Ubiquitin-dependent protein degradation is involved in various biological processes, and accumulating evidence suggests that E3 ubiquitin ligases play important roles in cancer development. Smad ubiquitin regulatory factor 1 (Smurf1) and Smurf2 are E3 ubiquitin ligases, which suppress transforming growth factor-β (TGF-β) family signaling through degradation of Smads and receptors for TGF-β and bone morphogenetic proteins. In addition, Smurf1 has been reported to promote RhoA ubiquitination and degradation and regulate cell motility, suggesting the involvement of Smurf1 in cancer progression. However, the regulation and biological function of Smurf1 and Smurf2 in cancer development remain to be elucidated. In the present study, we show the post-translational regulation of Smurf1 by Smurf2 and the functional differences between Smurf1 and Smurf2 in the progression of breast cancer cells. Smurf2 interacted with Smurf1 and induced its ubiquitination and degradation, whereas Smurf1 failed to induce degradation of Smurf2. Knockdown of Smurf2 in human breast cancer MDA-MB-231 cells resulted in increases in the levels of Smurf1 protein, and enhancement of cell migration in vitro and bone metastasis in vivo. Of note, knockdown of Smurf1, but not of Smurf2, enhanced TGF-β signaling in MDA-MB-231 cells, suggesting that increased an protein level of Smurf1 offsets the effect of Smurf2 knockdown on TGF-β signaling. These results indicate that two related E3 ubiquitin ligases, Smurf1 and Smurf2, act in the same direction in TGF-β family signaling but play opposite roles in cell migration.

Ubiquitin-dependent protein degradation plays key roles in various biological processes, including cell cycle progression, signal transduction, transcriptional regulation, receptor down-regulation, and endocytosis (1, 2). Protein ubiquitination is generally catalyzed by sequential activity of three different types of enzymes: an ubiquitin activation enzyme (E1), ubiquitin-conjugation enzymes (E2), and ubiquitin ligases (E3) (1). Among them, E3 ubiquitin ligases play a crucial role in recognition of target proteins and in subsequent protein degradation by the 26 S proteasomes (3).

Smad ubiquitin regulatory factor 1 (Smurf1) and Smurf2, a Smurf1-related protein, are HECT (homologous to the E6-accessory protein C terminus)-type E3 ubiquitin ligases that regulate transforming growth factor-β (TGF-β) and bone morphogenetic protein (BMP) signaling (4, 5). Smurf1 was originally identified as an E3 ubiquitin ligase for Smad1 and Smad5, intracellular signaling mediators for BMPs (4). Smurf2 interacts with Smad1 as well as Smad2, an intracellular signaling molecule for TGF-β, and induces their ubiquitin-mediated degradation (5, 6). Moreover, Smurf1 and Smurf2 induce ubiquitin-dependent degradation of receptors for TGF-β and BMPs (7–11). Through interaction with Smads and receptors for TGF-β and BMP, Smurf1 and Smurf2 attenuate TGF-β family signaling. However, Smurf2 has also been reported to bind to transcriptional co-repressor SnoN (Ski-related novel protein N) via Smad2 and to degrade SnoN (12). Thus, Smurf2 may enhance TGF-β signaling under certain conditions.

In addition to TGF-β/BMP signaling, Smurf1 and Smurf2 have other targets. For example, Smurf1 and Smurf2 interact with Runx2, a master regulator of osteoblast differentiation, and mediate its proteasomal degradation in osteoblasts (13, 14). Moreover, Smurf1 induces ubiquitination and turnover of MEKK2 to reduce bone formation in vivo (15).

Accumulating evidence suggests that Smurf1 and Smurf2 play important roles in cancer development. Smurf1 has been reported to promote RhoA ubiquitination and degradation and to regulate cell motility (16–19). Smurf2 can target RhoA for ubiquitination; however, in contrast to Smurf1, Smurf2 activity toward RhoA is restricted by the intramolecular interaction between the Smurfl C2 and HECT domains (20). Loukopoulos et al. (21) reported that the human SMURF1 gene is amplified in some cases of pancreatic adenocarcinoma. In addition, Fukuchi et al. (22) reported that high expression of Smurf2 correlates...
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with poor prognosis in patients with esophageal squamous cell carcinoma. On the other hand, up-regulation of Smurf2 has been reported to activate replicative senescence, which occurs through a novel mechanism independent of its ligase activity (23). Therefore, the roles of Smurf1 and Smurf2 in cancer development remain to be elucidated.

