphorylated at this site. It is worth mentioning that ER β isoforms may have functions different from those of ER β 1 (Leung et al., 2006). Because of the small sample size of the current IHC study, it has a limited capacity to reveal the clinical relevance of pS105 in BCa. Fortunately, two recent large-scale studies have helped addressed this important question. Expression of ER β 2 and ER β 5 isoforms predicts better survival in a cohort of 880 BCa patients (Shaaban et al., 2008). A report on 459 cases of BCa demonstrated that nuclear pS105-ER β -positivity is associated with better survival and correlates with immunopositivity of ER β 1 and ER β 2 (Hamilton-Burke et al., 2010). Collectively, these data uncover a significant degree of complexity in how phosphorylation regulates the function of ER β in breast carcinogenesis.

In this study, we reported, using an unbiased MS method, the identification and characterization of the first functional phosphorylation site, S105, located in the N-terminus of the human ER β . We also described the development of a specific antibody conducive for studying this PTM in IHC and IB analyses. Both *in vitro* and *in cellulo* experiments demonstrated that pS105 is a direct target of ERK1/2 and p38 MAPK. In ER α -positive (BT-474) and -negative (MDA-MB-231) BCa cell lines, pS105 promotes E2-induced ER β 1 transactivation and enhances the ability of ER β 1 to inhibit cell migration and invasion without affecting cell growth and cell-cycle progression. Our data suggest that S105 phosphorylation confers anti-cell invasion property to ER β 1. Further investigations are necessary to resolve the potential role of phosphorylation in regulating the function of ER β 1 and its isoforms.

- > First identified direct phosphorylation targets of human ER β at serine (S) 75, S87, and S105
- > ER β phosphorylation at S105 is activated by ERK1/2 and p38 signaling in MDA-MB-231 and BT-474 breast cancer cells.

- > Phospho-mimetic mutant (ER β 1-S105E) enhanced human ER β transactivation.
- > ER β 1-S105E inhibited cellular migration and invasion, but did not affect cell growth and cell-cycle progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Discovery of ERβ phosphorylation sites from *in vitro* phosphorylation by mass spectrometry

(A) Phosphorylation of recombinant ERβ by recombinant kinases (ERK1/2, p38, Src and PKA) in the presence of ATP *in vitro*. Myelin basic protein (MBP) was used as a positive control. P-ERβ: phospho-ERβ, p-MBP: phospho-MBP. (B) Upper panel, MS² spectrum of the tryptic peptide containing pS105 (QLSHLYAEPQKpSPWCEAR). A continuous b- and y-ion series was observed in the MS². Accurate mass measurement of the precursor (FT-MS) demonstrated a small mass deviation to the theoretical mass (<2 ppm). Lower panel, Confirmation of ERβ peptide identity in a consecutive neutral loss-triggered MS³. Database searching of MS³ spectrum revealed the same match (QLSHLYAEPQKpSPWCEAR), with a loss of H₂O on S105.

Figure 2. Validation of pS105-ERβ antibody and detection of human ERβ S105 phosphorylation *in vitro* and *in cellulo*

(A) Evaluation of pS105 antibody specificity in a breast specimen by immunohistochemistry. Immunopositive staining of pS105-ERβ in the nuclei and cytoplasm of epithelial cells can be abolished by overnight incubation with a phosphorylated peptide (p105 peptide, CAEPQK*SPW) but not with an unphosphorylated peptide (105 peptide, CAEPQKSPW) flanking the serine 105 site of human ERβ. (B) Evaluation of pS105 antibody specificity by immunoblotting assay. The pS105-specific band was only detected upon addition of ERK1 and ATP (+ATP) in the *in vitro* phosphorylation experiment using human recombinant ERβ1. The band was abolished when the antibody was pre-incubated

with the phosphorylated 105 peptide but not with the unphosphorylated 105 peptide. Signals from panER β antibody were used as a protein loading control. (C) *In vitro* phosphorylation of a recombinant ER β 1 with ERK1 and p38 in the absence (–ATP) or presence (+ATP) of ATP. Only the reaction mixture containing ATP yielded a single band with the correct size in immunoblotting analyses. (D) Detection of endogenous pS105 in (left) MDA-MB-231 and (right) BT-474 cells upon ERK1/2 activation by 10nM E2, and p38 activation by 0.7M NaCl. PD: PD 98059, a MEK inhibitor. SB: SB 203580, a p38 inhibitor. β -actin is a protein loading control. Representative pictures are shown from two to three independent experiments of similar results.

