Supplemental Data

Murine protein serine/threonine kinase 38 stimulates TGF-β signaling in a kinase-dependent manner via direct phosphorylation of Smad proteins

Hyun-A Seong, Haiyoung Jung, and Hyunjung Ha

Generation of an Inducible MPK38(KD) Cell Line—An inducible MPK38 shRNA HaCaT cell line [MPK38(KD)] was generated using the following oligonucleotides as described previously (25). The double-stranded oligonucleotides (forward, 5'-TCGAGGCAGGCAGACAATGGAGGATTTCAAGAGAATCCTCCATTGTCTGCCTGCTTTTTTA-3'; reverse, 5'-AGCTTAAAAAAGCAGGCAGACAATGGAGGATTCTCTTGAAATCCTCCATTGTCTGCCTGCC-3'; MPK38 sequence underlined) were cloned into the pSingle-tTS-shRNA vector (Clontech). HaCaT cells were transfected with pSingle-tTS-shRNA harboring MPK38-specific shRNA or pSingle-tTS-shRNA empty vector using WelFect-Ex^{TM} Plus (WelGENE, Daegu, Korea). Inducible MPK38(KD) stable clones were screened in the presence of 480 μg/ml G418 for 14 d until all control parental HaCaT cells had died. To confirm the knockdown of endogenous MPK38, stable clones were isolated, treated with 1 μg/ml doxycycline (Sigma, St. Louis, MO), a tetracycline analogue, for 72 h, and subjected to Western blotting by anti-MPK38 antibody.

Construction of Smad6 Mutants—Smad6 mutants were generated by PCR as described previously (25). In brief, wild-type human Smad6 was used as the template for amplification with either the Smad6 forward 5'-GATGGATCCATGTTCAGGTCCAAACGCTCGG-3' or reverse 5'-CTACTCGAGCTATCTGGGGTTGTTGAGGAGG-3' primer, in conjunction with one of the following mutant primers containing alterations in the nucleotide sequence of Smad6: for S364A, forward 5'-GACCTACCTCAGGGCGCCGGCTTCTGCCTGGGC-3', reverse 5'-GCCCAGGCAGAAGCCGGCGCCCTGAGGTAGGTC-3'; for S187A, forward 5'-CGGCTCAAGGAGCGCGCGCTGGACACGCTGCTG-3', reverse 5'-CAGCAGCGTGTCCAGCGCGCGCTCCTTGAGCCG-3'; for T176A, forward 5'-GAACTCAAAACCGTCGCGTACTCGCTGCTGAAG-3', reverse 5'-CTTCAGCAGCGAGTACGCGACGGTTTTGAGTTC-3'.

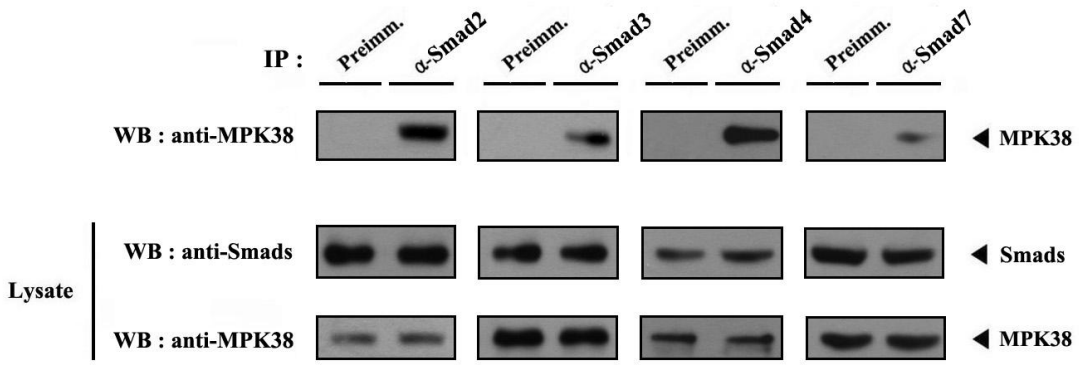
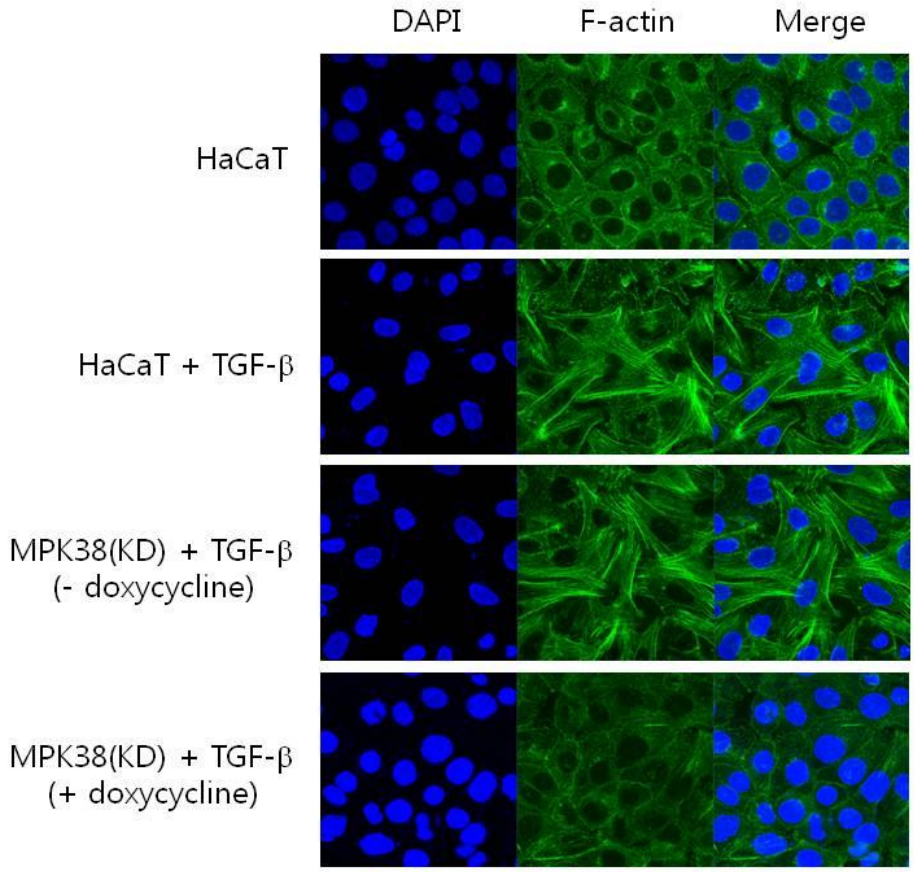