In the present study, we show the functional differences between Smurf1 and Smurf2 in the progression of breast cancer cells. Smurf2 interacts physically with Smurf1 and induces its ubiquitination and degradation, whereas Smurf1 fails to induce degradation of Smurf2. Knockdown of Smurf2 in human breast cancer MDA-MB-231 cells results in increased levels of the Smurf1 protein, leading to enhancement of cell migration in vitro and bone metastasis in vivo. The present findings thus provide evidence of functional interaction of Smurf2 with Smurf1 and of different activities between the two related E3 ligases in the development of cancer.

EXPERIMENTAL PROCEDURES

Cell Cultures—MDA-MB-231, a human breast cancer cell line, was obtained from the American Type Culture Collection (ATCC, Manassas, VA). 293FT was obtained from Invitrogen. MDA-MB-231 cells and 293T cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. 293FT cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.1 mM minimum essential medium with non-essential amino acids (Invitrogen), and 1 mM sodium pyruvate (Invitrogen).

Construction of Plasmids, Lentiviral Vector Production, and Infection—Wild-type (WT) and ligase inactive mutants (CA) of Smurf1 and Smurf2 were FLAG, 6Myc, or hemagglutinin epitope-tagged at their N termini and subcloned into pcDNA3 vector or pcDEF3 vector as described previously (24). 6Myc-Smad2, 6Myc-Smad7, and FLAG-tagged ubiquitin (FLAG-Ub) were described previously (24).

Micro-RNA (miRNA) expression vectors were constructed using the BLOCK-It Pol II miIRNA expression vector kit (Invitrogen) by annealing each oligonucleotide (supplemental Table 1) followed by ligation into the pcDNA6.2-GW/EmGFP-miR vector, which was prelinearized and sequenced. miRNA coding regions were then transferred to the lentivector construct pCSII-CMV-RfA (25) by the Gateway system (Invitrogen).

To produce lentiviruses expressing miR-Smurfl or miR-Smurf2, 293FT cells were transfected using Lipofectamine 2000 (Invitrogen) with three plasmids: an miRNA vector construct, a VSV-G and Rev-expressing construct (pCMV-VSVG-RSV-Rev), and a packaging construct (pCAG-HIVgp). The culture supernatants were collected 48 h after transfection, and viral particles were concentrated by centrifugation. For lentiviral infection, 7 × 10^5 MDA-MB-231 cells/dish in 10-cm culture dishes were infected with lentivirus vectors at 100 plaque-forming units/cell.

Small interfering RNA (siRNA) and Oligonucleotides—siRNAs were introduced into cells (26) using the Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer’s instructions with 30 pmol of siRNA and 5 μl of transfection reagent per well of a 6-well plate for MDA-MB-231 cells. Expression vectors were co-transfected with siRNAs. The siRNA duplex corresponding to human Smurf1 (5′- GCCA-GAUAUGAAAGAUUGGAGAA-3′) was purchased from Invitrogen. Stealth control siRNA (Invitrogen, Stealth™ RNAi Negative Control Low GC Duplex) was used as a control. Annealing was performed as described previously (27).

Real-time RT-PCR—Quantitative real-time RT-PCR was performed as described. MDA-MB-231 cells were seeded at a concentration of 1 × 10^5 cells/well in 6-well plates. Two days after seeding, total RNA was extracted from cells using TRIzol reagent (Invitrogen). First-strand cDNA was synthesized with Primers reverse transcriptase (TaKaRa Bio, Shiga, Japan) and oligo(dT). Real-time PCR was performed using SYBR Green PCR master mix (Invitrogen) and the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). PCR primers used are listed in supplemental Table 2. The specificity of detected signals was confirmed by a dissociation curve consisting of a single peak. All samples were run in duplicate in each experiment. Values were normalized by human HPRT1.

Cell Migration Assay—To assess cell motility, chamber migration assays were performed using a cell culture insert (8-μm pore size, 12-well format; BD Biosciences). MDA-MB-231 cells were seeded in duplicate at a density of 5 × 10^4 cells/well/ chamber. Twenty hours later, cells that had not moved to the lower wells were removed from the upper face of the filters using cotton swabs, and cells that had moved to the lower surface of the filter were stained with a Diff-Quik kit (Sysmex, Hyogo, Japan). Cell migration was quantified by visual counting after being photographed. Experiments were performed in duplicate. Mean values for three random fields were obtained for each well.