Figure 3. Ligand-dependent ERE transactivation of ERβ

ERβ1 wild-type or mutants (S105A and S105E) were transfected in (A) HEK-293, (B) MDA-MB-231, and (C) BT-474 cells and S105E enhanced the ligand-dependent transactivation of ERE in both cell lines. Ctl: 0.1% DMSO; E2: Estradiol-17 beta. Values are the average of triplicate samples from an independent experiment that was repeated thrice with similar results. Mean $\pm$ SD, * p <0.05 compared with ERβ1 with E2 treatment.

Figure 4. S105E inhibited cell migration and invasion but not growth or cell cycle in MDA-MB-231 and BT-474 cells

(A) S105E did not affect the cell growth by MTS assay. Control vector (Ctl vec) was used as a negative control. Cell viability was compared with that of Ctl vec. Values are mean $\pm$ SD of three independent experiments, triplicate for each experiment. (B) S105E did not affect the cell cycle in flow-cytometry analysis. Values are mean $\pm$ SD of three independent experiments. (C) S105E inhibited cell migration in the wound-healing assay. (D) S105E inhibited cell invasion in the Matrigel transwell assay. Values are mean $\pm$ SD of three independent experiments for (C) and (D), * $p < 0.05$, ** $p < 0.01$ compared with Ctl vec.

Figure 5. Representative immunohistochemical staining of pS105-ER β in human breast specimens

Positive pS105-ER β staining was detected in both nuclei (stronger) and cytoplasm (weaker) of (A) luminal and myoepithelial cells in the benign duct, (B) epithelial cells in the benign lobule, cancer cells in (C) ductal carcinoma in situ (DCIS) and (D) invasive cancer. Red dotted arrow indicated unstained cells. ER β 1 was found in the nuclei and cytoplasm of cells in (E) normal lobules and weak ER β 1 staining was detected in (F) invasive cancer. Magnification: 200 $\times$, scale: 100 μ m.

Table 1

Quantification of (A) pS105-ER β and (B) ER β 1 (GC17) immunostaining in the human breast specimens.

A. pS105-ERβ staining				
	Breast (n=25)			
	Benign	Cancer	Gr I/II	Gr III
Nucleus	7.0 $\pm$ 0.2	7.0 $\pm$ 0.1 ^a	7.0 $\pm$ 0.1	7.1 $\pm$ 0.1
Cytoplasm	6.5 $\pm$ 0.3	6.6 $\pm$ 0.1	7.0 $\pm$ 0.1	6.3 $\pm$ 0.1
B. ERβ1 (GC17) staining				
	Breast (n=25)			
	Benign	Cancer	Gr I/II	Gr III
Nucleus	6.7 $\pm$ 0.2	4.5 $\pm$ 0.5 ^b	4.3 $\pm$ 0.7 ^c	4.6 $\pm$ 0.6 ^b
Cytoplasm	6.4 $\pm$ 0.2	5.0 $\pm$ 0.3 ^b	5.0 $\pm$ 0.5 ^c	5.0 $\pm$ 0.3 ^b

Allred score (0–8) represents the sum of the staining intensity (0–3) and coverage of positive stain (0–5).

GS, Gleason Score; Gr, grade.

^a p<0.01 compared to cytoplasmic staining.

^b p<0.01 compared to normal tissue.

^c p<0.05 compared to normal tissue.