1. Supplementary Material

1.1 Supplementary Methods

1.1.1. Reagents and plasmids

ALK5 inhibitors, EW-7197, SB-505124, IN-1130 and LY-2157299 were synthesized by Dr. Dae-Kee Kim (Ewha Womans University, Seoul, Korea). Recombinant human TGF-β1 was purchased from R&D Systems.

1.1.2. Protein Kinase Assay

A radiometric protein kinase assay (^{33}PanQinase® Activity Assay) was used for measuring the kinase activity of TGFB-R1 in ProQinase (Freiburg, Germany) (Park et al., 2011b). All kinase assays were performed in 96-well FlashPlates™ from Perkin Elmer (Boston, MA, USA) in 50 μl reaction volume. The reaction cocktail contained ATP solution, assay buffer, test sample, and enzyme/substrate mixture. Appropriate buffer, enzymes, and substrates for each kinase were used according to manufactures. All assays were performed with a BeckmanCoulter Biomek 2000/SL robotic system. Incorporation of ^{33}Pi (counting of "cpm") was determined with a microplate scintillation counter (Microbeta, Wallac). The inhibitory activity of EW-7197 (10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} mol/L) was calculated as "residual kinase activity". The median value of the cpm at full activity of a protein kinase in the absence of any inhibitor was taken as "high control". And the median value of the cpm in the absence of a protein kinase but presence of the substrate defined as "low control". The difference between high and low control of each kinase was taken as 100% activity.

Residual kinase activity (%) = 100 X [(cpm of compound – low control) / (high control-low control)]

1.1.3. Cytotoxicity assay

Cells were seeded in 96 well plate and treated with indicated concentrations of EW-7197 in 0.2% HI-FBS medium for 72 h. Cells were dried after incubation with 10% TCA in media. Then, cells were incubated with 0.4% SRB (Sulforhodamine B) in 1% acetic acid for 30 min. After washing with 1% glacial acetic acid, bounded dye was released in 10 mM Tris buffer (pH 10.5) for 30 min. Absorbance was measured at 570nm.

1.1.4. H&E staining and Immunohistochemistry

Formalin-fixed and paraffin-embedded sections of primary tumors were dewaxed in OTTIX bath (Diapath, Martinengo, Italy) and stained with hematoxylin (Sigma Aldrich, St. Louis, Missouri, USA) and eosin (Diapath, Martinengo, Italy) according to the manufacturer's instruction. Images were captured with phase-contrast microscopy (Carl Zeiss, Oberkochen, Germany). For immunohistochemistry, dewaxed slides were incubated with blocking solution (TBST/Tween-20 including 3% bovine serum albumin). After washing with TBST/tween-20, slides were stained with PE-anti-mouse Cd8α (CD8 antigen, alpha chain) (BD Transduction Laboratories, San Diego, CA) and nuclei were counterstained with DAPI. Sections were mounted under glass coverslips using VectaMount (Vector Laboratoris, Burlingame, CA). Fluorescence was visualized and analyzed by LSM 510 META laser confocal microscopy system (Carl Zeiss, Oberkochen, Germany). Cd8α-positive area was calculated as % of field with Image J program.

1.2 Supplementary figure legend

1.2.1 Supplementary Figure 1.

(A) Blockade of Smad2 phosphorylation by various ALK5 inhibitors. 4T1 cells starved in 0.2% HI-FBS medium for 24 h, and were treated with the indicated chemicals for 2 h with or without TGF-β1 (2 ng/ml) in 0.2% HI-FBS medium. (B, C) Blockade of Smad2 phosphorylation by EW-7197. NMUMG (B) and MDA-MB-231 (C) cells were incubated with 0.2% HI-FBS medium for 24 h, and treated with TGF-β1 (2 ng/ml) in the presence or absence of ALK5 inhibitors in 0.2% HI-FBS medium for 2 h. Lysates from the cells were analyzed by Western blotting. EW, SB, LY, IN indicate EW-7197, SB-505124, LY-2157299, and IN-1130, respectively.

1.2.2 Supplementary Figure 2.

(A) Representative images of wound healing assay of MDA-MB-231 cells. Right after the wound was made on the MDA-MB-231 cell monolayer, the cells were treated with TGF-β1 (2 ng/ml) with or without ALK5 inhibitors for 53 h. Total magnification was × 100. Scale bar indicates 100 μm. Veh, SB, and EW indicate vehicle, SB-505124, and EW-7197, respectively. (B-C) Effects of EW-7197 on cell cytotoxicity (described in the Supplementary Methods). 4T1 (B) and MCF10A (C) cells were treated with indicated concentrations of EW-7197 in 0.2% HI-FBS medium for 72 h. The cell viability was determined by SRB (Sulforhodamine B) assay (described in the Supplementary Methods). Data represent the mean ± SD (n=3).

1.2.3. Supplementary Figure 3.

Inhibitory effects of various ALK5 inhibitors on TGFβ1-induced EMT in MCF10A cells. MCF10A cells were treated with TGFβ1 (2 ng/ml) for 96 h in serum-reduced

media (1% heat inactivated-horse serum (HI-HS)) in the presence or absence of the indicated ALK5 inhibitors. Medium was replaced every other day. Cell morphology was observed by phase-contrast microscopy (total magnification: × 100, scale bar: 100 μm).

