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EGF Inhibits Wnt/β-Catenin-Induced Osteoblast Differentiation by Promoting β-Catenin Degradation

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ABSTRACT

Bone morphogenetic protein (BMP) and canonical Wnts are representative developmental signals that enhance osteoblast differentiation and bone formation. Previously, we demonstrated that epidermal growth factor (EGF) inhibits BMP2-induced osteoblast differentiation by inducing Smurf1 expression. However, the regulatory role of EGF in Wnt/β-catenin-induced osteoblast differentiation has not been elucidated. In this study, we investigated the effect of EGF on Wnt/β-catenin signaling-induced osteoblast differentiation using the C2C12 cell line. EGF significantly suppressed the expression of osteoblast marker genes, which were induced by Wnt3a and a GSK-3β inhibitor. EGF increased the expression levels of Smurf1 mRNA and protein. Smurf1 knockdown rescued Wnt/β-catenin-induced osteogenic marker gene expression in the presence of EGF. EGF treatment or Smurf1 overexpression did not affect β-catenin mRNA expression levels, but reduced β-catenin protein levels and TOP-Flash activity. EGF and Smurf1 promoted β-catenin ubiquitination. Co-immunoprecipitation and GST pull-down assays showed that Smurf1 associates with β-catenin. These results suggest that EGF/Smurf1 inhibits Wnt/β-catenin-induced osteogenic differentiation and that Smurf1 downregulates Wnt/β-catenin signaling by enhancing proteasomal degradation of β-catenin. J. Cell. Biochem. 116: 2849–2857, 2015.

KEY WORDS: WNT; β-CATENIN; EPIDERMAL GROWTH FACTOR; SMURF1; OSTEOBLAST

Ubiquitination-mediated protein degradation has been shown to regulate a variety of cellular processes, such as cell signaling transduction, cell cycle regulation, and gene transcription. Smad ubiquitination regulatory factor1 (Smurf1) is a HECT-type E3 ubiquitin ligase that is largely known for its role in bone cell differentiation. It has been demonstrated that Smurf1 negatively regulates the differentiation of osteoblast and osteoprogenitor cells by limiting the accumulation of crucial transcription factors, such as bone morphogenetic protein (BMP)-activated R-Smad (Smad1/5), Runx2, and JunB proteins via proteasomal degradation. Previous studies have demonstrated that overexpression of Smurf1 in pluripotent mesenchymal cells and pre-osteoblasts inhibits osteoblast differentiation. Smurf1-transgenic mice exhibit a lower bone mass phenotype due to reduced bone formation [Yamashita et al., 2005]. Smurf1-null mice exhibited an age-dependent increase in bone mass, and this deficiency protected tumor necrosis factor (TNF) α-transgenic mice from systemic bone loss [Guo et al., 2008]. Under inflammatory conditions, Smurf1 expression and activity is enhanced, which causes Smurf1-mediated protein degradation. It has been reported that Smurf1-deficient mice show attenuated inflammatory arthritis-induced bone loss, and that TNFα inhibits osteoblast differentiation and bone formation via upregulation of Smurf1 and Smurf2 expression in bone marrow stromal cells [Guo et al., 2008].

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However, which extracellular signals regulate the expression of Smurf1 has not been fully elucidated.

We previously demonstrated that epidermal growth factor (EGF) enhances Smurf1 expression and that Smurf1 mediates the inhibitory effect of EGF on BMP2-induced osteoblast differentiation [Lee et al., 2014]. EGFR ligands and their receptor, EGFR, are among the best-studied signaling networks that play crucial roles in tissue development and tumorigenesis [Zhu et al., 2011]. It has been well established that EGF stimulates osteoblast proliferation, but inhibits their differentiation in vitro [Antosz et al., 1987; Nicolas et al., 1990; Laflamme et al., 2010; Zhu et al., 2011]. Overexpression of EGF in mice exhibited a reduction in cortical bone thickness, even with endosteal and periosteal stromal cell accumulation [Chan and Wong, 2000]. Mice lacking EGFRs demonstrated impaired bone formation due to reduced numbers of osteoprogenitor/osteoblast cells, although in vitro experiments using EGFR-deficient cells showed accelerated osteoblast differentiation [Sibilia et al., 2003; Schneider et al., 2009]. These previous reports demonstrated that EGF suppresses osteogenic differentiation of osteoprogenitor cells, independent of its stimulating effect on cell proliferation, by inhibiting the expression of key transcription factors, Runx2 and osterix, through the upregulation of histone deacetylase expression. However, the underlying mechanisms by which EGF inhibits osteoblast differentiation are not fully understood.

Wnt signaling is a pivotal pathway that regulates the development and growth of many organs and tissues, including bone [Logan and Nusse, 2004]. Together with BMPs, Wnt/β-catenin signaling is the master regulator of mesenchymal stem cell differentiation toward osteogenic lineage and subsequent bone formation [Logan and Nusse, 2004; Longo et al., 2004; Galli et al., 2012]. Ubiquitin-mediated regulation participates in the Wnt/β-catenin signaling pathway. In the absence of a Wnt-signal, β-catenin is retained in the cytoplasm by a multi-protein degradation complex (destruction complex) composed of glycogen synthase kinase 3 (GSK3), casein kinase 1, APC, axin, and β-catenin. In these conditions, β-catenin is phosphorylated by GSK3, followed by β-TrCP-mediated ubiquitination, leading to proteasomal degradation. Canonical Wnt stimulation inhibits the activity of the destruction complex, and β-catenin then accumulates in the cytosol, translocates to the nucleus and interacts with T-cell factor (TCF)/lymphoid enhancer factor (LEF) family transcription factors to regulate Wnt target gene expression [He, 2003; Longo et al., 2004]. Although there has been rapid progress regarding the understanding of Wnt/β-catenin signaling and bone homeostasis, the mechanisms modulating Wnt/β-catenin signal-induced osteogenic differentiation are not fully understood. Recently, there was a report showing that under inflammatory conditions, β-catenin ubiquitination and degradation increases via the induction of Smurf1 [Chang et al., 2013]. However, the regulatory role of EGF and Smurf1 in Wnt/β-catenin signaling-induced osteoblast differentiation has not been explored.

