Novel Link between Estrogen Receptor α and Hedgehog Pathway in Breast Cancer

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Abstract. Ligand-dependent constitutive activation of the hedgehog (Hh) pathway is important in the development of various carcinomas including breast cancer. A link between estrogen receptor α (ERα) and the Hh pathway in human breast cancer is shown here for the first time. In ERα-positive cells, estrogen depletion decreased the expression of sonic hedgehog (Shh), a ligand of the Hh pathway, while estrogen supplementation triggered Shh up-regulation. This estrogen-induced Shh expression activated the Hh pathway in a ligand-dependent manner, and increased cell proliferation. These effects were suppressed by ERα inhibitors, including ICI 182,780 (ICI), the dominant negative form of ERα and small interfering RNA (siRNA) against ERα. Consistent with the in vitro data, a positive correlation between ERα and Shh expression was found in breast cancer tissues. These data suggest that ERα regulates the Hh pathway through Shh induction, and promotes breast cancer development.

The hedgehog (Hh) signaling pathway is a key mediator of many fundamental processes in embryonic development (1), and Hh signaling is crucial in some adult organs for stem cell maintenance and tissue repair (2, 3). The Hh pathway has also been found to be constitutively activated in many human cancer tissues (4-7), while Hh pathway activation increases proliferation of various tumor cells in a ligand-dependent manner (5, 8-11). Thus, the factors which lead to overexpression of the Hh ligand and activation of Hh signaling may represent a new therapeutic target for human carcinomas (12). In mouse breast tissue, the Hh pathway plays a critical role during ductal development (13), and disruption of Patched1, a Hh ligand receptor and suppressor of the Hh pathway, results in severe defects in ductal morphogenesis such as ductal hyperplasia, that closely resembles some hyperplasia in humans (14). Consistent with these data, Hh signaling is activated in human breast cancer tissues, and inhibition of the signaling activity attenuates tumor cell proliferation (15). Thus, it is important to elucidate the molecules that regulate Hh pathway activation in breast cancer; however, the detailed mechanisms that underlie Hh pathway activation in breast cancer remain unclear.

Estrogen is a crucial factor for physiological proliferation and differentiation of the normal mammary gland. On the other hand, estrogen is also considered as a stimulant for initiation and promotion of breast tumors. The biological effects of estrogen are mediated through two distinct intracellular receptors, estrogen receptor α (ERα) and ERβ, which belong to the superfamily of nuclear receptors that share a similar structure and mode of action. ERα plays important roles in the proliferation of ERα-positive breast cancer cells (16, 17), and ERα status is essential information in making decisions for endocrine therapy (18).

Thus, it has been generally considered that both Hh pathway activation and ERα activation participate in the carcinogenesis and growth of breast tumors. There are two representative mechanisms of Hh pathway activation, ligand-dependent and ligand-independent activation (1). Since aberrant Hh-ligand production has been seen in several carcinomas including breast cancer (8, 9, 15, 19), and mutations of Hh pathway components has been identified at low frequency in breast cancer (20-22), it is proposed that Hh pathway activation is mainly caused in a ligand-dependent manner in breast cancer. Thus, in the present study we focused on the mechanism of up-regulation of sonic hedgehog (Shh), a ligand of the Hh...
pathway in several carcinomas (8). Our previous study has also indicated a positive correlation between ERα status and Gli1 nuclear translocation, which is a marker of Hh pathway activation, suggesting a link between ERα status and Hh pathway activation (15). To prove this potential link, we selected two breast cancer cell lines, ERα-positive MCF-7 cells and ERα-negative MDA-MB-231 cells, which are representative cell lines and well-characterized at the molecular level were selected.

**Materials and Methods**

**Cell culture, reagents, and antibodies.** The MCF-7 cells and MDA-MB-231 cells were purchased from American Type Culture Collection and were maintained at 37°C under a humidified atmosphere of 5% CO2 and 95% air in RPMI1640 medium (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Life Technologies) and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin, Meiji-seika, Tokyo, Japan). The cells were incubated for 24 h in phenol-red free minimum essential medium (MEM; Invitrogen, Carlsbad, CA, USA) without FBS prior to all experiments (termed estrogen starvation). Thereafter, cells were principally cultured in MEM supplemented with 5% dextran-coated charcoal-treated FBS (DCC-FBS-MEM) in a humidified 95% air and 5% CO2 atmosphere. Stock solutions of 17β-estradiol (E2) purchased from Sigma (Deisenhofen, Germany), ICI 182,780 (ICI), a pure anti-estrogen from Tocris Cookson Ltd. (Ellisville, MO, USA) and cyclopamine (Cyc), a Hh pathway inhibitor from Toronto Research Chemicals (North York, Ontario, Canada) diluted in 99.5% ethanol were stored at –30°C. The recombinant human Shh N-terminal peptide (rhShh) and the rat anti-Shh NH2-terminal peptide antibody (aShh-Ab) were purchased from R&D Systems (Minneapolis, MN, USA). The anti-GAPDH (sc-25778), anti-Shh (sc-9024), anti-Gli1 (sc-6153), and anti-ERα (sc-8002) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and the mouse anti-phospho ERα (Ser118) from Cell Signaling Technology, Inc. (Danvers, MA, USA).