1.2.4. Supplementary Figure 4.

Inhibitory effects of various ALK5 inhibitors on TGFβ1-induced EMT in MCF10A cells. MCF10A cells were treated with TGFβ1 (2 ng/ml) for 96 h in serum-reduced media (1% HI-HS) in the presence or absence of the indicated ALK5 inhibitors. Medium was replaced every other day. The protein levels of E-CADHERIN and N-CADHERIN were analyzed by western blotting as described in the Material and Methods. β-ACTIN was used as endogenous control.

1.2.5. Supplementary Figure 5.

(A~C) Breast cancer model #1, described in the Material and Methods. (A) Effect of EW-7197 on lung metastasis of breast cancer in MMTV-c/Neu mice. Representative images of H&E stained lungs (total magnification: × 12.5 or 100, scale bar: 800 μm or 100 μm). Total tumor volume (B) and body weight (C) in MMTV/c-Neu mice. Data represent the mean ± SE (Veh: n=7, EW: n=10). (D and E) Body weight in breast cancer model #2 (D) and in breast cancer model #3 (E) (described in the Material and Methods). Data represent the mean ± SE (n=10/group in Model #2 or n=6~8/group in Model #3). Veh, LY and EW indicate artificial gastric fluid, LY-2157299 and EW-7197, respectively. (F) In breast cancer model #3 (described in the

Material and Methods), on day 28, two mice of each group were selected and treated with the indicated concentration of EW-7197. One mouse from each group was injected with TGF-β1 (50 ng/mouse) through *i.v.* after 30 min and another was not injected. 90 min after TGF-β1 injection, mice were sacrificed and lysates from primary mammary tumors were analyzed by western blotting as described in the Material and Methods.

1.2.6. Supplementary Figure 6.

(A, B) Breast cancer model #6 (described in the Material and Methods). (A) Inhibition of lung metastasis by EW-7197 was evaluated by analysis of luciferase activity in lungs of 4T1-luc orthotropic xenograft mice. Statistical significance was defined using one-way ANOVA with Dunnett's multiple comparison test. * indicates significance at $p<0.05$. (B) Effect of EW-7197 on primary tumor size. Data represent the mean ± SE (n=15/group). Veh and EW indicate artificial gastric fluid and EW-7197, respectively.

1.3. Supplementary Table

1.3.2 Supplementary Table 1. Antibodies for Western blot analysis.

Primary Antibody	Secondary Antibody
Anti-phospho-Smad2 (Millipore, Bedford, MA) Anti-phospho-Smad3 (Cell Signaling, Beverly, MA, USA) Anti-smad3 (AbFrontier, Seoul, Korea) Anti-Smad2/3 (BD Bioscience, San Diego, CA)	HRP-conjugated goat anti-mouse antibody (Santa Cruz Biotechnology, Santa Cruz, CA)
Anti-E-cadherin (BD Bioscience, San Diego, CA) Anti-N-cadherin (BD Bioscience, San Diego, CA)	HRP-conjugated goat anti-rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA)
Anti-Fibronectin (BD Bioscience, San Diego, CA) Anti-β-actin (Sigma Aldrich, St. Louis, Missouri, USA) Anti-Snail (Cell Signaling, Beverly, MA, USA) Anti-Vimentin (BD Bioscience, San Diego, CA)	HRP-conjugated goat anti-rat antibody (Cell Signaling, Beverly, MA, USA)

1.3.2 Supplementary Table 2. Primers for qRT-PCR or RT-PCR

qRT-PCR	Forward	Reverse
Mouse		
Cd8b1	GAATGTGAAGCCAGAGGACAGTG	GGGCAGTTGTAGGAAGGACATC
Gzmb	CAGGAGAAGACCCAGCAAGTCA	CTCACAGCTCTAGTCCTCTTGG
Prf1	ACACAGTAGAGTGTCGCATGTAC	GTGGAGCTGTTAAAGTTGCGGG
Ppia	TGGAGAGCACCAAGACAGACA	TACTAGGCAGATGGCCACAGG

RT-PCR	Forward	Reverse
Human		
CDH1	TCCATTTCTTGGTCTACGCC	CACCTTCAGCCATCCTGTTT
FN1	GGACTTCTTATGTGGTCGGA	GTTGGTAAACAGCTGCACGA
GAPDH	ACATCGCTCAGACACCATGG	GTAGTTGAGGTCAATGAAGGG
GAPDH	For detection of lung metastasis CCAGGGCTGCTTTTAACTCTGGTA	AGCATCGCCCCACTTGATTTTGGA
HMGA2	CAGCCCTATCACCTCATCTC	CCATTTCCTAGGTCTGCCTC
SNAI1	ACCTTCCAGCAGCCCTACGACC	GTGTGGCTTCGGATGTGCATC
SNAI2	CTGGTCAAGAAGCATTTCAACGCC	AAAGAGGAGAGAGGCCATTGGGTA
ACTB	AGCCATGTACGTTGCTATCCAG	CTTCTCCTTAATGTCACGCACG
Mouse		
Csn2	ACAGCTGCAGGCAGAGGAT	GAATGTTGTGGAGTGGCAGG
Gapdh	ATGTGTCCGTCGTGGATCTGA	TTGAAGTCGCAGGAGACAACC

1.3.3 Supplementary Table 3. Radiometric protein kinase assay

Concentration of EW-7197	10^{-8} mol/L	10^{-7} mol/L	10^{-6} mol/L	10^{-5} mol/L
Target	Residual kinase activity (%)			
TGFB-R1	35	4	1	0
ACV-R1	104	101	83	29

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