Supporting Information

Grugan et al. 10.1073/pnas.0914295107

SI Methods

Reagents. Unless otherwise noted, all chemicals and common reagents were purchased from Sigma or Thermo Fisher Scientific. Antibodies were obtained from the following sources: EGFR (Ab-12) were from NeoMarkers/Thermo Fisher Scientific, phospho-EGFR (Tyr1068) and phospho-Met (Tyr1234/1235) were from Cell Signaling Technology, Met antibody (C-28) was from Santa Cruz Biotechnology, p53 (Ab-6) was from Calbiochem, E-cadherin was from BD Biosciences, α-smooth muscle actin (IA4) was from Sigma, and GAPDH was from Chemicon/Millipore. Anti-mouse and anti-rabbit horseradish peroxidase (HRP)-conjugated antibodies were purchased from GE Healthcare Life Sciences. Recombinant human HGF was purchased from R&D Systems. PHA665752 was obtained from Tocris Bioscience. 2B8 (mouse anti-human HGF IgG1 generated from hybridoma) was obtained from AVEO Pharmaceuticals. Mouse IgG1 control antibody was obtained from R&D Systems.

Cell Culture. Primary human esophageal keratinocytes, designated EPC, are described in refs. 1 and 2. The isolation and characterization of the fibroblasts used in this study is described in ref. 1. The TT, HCE4, and TE series cells are all ESCC cell lines and are described in refs. 3 and 4.

Western Blot Analysis. For Western blot analysis, cells were harvested in lysis buffer [50 mM Tris·HCl (pH 8), 150 mM NaCl, 1% Nonidet P-40, 1% Triton X-100, 2 mM sodium orthovanadate, 10 mM sodium fluoride, 5 mM sodium pyrophosphate, protease inhibitor tablet (Roche)]. Thirty micrograms of protein were run on a 4–12% SDS/PAGE Bis-Tris gel (Invitrogen) and transferred to a poly(vinylidene difluoride) membrane (Immobilon-P; Millipore). Membranes were blocked in 5% nonfat milk (Bio-Rad Life Science) in PBS-T [1× PBS without Ca^{+2} and Mg^{+2} (Invitrogen) and 0.1% Tween 20] for 1 h at room temperature. Membranes were then probed with primary antibody diluted in 5% milk in PBS-T overnight at 4 °C, washed with PBS-T, and incubated with anti-mouse or anti-rabbit secondary antibodies (1:5,000 in PBS-T) for 1 h at room temperature and washed in PBS-T. The signal was visualized using an enhanced chemiluminescence solution (ECL Plus; GE Healthcare Life Sciences) and exposed to Blue Lite Autorad film (ISC-BioExpress).

Invasion Assays. For invasion assays, insert plates (8-μm pore size, 24-well insert) coated with growth factor reduced Matrigel matrix were used (BD Biosciences). Inserts were placed in a 24-well plate containing DMEM + 10% serum to stimulate cell invasion. In some cases, 10 ng/mL HGF was added to the chemoattractant. 1×10^5 cells in serum-free medium were placed in each insert. Twenty hours later, the cells remaining inside the insert were removed with a cotton swab and the invading cells on the insert bottom were labeled with 4 μg/mL Calcein AM dye (Invitrogen) in Hanks's Balanced salt solution (HBSS) (Invitrogen) for 30 min at 37 °C. The labeled cells were then read on a Biotek FLX800 multidetection microplate reader (BioTek) at 485 nm excitation and 528 nm detection. All experiments were performed in triplicate on three independent days. Error bars represent ± SEM, and Student's t test was used to determine significance (*, $P \leq 0.05$).

Immunohistochemistry. For tumor microarray (TMA) analysis, 73 paired paraffin blocks representing tumors containing esophageal squamous cell cancer as well as squamous dysplasia and the corresponding adjacent normal mucosa were provided us by Michiyuki Kanai (Kitano Hospital, Osaka, Japan). All of the clinical materials were procured via surgery from informed-consented Japanese patients (62 male, 13 female) in accordance with Institutional Review Board standards and guidelines. We constructed four TMAs of paired normal esophageal mucosa and tumor (SCC) from 73 patients (totaling 292 cores, each patient was represented by 4 cores, 2 normal mucosa, and 2 SCC cores). Antibody staining was done according to standard protocols described in ref. 1. To score the Met staining of tumor microarrays, the slides were evaluated by an expert pathologist using the following scale: 0.5 = marginal, 1= moderate, 2 = intense staining.

1. Okawa T, et al. (2007) The functional interplay between EGFR overexpression, hTERT activation, and p53 mutation in esophageal epithelial cells with activation of stromal fibroblasts induces tumor development, invasion, and differentiation. *Genes Dev* 21:2788–2803.
2. Andl CD, et al. (2003) Epidermal growth factor receptor mediates increased cell proliferation, migration, and aggregation in esophageal keratinocytes in vitro and in vivo. *J Biol Chem* 278:1824–1830.
3. Mizushima T, et al. (2002) Wnt-1 but not epidermal growth factor induces β-catenin/T-cell factor-dependent transcription in esophageal cancer cells. *Cancer Res* 62:277–282.
4. Okano J, Gaslightwala I, Birnbaum MJ, Rustgi AK, Nakagawa H (2000) Akt/protein kinase B isoforms are differentially regulated by epidermal growth factor stimulation. *J Biol Chem* 275:30934–30942.