In the present study, we investigated whether EGF/Smurf1 inhibited Wnt/β-catenin-induced osteogenic differentiation and whether Smurf1 downregulated Wnt/β-catenin signaling by enhancing the proteasomal degradation of β-catenin.

MATERIALS AND METHODS

REAGENTS, ANTIBODIES, AND CELL CULTURE

Dulbecco's modified Eagle's medium (DMEM) was purchased from Hyclone (Logan, UT), and fetal bovine serum (FBS) was obtained from BioWhittaker (Walkersville, MD). Easy-BLUE™, for total RNA extraction, was ordered from iNtRON Biotechnology (Sungnam, Korea). The Alkaline Phosphatase staining kit, cycloheximide, MG132, *p*-nitrophenyl-phosphate and *p*-nitrophenol were purchased from Sigma (St. Louis, MO). For first-strand cDNA synthesis, AccuPower RT PreMix was purchased from Bioneer (Daejeon, Korea), and SYBR premix EX Taq was purchased from Takara (Otsu, Japan). The PCR primers were synthesized by Cosmogenetech (Seoul, Korea). Lipofectamine™ reagent was purchased from Invitrogen (Carlsbad, CA). Anti-Flag antibody and Wnt3a were purchased from R&D Systems (Minneapolis, MN). Anti-Smurf1, anti-β-catenin, anti-β-actin antibodies, goat anti-rabbit, and anti-mouse HRP-conjugated IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). For Western blot analysis, Supex reagent was ordered from Dyne-Bio (Sungnam, Korea). The TCF/LEF reporter plasmid, TOP-Flash, was purchased from Upstate Biotechnology (Lake Placid, NY). The Dual-Glo luciferase assay kit was purchased from Promega (Madison, WI). SB216763, the GSK-3β inhibitor, was purchased from Cayman chemical (Ann Arbor, MI). Smurf1 expression plasmids (flag-tagged Smurf1-C699A, GST-Smurf1 wild-type) were purchased from Addgene, Inc. (Cambridge, MA).

HEK293T and C2C12 cells were cultured in DMEM supplemented with 10% FBS and antibiotics (100 U/ml of penicillin and 100 μg/ml of streptomycin).

REVERSE TRANSCRIPTION–POLYMERASE CHAIN REACTION (RT–PCR)

To evaluate mRNA expression, quantitative RT-PCR was performed. Total RNA was extracted using easy-BLUE™ RNA extraction reagents. cDNA was synthesized from total RNA with the AccuPower™ RT PreMix. Real-time-PCR was performed to observe mRNA expression levels of Smurf1 using SYBR premix EX Taq using an AB 7500 Fast Real-Time system (Applied Biosystems, Foster City, CA). Each sample was analyzed in triplicate, and target genes were normalized to the reference housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Fold differences were then calculated for each treatment group using C_T values normalized to the control. The PCR primer sequences used for real-time PCR were as follows: Smurf1 (f) 5′-AGCATCAAGATCCGTCTGACA-3′, (r) 5′-CCAGAGCCGTCCACAACAAT-3′; Dlx5 (f) 5′-TCTCTAGGACTGACGCAAACA-3′ and (r) 5′-GTTACACGCCATAGGGTCGC-3′; β-catenin (f) 5′-GGTGCTGACTATCCAGTTG-3′ and (r) 5′-GGCAGAGTAAAGTATTCACCC-3′; Runx2 (f) 5′-TTCTCCAACCCACGAATGCAC-3′ and (r) 5′-CAGGTACGTGTGGTAGTGAGT-3′; alkaline phosphatase (ALP) (f) 5′-CCAACTCTTTTGTGCCAG-3′ and (r) 5′-GGCTACATTGGTGTTGAGCTTTT-3′; osterix (OSX) (f) 5′-CCCACCCTTCCCTCACTC-3′ and (r) 5′-CCTTGTACCACGAGCCAT-3′; osteocalcin (OCN) (f) 5′-CTGACAAAGCCTTCATGT-3′ and (r) 5′-GCGCCGGAGTCTGTTCAC-3′ and GAPDH (f) 5′-TCAATGACAACTTTGTCAAGC-3′ and (r) 5′-CCAGGGTTTCTTACTCCTTGG-3′.

WESTERN BLOT ANALYSIS AND IMMUNOPRECIPITATION

After treatment, whole cell lysates were prepared for Western blot analysis. For the whole cell lysate preparation, cells were lysed in

buffer consisting of 10 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM EDTA (pH 8.0), 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM sodium fluoride, 0.2 mM sodium orthovanadate, 1 mM PMSF, 1 μg/ml aprotinin, 1 μM leupeptin, and 1 μM pepstatin. The lysate was briefly sonicated and centrifuged at 16,000*g* for 10 min. The supernatant was then used for immunoblot analysis. For immunoprecipitation, the cells were washed with ice-cold phosphate buffered saline (PBS) and scraped into immunoprecipitation buffer consisting of 20 mM Tris–Cl (pH 7.5), 150 mM NaCl, 1 mM EDTA (pH 8.0), 2% Brij35, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM PMSF, 1 μg/ml aprotinin, 1 μM leupeptin, and 1 μM pepstatin. After brief sonication, 1 mg of each protein sample was used for immunoprecipitation with the appropriate primary antibodies and protein G agarose beads. The bead pellet was washed five times with immunoprecipitation buffer, denatured by boiling in 2× SDS sample buffer, and then subjected to SDS–PAGE and immunoblot analysis. The proteins were gel separated and transferred onto a PVDF membrane. The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween20, then incubated with the indicated primary antibody, and subsequently incubated with HRP-conjugated secondary antibody. Immune complexes were visualized using Supex reagent and luminescence was detected with a MicroChemi imaging system (DNR; Jerusalem, Israel).