Cell Proliferation Assay—MDA-MB-231 cells were seeded in duplicate at a density of 1 × 10^4 cells/well in 24-well plates and cultured for up to 96 h. Cells were trypsinized and counted at the times indicated using a Coulter counter (Beckman Coulter). Cell culture medium was replenished every second day.

Transfection of cDNAs, Immunoprecipitation, and Immunoblotting—Transfection of DNA was performed using FuGENE 6 (Roche Diagnostics) according to the manufacturer’s recommendations. Cells were lysed using Igepal CA630 lysis buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Igepal CA630, 1 mM phenylmethylsulfonyl fluoride, and 100 units/ml aprotinin. After centrifugation, protein concentrations of cell lysates were quantified with a DC protein assay kit (Bio-Rad Laboratories). In some experimental settings, cells were treated with 10 μM MG132 (Peptide Institute, Osaka, Japan) prior to harvesting to inhibit proteasomal degradation. For immunoprecipitation, cell lysates and antibodies were incubated anywhere from 2 h to overnight. Immune complexes were then precipitated with protein G-Sepharose beads. SDS-polyacrylamide gel electrophoresis and immunoblotting were performed as described previously (28), except for the use of an LAS-3000 mini lumino-image analyzer (Fujifilm, Tokyo). To co-immunoprecipitate Smurf2 by the anti-Smurf1 antibodies, MDA-MB-231 cells were lysed in TNTE buffer (20 mM Tris-
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HCl, pH 7.5, 120 mM NaCl, 1 mM EDTA, and 0.5% Triton-X 100) supplemented with protease inhibitors. Cell lysates were first incubated with 20 μl of protein G-Sepharose beads (pre-blocked with 100 mg/ml bovine serum albumin) for 1 h with gentle rotation. The cleared supernatants were then incubated with 4 μg of the anti-Smurf1 antibodies and protein G-Sepharose beads for 4 h. Normal goat IgG was used as negative control. The beads were intensively washed with TNTE buffer, and bound proteins were separated by SDS-polyacrylamide gel electrophoresis and subjected to immunoblot analysis.

The following antibodies were used: anti-Smurfl F-20 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-FLAG M2 (Sigma), anti-Myc 9E10 (BD Biosciences), anti-hemagglutinin (Invitrogen, San Diego, CA), anti-α-tubulin DM1A (Sigma), anti-β-actin (Sigma), anti-phospho-Smad2 (Cell signaling, Beverly, MA), and anti-Smad2/3 (BD Biosciences). Anti-Smurfl anti-serum was prepared as follows: a 16-mer peptide, CDPRLAER-RVRSQRHR, which corresponds to residues 224–239 of human Smurf2, was synthesized and conjugated to keyhole limpet hemocyanin (Calbiochem) using succinimidyl 4-(p-maleimidophenyl)butyrate (Pierce). The conjugate was injected into rabbits in Freund’s complete adjuvant, and antiserum was collected from which anti-Smurfl antibody was affinity-purified.

Protein Stability Assay—293T cells were transiently transfected with expression plasmids for the indicated proteins. After 24 h, cells were incubated for the indicated times with 10 μg/ml of cycloheximide (Sigma). Cells were lysed in lysis buffer as described above, and the cell extracts were analyzed by immunoblotting using anti-Myc, anti-FLAG, and anti-β-actin antibodies.

Intracardiac Experimental Metastasis Model—All animal procedures were performed in the animal experiment room of the Japanese Foundation for Cancer Research (JFCR) according to the guidelines proposed by the Science Council of Japan. Female BALB/c nu/nu mice (5 weeks old) were purchased from Charles River Japan, Inc. (Kanagawa, Japan). Mice were maintained under specific pathogen-free conditions.

Subconfluent MDA-MB-231 cells (2 × 10⁵) were suspended in 200 μl of sterile Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and injected into the left ventricle of the heart with a 26-gauge needle under anesthesia with diethyl ether. Mice were imaged for luciferase activity immediately after injection to exclude any mice that had not been successfully xenografted.

Radiographic Analysis of Bone Metastasis—Development of bone metastasis was monitored by x-ray radiography once a week for up to 9 weeks. Mice were anesthetized (50 mg/kg sodium pentobarbital), placed in a prone position on wrapped film (Fujifilm), and exposed to x-irradiation at 23 kV for 90 s using a soft x-ray apparatus (SOFRON; Sofron Co., Tokyo). Film was developed using a Fuji medical film Processor (SEPROS SV; Fujifilm) and inspected for visible bone lesions.

Statistical Analysis—For quantitative real-time RT-PCR, cell migration analysis, and in vivo experiments, the results are expressed as means ± S.D. Student’s t test was used to calculate the significance of differences between the two samples. For multiple comparisons of the data, the Tukey-Kramer method was used to calculate the significance of the differences.

RESULTS

Knockdown of Smurf2 Enhances Cell Migration of MDA-MB-231 Cells—To explore the functional difference between Smurf1 and Smurf2 in cancer cells, we established human breast cancer MDA-MB-231 cells that stably express miR-control (MDA control cells), miR-Smurfl (MDA-S1KD cells), and miR-Smurfl (MDA-S2KD cells) through use of a lentivirus system. At least a 70% reduction in mRNA levels of Smurf1 (Fig. 1A, upper panel) and Smurf2 (Fig. 1A, lower panel) in MDA-S1KD cells and MDA-S2KD cells, respectively, was confirmed by real-time RT-PCR. Expression level of Smurf2 in MDA-S1KD cells and that of Smurf1 in MDA-S2KD cells did not differ compared with those in MDA-control cells.

Because both Smurf1 and Smurf2 are known to inhibit TGF-β signaling, we first examined the effect of Smurf1 or Smurf2 knockdown on TGF-β signaling in MDA-MB-231 cells. As shown in Fig. 1B, knockdown of Smurf1 enhanced induction of the TGF-β target genes, Smad7 (upper panel) and PAI-1 (lower panel). However, unexpectedly, knockdown of Smurf2 failed to do so.

Cell migration is a critical metastatic event that occurs in various epithelial cells during cancer progression. As Smurf1 has been reported to induce cell migration of cancer cells through degradation of RhoA (19), we next examined whether Smurf2 also affects the cell migration of MDA-MB-231 cells. Cell migration was examined using a chamber migration assay. As expected, Smurf1 knockdown prevented cell migration of MDA-MB-231 cells (Fig. 1C). Interestingly, knockdown of Smurf2 significantly enhanced migration of MDA-MB-231 cells, suggesting a functional difference between Smurf1 and Smurf2 in affecting migration of the breast cancer cells. Similar results were obtained using other miRNA target sequences (data not shown). Of note, the cell growth rate of MDA-S1KD cells and that of MDA-S2KD cells were not different compared with that of MDA-control cells (Fig. 1D), indicating that neither Smurf1 nor Smurf2 knockdown affected cell growth of MDA-MB-231 cells.

Smurf2 Interacts with Smurf1 and Induces Its Ubiquitination and Degradation—To elucidate the mechanisms underlying enhanced cell migration of MDA-S2KD cells, we examined whether Smurf2 affects the expression or the function of Smurf1 in the cells. Although knockdown of Smurf2 did not affect the mRNA level of Smurf1 in MDA-MB-231 cells (see Fig. 1A), Smurf2 knockdown increased the expression level of Smurf1 protein (Fig. 2A). Of note, Smurf1 knockdown did not affect the protein level (Fig. 2A) and the mRNA level (see Fig. 1A) of Smurf2. These results suggest that Smurf2 induces degradation of Smurf1 protein, although Smurf1 is unable to induce degradation of Smurf2. As Smurf1 up-regulates cell migration of cancer cells through degradation of RhoA (19), we also investigated the levels of RhoA and the activation status of RhoA in MDA-S1KD cells and MDA-S2KD cells. No substantial change in the total levels of RhoA and the activation status of RhoA were observed in Smurf1 or Smurf2 knockdown cells (supplemental Fig. S1 and data not shown), presumably because the activity of Smurf1
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FIGURE 1. The effects of knockdown of Smurf1 and Smurf2 on migration of MDA-MB-231 cells. A, expression levels of Smurf1 mRNA (upper panel) and Smurf2 mRNA (lower panel) in MDA-control cells, MDA-S1KD cells, and MDA-S2KD cells were measured by quantitative real-time RT-PCR. B, effects of Smurf1 or Smurf2 knockdown on the transcription of Smad7 (upper panel) and PAI-1 (lower panel) were determined by quantitative real-time RT-PCR. MDA-MB-231 cells were treated with TGF-β (1 ng/ml) for 1 h (upper panel) or 24 h (lower panel). Each value was normalized to the expression of HPRT1 and represented a mean of duplicate determination. C, the effects of Smurf1 or Smurf2 knockdown on cell migration of MDA-MB-231 cells were determined by a chamber migration assay. MDA-control cells, MDA-S1KD cells, and MDA-S2KD cells were seeded in the upper chambers of 8-μm pore culture inserts. Twenty hours later, cells that migrated through the membranes were fixed, stained, and then counted. Representative pictures of the staining of migrated cells are shown (upper panel). The graph shows the number of cells that invaded through the membrane (lower panel). D, effects of Smurf1 or Smurf2 knockdown on cell growth of MDA-MB-231 cells. MDA-control cells, MDA-S1KD cells, and MDA-S2KD cells were cultured for 24, 48, or 96 h, and cell numbers were counted.

Smurf1 in transfected 293T cells. As shown in Fig. 2D, Smurf2 induced ubiquitination of the catalytically inactive mutant of Smurf1, Smurf1(CA), suggesting that Smurf2 acts as an E3 ubiquitin ligase for Smurf1. Smurf1 also induced ubiquitination of Smurf1 itself in trans. Although both Smurf1 and Smurf2 ubiquitinated Smurf1 (Fig. 2D) and Smad7 (supplemental Fig. S2) (7, 8) at the same intensity, Smurf1 and Smurf2 failed to induce ubiquitination of Smurf2(CA) (Fig. 2D). Co-expression of Smurf2(WT) with Smurf1(CA) produced a significant, dose-dependent decrease in steady-state levels of Smurf1 protein (Fig. 2E). In contrast, Smurf2(CA) had little effect on Smurf1 protein levels. To confirm that the reduced abundance of Smurf1(CA) in response to Smurf2 expression resulted from increased proteolysis, we measured the half-life of Smurf1(CA) in the absence or presence of co-expressed Smurf2(WT) or Smurf2(CA). Expression of Smurf2(WT) enhanced the turnover of Smurf1(CA), whereas co-expression of Smurf2(CA) had no effect (Fig. 2F). These results indicate that Smurf2 specifically induces degradation of Smurf1 depending on its ligase activity.

Decreased Smurf1 Cancels the Enhancement of Cell Migration in Smurf2 Knockdown MDA-MB-231 Cells—To explore whether knockdown of Smurf2 induces cell migration by increasing the expression of Smurf1 protein, we examined the effect of Smurf1 knockdown on migration of MDA-S2KD cells. Smurf1 was knocked down by siRNA transfection, and expression of the mRNAs (Fig. 3A) of proteins (supplemental Fig. S3) of Smurf1 and Smurf2 in the transfected MDA-control cells and MDA-S2KD cells was confirmed by real-time RT-PCR and immunoblotting, respectively. As shown in Fig. 3B, knockdown of Smurf1 prevented migration of MDA-S2KD cells down to the level of the transfected MDA-control cells, suggesting that Smurf2 reduces migration of MDA-MB-231 cells through degradation of the Smurf1 protein. We also examined the expression levels of known substrates of Smurfs, including Smad1, Smad2, Smad7, SnoN, and TβR-I. Although Smad7 was slightly increased upon knockdown of Smurf1 or Smurf2, the other...
**FIGURE 2. Ubiquitination and degradation of Smurf1 by Smurf2.** A, expression levels of endogenous Smurf1 (upper panel) and Smurf2 proteins (middle panel) in MDA-control cells, with or without Smurf1 siRNA transfection, and MDA-S2KD cells were examined by immunoblotting. α-tubulin expression was examined as an internal control (lower panel). B, interaction of Smurf2 with Smurf1 in transfected 293T cells. 293T cells were transfected with the indicated plasmids. 6Myc-Smad2 was used as a positive control to compare the intensity of interaction. The top panel shows the interaction, and the lower two panels show the expression of each protein. Asterisks indicate nonspecific bands. C, interaction of endogenous Smurf2 with endogenous Smurf1 in MDA-MB-231 cells. The cells were treated with 10 μM MG132 for 12 h. