

# Silencing of *EPHB2* promotes the epithelial-mesenchymal transition of skin squamous cell carcinoma-derived A431 cells

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**Abstract.** Erythropoietin-producing hepatocellular (Eph) receptors and their ligand ephrins serve crucial roles in the interactions among epithelial cells. Eph receptor/ephrin signaling regulates cell functions, including proliferation, differentiation and migration, via these cell-cell interactions. We reported previously that *EPHB2*, a member of the Eph receptor family, was highly expressed in chemically induced cutaneous squamous cell carcinoma (cSCC) tissues in mice. Although the higher expression level of *EPHB2* has been observed in various human cancers, its roles in the development and progression of cancers are still unclear. In the present study, the functional implications of *EPHB2* in the acquisition of malignant phenotypes of cSCC cells was investigated. Silencing of *EPHB2* in the human cSCC cell line A431 induced epithelial-mesenchymal transition (EMT)-like morphological changes accompanied by a significant upregulation of epithelial-mesenchymal transition-associated genes such as zinc finger E-box binding homeobox 1/2. In addition, silencing of *EPHB2* suppressed anchorage-independent cell growth under 3D culture conditions. Consistent with these observations, *EPHB2* exhibited higher levels of expression in tumor spheres formed under 3D culture conditions than in cells cultured in adherent form, and the expression pattern of

EMT markers indicated that EMT was suppressed in tumor spheres. The results of the present study indicated that *EPHB2* serves a pivotal role in promoting the anchorage-independent growth of A431 cells through the suppression of EMT.

## Introduction

Erythropoietin-producing hepatocellular (Eph) receptors and their ligand ephrins are membrane-bound molecules, which play crucial roles in cell-cell communication (1). Eph receptors constitute the largest family of receptor tyrosine kinases, and the Eph receptors and ephrins are divided into subclasses A and B based on their structure and binding affinities for each other (2). In humans, 16 Eph receptors, including 10 EphA and 6 EphB, and 9 ephrins, including 6 ephrin-A and 3 ephrin-B, have been identified (3). EphA receptors bind to ephrin-As, and EphB receptors bind to ephrin-Bs, with a few exceptions (4). As both Eph receptors and ephrins are expressed on the cell surface, it is necessary for cells to contact each other to generate Eph/ephrin signals. When they bind each other, both Eph receptors and ephrins emit signals for regulation of a wide range of cell phenotypes, including cell morphology, motility, adhesion, and invasion (4,5).

The roles of the Eph/ephrin system in the development and progression of cancer vary among the molecules and cancer types. Upregulated expression of Eph receptors and ephrin has been observed in many types of cancers, and their downregulation in cancers has also been reported (5). For example, higher expression of *EPHB2* has been reported to be associated with higher stage of cervical cancer (6) and colorectal cancer (7), and higher expression of *EPHB4* with poor prognosis of ovarian cancer (8) and colon cancer (7). On the other hand, downregulation of *EPHA1* in skin cancer (9) and colorectal cancer (10), and EphB receptors in colorectal cancer (11) and Ephrin-A5 (EFNA5) in glioblastoma (12) have been reported.

Recently we have found that the expression levels of *EPHB2* are significantly higher in mouse cutaneous

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squamous cell carcinoma (cSCC) obtained by treating murine dorsal skin using a two-stage carcinogenesis protocol with 7.12-dimethylbenz(a)anthracene (DMBA) and 12-O-tetradecanoylphorbol-13-acetate (TPA) than in normal skin (13). In addition, genomic DNA near the EPHB2 gene has been shown to be hypomethylated in skin cancer tissues (13). Overexpression of EPHB2 has also been observed in human cSCC (14), and knockdown of EPHB2 in cSCC cells derived from cSCC surgical specimens resulted in suppression of cSCC cell growth *in vitro* and *in vivo* (14). It has also been demonstrated that knockdown of EPHB2 suppressed the expression of genes involved in cell viability, migration, and invasion (14). These findings indicate that EPHB2 possesses oncogenic properties in epithelial tumors.

Many recent studies have suggested that Eph/ephrin systems have pivotal roles in epithelial-mesenchymal transition (EMT) processes (15). Cells undergoing EMT show reduction in cell-cell adhesion caused by reduced expression of E-cadherin on the cell surface, and gain mesenchymal phenotypes with spindle-shaped morphology and increased potential for migration and invasion (16). Therefore, EMT has been implicated in the acquisition of an invasive phenotype by cancer cells. Overexpression of EPHA2 and reduced expression of E-cadherin are associated with higher stage of gastric cancer (17) and colorectal cancer (18). On the other hand, higher expression levels of EPHB3 and E-cadherin were reported to be associated with lower stage of esophageal adenocarcinoma (19). In cervical cancer, forced expression of EPHB2 induced EMT signature, and silencing of EPHB2 resulted in the opposite phenotype (6).