TOP-FLASH ASSAY

C2C12 cells were plated into 96-well plates at a density of 2×10^4 cells/well. The cells were transiently transfected with 0.2 μg of TOP-Flash plasmid. To normalize the transfection efficiency, Renilla luciferase plasmid was used as an internal control. When indicated, cells were transfected with wild-type or mutant Smurf1 expression plasmids. After 24 h, the cells were treated with SB216763 (10 μM), a GSK inhibitor, in the presence or absence of EGF for 24 h. Luciferase activity was measured using a Dual-Glo luciferase assay kit. Relative luciferase activity was calculated after normalization to Renilla luciferase activity.

GST PULL-DOWN ASSAY

The GST-Smurf1 (pGEX4T1-Smurf1-wt) or GST plasmid was expressed in *E. coli* DH5α cells. After bacterial lysis, GST proteins were captured on glutathione-sepharose beads and washed in lysis buffer before use. Nuclear proteins containing β-catenin were obtained by transiently transfecting HEK293T cells with β-catenin expression vectors, followed by incubation for 24 h. Then, 0.5 μg of the indicated GST protein or GST-Smurf1 and 20 μg of cell lysates were mixed and incubated for 90 min at 4°C. After washing with binding buffer, pulled-down glutathione-sepharose beads were denatured by boiling in 2× SDS sample buffer and were then subjected to SDS–PAGE and immunoblot analysis [Yeung et al., 2013].

CYCLOHEXIMIDE (CHX) CHASE MEASUREMENTS OF β-CATENIN HALF-LIFE

C2C12 cells were transiently transfected with either β-catenin alone or together with Smurf1 or EGF treatment. At 16 h post-transfection and incubation with EGF, culture medium was replaced by DMEM supplemented with 10% FBS, 1% penicillin-streptomycin and CHX (10 μg/ml). Cells were lysed at 0, 2, 4, 8, 12, and 24 h following CHX treatment, and the resulting lysates were analyzed by Western blotting using β-catenin and Smurf1 antibodies.

SMURF1 KNOCK-DOWN BY SMALL INTERFERING RNA (siRNA)

ON-TARGETplus Smurf1 siRNA and non-targeting control siRNA were purchased from Dharmacon (Lafayette, CO). Transient transfection of siRNA into C2C12 cells was performed using Dharmafect according to the manufacturer's instructions.

ALKALINE PHOSPHATASE (ALP) STAINING AND ALP ACTIVITY ASSAY

ALP staining of C2C12 was performed using an ALP staining kit according to the manufacturer's instructions. The ALP assay was performed as previously described [Yu et al., 2013]. Briefly, after C2C12 differentiation, lysates were clarified by centrifugation and 10 μl of cell extracts was added to each well (96-well plate) containing 200 μl *p*-nitrophenyl phosphate. After incubation for 10–60 min depending on the ALP activity in the extracts at 37°C, ALP activity was determined by measuring absorbance at 405 nm using a 96-well plate reader. *p*-Nitrophenol was used as a standard. ALP activity was then normalized to total protein amount.

STATISTICAL ANALYSIS

All of the quantitative data are represented as the mean ± SD. Statistical significance was analyzed by Student's *t*-test or multiple-comparison using Prism6. A *P*-value of less than 0.05 was considered statistically significant.

RESULTS

EGF SUPPRESSES WNT3A/β-CATENIN SIGNALING-INDUCED OSTEOBLAST DIFFERENTIATION IN C2C12

We first examined whether EGF has a regulatory role in Wnt3a/β-catenin signaling-induced osteogenic differentiation in our culture system. C2C12 cells in the absence or presence of EGF were subjected to osteogenic differentiation with Wnt3a or SB216763 (SB) for 72 h. EGF strongly suppressed Wnt3a or SB-induced ALP activity in a dose-dependent manner (Fig. 1A). EGF also strongly suppressed SB-induced osteoblast marker gene expression at a concentration of 10 ng/ml (Fig. 1B), indicating that EGF exerts a strong inhibitory effect on Wnt3a/β-catenin-induced osteoblast differentiation.

EGF DOWNREGULATES β-CATENIN PROTEIN LEVELS IN A SMURF1-DEPENDENT MANNER

To further explore whether EGF antagonizes the transcriptional activity of β-catenin, the effect of EGF was studied in C2C12 cells using the TOP-Flash reporter assay system. To enhance the transcriptional activity of β-catenin, C2C12 cells were treated with the GSK3 inhibitor. SB significantly increased luciferase activity, whereas EGF significantly abolished SB-induced TOP-Flash activity (Fig. 2A). The corresponding results were also observed by Western blot analysis. The levels of β-catenin protein increased with SB treatment (Fig. 2B); however, when EGF was added, SB-induced increment of β-catenin protein expression was abolished.

