Supplementary Materials and Methods

Antibodies and reagents. Mouse anti-human HB-EGF monoclonal antibody (MAB259), goat anti-human HB-EGF polyclonal neutralizing antibody (AF-259), goat anti-human PDGFRβ polyclonal antibody (AF-385) and PDGF-DD were purchased from R&D Systems (Minneapolis, MN, USA). Anti-phosphotyrosine antibody (4G10) was purchased from Upstate Technology (Lake Placid, NY, USA). Anti-EGFR antibody (1005) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). PDGF-AA, PDGF-AB, PDGF-BB and PDGF-CC were purchased from PeproTech (Rocky Hill, NJ, USA). Rabbit anti-human PDGF-BB polyclonal antibody (ab9704) was purchased from Abcam (Cambridge, UK). Anti-actin monoclonal antibody (MAB1501) was purchased from Millipore (Billerica, MA, USA). Anti-EGFR antibody (Ab-1), Kinase inhibitors (AG1295, PD173074, AGL2263 and TGFβ-RI kinase inhibitor II (Cat. #616452)) were purchased from Calbiochem (Darmstadt, Germany). Imatinib mesylate was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). CRM197 was prepared as described (1).

Immunohistochemistry. Tissues were obtained from patients with invasive cervical
cancers, SILs and normal cervices. The clinicopathological features of the patients and samples are shown in Supplemental Tables 1 and 2. Frozen tissues embedded in Tissue-Tek OCT Compound (Sakura Finetechanical, Tokyo, Japan) were sectioned serially at 4 μm and fixed with cold acetone for 10 min. Endogenous peroxidase activity was blocked with 0.03% hydrogen peroxide in methanol for 10 min, followed by treatment with a blocking agent (Block Ace Powder; Yukijirushi, Sapporo, Japan) according to the manufacturer’s protocol. Sections were incubated with 1:100-diluted anti-human HB-EGF mouse monoclonal antibody for 24 h at 4°C, and then treated with biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA) at room temperature for 30 min. Subsequently, the sections were incubated with the Vectastain Elite ABC Reagent (Vector Laboratories) and positive reactions were visualized using DAB-Buffer tablets (Merck, Tokyo, Japan). For negative controls, the primary antibody was omitted. All sections were counterstained with H&E. For mouse xenograft tumors, tissues were fixed with 4% paraformaldehyde for 12 h, embedded in OCT Compound and stained with the goat anti-human HB-EGF polyclonal antibody.

**Laser microdissection.** Frozen tissues embedded in OCT compound were sectioned
serially at 8 μm, fixed with ethanol/acetic acid at 19:1 (v/v) for 3 min, and washed with RNase-free water for 1 min. The tissues were treated with 0.05% toluidine blue solution, followed by two washes with RNase-free water. The tissues were air dried and dissected to divide the region of cancer epithelium from that of the stroma using a laser microdissection system (AS LMD; Leica, Wetzlar, Germany).

**RNA extraction and qRT-PCR.** RNA from cultured cells or microdissected tissue samples was prepared using an RNeasy RNA Purification Kit (Qiagen, Valencia, CA, USA) and cDNA was prepared using a ReverTra Ace-α Reverse Transcription Kit (Toyobo, Tokyo, Japan). mRNAs of HB-EGF and other EGF family ligands were measured by qRT-PCR using TaqMan-labeled specific probes for each ligand (Applied Biosystems, Foster City, CA, USA) and an ABI Prism 7900 Sequence Detection System (Applied Biosystems). Data were normalized by the expression level of GAPDH for each sample, and mRNA quantities of HB-EGF and other EGF family ligands are indicated relative to the quantity of HB-EGF mRNA expressed in HeLa cells cultured to confluence in DMEM supplemented with 10% FBS.

**Retrovirus vectors and cDNAs.** The retrovirus vectors pCX4bsr, pCX4pur and
pCX4zeo were described previously (2). pCX4bsr/SV40 T and pCX4hyg/mCAT (mouse cationic amino acid transporter) were provided by Dr. Tsuyoshi Akagi (KAN Research Institute, Kobe, Japan). A cDNA encoding hTERT provided by Dr. Norimitsu Inoue (Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan) was subcloned into pCX4pur. A cDNA for human EGFR was described previously (3). A cDNA for human PDGFRβ was provided by Dr. Koutaro Yokote (Chiba University, Chiba, Japan). Dominant-negative mutants of EGFR (dnEGFR) and PDGFRβ (dnPDGFRβ) were constructed by deleting the cytoplasmic domains of EGFR (amino acids 654–1187) and PDGFRβ (amino acids 603–1106), respectively, as described previously (4, 5). These mutants were cloned into pCX4zeo.

**Primary culture of CCFs.** Pieces of surgically removed cancerous tissues were washed 10 times with ice-cold PBS and cut into fragments measuring about 2 × 5 × 5 mm under sterile conditions. These fragments were incubated in 0.05% trypsin solution at 37°C for 30 min, followed by culture in DMEM supplemented with 20% FBS and antibiotics. After several days of culture, fibroblast colonies were picked out and isolated fibroblasts were collected.
Immortalization of CCF1 cells. CCF1 cells were transiently transfected with a cDNA for mCAT, the ecotropic retrovirus receptor, by electroporation using a Gene Pulser II (Bio-Rad, Hercules, CA, USA), and then infected with retroviruses encoding SV40 T and hTERT at 2 days after transfection. The retrovirus infection was performed as described previously (2). Cells were selected with medium containing blasticidin S (10 μg/mL) and puromycin (2 μg/mL) for 1 week. The immortalized cells were designated CCF-TT cells.