**Expression vectors.** cDNAs encoding human ERα or dominant negative human ERα (S554fs) were cloned into the pSG5 expression vector as described previously (27, 28). These cDNAs and ERE-tk-luc, a consensus estrogen response element upstream of luciferase, were kindly provided by Dr Norio Wake (Kyushu University, Fukuoka, Japan). The pRL-SV40 was purchased from Promega (Madison, WI, USA). The pIRE2-hShh-EGFP (pShh-GFP) and pcdNA3.1/His-hGli1 (pGli1) were kindly provided by Dr. Aubie Shaw (Division of Urology, Department of Surgery, University of Wisconsin, Madison, WI, USA) (29) and Dr. H. Sasaki (Center for Developmental Biology, Riken, Kobe, Japan) (30), respectively.

**Real-time reverse transcription-PCR.** The total RNA was extracted by RNeasy mini kit (Qiagen, Valencia, CA, USA) and quantified by spectrophotometry (Ultrospec 2100 Pro; Amersham Pharmacia Biotech, Cambridge, UK). The RNA (700 ng) was treated with DNase, and reverse transcribed to cDNA with the Quantitect Reverse Transcription Kit (Qiagen) according to the manufacturer’s protocol. The reactions were run with SYBR Premix Ex Taq (Takara Bio. Inc., Otsu, Japan) on a DNA Engine Opticon 2 System (MJ Research, Waltham, MA, USA). The pShh-GFP or pGli1 were serially diluted in 10-fold increments and amplified with the primer pairs to generate a standard curve for Shh or Gli1. Standard curves for pS2 and β-actin were generated using cDNA from MCF-7 cells treated with E2 for 16 h. Each sample was run in triplicate. All the primer sets amplified fragments less than 200 bp long. Sequences of the primers used were β-actin, forward, 5’-TTG CCG ACA GGA TGC AGA AGG A-3’, reverse, 5’-AOG TGG ACA GCG AGG CCA GGA T-3’; Shh, forward, 5’-GTG TAC TAC GAG TCC AAG GCA C-3’, reverse, 5’-AGG AAC TCG CTG TAG AGC AGC A-3’; Gli1, forward, 5’-GGT TCA AGA GCC TGG GCT GTG T-3’, reverse, 5’-GGC AGT CCT AGT AGT GAT C-3’; ERα, forward, 5’-GGA GGG CAG GGG TGA A-3’, reverse, 5’-GGC CAG GGT TGA A-3’, Erk, forward, 5’-GGC CAT GGA CTT CAC TCC CCC TAC AGA GA G-3’, reverse, 5’-CTC TGG GAC TAA TCA CCG TGC TG-3’. The amount of each target gene in a given sample was normalized to the level of β-actin in that sample.

**Immunoblotting.** Whole-cell extraction was performed with M-PER Reagents (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer’s instructions. The protein concentration was determined with Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA), and the whole-cell extract (100 μg) was separated by electrophoresis on SDS-polyacrylamide gel, and transferred to Protran nitrocellulose membranes (Dassel, Germany). The blots were then incubated with anti-Shh (1:100), Gli1 (1:200), GAPDH (1:1000), ERα (1:500), or phospho ERα (1:500) primary antibody overnight at 4°C followed by incubation in horse radish peroxidase (HRP)-linked secondary antibody (Amersham Biosciences, Piscataway, NJ, USA) at room temperature for 1 h. The immunocomplexes were detected with the ECL plus Western Blotting Detection System (Amersham Biosciences) and visualized with a Molecular Imager FX (Bio-Rad). GAPDH was used for protein loading control. The Shh protein level was quantified with the 19 kDa fragment, as it is the physiologic active form of Shh (31).