We previously demonstrated that EGF upregulates Smurf1 expression in C2C12 cells [Lee et al., 2014]. Therefore, we next

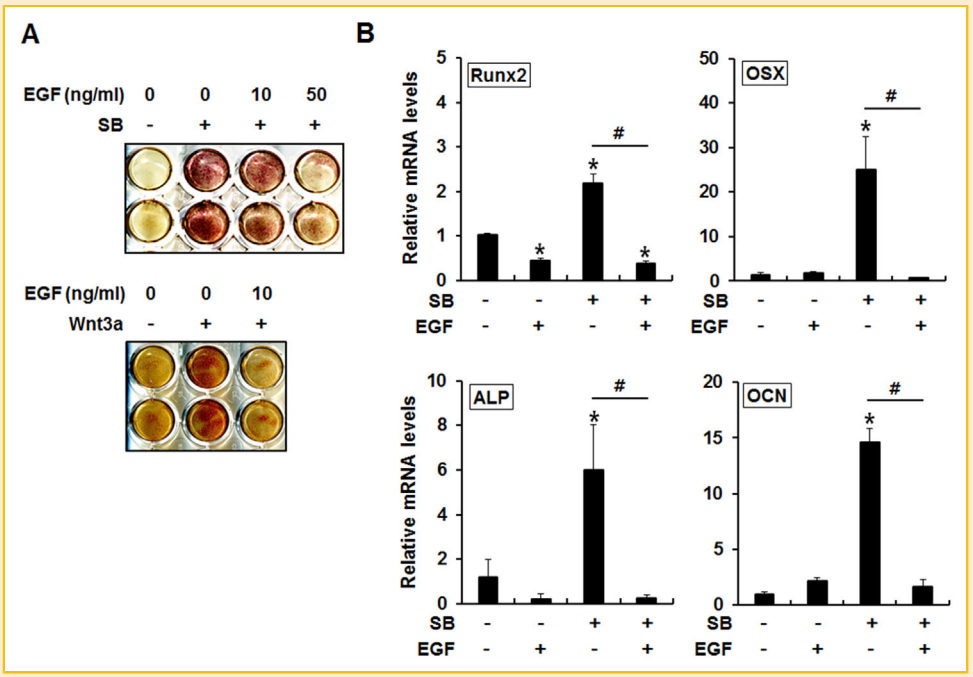

Fig. 1. EGF suppresses Wnt/β-catenin-induced osteoblast differentiation. (A) EGF inhibits ALP activity in C2C12 cells under SB216763 (SB) or Wnt3a-induced osteogenic differentiation. Cells were cultured with SB (10 μM) or Wnt3a (50 ng/ml) in the presence and absence of EGF (dosed as indicated) for 3 days. The negative control was left untreated and analysis was performed by ALP staining. (B) C2C12 cells were cultured in the presence and absence of SB with or without EGF (10 ng/ml) for 2 days. Total RNA was extracted, and the levels of osteogenic markers were evaluated by real-time PCR. The indicated gene expression is presented as the mean ± SD of triplicate. An * indicates that $P < 0.05$ compared to the non-treatment control, while # denotes that $P < 0.05$ for the indicated pairs.

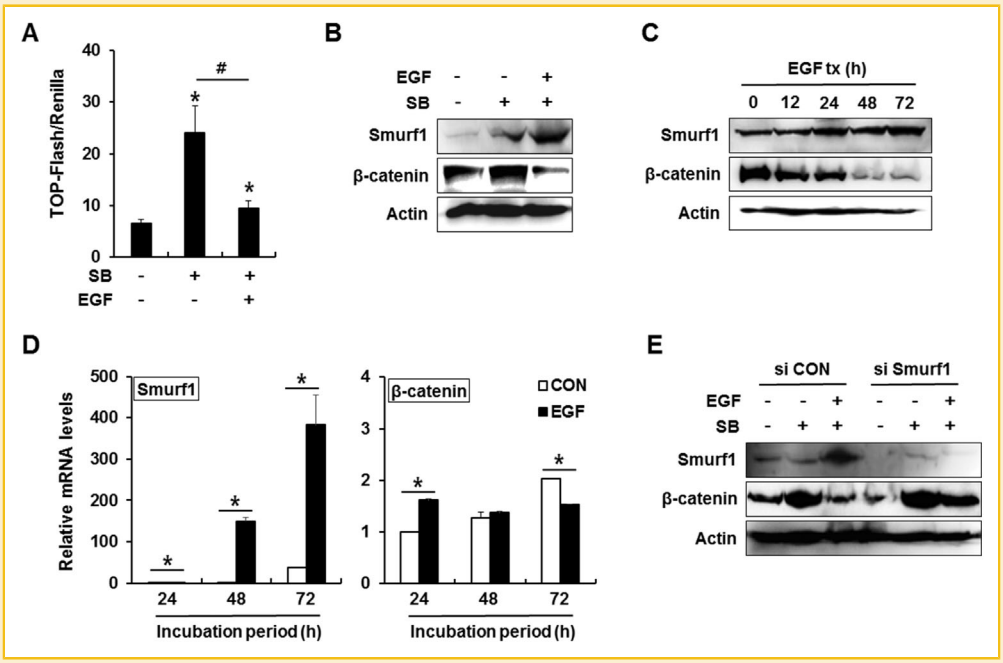

Fig. 2. EGF downregulates β-catenin protein levels in a Smurf1-dependent manner. (A) C2C12 cells were transiently transfected with TOP-Flash and incubated in the presence and absence of SB or EGF for 48 h. The data are shown as the activity relative to Renilla luciferase activity and represent the mean ± SD of six independent experiments. An * indicates $P < 0.05$ compared to the TOP-Flash alone, while # denotes that $P < 0.05$ for the indicated pairs. (B) C2C12 cells were incubated in the presence and absence of SB and/or EGF for 48 h, and the protein levels of β-catenin and Smurf1 were determined by Western blot analysis. (C, D) C2C12 cells were cultured in the presence or absence of EGF for the indicated periods, and the expression levels of β-catenin and Smurf1 were evaluated by Western blot analysis (C) and real-time PCR (D). An * denotes that $P < 0.05$ for the indicated pairs. (E) C2C12 cells were transfected with control siRNA (siCON) or Smurf1 siRNA (siSmurf1). After 24 h, cells were incubated with or without SB and EGF for 48 h. The protein levels of β-catenin and Smurf1 were determined by Western blot analysis.

examined whether there was any correlation between the expression levels of Smurf1 and β-catenin. C2C12 cells were incubated in the presence of EGF for 12, 24, 48, and 72 h. The Western blot results showed that EGF time-dependently increased the Smurf1 protein levels, while it decreased the β-catenin protein levels (Fig. 2C). Quantitative PCR results confirmed that EGF increased Smurf1 mRNA levels (Fig. 2D, left panel); however, the β-catenin mRNA levels were unaffected (Fig. 2D, right panel), implying that EGF-induced Smurf1 expression attenuates β-catenin at the protein level.