Nevertheless, the role of EPHB2 in EMT in SCC is still unclear. In the present study, we conducted functional analysis to elucidate whether EPHB2 is involved in the regulation of EMT in SCC.

## Materials and methods

**Cell lines and culture conditions.** The human skin squamous carcinoma-derived cell line A431 was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in DMEM (Nacalai Tesque, Kyoto, Japan) supplemented with heat-inactivated fetal bovine serum (FBS; Nichirei Bioscience, Tokyo, Japan) at a final concentration of 10%, 100 IU/ml of penicillin (Thermo Fisher Scientific, Inc., Waltham, MA, USA), and 100  $\mu$ l/ml of streptomycin (Thermo Fisher Scientific, Inc.). The cells were maintained at 37°C in a CO<sub>2</sub> incubator with a controlled humidified atmosphere composed of 95% air and 5% CO<sub>2</sub>.

**Analysis of cell viability.** Cells were seeded in 96-well plates at a density of 1x10<sup>3</sup> cells per well, and cultured for 24 h. The cells were then transfected with control siRNA or EPHB2 siRNA using Lipofectamine 3000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Cell viability was measured by WST8 assay using Cell Count Reagent CF (Nacalai Tesque) at 24, 48, and 72 h after transfection.

**Wound-healing migration assay.** Cells were seeded in 24-well plates at a density of 2x10<sup>5</sup> cells per well, and transfected with control siRNA or EPHB2 siRNA at 24 h after seeding.

Forty-eight h after transfection, cell layers were wounded using a Cell Scratcher Scratch stick (AGC Technoglass, Shizuoka, Japan) and medium was replaced with 500  $\mu$ l of fresh medium. After 24 h, cells were photographed by phase-contrast microscopy, and the width of the wounded area was measured. The percentage gap size was calculated by dividing the width at 24 h by the width at 0 h.

**Matrigel invasion assay.** Cells were seeded into the upper chambers of BD Matrigel invasion chambers (BD Biosciences, San Jose, CA, USA) at a density of 2x10<sup>5</sup> cells per well, and immediately transfected with control siRNA or EPHB2 siRNA. The lower wells were filled with culture medium. After 24 h, non-invading cells on the upper surface of the membrane were removed, and invading cells were fixed and stained with Diff-Quik solutions (Sysmex, Kobe, Japan). The number of invading cells in 10 microscopic fields was counted for each membrane under a light microscope at x200 magnification.

**Analysis of sphere formation efficiency.** Cells were seeded in ultra-low attachment surface 24-well plates (Corning Incorporated, Corning, NY, USA) at a density of 4x10<sup>3</sup> cells per well, with MEGM Bullet kit serum-free medium supplemented with 0.4% BPE, 0.1% hEGF, 0.1% hydrocortisone, 0.1% GA1000, 0.1% insulin, 1% l-glutamine (Lonza, Walkersville, MD, USA). To examine the effect of EPHB2 on sphere formation efficiency, cells were immediately transfected with control siRNA or EPHB2 siRNA. Five days after seeding, the number of spheres >100  $\mu$ m in diameter in eight microscopic fields at x50 magnification was counted. Representative images were photographed at x200 magnification.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was extracted from cells using RNeasy mini kits (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's instructions. For cDNA synthesis, 500 ng of total RNA was reverse transcribed using iScript cDNA synthesis system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). qPCR was performed using a SYBR Premix Ex Taq™ system according to the manufacturer's recommendations (Takara Bio, Inc., Otsu, Japan). A mixture of cDNA derived from total RNA of cells was used as a reference. Then, a dilution series of the cDNA mixture was prepared and used in RT-qPCR as the template to obtain a standard curve for each gene. Three independent measurements were performed and the relative amounts of the PCR products were quantified via extrapolation from a standard curve. The sequences of primers used for qPCR-based amplification were as follows: EPHB2, 5'-TGAGTGGCCTCAGATGGTCAA-3' (sense) and 5'-AGG GCAGGGTATCACAGTGAATG-3' (antisense); CDH1, 5'-AATTCCTGCCATTCTCTGGGGA-3' (sense) and 5'-TCT TCTCCGCTCTTCTTTC-3' (antisense); FN, 5'-CAGTGG GAGACCTCGAGAAG-3' (sense) and 5'-TCCCTCGGAACA TCAGAAAC-3' (antisense); SNAIL, 5'-CCATGCTCCTCT TTGCTCTC-3' (sense) and 5'-TACAAAAACCCACGCAGACA-3' (antisense); ZEB1, 5'-GCCAACAGACCAGACAGT GTT-3' (sense) and 5'-TCTTGCCCTTCCTTTCCTG-3' (antisense); ZEB2, 5'-CAAGAGGCGCAAACAAGC-3' (sense) and 5'-AACCTGTGTCCACTACATTGTCA-3' (antisense);