Establishment of human cells susceptible to ecotropic retrovirus infection. ME180 and CCF-TT cells were transiently transfected with a cDNA for mCAT using the FuGENE6 transfection reagent (Roche, Mannheim, Germany), and then infected with a retrovirus encoding mCAT at 2 days after transfection. Cells were selected with medium containing hygromycin (200 μg/mL) for 1 week. The established cells, designated ME180eco and CCF-TTeco cells, were infected with a retrovirus encoding dnEGFR or dnPDGFRβ, and then selected with medium containing zeocin (100 μg/mL) for 1 week.

2D agar culture. 2D agar cultures were carried out as described previously (2) with some modifications. Briefly, cells were cultured on top of semisolid medium containing 0.5% agarose and 5% FCS and overlaid with DMEM containing 5% FCS in the presence of
various concentrations of recombinant HB-EGF for 1 week.

**Fluorescence cell marking.** Green fluorescent protein (GFP)-labeled ME180 (ME180/GFP) cells were prepared by transfection of ME180 cells with a GFP expression vector using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Ds-Red-labeled MEF-HB^+/+ (MEF-HB^+/+Red) and Ds-Red-labeled MEF-HB^-/- (MEF-HB^-/-Red) cells were prepared by infection with a retrovirus vector as described (6).

**Fluorescence optical whole-body imaging.** Measurement of the tumor size by the fluorescence of GFP/ME180 cells was determined by fluorescence optical whole-body imaging as described (7, 8).

**Immunoprecipitation.** Cell lysates were incubated with an anti-EGFR antibody (Ab-1) or anti-PDGFRβ antibody together with protein G-Sepharose (GE Healthcare, Buckinghamshire, UK) for 3 h at 4°C. The immunoprecipitated samples were washed three times with lysis buffer and probed with an anti-EGFR antibody (1005), anti-PDGFRβ antibody or anti-phosphotyrosine antibody.

**Western blot analysis.** Whole cell lysates were used for western blot analysis as
described (2).
References


**Supplementary Table S1.** Characteristics and cancer scores of the 14 patients with cervical cancer. The histology, clinical staging and scoring of HB-EGF immunostaining in the cancer epithelium or stroma are shown. HB-EGF immunostaining was scored according to the intensity of staining: 0, 1+, 2+ and 3+. The relative area of HB-EGF immunostaining was scored into four categories: 1 (0–25%), 2 (26–50%), 3 (51–75%) and 4 (76–100%). The sum of the intensity and percentage scores was used as the total staining score.

**Supplementary Table S2.** Characteristics and cancer scores of six patients with precancerous lesions and normal cases. The histology and scoring of immunostaining in the epithelium or stroma are shown, as described in Supplementary Table S1.
Supplementary Figure Legends

Supplementary Figure S1. H&E and immunohistochemical staining of uterine cervical adenocarcinoma tissue sections and uterine cervical small cell carcinoma tissue sections.

a, b, Adenocarcinoma; c, d, small cell carcinoma of the uterine cervix. Stromal cells contacting the cancerous epithelial cells were strongly stained (arrows)

Supplementary Figure S2. CaSki cell growth in collagen gel was enhanced by coculture with CCF1 cells, and CRM197 (500 ng/mL) blocked the enhanced growth of CaSki cells cocultured with CCF1 cells. **P < 0.01 and ***P < 0.005.

Supplementary Figure S3. Promotion of ME180 cell growth by recombinant HB-EGF in the absence of CCF1 cells. ME180 cells alone (2 × 10³) were cultured in 2D agar with the medium containing the indicated concentrations of recombinant HB-EGF (rHB-EGF) for 1 week as described in the Supplementary Materials and Methods. **P < 0.01 and ***P < 0.005.

Supplementary Figure S4. A, Expression of HB-EGF receptors and PDGF-BB receptor. The expression of HB-EGF receptors (EGFR and ErbB4) and PDGF-BB receptor (PDGFRβ) was detected by western blotting. β-actin was used as an internal
loading control. MCF-7, a breast cancer cell line, was used as a positive control for ErbB4 expression. B, Activation of EGFR by coculture. EGFR immunoprecipitates from ME180 cells cultured with or without CCF1 were probed with an anti-EGFR antibody or anti-phosphotyrosine antibody. β-actin was used as an internal loading control.

Supplementary Figure S5. HB-EGF is specifically induced by CM from cervical cancer cell lines. CCF1 cells were cultured with CM from various cervical cancer cell lines for 6 h, and EGFR ligand mRNA levels were measured by real-time qRT-PCR. CM from CCF1 cells was used as a control. EGFR ligand mRNA levels shown in ordinate are relative to the quantity of HB-EGF mRNA expressed in HeLa cells as described in the Supplementary Materials and Methods. The bars represent the mean ± SD for triplicate determinations.

Supplementary Figure S6. Activation of PDGFRβ by coculture. PDGFRβ immunoprecipitates from CCF1 cells cultured with or without ME180 cells were probed with an anti-PDGFRβ antibody or anti-phosphotyrosine antibody. Full-length and degraded (Deg.) PDGFRβ are indicated by arrowheads and brackets, respectively.
β-actin was used as an internal loading control.