FIG. S1. ***In vivo*** **interaction between MPK38 and Smad proteins.** Cell lysates from HEK293 cells were immunoprecipitated with rabbit preimmune serum (preimm.) or the indicated anti-Smad antibodies and blotted with an anti-MPK38 antibody to determine the endogenous association between MPK38 and Smad proteins (*top panels*). The expression levels of Smads and MPK38 in total cell lysates (*Lysate*) were analyzed by Western analysis using anti-Smad and anti-MPK38 antibodies, respectively.

A


DAPI
F-actin
Merge
HaCaT
HaCaT + TGF-β
MPK38(KD) + TGF-β
(- doxycycline)
MPK38(KD) + TGF-β
(+ doxycycline)

B

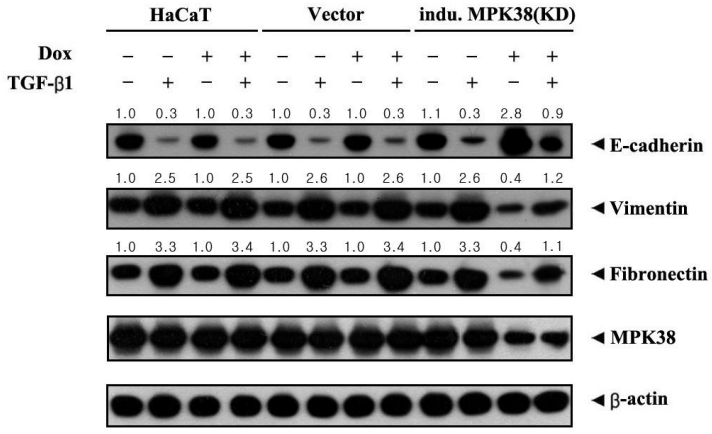
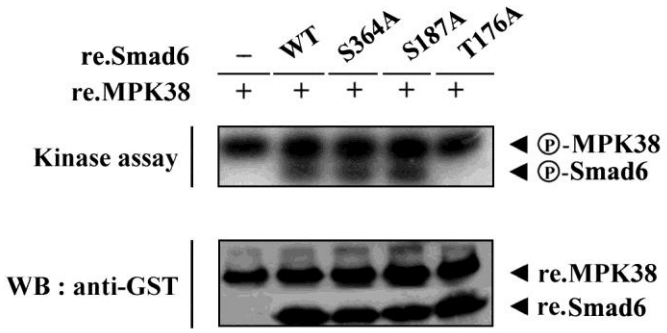

HaCaT
Vector
indu. MPK38(KD)
Dox
TGF-β1
1.0 0.3 1.0 0.3 1.0 0.3 1.0 0.3 1.1 0.3 2.8 0.9
◄ E-cadherin
1.0 2.5 1.0 2.5 1.0 2.6 1.0 2.6 1.0 2.6 0.4 1.2
◄ Vimentin
1.0 3.3 1.0 3.4 1.0 3.3 1.0 3.4 1.0 3.3 0.4 1.1
◄ Fibronectin
◄ MPK38
◄ β-actin

