The Hedgehog Pathway Is a Possible Therapeutic Target for Patients with Estrogen Receptor-negative Breast Cancer

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Abstract. *Understanding the expression patterns of estrogen receptor-α (ERα) is essential for determining therapeutic strategies for patients with breast cancer. The prognosis of patients with ERα-negative breast cancer is still poor. We have previously shown that Hedgehog (Hh) signaling is constitutively activated in breast cancer and that Hh signaling could be a new therapeutic target. Therefore, in this study, whether or not Hh signaling could be utilized as a therapeutic target for patients with ERα-negative breast cancer was examined. For this purpose, three ERα-negative breast cancer cell lines were used in which Hh pathway-related molecules such as the ligand Patched1 and the transcriptional factor Gli1 as target cells are expressed. Cyclopamine, an inhibitor of the Hh pathway, significantly suppressed both the cell proliferation and invasion ability of these cancer cells. In addition, the knockdown of Gli1 by RNA interference in these cells also significantly reduced both cell proliferation and invasion ability. Since our previous data have shown a constitutive activation of the Hh pathway in surgically-resected ERα-negative breast cancer specimens, the Hh pathway, especially Gli1, may be a useful therapeutic target for patients with ERα-negative breast cancer.*

Estrogen is not only a crucial factor for physiological proliferation and differentiation of the normal mammary gland, but it is also considered as a stimulant for initiation and promotion of breast cancer. The biological effects of estrogen are mediated through two distinct intracellular receptors, estrogen receptor-α (ERα) and ERβ. ERα plays an important role in the proliferation of ERα-positive breast cancer cells (1, 2), ERα status is essential in making decisions for endocrine therapy (3) and various antiestrogen agents such as tamoxifen have been developed. As a result, the therapeutic strategy for patients with ERα-positive breast cancer is now standardized and their survival prognosis has clearly improved in the last decade. On the other hand, few effective treatments are available for patients with chemotherapy-resistant ERα-negative -breast cancer (4). Therefore, wide therapeutic targets, effective not only for ERα-positive cases, but also for ERα-negative cases are needed. Recently, we have investigated a new therapeutic target, the Hedgehog (Hh) pathway in breast cancer (5).

The Hh signaling pathway is a key mediator of many fundamental processes in embryonic development and it acts as a morphogen, mitogen and inducing factor of developing organs (6-8). The Hh pathway is a highly coordinated and integrated network and consists essentially of Hh proteins (Sonic Hh, Indian Hh and Desert Hh), the transmembrane receptor Patched (Patched1 and Patched2), the transmembrane protein Smoothened (Smo), and the transcription factor Gli genes (Gli1, Gli2 and Gli3) (6-8). In the absence of Sonic Hh (Shh), Glis forms a large protein complex with other proteins, the kinesin-like Costal2 and the serine-threonine kinase Fused and is sequestered in the cytoplasm (6, 9, 10). In the presence of Shh, a full length Glis released from the large protein complex is transported into the nucleus to activate Hh target genes (6, 9, 10). *Gli1* is one of the target genes of Glis (11). Therefore, Gli1 is a marker of Hh pathway activation (9, 12, 13). Evidence also suggests that properly regulated Hh signaling is required in some adult organs for stem cell maintenance or tissue repair (14, 15). In the mouse model, the Hh pathway plays a critical role during ductal development in the mammary gland (12) and disruption of the Patched1 *(Ptch1)* or *Gli2* genes results in severe defects in ductal morphogenesis such as ductal hyperplasia that closely resembles some hyperplasia in humans (13). A number of observations have

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indicated a contribution of Hh signaling to cell proliferation in various types of cancer cells (15-19). Hh signaling is also thought to contribute to cellular invasion (20, 21). We have previously shown that the Hh pathway is constitutively activated in breast cancer tissues and that it may be a new therapeutic target for Hh pathway-activating breast cancer (5). However, information about the relationship between Hh pathway activation and ERα status is lacking.

The present study focused on whether the Hh pathway could be a new therapeutic target against ERα-negative breast cancer, using the ligand Ptch1 and the transcriptional factor Gli1 as Hh pathway-related molecules.

Materials and Methods

Cell culture, reagents, and antibodies. The ERα-negative human breast carcinoma cell lines (MDA-MB-231, SK-BR-3, HCC-38 cells) and an ERα-positive human breast carcinoma cell line (MCF-7) (5) were purchased from American Type Culture Collection (ATCC, Manassas VA, USA) and were maintained at 37˚C under a humidified atmosphere of 5% CO_2 and 95% air in RPMI-1640 medium (Nacalai tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS; Life Technologies Grand Island, NY, USA) and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin; Meijiseika, Tokyo, Japan). Cyclopamine, a small-molecule Smo antagonist used as a blockade of Hh signaling (21-23) was purchased from Toronto Research Chemicals (North York, Ontario, Canada), and was diluted in 99.5% ethanol as a stock solution and stored at –30˚C.

Reverse transcription-polymerase chain reaction (RT-PCR). The total RNA was extracted from the cultured cells by the guanidinium thiocyanate-phenol-chloroform single-step method. For the reverse transcription reaction, the $pd(N)_6$ Random Hexamer (GE Healthcare UK Ltd, Buckinghamshire, UK) was used for priming. *Gli1* forward (5'-TCT GCC CCC ATT GCC CAC TTG-3') and reverse (5'-TAC ATA GCC CCC AGC CCA CTT G-3') primers yielded a 480-bp product. *Ptch1* forward (5'-CGG CGT TCT CAA TGG GCT GGT TTT-3') and reverse (5'-GTG GGG CTG CTG TCT CGG GTT CG-3') primers yielded a 376-bp product. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) forward (5'-CCA CCC ATG GCA AAT TCC ATG GCA-3') and reverse (5'-TCT AGA CGG CAG GTC AGG TCC ACC-3') primers gave rise to a 593-bp product. The amplification conditions comprised an initial denaturation for 2 min at 95˚C followed by 30 cycles of 94˚C for 30 s, 58˚C for 30 s, and 72˚C for 1 min. Amplification of each gene was in the linear range. The RT-PCR products were separated on ethidium bromide-stained 2% agarose gels. Semi-quantitative analysis was carried out with a Molecular Imager FX Pro (Bio-Rad Laboratories, Hercules, CA, USA).

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. 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). Standard curves for *18S* ribosomal RNA (*18S*) were generated using cDNA *Gli1* transfected MK-1, gastric cancer cell line cells established in our laboratory. Each sample was run in triplicate. All the amplified fragments were less than 200 bp long. The sequences of the primer used were *18S*, forward, 5'-GAT ATG CTC ATG TGG TGT TG-3', reverse, 5'- AAT CTT CTT CAG TCG CTC CA-3'; *Gli1*, forward, 5'-CTC GGG CAC CAT CCA TTT CTA C-3', reverse, 5'-ATT GCC AGT CAT TTC CAC ACC A-3'. The quantity of each target gene in a given sample was normalized to the level of 18S in that sample.

Immunostaining of cell lines. The SK-BR-3, MDA-MB-231, HCC-38 and MCF-7 cells (2×10^4/well) were seeded onto pre-underlaid poly-L-lysine coated glass coverslips (Asahi Techno Glass Corporation, Chiba, Japan) in 24-well plates, and were incubated overnight in 10% FBS-RPMI. The cells were fixed in 4% paraformaldehyde followed by permeabilization with 0.2% Triton® X-100 and then incubated with primary antibody followed by secondary antibody. 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 (Axioimager A1; Carl Zeiss Imaging, Tokyo, Japan). Exposure time for recording was manually fixed at 12 ms, 25 ms, and 3 ms for *Ptch1, Gli1,* and DAPI, respectively. The antibodies and dilutions used were as follows: rabbit anti-Ptc at 1:100, goat anti-Gli at 1:100, AlexaFluor 488 chicken anti-rabbit IgG at 1:1000 (Molecular Probes, Eugene, OR, USA), and AlexaFluor 594 donkey anti-goat IgG at 1:1000 (Molecular Probes).

Small interfering RNA (siRNA) against Gli1. SK-BR-3 (5.0×10^5) cells and MDA-MB-231 (5.0×10^5) cells were transfected with siRNA (100 nM) against *Gli1* (ON-TARGET*plus* SMART pool, L-003896) and negative control siRNA (ON-TARGET*plus* siCONTROL Non-targeting Pool, D-001810) purchased from Dharmacon RNA Technologies (Chicago IL, USA) by nucleofection with Nucleofector II (Amaxa GmbH, Koeln, Germany) as per the manufacturer's instructions and plated in a 25 cm^2-flask for 48 h in 10% FBS-RPMI, and then used for real-time RT-PCR, proliferation assay and invasion assay.

Matrigel invasion assay. The invasiveness of the breast cancer cells was assessed based on the invasion of cells through Matrigel-coated transwell inserts. In brief, the upper surface of a filter (pore size, 8.0 μm; BD Biosciences, Heidelberg, Germany) was coated with basement membrane Matrigel (BD Biosciences) at a concentration of 2 mg/ml, and incubated at 4˚C for 3 h; unbound material was aspirated. The cells were suspended in RPMI-1640 with 10% FBS containing the desired dose of reagents. SK-BR-3 (1.0×10^5) cells and MDA-MB-231 (3.0×10^4) cells were then added to the upper chamber and incubated in a water-saturated 5% CO_2 atmosphere at 37˚C for 16 h. After incubation, the filter was fixed with 70% ethanol and stained with Diff-Quik reagent (International Reagents, Kobe, Japan). The cells on the upper surface were then completely removed by wiping with a cotton swab. Cells that had migrated from the upper to the lower side of the filter were counted under a light microscope (BX50; Olympus Corp., Tokyo, Japan) at a magnification of ×100. Tumor cell invasiveness was defined as the total number of cells in five randomly selected microscopic fields. Each experiment was performed in triplicate wells.

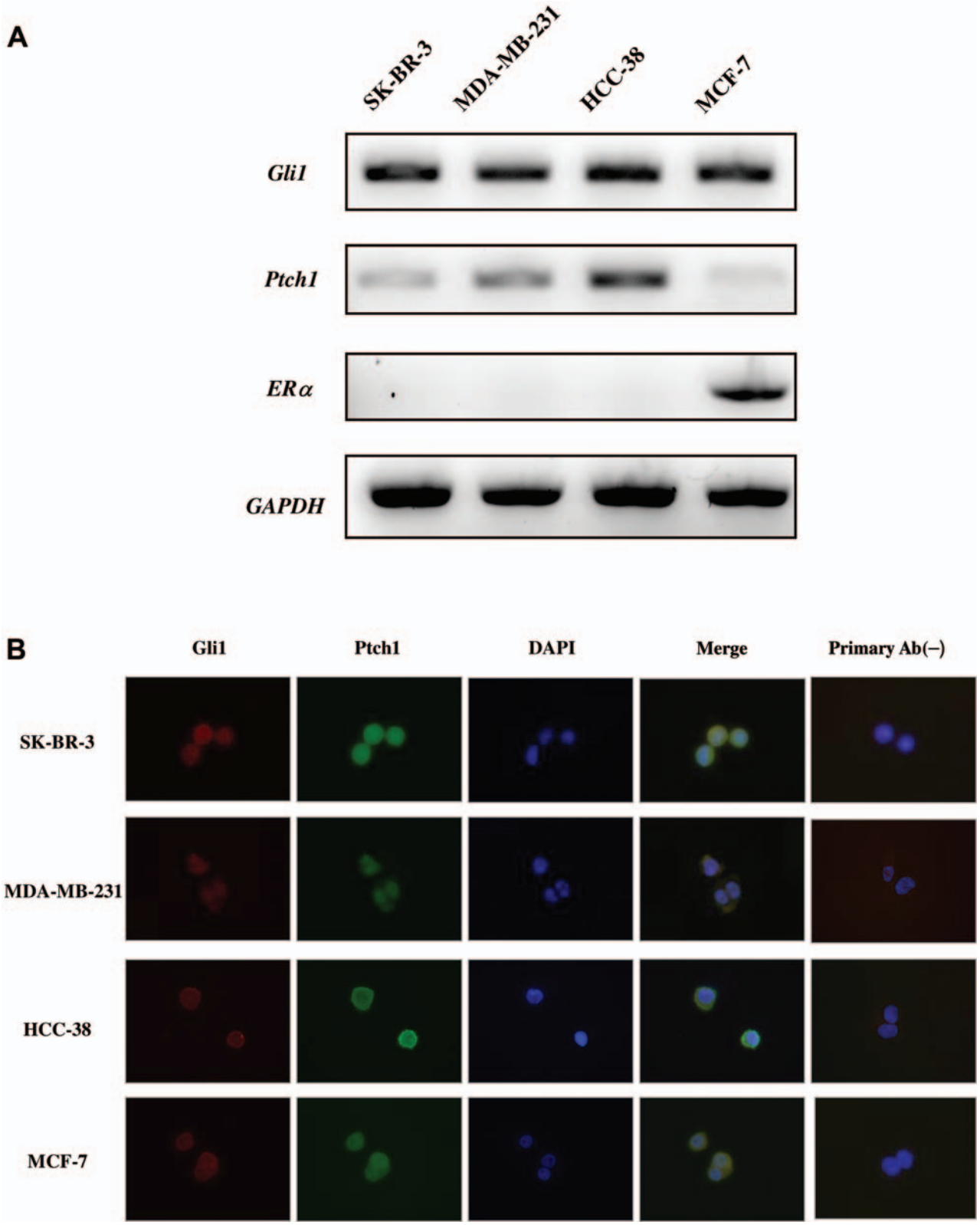

Figure 1. *Expression of Hh pathway components in human breast cancer cells. A, mRNA expression of Gli1 and Ptch1 in SK-BR-3, MDA-MB-231, HCC-38 and MCF-7 cells. B, Immunofluorescence staining with antibodies against Gli1 (red signal) and Ptch1 (green signal) proteins. The blue signal represents nuclear DNA staining by DAPI. Ab, primary Ab(–), no addition of primary antibody.*

Proliferation assay. The SK-BR-3 (5×10^3/well), MDA-MB-231 (3×10^3/well) and HCC-38 (5×10^3/well) were seeded in 96-well plates in complete culture medium and were incubated overnight. The medium was changed to 5% FBS containing the indicated doses of cyclopamine. After 72 h incubation, cell proliferation was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazoliumbromide (MTT) assay. Each experiment was carried out in triplicate.

Clinical samples. Ten patients with primary breast carcinoma underwent resection at the Department of Surgery and Oncology, Kyushu University (Fukuoka, Japan) between April 2004 and August 2006. All ten patients gave informed consent before surgical treatment and were entered into the present study. All the tumor and normal tissues were frozen at –80˚C, examined histopathologically and classified using the Tumor-Node-Metastasis classification. The total mRNA of these specimens was extracted using the RNeasy mini kit (Qiagen) as per the manufacturer's recommendation. The standard curve for *β-tubulin* was generated using cDNA *Gli1*-transfected MK-1 cells. Each sample was run in triplicate. All the amplified fragments were less than 200 bp long. The sequences of the primers used were *β-tubulin*, forward, 5'-CCG TGT CTG ACA CCT TGG GT-3', reverse, 5'-ATC AGC AAG ATC CGG GAA GAG-3' and *Gli1*, forward, 5'-CTC GGG CAC CAT CCA TTT CTA C-3', reverse, 5'-ATT GCC AGT CAT TTC CAC ACC A-3'. The quantity of each target gene in a given sample was normalized to the level of *β-tubulin* in that sample.

Statistical analysis. The Student's *t*-test was used for statistical analysis unless otherwise indicated. The Wilcoxon *t*-test was used for the clinical samples. All the calculations were carried out with StatView 5.0 J software (Abacus Concepts, Berkeley, CA, USA). An asterisk indicates $p<0.05$ and *p*-values less than 0.05 were considered significant.

Results

The expression of Hh pathway-related molecules in ERα-negative breast cancer cells. It was confirmed that the MDA-MB-231, SK-BR-3 and HCC-38 cells did not express ERα (Figure 1A), and all these ERα-negative cell lines expressed Ptch1 and Gli1 at both the mRNA (Figure 1A) and protein levels (Figure 1B).

Effect of blockade of Hh signaling on proliferation of ERα-negative breast cancer cells. When the cancer cells were cultured with cyclopamine at different concentrations (1 to 100 μM) for 6 h, cyclopamine concentrations over 10 μM significantly suppressed *Gli1* mRNA expression in all of the ERα-negative breast cancer cells compared to 10 μM ethanol vehicle (Figure 2A). When these cells were cultured with cyclopamine for 72 h, cyclopamine suppressed the cell proliferation in a dose-dependent manner (Figure 2B). However, when the cells were cultured with cyclopamine (1 to 100 μM) for 24 h, cyclopamine did not affect cell proliferation (data not shown).

In order to confirm a contribution of Hh signaling to the proliferation of the ERα-negative breast cancer cells, *Gli1* was silenced by RNA interference. Transfection of siRNA targeting *Gli1* resulted in a 90%-reduction of *Gli1* mRNA expression in SK-BR-3 and MDA-MB-231 cells compared to control siRNA (Figure 3A), and knockdown of *Gli1* resulted in significant suppression of proliferation compared with controls (Figure 3B).

Table I. *Clinicopathological features of 10 ER-negative specimens*.

Age (years)	Histology	pTMN* stage	ER / PgR status	HER2 / neu status	pTNM stage
73	Papillo-tubular	T4bN1M0–	– / –	–	IIIB
53	Scirrhous	T2N2M0	– / –	3+	IIIA
56	Papillo-tubular	T3N2M0	– / –	2+	IIIB
47	Papillo-tubular	T2N0M0	– / –	–	IIA
30	Solid-tubular	T2N0M0	– / –	–	IIA
64	Papillo-tubular	T2N1M0	– / –	3+	IIB
75	Papillo-tubular	T2N0M0	– / –	3+	IIA
47	Scirrhous	T1cN1M0	– / –	3+	IIA
66	Solid-tubular	T2N0M0	– / –	–	IIA
67	Solid-tubular	T2N0M0	– / –	3+	IIA

*According to the TNM classification system of International Union Against Cancer. pTMN, pathological tumor-node-metastasis. PgR, progesterone receptor; HER2, human epidermal growth factor receptor type 2.

Blockade of Hh signaling and the invasive ability of ERα-negative breast cancer cells. When SK-BR-3 cells or MDA-MB-231 cells were incubated with cyclopamine for 16 h, cyclopamine reduced the invasion ability in a dose-dependent manner (Figure 4A). In order to confirm a contribution of Hh signaling to the invasion of the ERα-negative breast cancer cells, *Gli1* was silenced by RNA interference. Knockdown of *Gli1* significantly reduced the invasion ability compared with that of control siRNA-transfected cells (Figure 4B).

Gli1 mRNA expression in ERα-negative human breast cancer tissues. Real-time RT-PCR analysis was used for the quantitative analysis of *Gli1* mRNA expression. The clinicopathological features of 10 ER-negative specimens were shown Table I. The level of *Gli1* mRNA expression in the ERα-negative breast cancer tissues was significantly higher than that in the normal tissues (Figure 5).

Discussion

For the first time, it was shown that Hh signaling contributed not only to proliferation, but also to invasion in ERα-negative breast cancer cells. This finding indicated that Hh signaling could be useful as a therapeutic target for patients with ERα-negative breast cancer.

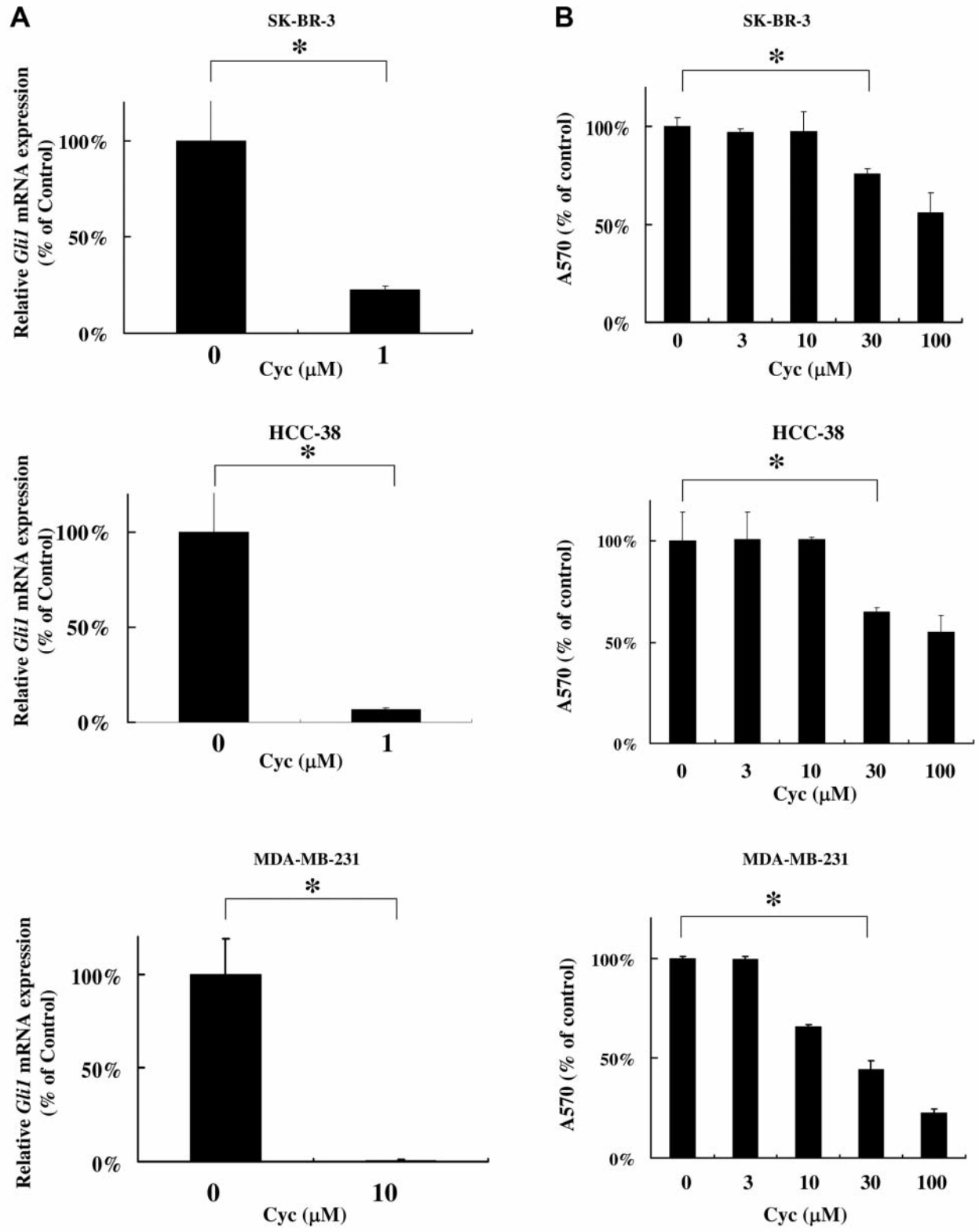

Figure 2. *Effect of cyclopamine on Gli1 mRNA expression and cell proliferation. A, Gli1 mRNA expression in ERα-negative breast cancer cells after treatment with ethanol vehicle or cyclopamine (Cyc) for 6 h. Mean ± s.d., *p<0.05. B, ERα-negative breast cancer cell proliferation after treatment with 100 μM ethanol vehicle or various concentrations (1 to 100 μM) of cyclopamine at 37˚C for 72 h. After incubation, cell viability was measured using MTT assay at an absorbance at 570 nm. Mean ± s.d. *p<0.05.*

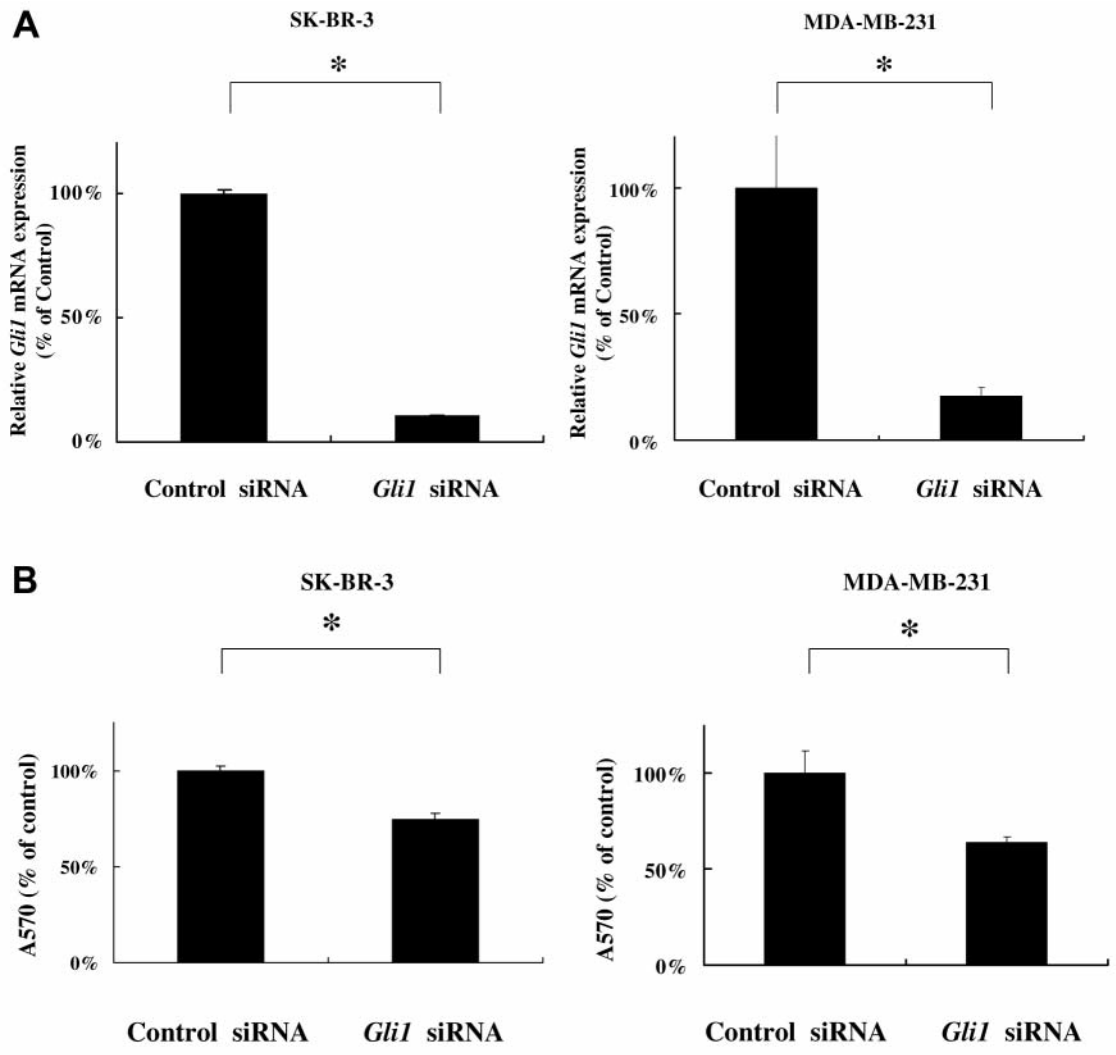

Figure 3. *Effect siRNA of Gli1 on proliferation in ERα-negative breast cancer cells. A, mRNA expression; B, cell proliferation. Cells were maintained at 37˚C for 72 h. Cell viability was measured using MTT assay detected at an absorbance at 570 nm. Mean ± s.d., *p<0.05.*

It was important that the Hh pathway was shown to be activated in most of the 52 breast cancer specimens examined in our previous study and that cyclopamine could suppress the proliferation of several breast cancer cell lines (5). These findings suggested that the Hh pathway may be activated independently of the status of ERα expression. If this is the case, cyclopamine may be able to suppress the proliferation of ERα-negative breast cancer (5). In order to examine this possibility, we re-analyzed our previous data in detail concerning the relationship between Hh signaling and the status of ERα expression. As we expected, this analysis revealed that the Hh pathway was constitutively activated in all of the 13 ERα-negative breast cancer specimens as it was also in the three ERα-negative breast cancer cell lines examined in the present study as shown by *Gli1* expression (Figure 1). However, the degree of Hh signaling activation was found to be significantly higher in the 39 ERα-positive breast cancer specimens than in the 13 ERα-negative breast cancer specimens, when nuclear translocation of *Gli1* was used as a marker of Hh pathway activation (5). Cyclopamine suppressed the proliferation of the three ERα-negative breast cancer cell lines in the present study (Figure 2B). Since cyclopamine may be not precisely specific against Hh signaling, in order to confirm the contribution of Hh signaling to cell proliferation, we silenced *Gli1*, which is a transcriptional factor, and is itself a transcriptional target of Hh signaling in ERα-negative breast cancer cells. Knockdown of *Gli1* also induced a significant suppression of proliferation in the ERα-negative breast cancer cells (Figure 3B).

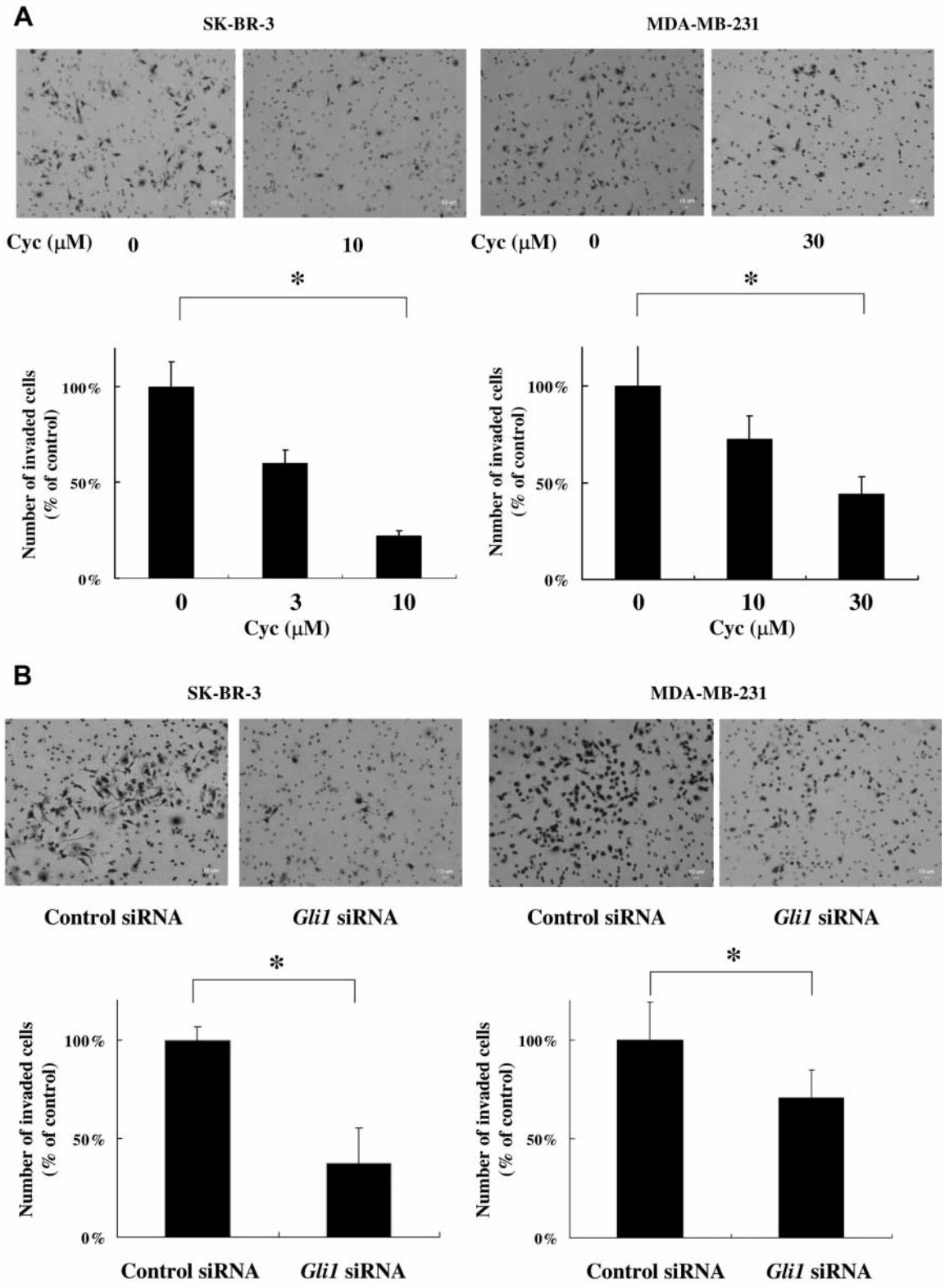

Figure 4. *Effect of cyclopamine and siRNA of Gli1 on invasion of ERα-negative breast cancer cells. Invasion of MDA-MB-231 and SK-BR-3 cells, A, after incubation with or without cyclopamine (Cyc; B, after control or siRNA transfection. Cells were maintained at 37˚C for 16 h. ×40 magnification. Mean ± s.d., *p<0.05. The migration ability of the breast cancer cells was assessed in the same way as described above, except for using non-Matrigel-coated transwell inserts.*

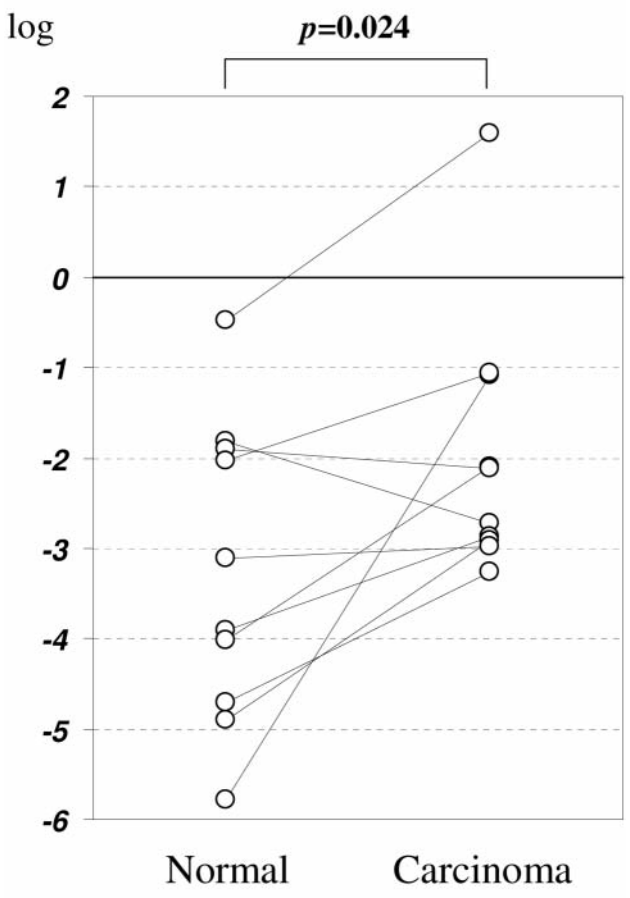

Figure 5. *Gli1 mRNA expression in surgically resected breast cancer tissues. Paired open circles are relative Gli1 mRNA expression in normal or cancer tissues of the same sample. These data were normalized to the corresponding β-tubulin mRNA expression.*

The contribution of Hh signaling to invasiveness of the ERα-negative breast cancer cells was also demonstrated (Figure 4). Recently, an association of cell invasion with Hh signaling has been demonstrated in pancreatic cancer cells (20, 21). In fact, our previous study indicated a positive relationship between invasive ability and the degree of Hh signaling activation since the degree of Hh signaling activation of invasive ductal type carcinomas was higher than that of non-invasive ductal type carcinomas (5). Although the molecular mechanism remains unclear, our recent data have suggested participation of increased Hh pathway-mediated matrix metalloproteinase (MMP)-9 expression in the enhanced invasive ability of human pancreatic cancer cells (21).

In conclusion, blockade of Hh signaling is useful for treatment for patients with ERα-negative breast cancer, which is supported by our previous data showing a frequent activation of Hh signaling in ERα-negative breast cancer specimens.

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