PP197 EGF Suppresses BMP-induced Osteogenic Differentiation through the Up-regulation of Smurf1 Expression

Hye-Lim Lee, Kyung Mi Woo, Hyun-Mo Ryoo, Jeong-Hwa Baek

Department of Molecular Genetics, School of Dentistry, Seoul National University, Seoul, Korea

Abstract

Although EGF has been known to inhibit osteoblast differentiation, its molecular mechanism has not been clearly elucidated. Smurf1 acts as a negative regulator of BMP signaling by inducing ubiquitination and subsequent proteasomal degradation of BMP type I receptor and R-Smads. In this study, we investigated the effect of EGF on the expression of Smurf1 and the role of Smurf1 in EGF inhibition of BMP2-induced osteogenesis. EGF increased Smurf1 expression which was blocked by treatment with a specific inhibitor of EGFR tyrosine kinase, JNK or ERK. Reporter assay using the constructs that contained the mouse Smurf1 promoter sequence, demonstrated that AP-1 and Runx2 are the transcription factors activated by JNK and ERK, respectively. EGF treatment or Smurf1 overexpression suppressed BMP2-induced expression of osteogenic marker genes, whereas knockdown of Smurf1 partially rescued the expression of these genes in EGF-treated cells. Taken together, these results suggest that the JNK/c-Jun and ERK/Runx2 signaling pathways play an important role in the regulation of Smurf1 expression by EGF and that Smurf1 partially mediates the inhibitory effect of EGF on osteogenic differentiation.

Results

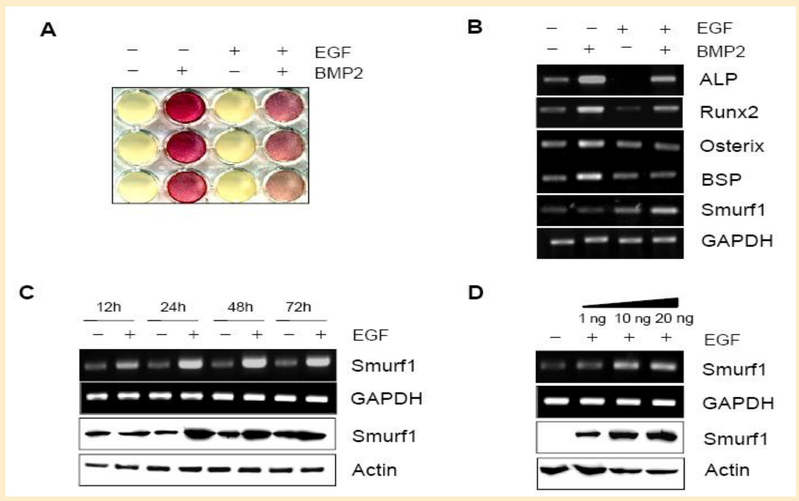

FIG. 1. EGF suppresses BMP2-induced osteoblast differentiation and enhances the expression of Smurf1 in C2C12 cells. (A, B) C2C12 cells were incubated in the presence of the indicated reagents for 24 h. Then, ALP staining (A) or RT-PCR (B) was performed. The concentrations of EGF and BMP2 were 10 ng/ml and 100 ng/ml, respectively. (C) Cells were incubated in the presence or absence of EGF for the indicated periods. RT-PCR (*upper panel) or* western blot (*down panel*) were performed. (D) Cells were incubated for 24 h in the presence of EGF, and RT-PCR and western blot analysis were performed. The concentrations of EGF used were 1,10 and 20 ng/ml.

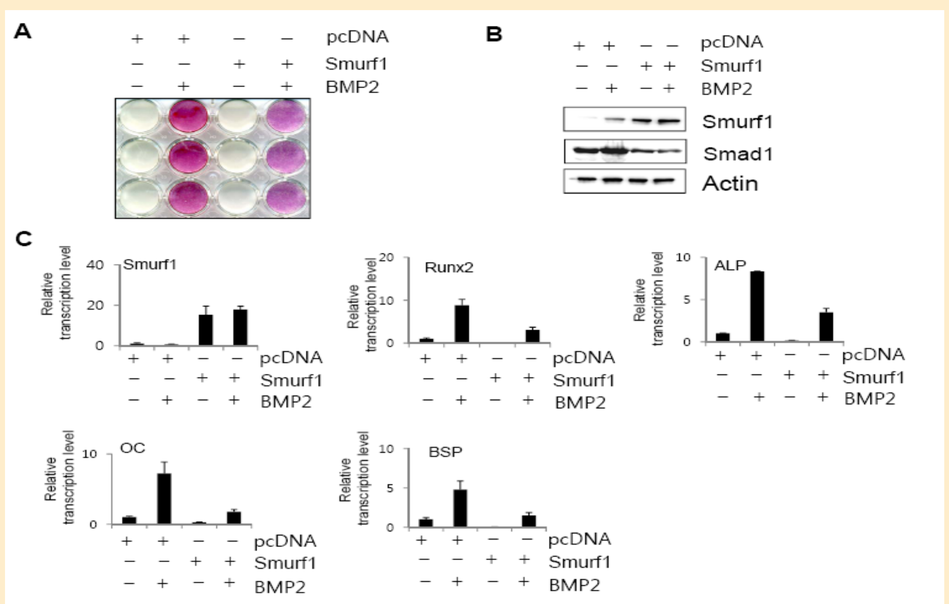

FIG. 2. Smurf1 overexpression reduces Smad1 protein levels and inhibits BMP2-induced osteoblast differentiation. C2C12 cells were transiently transfected with pcDNA or Smurf1 expression vector and incubated in the presence or absence of BMP2 for 24 h. ALP staining (A), western blot (B) or quantitative RT-PCR (C) was performed.