Thus, we next explored whether Smurf1 knockdown blocks EGF inhibition of β-catenin protein expression. Cells were transiently transfected with control siRNA or Smurf1 siRNA and incubated overnight, followed by a 48 h incubation with SB in the presence or absence of EGF. Smurf1 siRNA silenced Smurf1 expression that was induced by EGF as determined by Western blot. Knockdown of Smurf1 reversed the inhibitory effect of EGF on the β-catenin protein levels (Fig. 2E).

OVEREXPRESSION OF SMURF1 SUPPRESSES WNT/β-CATENIN-INDUCED OSTEOBLAST DIFFERENTIATION, WHEREAS SMURF1 KNOCKDOWN ATTENUATES EGF-MEDIATED SUPPRESSION OF OSTEOBLAST DIFFERENTIATION

Because EGF exerted an inverse regulatory effect on the Smurf1 and β-catenin expression levels, we next examined whether the increased expression of Smurf1 itself suppresses Wnt/β-catenin-induced osteoblast differentiation. C2C12 cells were transiently transfected with pcDNA or a Smurf1 expression plasmid and incubated overnight. Smurf1 overexpression was confirmed by quantitative RT-PCR. Smurf1 overexpression reduced SB-induced ALP activity (Fig. 3A) and the expression levels of osteogenic marker genes (Fig. 3B). Furthermore, Smurf1 overexpression also attenuated Wnt3a-induced ALP activity and bone marker gene expression (Fig. S1). These results indicate that an increase in Smurf1 levels suppresses Wnt/β-catenin-induced osteogenic differentiation.

We next verified whether Smurf1 knockdown blocks EGF inhibition of Wnt/β-catenin-induced osteogenic differentiation. Cells were transiently transfected with control siRNA or Smurf1 siRNA and incubated overnight, followed by a 48 h incubation in the presence of SB or EGF. The silencing efficiency of Smurf1 siRNAs was confirmed using RT-PCR. In control siRNA-transfected cells, EGF treatment strongly suppressed SB-induced ALP activity and osteoblast marker gene expression (Fig. 3C and D). Knockdown of Smurf1 further elevated the expression levels of Dlx5, ALP, and OCN, which were induced by SB (Fig. 3D). Furthermore, Smurf1 silencing rescued SB-induced ALP activity (Fig. 3C). These results indicate that Smurf1 plays a significant role in the EGF inhibition of Wnt/β-catenin-induced osteoblast differentiation.

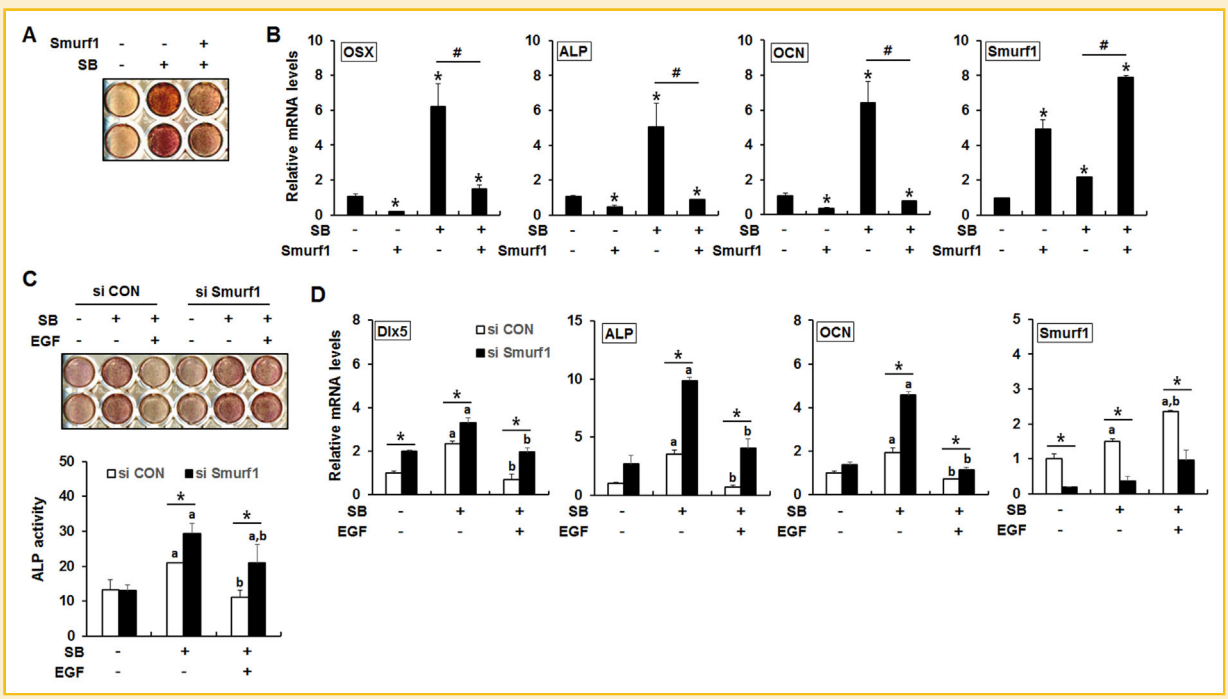

Fig. 3. Overexpression of Smurf1 suppresses Wnt/β-catenin-induced osteoblast differentiation, whereas Smurf1 knockdown attenuates EGF-mediated suppression of osteoblast differentiation. C2C12 cells were transfected with the indicated plasmid (pcDNA and Smurf1) or siRNAs (siControl and siSmurf1) and incubated for 24 h. The plasmid-transfected cells were then incubated for an additional 48 h in the presence and absence of SB. ALP staining (A) and real-time PCR of bone marker genes (B) were performed. An * indicates that $P < 0.05$ compared to vehicle-treated pcDNA-transfected cells, while # denotes $P < 0.05$ for the indicated pairs. The siRNAs-transfected cells were incubated with and without SB and EGF for 48 h. ALP activity assays (C) and real-time PCR of bone marker genes (D) were performed. An * denotes that $P < 0.05$ for the indicated pairs. [a] indicates $P < 0.05$ as compared to vehicle-treated cells, and [b] indicates $P < 0.05$ compared to SB-treated cells.