**Dual luciferase assay.** The MCF-7 cells in 24-well plates were transfected with plasmids with TransFast transfection reagent according to the manufacturer’s instructions. The cells in each well were co-transfected with 10 ng of pRL-SV40 (Promega) and 1 μg ERE-tk-Luc. After estrogen starvation, E2 and ICI or Cyc were added to each well, and luciferase assays were performed 6 h later with the dual luciferase assay kit (Promega) according to the manufacturer’s instructions. The luciferase activities were normalized to the Renilla luciferase activity.
(0.1% ethanol), or indicated doses of E2, ICI, or Cyc for 24 h in 5% DCC-FBS-MEM. The cells were fixed in 4% paraformaldehyde followed by permeabilization with 0.2% Triton X-100, and then incubated with primary followed by secondary antibodies. The cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich Corp., St. Louis, MO, USA). After mounting in Vectorshield Mounting Medium (Vector Laboratories, Burlingame, CA, USA), the samples were examined by fluorescence microscopy (BX 50; Olympus Corp., Tokyo, Japan). The exposure time for recording was manually fixed at 2 msec, 1.8 msec, and 0.2 msec for Shh, P-ERα, and DAPI, respectively. The antibodies and dilutions used were as follows: rabbit anti-Shh (1:200), mouse anti-phospho ERα (1:100), AlexaFluor 488 goat anti-rabbit IgG and AlexaFluor 594 goat anti-mouse IgG (1:400; Molecular Probes, Eugene, OR, USA).

**Transient cell transfection.** The MCF-7 cells (1x10^6/well) seeded in a 25 cm² flask were transfected with 9 μg of dominant negative ERα (DNER) or pSG5 plasmid control, with TransFast Reagent (Promega). The transfected cells were incubated in 10% FBS-RPMI for 16 h. After estrogen starvation, the transfected cells were treated with the indicated reagents in 5% DCC-FBS-MEM for 24 h, and then used for immunoblotting.

**Small interfering RNA (siRNA) against ERα.** The MCF-7 cells (2.0x10^6 cells) were transfected with siRNA (100 nM) against ERα by nucleofection with NucleoFector II (Amaxa GmbH, Koeln, Germany) as per the manufacturer’s instructions, and plated in a 25 cm² flask for 12 h in 10% FBS-RPMI. After estrogen starvation, the cells were treated with E2 for 24 h, and then used for immunoblotting. The Validate stealth RNAi against ERα, and the stealth RNAi-negative control (Invitrogen) were used.

**Proliferation assay.** The cells (2x10^4/well) seeded in 48-well plates in complete culture medium were incubated overnight. After estrogen starvation the medium was changed to 5% DCC-FBS-MEM containing the indicated doses of reagents. After 72 h incubation, the cells were harvested by trypsinization, and cells were counted with a Coulter counter (Beckman Coulter, Fullerton, CA, USA).

**Clinical samples.** Samples from 14 patients with primary breast carcinoma selected on the basis of ERα status who underwent resection at the Department of Surgery and Oncology, Kyushu University (Fukuoka, Japan), between February 2003 and August 2003 were included. Eight specimens were ERα-positive and 6 were ERα-negative. All 14 patients gave informed consent before surgical treatment. All the surgical specimens were frozen at –80°C, examined histopathologically, and classified using the tumor-node-metastasis (TNM) classification system of the International Union Against Cancer (UICC, Fifth edition, 1997). The total mRNA of these specimens was extracted using the RNeasy mini kit (Qiagen) as per the manufacturer’s recommendation.

**Statistical analysis.** The Student’s t-test was used for statistical analysis unless otherwise indicated. All the calculations were carried out with StatView 5.0 J software (Abacus Concepts, Berkeley, CA, USA). P-values less than 0.05 were considered significant.

**Results**

**Hh pathway activation.** Hh activity with or without estrogen in the breast cancer cell lines was studied. Minimum essential medium supplemented with dextran-coated charcoal-treated fetal bovine serum (DCC-FBS-MEM) was used as the estrogen-depleted medium. When the ERα-positive MCF-7 cells were cultured with DCC-FBS-MEM instead of conventional FBS-MEM, pS2 mRNA and P-ERα protein expression increased (Figure 2A). Gli1 mRNA expression in the MCF-7 cells was also increased, in sharp contrast to the MDA-MB-231 cells (data not shown). The E2-induced activation of ERα and the Hh pathway were almost completely inhibited by ICI (Figure 2B).
Co-incubation with Cyc completely prevented the E2-induced $Gli1$ mRNA elevation in the MCF-7 cells (Figure 2B), but did not affect the $pS2$ mRNA expression and ERα transcriptional activity (Figure 2A).