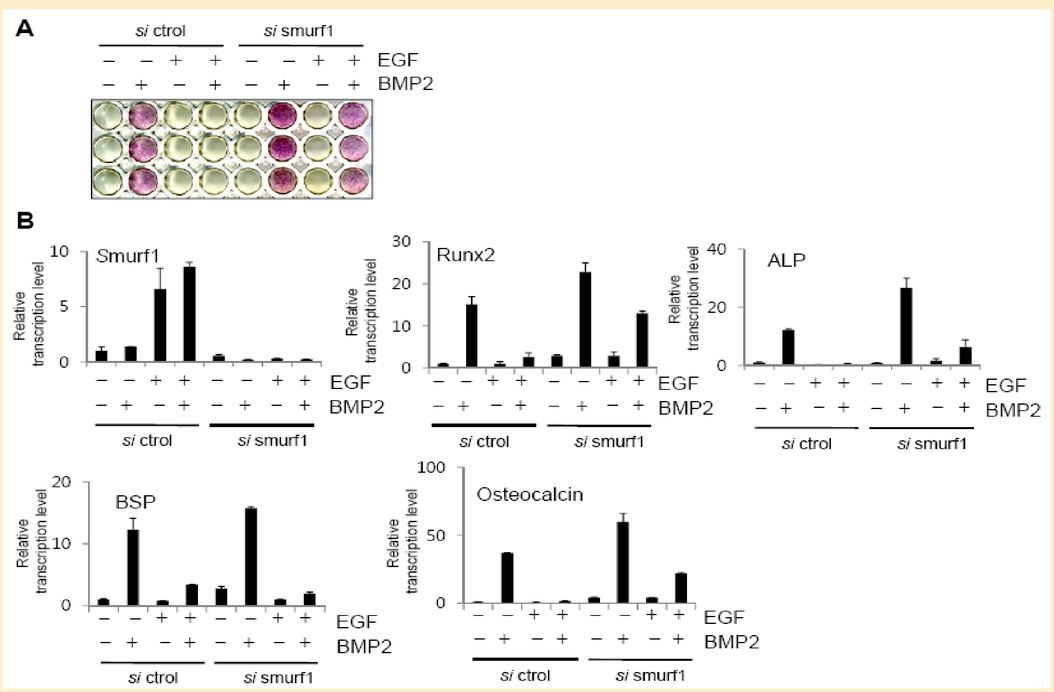

FIG. 3. EGF inhibition of osteoblast differentiation is partially suppressed by the knockdown of Smurf1. C2C12 cells were transiently transfected with Smurf1 siRNA (*si* Smurf1) or control *si*RNA (*si* ctrol) and incubated in the presence of the indicated reagents for 24 h. ALP staining (A) and quantitative RT-PCR (B) were performed. Efficiency of Smurf1 knockdown was confirmed by RT-PCR.

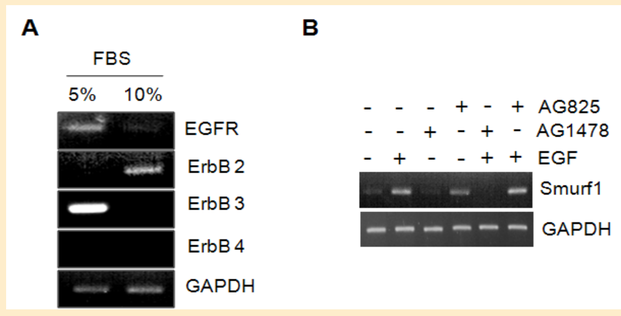

FIG. 4. EGFR is involved in EGF stimulation of Smurf1 expression. (A) C2C12 cells were incubated in osteogenic medium (5% FBS) or growth medium (10% FBS) for 24 h and the expression levels of EGF receptors were examined by RT-PCR. (B) Cells were incubated in the presence of the indicated reagents for 24 h. *(*EGF,10 ng/ml; AG1478, 20 μM, EGFR-specific tyrosine kinase inhibitor; AG825,10 μM, ErbB2 specific tyrosine kinase inhibitor)