DOWNREGULATION OF β-CATENIN PROTEIN LEVELS BY EGF/SMURF1 DEPENDS ON THE E3 LIGASE ACTIVITY OF SMURF1 AND PROTEASOMAL DEGRADATION

Given our results that EGF downregulates β-catenin protein expression through Smurf1, we hypothesized that EGF-induced Smurf1 expression promotes β-catenin degradation to inhibit osteoblastic differentiation.

To explore the mechanism by which Smurf1 decreases β-catenin protein levels, the inhibitory effect of Smurf1 was first studied using a TOP-Flash reporter assay. Cells were transfected with wild type (Smurf1) or an E3 ligase-inactive mutant of Smurf1 (Smurf1-CA, C699A) [Fei et al., 2013], and TOP-Flash activity was examined following GSK-3 inhibitor stimulation (Fig. 4A). Enhancing Smurf1 levels by overexpression markedly reduced SB-induced TOP-Flash activity. In contrast, no significant change of TOP-Flash activity was obtained in Smurf1-CA cells compared with SB alone. Western blot analysis revealed that Smurf1 overexpression decreased β-catenin protein expression, while Smurf1-CA overexpression caused no significant change to the β-catenin protein levels (Fig. 4B). These results suggest that the downregulation of β-catenin protein levels by EGF/Smurf1 depends on the E3 ligase activity of Smurf1.

Next, to determine whether the downregulation of β-catenin protein levels by EGF/Smurf1 depends on proteasomal degradation, C2C12 cells were cultured with Smurf1 or EGF in the presence or absence of a proteasome inhibitor, MG132. Western blot analysis revealed that MG132 potently blocked the β-catenin degradation that was induced by EGF treatment or Smurf1 overexpression (Fig. 4C).

Because the above results indicated that the effect of EGF/Smurf1 on β-catenin protein levels depends on the E3 ligase activity of Smurf1 and proteasomal degradation, we next examined whether EGF or Smurf1 decreased the stability of β-catenin protein. C2C12 cells were incubated for 24 h in the presence or absence of EGF treatment or Smurf1 overexpression, followed by incubation with CHX (an inhibitor of de novo protein synthesis) for 2, 4, 8, and 12 h. Western blot analysis revealed that EGF treatment or Smurf1 overexpression accelerated β-catenin degradation compared to control cells (Fig. 4D). These data indicated that the inhibition of β-catenin by EGF/Smurf1 is the result of increased degradation.

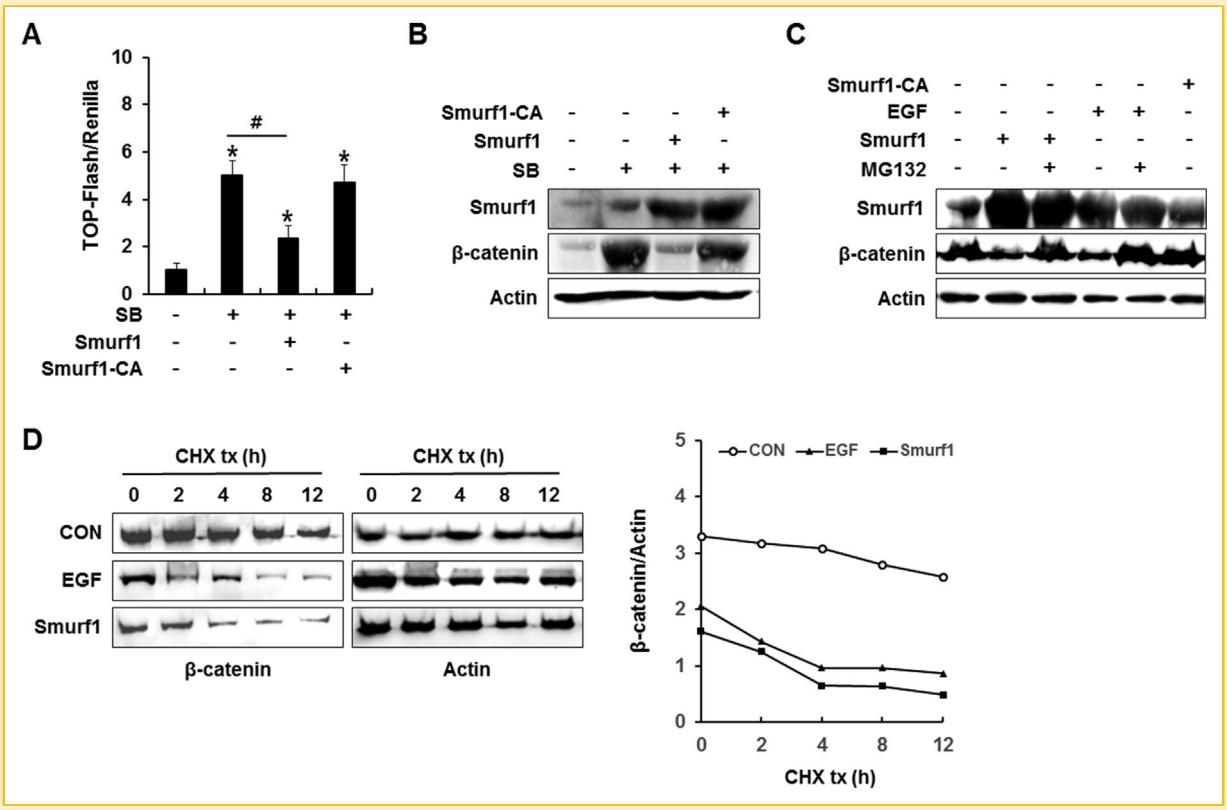

Fig. 4. Downregulation of β-catenin protein levels by EGF/Smurf1 depends on the E3 ligase activity of Smurf1 and proteasomal degradation. (A) C2C12 cells were transiently transfected with a TOP-Flash reporter plasmid with or without wild type Smurf1 or mutant Smurf1 (Smurf1-CA; Smurf1 C699A) plasmid, and then incubated with SB for 48 h. The data are shown as activity relative to Renilla luciferase activity and represent the mean ± SD of six independent experiments. (B) C2C12 cells were transfected with pcDNA, Smurf1, or Smurf1-CA plasmids. After 24 h, the transfected cells were incubated with SB for 48 h and the protein levels of β-catenin and Smurf1 were evaluated by Western blot analysis. (C) C2C12 cells were transiently transfected with the indicated plasmids (β-catenin, Smurf1, or Smurf1-CA) and incubated for 24 h in the presence of MG132 (5 μM) and EGF. The protein levels of β-catenin and Smurf1 were determined. (D) C2C12 cells were transfected with Smurf1 or pcDNA, treated with vehicle (CON) or EGF for 24 h and then incubated for the indicated time periods with cycloheximide (CHX, 10 μg/ml). Western blot analysis and densitometry analysis were performed, and the band intensity of β-catenin was normalized to the actin.

SMURF1 ASSOCIATES WITH β-CATENIN AND ENHANCES UBIQUITINATION OF β-CATENIN

We next investigated whether Smurf1 enhanced the ubiquitination of β-catenin. C2C12 cells were transiently transfected with β-catenin, Smurf1 or flag-tagged ubiquitin and incubated in the presence or absence of EGF for 24 h. Immunoprecipitation of cell lysates with β-catenin antibody and subsequent immunoblotting with flag antibody demonstrated that EGF treatment or overexpression of Smurf1 increased β-catenin ubiquitination (Fig. 5A). These data suggest that EGF/Smurf1 reduced β-catenin protein levels by enhancing the ubiquitination of β-catenin.

Finally, to test whether Smurf1 physically associates with β-catenin, we performed co-immunoprecipitation assays using C2C12 cells overexpressing β-catenin and either wild-type or mutant Smurf1 constructs in the presence of a proteasomal inhibitor. As shown in Fig. 5B, Smurf1 protein bands were detected in samples that were immunoprecipitated with β-catenin antibody, but not in samples that were precipitated with control IgG. Given that both Smurf1 and Smurf1-CA interact with β-catenin, we speculate that the E3 ligase activity of Smurf1 is not required for this interaction.

To further confirm the interaction of Smurf1 with β-catenin in vitro, a GST pull-down assay was performed using protein lysates from HEK293T cells expressing β-catenin and either GST or GST-Smurf1 protein. Immunoblotting with a β-catenin antibody showed that β-catenin associates with Smurf1-GST (Fig. 5C).

DISCUSSION

BMPs and Wnt/β-catenin proteins are the canonical developmental signals that enhance osteoblast differentiation and bone formation. We previously reported that Smurf1 plays a role in EGF inhibition of BMP2-induced osteogenic differentiation. In the present study, we provide solid evidence to demonstrate that ubiquitination and proteasomal degradation of β-catenin is regulated by Smurf1, suggesting that Smurf1 also executes a regulatory function in the Wnt/β-catenin signaling pathway. Our study demonstrates that EGF-induced Smurf1 inhibits Wnt/β-catenin-induced osteogenic differentiation and that Smurf1 downregulates Wnt/β-catenin signaling via enhancing proteasomal degradation of β-catenin.

As previously mentioned, it has been demonstrated that EGF signaling enhances proliferation and impairs osteogenic differentiation in both in vitro and in vivo studies [Sibilia et al., 2003; Schneider et al., 2009; Laflamme et al., 2010]. Smurf1 knockout mice were reported to have an increased bone mass phenotype due to the enhanced ostogenic differentiation [Zhao et al., 2004; Zhao et al., 2010]. To our knowledge, this study is the first to demonstrate an interaction between Wnt signaling and EGF; specifically, EGF suppression of Wnt/β-catenin signaling-induced osteogenic differentiation. Moreover, we identified that Smurf1 mediates the inhibitory effect of EGF on Wnt/β-catenin signaling and osteogenic differentiation.

The role of Smurf1 in the Wnt/β-catenin pathway was explored by multiple methods. First, we performed TOP-Flash reporter assays and found that Smurf1, but not Smurf1-CA, significantly decreased the transcriptional activity of β-catenin. Second, overexpression of Smurf1, but not Smurf1-CA, significantly reduced β-catenin protein levels. Third, depletion of Smurf1 by siRNA increased β-catenin protein levels. Finally, overexpression of Smurf1 abolished Wnt/β-catenin-induced osteogenic differentiation. The inhibitory effect of EGF on osteogenic differentiation was rescued by the depletion of Smurf1.