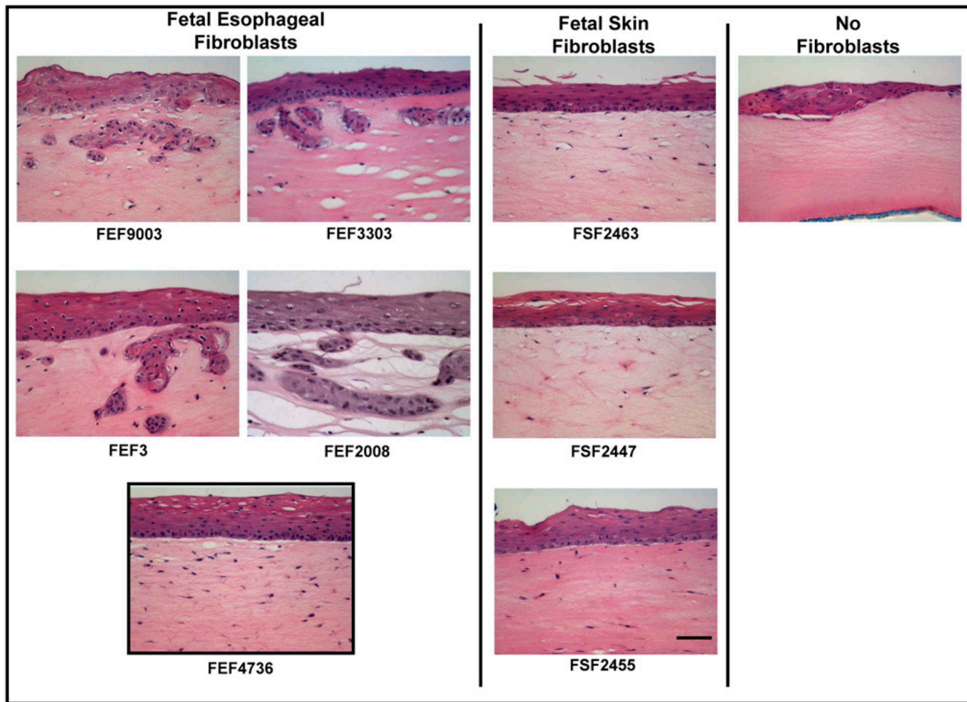

Fig. S1. Invasion of genetically transformed esophageal epithelial cells occurs into matrices of fetal esophageal fibroblasts. H&E-stained sections of organotypic culture of EPC-hTERT-EGFR-p53^{R175H} seeded above matrices containing the indicated fibroblast sample or no fibroblasts at all. (Scale bar: 100 μm.)

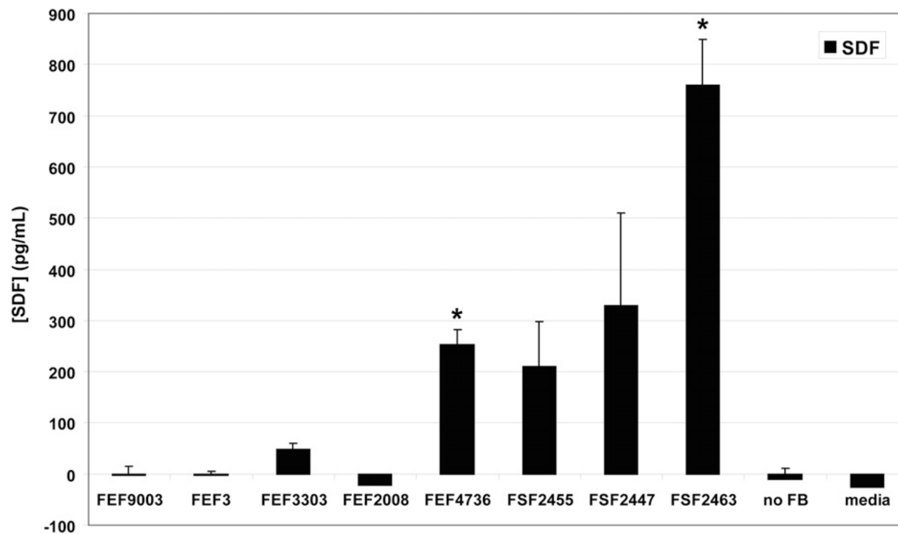

Fig. S2. SDF-1 is secreted by fibroblasts that are not permissive to invasion of transformed esophageal epithelial cells. Level of human SDF-1 detected in conditioned media collected from the indicated fibroblast sample set up in organotypic culture and measured by ELISA. Error bars represent ± SEM. *, $P \leq 0.05$ compared with no fibroblast sample.

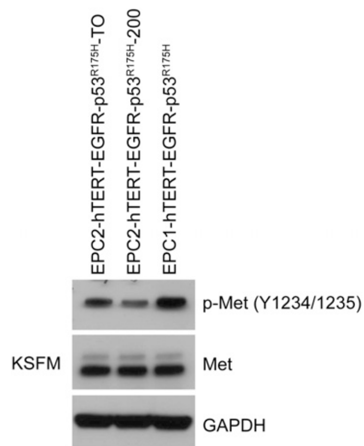

Fig. S3. Increased Met activation in three independent EPC-hTERT-EGFR-p53^{R175H} cell lines. Levels and phosphorylation status of Met and level of GAPDH were measured by Western blotting in whole-cell lysates of EPC-hTERT-EGFR-p53^{R175H}-TO (main cell line used in studies), EPC-hTERT-EGFR-p53^{R175H}-200 (independently generated cell line of same genotype and parental cell), and EPC1-hTERT-EGFR-p53^{R175H} (different parental esophageal primary cell with identical genotype as main cell line used).