FIG. S2. **Effect of MPK38 on TGF-β-induced EMT in HaCaT cells.** Parental HaCaT and inducible MPK38(KD) cells were treated with 10 ng/ml TGF-β1 for 48 h in the presence (+) or absence (-) of doxycycline (1 μg/ml, 72 h) and labeled against F-actin (*A*). Immunofluorescence images revealed stress fiber formations in HaCaT cells in response to TGF-β1. Nuclei were stained by 4’,6-diamidino-2-phenylindole (DAPI). Effect of MPK38 knockdown on the expression of EMT markers (E-cadherin, Vimentin, and Fibronectin) was examined by Western blotting (*B*). Inducible silencing of endogenous MPK38 expression by doxycycline (Dox) was assessed by immunoblotting using an anti-MPK38 antibody (*4th panel*), and β-actin was used as a loading control. *indu.*, inducible.

A


re.Smad6
–
WT
S364A
S187A
T176A
re.MPK38
+ + + + +
Kinase assay
◀ Ⓟ-MPK38
◀ Ⓟ-Smad6
WB : anti-GST
◀ re.MPK38
◀ re.Smad6

B

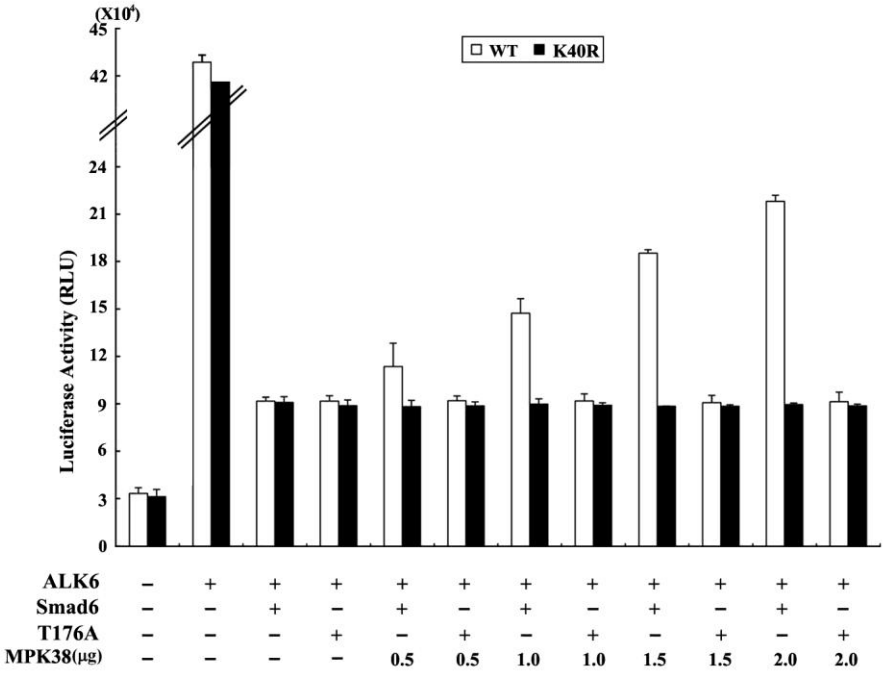

(X10⁴)
45
42
24
21
18
15
12
9
6
3
0
Luciferase Activity (RLU)
□ WT ■ K40R
ALK6 – + + + + + + + + + + +
Smad6 – – + – + – + – + – + –
T176A – – – + – + – + – + – +
MPK38(μg) – – – – 0.5 0.5 1.0 1.0 1.5 1.5 2.0 2.0

FIG. S3. **Effect of MPK38-mediated phosphorylation of Smad6 at Thr^{176} on BMP–induced transcriptional activity.** *A*, Identification of MPK38 phosphorylation sites on Smad6. Purified recombinant MPK38 was assayed for its kinase activity in the presence of kinase buffer containing approximately 3 μg of recombinant wild-type Smad6 and one of its substitution mutants, S364A, S187A, or T176A, as substrates. The *circled P*-MPK38 and *circled P*-Smad6 indicate the autophosphorylated MPK38 and phosphorylated Smad6, respectively. *B*, Murine myoblast C2C12 cells were transfected with 0.3 μg of 6XOSE-Luc plasmid, increasing amounts of wild-type (WT) and kinase-dead (K40R) MPK38, and wild-type and mutant form (T176A) of Smad6 (each 0.1 μg), as indicated, in the presence or absence of ALK6 (0.5 μg), a constitutively active form of BMP type 1B receptor. *re.*, recombinant.