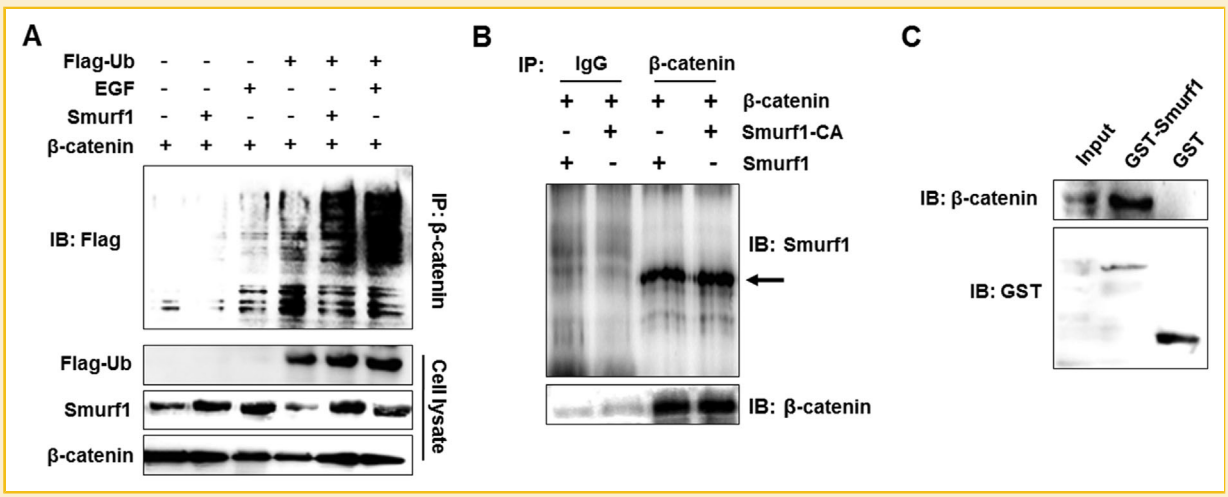

Fig. 5. Smurf1 associates with β-catenin and enhances the ubiquitination of β-catenin. (A) C2C12 cells were transfected with the indicated plasmids (Flag-tagged ubiquitin (Flag-Ub), β-catenin, or Smurf1) and incubated in the presence or absence of EGF for 24 h. Sixteen hours before cell harvest, the cells were treated with MG132. Cell lysates were then prepared and immunoprecipitated (IP) with a β-catenin antibody. The IP were analyzed by immunoblotting (IB) with the Flag antibody. The cell lysates were also analyzed by IB with β-catenin, Flag and Smurf1 antibodies. (B) C2C12 cells were transfected with the indicated Smurf1 expression plasmids along with β-catenin for 24 h, and the cell lysates were IP with β-catenin or control IgG, followed by IB with Smurf1 and β-catenin antibodies. (C) HEK293T cells were transfected with β-catenin for 24 h. Protein lysates (input) from HEK293T cells expressing β-catenin were mixed with GST as a control (GST) or GST fused to Smurf1 (GST-Smurf1) on beads. The beads were then pulled down by centrifugation and used for IB with β-catenin or GST antibodies.

Another major finding of this study is that Smurf1 physically associates with β-catenin and enhances its ubiquitination, which in turn accelerates proteasomal degradation of β-catenin. There have been reports that Smurf1 negatively regulates osteogenic differentiation by enhancing the proteasomal degradation of Smad1/5, Runx2, MEKK2, and JunB [Xing et al., 2010; Lee et al., 2013]. Accumulating evidence has shown that protein ubiquitination plays various roles in regulating Wnt/β-catenin signaling. For example, the tumor suppressor gene CYLD regulates Wnt/β-catenin signaling through the ubiquitination of the cytoplasmic effector Dishevelled [Tauriello et al., 2010]. The EDD E3 ubiquitin ligase ubiquitinates β-catenin, leading to the destabilization of β-catenin [Hay-Koren et al., 2011]. In the context of the antagonizing role of Smurf1 in the Wnt signaling pathway, Fei et al. recently reported that Smurf1-mediated non-proteolytic poly-ubiquitination of Axin negatively regulates Wnt/β-catenin signaling transduction [Fei et al., 2013]. In the present study, we identified that EGF-induced Smurf1 functions as an E3 ligase to promote the ubiquitination of β-catenin.

The potential physical interaction of Smurf1 with β-catenin was tested in the present study using co-immunoprecipitation and GST pull-down assays. The data shows that Smurf1 co-immunoprecipitated with β-catenin in vitro and in vivo. Furthermore, Smurf1′s E3 ligase activity is required for the downregulation of β-catenin protein levels, given that Smurf1, but not Smurf1-CA, reduced TOP-Flash activity and the β-catenin protein levels. In addition, the proteasome inhibitor MG 132, reversed the effect of EGF and Smurf1 on β-catenin degradation, indicating that EGF promotes β-catenin degradation through induction of Smurf1.

β-catenin does not possess a PPXY motif, which is the typical recognition motif for Smurf1 and other Nedd4 family ligases [David et al., 2013]. However, other domains or amino acid residues of the protein substrate besides the common motif could interact with Smurf1. WFS1 (Wolfram syndrome protein), for example, interacts with and is ubiquitinated by Smurf1, resulting in proteasomal degradation, although the proline-rich motifs were absent in this protein [Guo et al., 2011]. Given our observations using co-immunoprecipitation and GST pull-down assays demonstrating that Smurf1 associates with β-catenin and accelerates β-catenin ubiquitination and proteasomal degradation, further study is justified to determine which region of β-catenin is critical for Smurf1-mediated degradation.

In summary, EGF suppresses Wnt/β-catenin-induced osteoblast differentiation. EGF downregulates β-catenin protein levels in a Smurf1-dependent manner. Smurf1 suppresses Wnt/β-catenin-induced osteoblast differentiation. Although a PPXY motif is absent in β-catenin, Smurf1 physically associates with β-catenin and promotes its ubiquitination and proteasomal degradation. Downregulation of β-catenin protein levels by EGF/Smurf1 depends on Smurf1′s E3 ligase activity. Taken together, the data suggests that Smurf1 plays a role in EGF-inhibited osteogenic differentiation by acting as a biologically relevant E3 ligase to promote the ubiquitination and degradation of β-catenin.

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