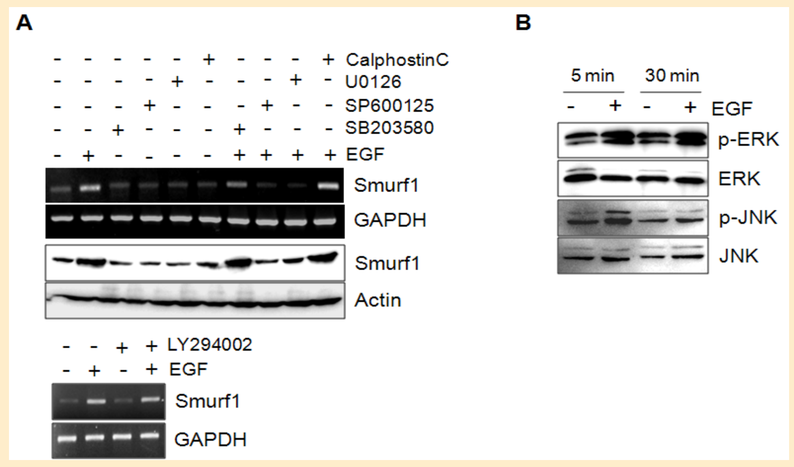

FIG. 5. Smurf1 induction is suppressed by the inhibition of JNK and ERK but not by the inhibition of PI3K, p38 MAPK and PKC. (A) C2C12 cells were incubated in the presence of the indicated reagents for 24 h, and RT-PCR and western blot analysis were performed. The concentrations of the reagents were as follows: 10 ng/ml EGF, 10 μM SP600125 (JNK inhibitor), 10 μM SB203580 (p38 inhibitor), 10 μM U0126 (ERK inhibitor), 10 μM LY294002 (PI3K inhibitor) and 0.5 μM CalphostinC (PKC inhibior). (B) EGF activated ERK and JNK. Serum-starved C2C12 cells were incubated in the presence of EGF for 5 and 30 min and western blot analysis was performed.

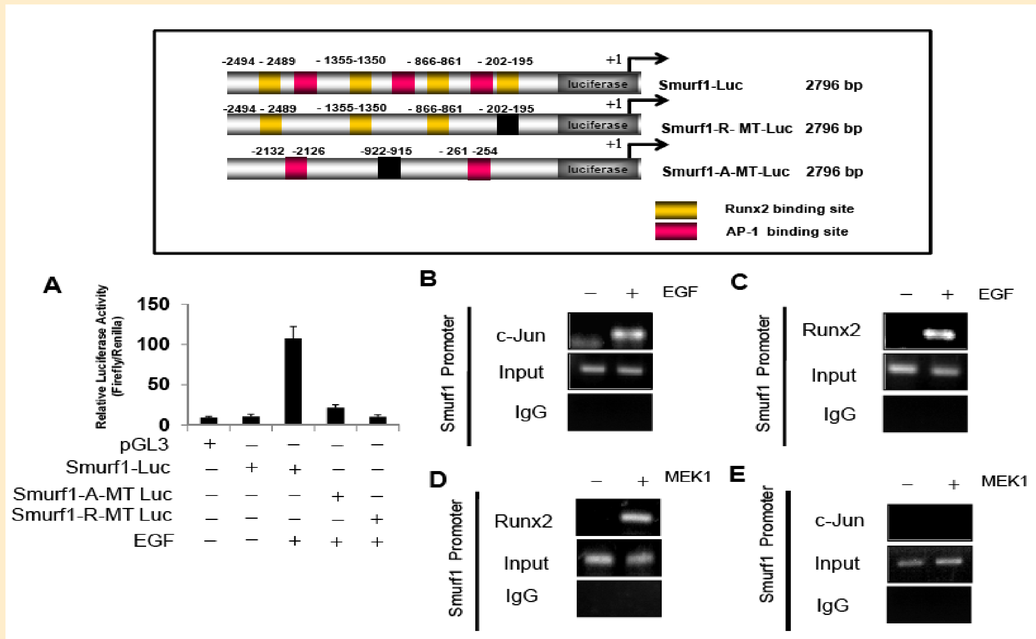

FIG. 6. EGF stimulates Smurf1 transcription through the enhancement of the binding of c-Jun and Runx2 to their respective binding elements on the mouse Smurf1 promoter. (Upper panel) Schematic illustrations of putative binding elements for the AP-1 and Runx2 on the mouse Smurf1 promoter constructs. Among the putative bindind elements, functional AP-1 *cis*-acting elements resides at -922 to -915 bp and functional Runx2 bindind site is present at -202 to -195 bp on the mouse Smurf1 promoter. (A) Reporter assays were performed using C2C12 cells which were transiently transfected with the indicated reporters. Smurf1-R-MT-luc and Smurf1-A-MT-luc are mutant reporters that contained mutations in Runx2 binding elements and the AP-1 binding elements, respectively. (B-E) C2C12 cells were either incubated with EGF or transiently transfected with constitutively active MEK1 (MEK1) expression vectors for 24 h. Chromatin immunoprecipitation was then performed with c-Jun antibody (B, E), Runx2 antibody (C, D) or normal IgG. Immunoprecipitated DNA fragments were amplified by PCR using primers which amplify the Smurf1 promoter region containing the AP-1 binding motif (B, E) or the Runx2 binding motif (C, D).

Conclusion

Our data suggest that the JNK/c-Jun and ERK/Runx2 signaling pathways mediate EGF-induced Smurf1 expression and that Smurf1 partially plays a role in the suppressive effect of EGF on BMP-induced osteoblast differentiation.