### Shh expression

The ligand dependency of Hh pathway activation in human breast cancer was confirmed by culturing the MCF-7 cells in 5% DCC-FBS-MEM supplemented with or without recombinant human Shh peptide (rhShh). Incubation with rhShh markedly increased the $Gli1$ mRNA expression compared to the rhShh-free cultures (Figure 3A), suggesting that Hh activation is ligand-dependent in MCF-7 cells. Next, the ligand-dependence of E2-induced Hh pathway activation was demonstrated by culturing the MCF-7 cells in 5% DCC-FBS-MEM with E2 in the presence or absence of antibodies directed against Shh (αShh-Ab). While the $Gli1$ mRNA expression significantly increased with E2, the rise in $Gli1$ mRNA was significantly less in the presence of αShh-Ab (Figure 3B).

The correlation between E2 stimulation and Shh production was then examined. When the MCF-7 cells were cultured in DCC-FBS-MEM instead of FBS-MEM, Shh mRNA expression was significantly decreased, as was Shh and P-ERα protein expression (Figure 4A). Supplementation with E2 in DCC-FBS-MEM significantly increased Shh mRNA expression in the MCF-7 cells, in contrast to the MDA-MB-231 cells (Figure 4B). The elevation of Shh mRNA expression in the MCF-7 cells induced by E2 was completely decreased to control levels by co-incubation with ICI (Figure 4B), while there was no effect with the addition of Cyc, on the increased Shh mRNA expression. The E2-induced elevation of Shh expression and inhibition by ICI were also detected at the protein level in the MCF-7 cells, but not in the MDA-MB-231 cells (Figure 4C). E2-induced elevation of Shh expression was also visualized by immunofluorescence staining in MCF-7 cells (Figure 4D). After E2 stimulation, Shh protein staining was higher than that seen in control cells consistent with the
Figure 4. Shh expression in MCF-7 and MDA-MB-231 cells. (A) Shh mRNA or Shh and P-ERα protein expression in MCF-7 cells cultured in 5% FBS-MEM (FBS) or 5% DCC-FBS-MEM (DCC-FBS) (estrogen-depleted) medium. The cells were cultured for 16 h to measure Shh mRNA expression or for 24 h to detect Shh and P-ERα protein expression. (B) Shh mRNA expression in MCF-7 cells or MDA-MB-231 cells treated with indicated reagents for 16 h. (C) Shh protein expression in MCF-7 cells or MDA-MB-231 cells treated with indicated reagents for 24 h. (D) Immunofluorescent staining of MCF-7 cells treated with indicated reagents for 24 h. Magnification, 400x; Bars, 20 μm. (E) Shh or P-ERα protein expression in MCF-7 cells transfected with dominant negative ERα (DNER) or control plasmid (pSG5) in the presence or absence of E2. After transfection with DNER or pSG5, MCF-7 cells were treated with E2 for 24 h. (F) ERα mRNA expression in MCF-7 cells transfected with ERα-siRNA (#1 or #2) or control siRNA (Cont.) at 100 nM (F, left). ERα protein expression in MCF-7 cells was confirmed by immunoblotting (F, left inset). Shh or P-ERα protein expression in MCF-7 cells transfected with ERα-siRNAs or control siRNA and treated with or without E2 for 24 h (F, right).

Results are expressed as mean±S.D. *p<0.05; **p<0.01; n.s., not significant. GAPDH was shown as protein loading control.
observation that nuclear P-ERα staining (Red) showed a higher signal after E2 stimulation than in the control cells. These E2-induced elevations of P-ERα and Shh protein intensity were suppressed to control level by ICI.

To further confirm that activated ERα increased Shh expression, the Shh protein levels in the dominant-negative form of ERα (DNER)-transfected MCF-7 cells was measured. The Shh protein level in the DNER-transfected MCF-7 cells was low compared to that of control cells in the presence of E2 (Figure 4E), suggesting that the E2-induced Shh production was a consequence of ERα activation. These results were supported by the following data using small interfering RNA against ERα (ERα-siRNA). Knockdown of ERα was confirmed at both mRNA and protein level (Figure 4F, left and inset). In the presence of E2, the Shh protein level of the ERα-siRNA-transfected MCF-7 cells decreased, in contrast to that of the control siRNA-transfected cells (Figure 4F, right).