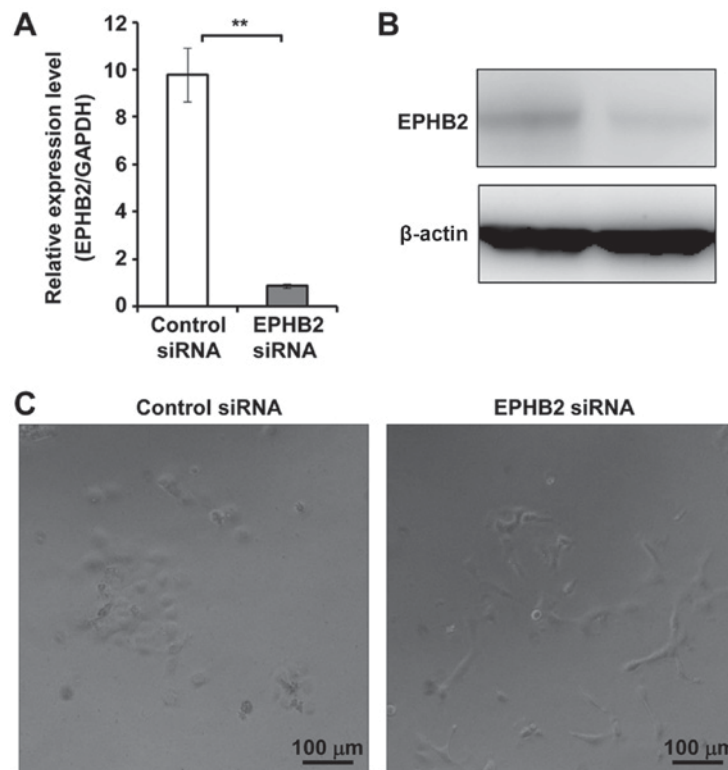


Figure 1. Knockdown of EPHB2 resulted in the epithelial-mesenchymal transition-like phenotype in A431 cells. (A and B) siRNA-mediated silencing of EPHB2. A431 cells were transfected with EPHB2 siRNA or with control siRNA. At 48 h post-transfection, total RNA and cell lysates were prepared and analyzed by (A) reverse transcription-quantitative polymerase chain reaction and (B) immunoblotting. Data are presented as the mean  $\pm$  standard deviation of triplicate experiments. \*\* $P < 0.01$ , as indicated. (C) Representative images of the cells at 48 h post-transfection (scale bars, 100  $\mu$ m). EPHB2, erythropoietin-producing hepatocellular B2; siRNA, small interfering RNA.

TWIST, 5'-ACGAGCTGGACTCCAAGATGGCAAG-3' (sense) and 5'-CATCCTCCAGACCGAGAAGGCGTAG-3' (antisense); CD44, 5'-GCAGTCAACAGTCGAAGAAGG-3' (sense) and 5'-TGTCCTCCACAGCTCCATT-3' (antisense); CD133, 5'-TTGCAATCTCCCTGTT-3' (sense) and 5'-TTGCTATCTGCCAGTT-3' (antisense); GAPDH, 5'-GCACCGTCAAGGCTGAGAAC-3' (sense) and 5'-TGGTGAAGACGCAGTGGA-3' (antisense). GAPDH was used as a reference.

**Immunoblotting.** Cells were lysed in RIPA buffer containing protease inhibitor cocktail (Nacalai Tesque). The protein concentration of lysates was measured using Bio-Rad DC kits (Bio-Rad Laboratories, Inc.). Cell lysates (20  $\mu$ g of protein) were separated by 4-12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then electroblotted onto Immobilon-P membranes (EMD Millipore, Billerica, MA, USA). Membranes were blocked with Blocking-one (Nacalai Tesque) overnight at 4°C, and incubated with goat polyclonal anti-EPHB2 antibody (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) or with mouse anti- $\beta$ -actin antibody (A5441; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 4°C. After 24 h of incubation, membranes were washed with PBS containing 0.1% Tween-20 (PBS-T), followed by incubation with horseradish peroxidase-conjugated secondary antibody for rabbit or mouse (GE Healthcare Life Science, Little Chalfont, UK), for 1 h at room temperature. The membranes were washed extensively with PBS-T, and treated with Chemi-Lumi-One Super (Nacalai Tesque) to visualize immunoreactive signals using LAS4000 (Fujifilm, Tokyo, Japan).

**Statistical analysis.** Statistical analyses were performed using Student's t-test with JMP software version 11 (SAS Institute, Inc., Cary, NC, USA). Data are presented as the mean  $\pm$  standard deviation from at least three independent experiments.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Knockdown of EPHB2 resulted in epithelial-mesenchymal transition (EMT)-like morphological changes of A431 cells.** To determine whether EPHB2 could be involved in the regulation of certain cellular process, we performed siRNA-mediated knockdown of EPHB2 in SCC-derived A431 cells. As EPHB2 mRNA and protein expression were sufficiently suppressed by transfection with EPHB2 siRNA (Fig. 1A and B), we performed phenotype analysis of cells with EPHB2 knockdown. As shown in the representative photographs, cells with EPHB2 knockdown were elongated and isolated compared to control cells showing a cobblestone-like morphology (Fig. 1C). These phenotypic changes suggested that silencing of EPHB2 induced epithelial-mesenchymal transition (EMT). Meanwhile, knockdown of EPHB2 in A431 cells had a negligible effect on cell viability (Fig. 1D), migration (Fig. 1E) and invasion (Fig. 1F).

**EPHB2 knockdown cells showed EMT-specific gene expression patterns.** As silencing of EPHB2 induced EMT-like morphological changes in A431 cells, the expression patterns

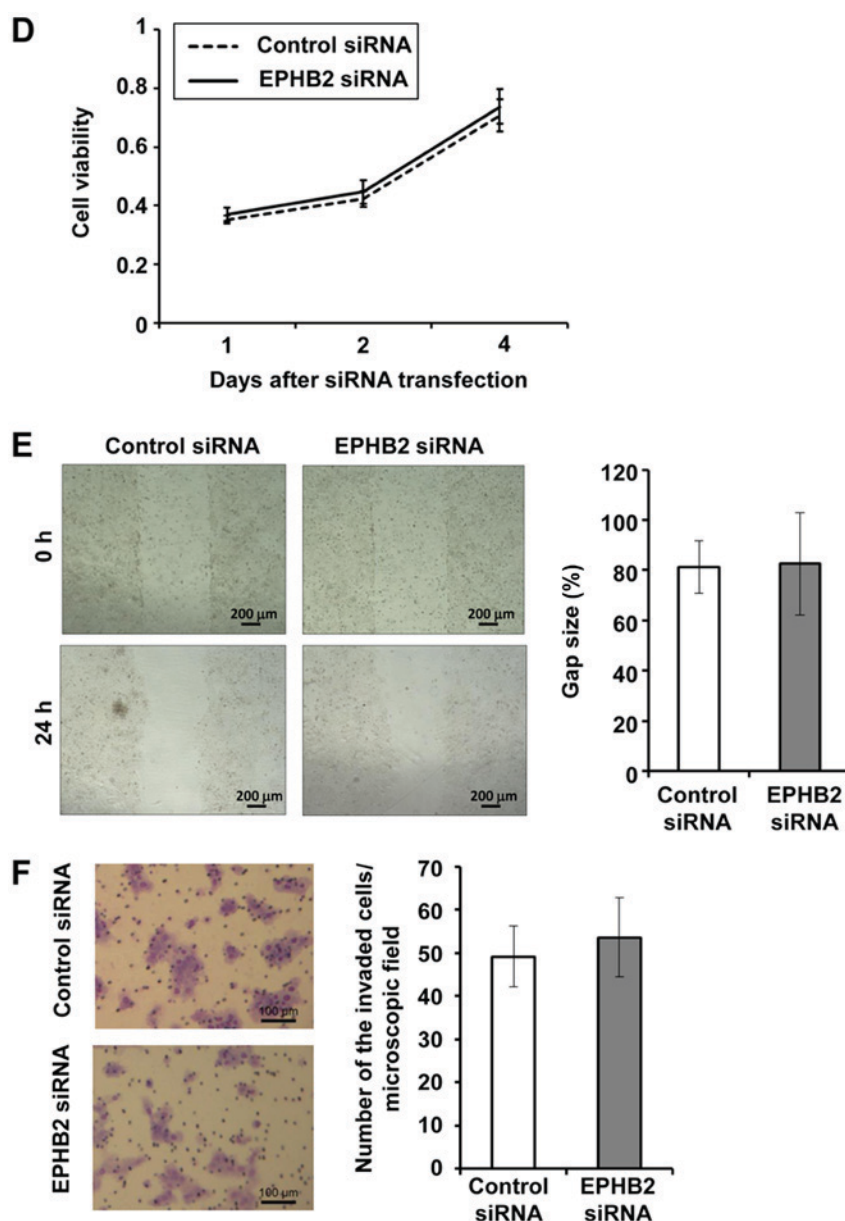


Figure 1. Continued. Knockdown of EPHB2 resulted in the epithelial-mesenchymal transition-like phenotype in A431 cells. (D) Cell viability was measured by WST8 assay at the indicated times following transfection with control siRNA (dashed line) or EPHB2 siRNA (solid line). (E) Wound-healing assay. At 48 h post-transfection with control siRNA or EPHB2 siRNA, the cell layer was scratched, and the medium was replaced with fresh medium (scale bars, 200  $\mu$ m). At 24 h post-scratching, the gap size was measured. Data are presented as the mean  $\pm$  standard deviation of triplicate experiments. Representative images of the wounded area are shown. (F) Matrigel invasion assay. Cells were seeded on invasion chambers, and immediately transfected with control siRNA or EPHB2 siRNA (scale bars, 100  $\mu$ m). Following 24 h, the invading cells were fixed and stained, and the number of stained cells was counted. The mean  $\pm$  standard deviation of 10 measurements and representative images of the invading cells are presented. EPHB2, erythropoietin-producing hepatocellular B2; siRNA, small interfering RNA.

of EMT-specific markers were examined. As shown in Fig. 2, the expression level of the epithelial marker E-cadherin (CDH1) was significantly decreased and the mesenchymal marker fibronectin (FN) was significantly increased in EPHB2 knockdown cells at 48 h after siRNA transfection. In addition, expression of transcription factors ZEB1 and ZEB2, that are EMT inducer, were significantly increased in EPHB2 knockdown cells. Expression level of another EMT inducer TWIST also tended to increase in EPHB2 silenced cells, but the difference was not significant. On the other hand, the expression of SNAI1, which is also one of the EMT inducer, was significantly suppressed in EPHB2 knockdown cells. The expression

profiles of the EMT markers, except for SNAI1, indicated that knockdown of EPHB2 induces EMT in A431 cells.

*Knockdown of EPHB2 resulted in reduced tumor sphere formation.* Accumulating evidence suggests that EMT is involved in the acquisition of cancer stem cell properties by tumor cells (20). To investigate whether silencing of EPHB2 could promote formation of cancer stem cells in A431 cells, sphere forming efficacy was analyzed. Cells transfected with EPHB2 siRNA or control siRNA were grown in suspension culture with serum-free sphere medium for 5 days, and the number of spheres >100  $\mu$ m in diameter was counted. In



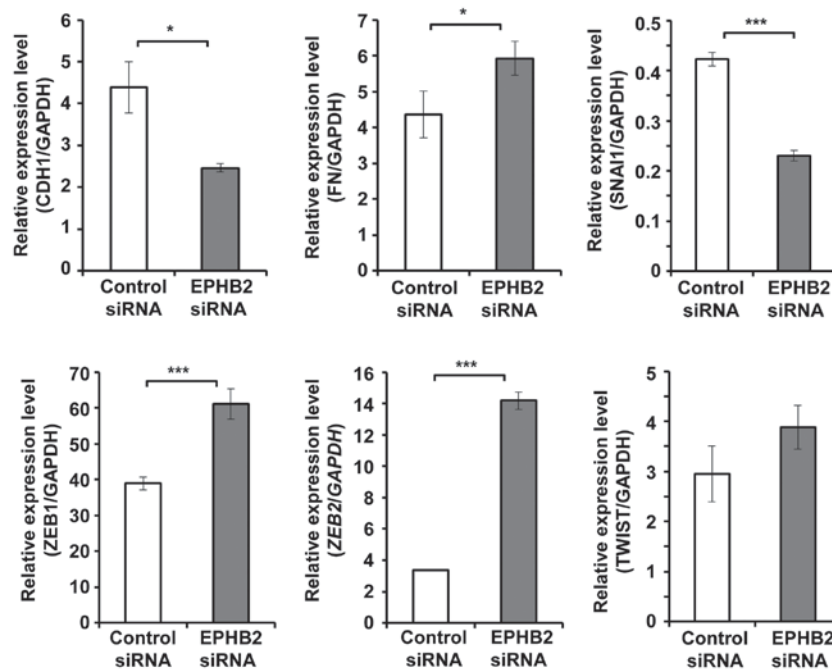


Figure 2. EPHB2-depleted cells exhibit EMT-specific gene expression patterns. A431 cells were transfected with EPHB2 or control siRNA. At 48 h post-transfection, total RNA was prepared and analyzed by reverse transcription-quantitative polymerase chain reaction to detect EMT markers. Data are presented as the mean  $\pm$  standard deviation of triplicate experiments. \* $P < 0.05$  and \*\*\* $P < 0.001$ . EPHB2, erythropoietin-producing hepatocellular B2; siRNA, small interfering RNA; EMT, epithelial-mesenchymal transition; CDH1, E-cadherin; FN, fibronectin; SNAI1, snail family transcriptional repressor 1; ZEB, zinc finger E-box binding homeobox; TWIST, Twist family basic-helix-loop-helix transcription factor 1.

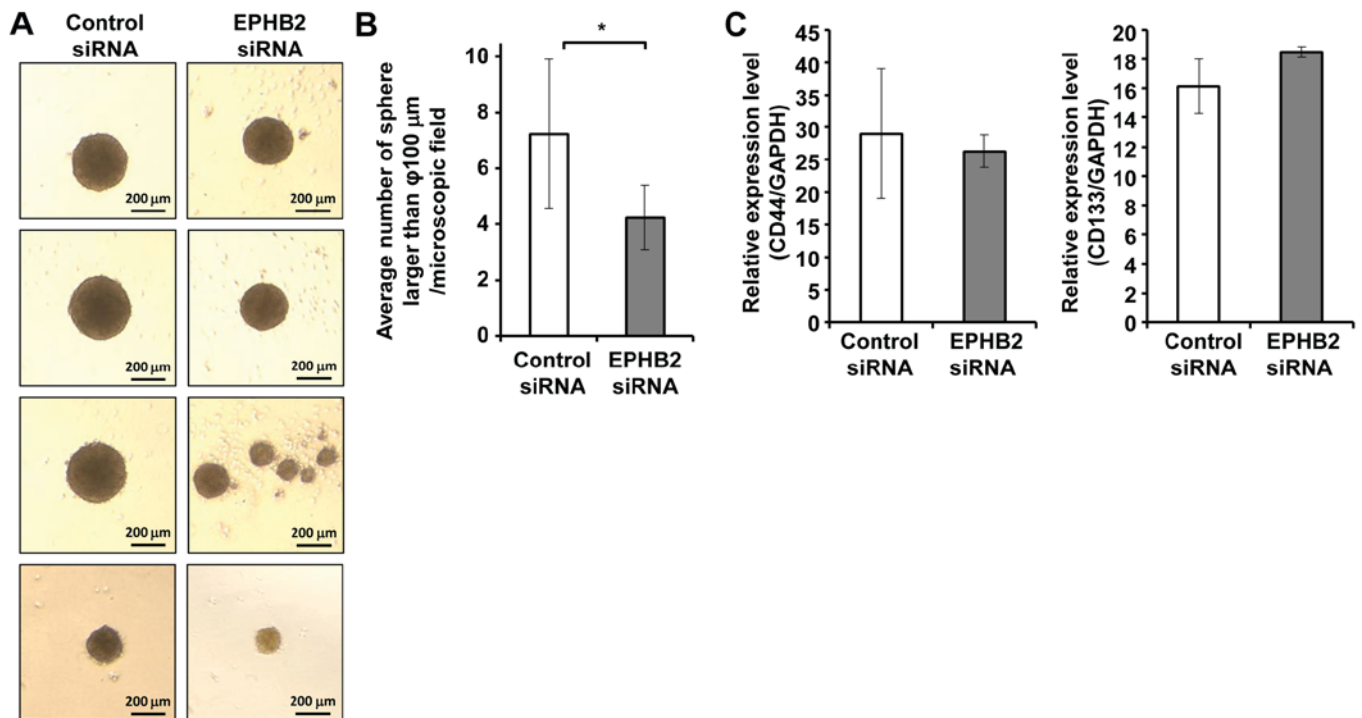


Figure 3. Knockdown of EPHB2 reduces the number of tumor spheres. Cells were plated on ultra-low attachment 24-well plates, with MEGM BulletKit serum-free medium, and transfected with control or EPHB2 siRNA. At 5 days post-transfection, spheres were photographed and the number of spheres were analyzed. (A) Representative images of spheres (scale bars, 200  $\mu$ m). (B) The number of spheres  $>100 \mu$ m in diameter. The mean values were obtained by counting the spheres in 8 microscopic fields of each group. \* $P < 0.05$ , as indicated. (C) At 48 h post-transfection, total RNA was extracted from the spheres and analyzed by reverse transcription-quantitative polymerase chain reaction. Data are presented as the mean  $\pm$  standard deviation of triplicate experiments performed. EPHB2, erythropoietin-producing hepatocellular B2; siRNA, small interfering RNA; CD, cluster of differentiation.

contrast to our prediction, the number of spheres formed in EPHB2 silenced cells was significantly lower than that

in control cells (Fig. 3A and B). On the other hand, no clear differences were observed in the expression levels of cell

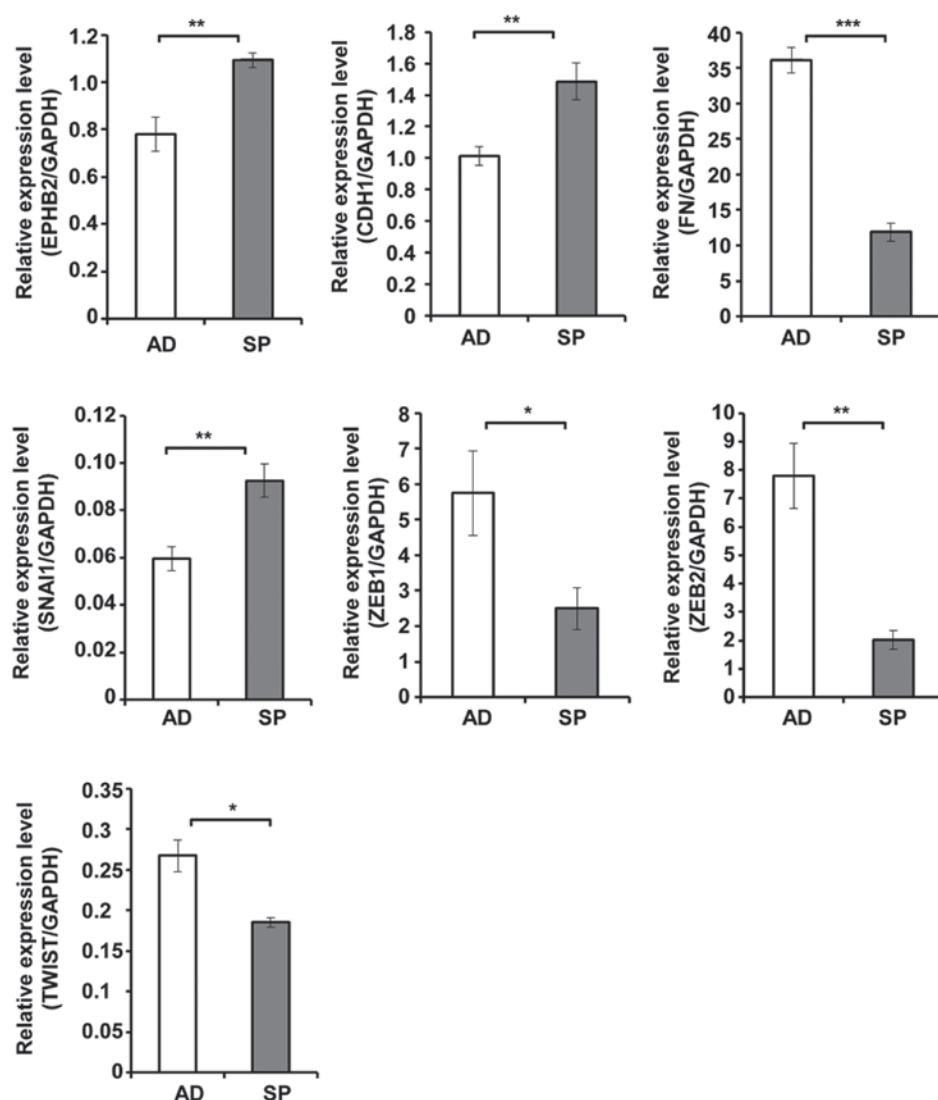


Figure 4. Spheres formed using A431 cells exhibit upregulated *EPHB2* expression and reduced epithelial-mesenchymal transition-specific gene expression. Cells were cultured under normal adherent conditions or sphere forming conditions for 5 days. Total RNA was extracted from AD and SP, and analyzed by reverse transcription-quantitative polymerase chain reaction. Data are presented as the mean  $\pm$  standard deviation of triplicate experiments. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ , as indicated. AD, adherent cells; SP, tumor spheres; *EPHB2*, erythropoietin-producing hepatocellular B2; *CDH1*, E-cadherin; *FN*, fibronectin; *SNAI1*, snail family transcriptional repressor 1; *ZEB*, zinc finger E-box binding homeobox; *TWIST*, Twist family basic-helix-loop-helix transcription factor 1.

stemness markers between *EPHB2* silenced cells and control cells (Fig. 3C).

*Tumor spheres showed increased expression of EPHB2 and reduced expression of EMT markers compared to cells cultured in adherent form.* To elucidate the roles of *EPHB2* in tumor sphere formation and EMT, the gene expression patterns of tumor spheres and adherent cells were also analyzed. As shown in Fig. 4, the expression level of *EPHB2* was significantly higher in tumor spheres compared to cells cultured in adherent form. Consistent with this observation, the expression patterns of all EMT markers except *SNAI1* indicated suppression of EMT in tumor spheres compared to adherent cells (Fig. 4).

## Discussion

The results of the present study indicated that silencing of *EPHB2* induced EMT-like morphological changes

accompanied by the up- and the down-regulation of the mesenchymal and the epithelial marker gene expression, respectively. In addition, *EPHB2* silencing resulted in reduced efficiency of tumor sphere formation. Accordingly, tumor spheres derived from A431 showed increased *EPHB2* expression level, along with decreased expression of genes characteristic of cells undergoing EMT.

As activation of EMT has been implicated in the acquisition of stem cell properties by cancer cells (20), and this makes cancer cells anoikis resistant and enables them to survive under non-adherent conditions (21,22), we expected that anchorage-independent cell growth would be enhanced in *EPHB2* knockdown cells. In contrast to our prediction, however, *EPHB2* knockdown cells formed significantly fewer tumor spheres than control cells. The lack of significant differences in expression levels of cell stemness markers between tumor spheres and adherent cells indicated that the increased number of tumor spheres in *EPHB2* knockdown cells might not be due to an increased stemness. Alternatively, it might

be attributed to their decreased cell-cell adhesion along with the promotion of EMT. This hypothesis was supported by the observations that EPHB2 expression level was increased and the expression profiles characteristic of EMT were suppressed in tumor spheres.

Previously, it has been reported that expression levels of EPHB2 were increased in both mouse cSCC (13,14) and human cSCC (14). Farshchian *et al* (14) demonstrated that depletion of *EPHB2* in cSCC cells reduces their proliferation, invasion and migration abilities in association with the down-regulation of genes implicated in these oncogenic processes. The present findings indicated that EPHB2 could promote anchorage-independent cell growth by increasing cell-cell adhesion through the suppression of EMT processes. Taken together, these findings strongly suggest that EPHB2 has oncogenic functions, promoting the growth and acquisition of malignant phenotypes in cSCC.

Among the EMT markers examined, expression pattern of SNAI1 was opposite to the other markers in EPHB2 knockdown cells and spheres. Similar to ZEB1/2 and TWIST, it has been well-known that SNAI1 contributes to the induction of EMT mainly through the repression of CDH1 transcription. However, the regulatory mechanisms behind the transcription of these EMT inducers are dependent on cellular context and signaling. For example, it has been reported that Insulin-like growth factor receptor signals induces SNAI1 and ZEB1 via NF- $\kappa$ B activation, whereas this signal augments Twist and ZEB1 via Ras/Raf/ERK signal (23). Under our experimental conditions, therefore, knockdown of EPHB2 might potentially activate certain signal pathway(s) linked to the transcriptional activation of ZEB1/2 and Twist, but to SNAI1.

In contrast to our current findings, it has been reported that EPHB2 activates EMT in cervical cancer cells (6) and breast cancer cells (24). In breast cancer cells, activation of wild-type p53 inhibited basal and TGF- $\beta$ -induced EMT by reducing the expression level of EPHB2 (24). This discrepancy between our results and those reported previously may be due to the variety of mutations in the molecules involved in EMT processes. Indeed, A431 cells carry a missense mutation in p53 gene at exon 8 (25). As observations in breast cancer indicated that there is cross-talk among EPHB2, TGF- $\beta$ , and p53 (24), the mutated p53 may affect the roles of EPHB2 in induction of EMT in A431 cells. In addition, although EMT has been implicated in promotion of invasion and migration activity of tumor cells, the present results indicated that silencing of EPHB2 induced EMT-like phenotypes but did not affect the invasion and migration abilities of the cells. This result may also have been due to genetic variation of genes related to cell functions, including EMT, among cells. To adequately address this issue, further analyses using many types of cell lines harboring different types of genetic variations are required.

In conclusion, the present study showed that knockdown of EPHB2 induces EMT-like phenotypes in A431 cells, and that spheres formed by A431 showed increased expression of EPHB2 and suppressed gene expression patterns characteristic of cells undergoing EMT. These findings indicated that EPHB2 is involved at least partially in suppression of EMT processes in A431 cells.

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## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contribution

KF and HN conceived and designed the study. KF, YI, TT, MY, DW, JH and TO performed the experiments. NF performed the statistical analysis. MS made substantial contributions to the analysis and interpretation of data. KF and MS wrote the paper. TO, HN, MS and NF critically revised the paper for important intellectual content and gave final approval of the paper to be published. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

Not applicable.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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