**Effect of Shh induced by estrogen on cell proliferation in ERα-positive cells.** Supplementation of E2 in 5% DCC-FBS-MEM increased the proliferation activity of MCF-7 cells in a dose-dependent manner, which was inhibited by ICI (Figure 5A, left and middle, respectively) and Cyc (Figure 5A, right). The αShh-Ab also inhibited proliferation of MCF-7 cells in the presence of E2 (Figure 5B, right), while consistent with the above data, rhShh increased proliferation of MCF-7 cells in a dose-dependent manner (Figure 5B, left). Thus, these data suggested that E2-induced Shh production was biologically significant, and activated MCF-7 cell proliferation in a ligand-dependent manner.

**ERα status correlation with Shh mRNA expression in breast cancer tissues.** Real-time RT-PCR analysis was used for the quantitative analysis of Shh expression in breast carcinoma tissue specimens. There was no significant correlation between the ERα status and the major clinicopathological parameters in these specimens (Table I). The level of Shh mRNA expression in ERα-positive tissues was significantly higher than that in ERα-negative tissues (Figure 6).

**Time-course analysis of pS2 and Shh mRNA expression.** The MCF-7 cells were treated with E2 and pS2 and Shh mRNA expression were analyzed by real-time RT-PCR. Shh mRNA expression significantly increased from 16 h onwards after E2 treatment (Figure 7A), whereas pS2 mRNA significantly increased from 4 h after E2 treatment (Figure 7B).

**Discussion**

Here for the first time ERα regulation of Hh pathway activation in an autocrine manner through up-regulation of Shh expression in ERα-positive breast cancer, is reported. In
the present study, E2 increased ERα activation, Shh expression, and Hh pathway activation only in the ERα-positive MCF-7 cells. Blockade of ERα activation by ICI or transfection with DNER or ERα-siRNA decreased the increase in Shh expression and Hh pathway activation induced by E2 in the ERα-positive MCF-7 cells. The data from surgically resected specimens indicated that this pathway consisting of ERα activation, Shh expression, and Hh pathway activation may be operating even in breast cancer tissue.

The data obtained from the two cell lines indicated that ERα is one of the regulators of ligand-dependent Hh pathway activation in breast cancer. Namely, ERα may be contributing to Shh production in ERα-positive breast cancer cells. The time-course analysis indicated that activation of ERα preceded Shh mRNA expression (Figure 7). Furthermore, incubation of MCF-7 cells with cycloheximide, a protein synthesis inhibitor, attenuated the ability of ERα to induce Shh mRNA expression (data not shown). These data suggested that ERα indirectly regulated Shh expression.

Our previous data showed that the Hh pathway was constitutively activated not only in MCF-7 cells but also in MDA-MB-231 cells (15), thus Hh pathway activation is also found even in ERα-negative cells. It is still unclear how the Hh pathway is activated in ERα-negative breast cancer cells.

Table I. Clinicopathological features in the 14 breast specimens.

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Major clinicopathological parameters were comparable in both groups. There were no significant differences in other parameters, such as age, HER2/neu, nuclear atypia, and vessel invasion (data not shown). *According to the TNM classification system. †Mann-Whitney U-test; n.s., not significant.

Several mechanisms may be operating dependently or independently in Hh pathway activation in different cells. For example, we have recently reported that nuclear factor κB activation increased Shh expression, which activated the Hh pathway in an autocrine manner and enhanced cell

Figure 6. Shh mRNA expression in resected breast cancer tissues. A scatter plot of relative Shh mRNA expression in ERα-negative or ERα-positive breast cancer groups are shown after normalization to the corresponding β-actin mRNA expression. Filled circles, mean of experiments; bars, SD; open circles, relative Shh mRNA expression of each sample. *p<0.05.

Figure 7. Time-course analysis of Shh and pS2 mRNA expression in MCF-7 cells treated with E2. MCF-7 cells were treated with E2 for indicated number of hours. Relative Shh or pS2 mRNA expression is shown after normalization to the corresponding β-actin mRNA expression as fold change relative to control. Columns, mean of triplicate experiments; bars, SD. *p<0.05.
proliferation in pancreatic cancer (12). The present data support a causal link between ERα activation and Shh expression, even in primary breast cancer tissue. Thus, our findings that ERα links with the Hh pathway appear to be important in the clinical setting, because both ERα and the Hh pathway play important roles in the development of breast cancer, and are also useful therapeutic targets.

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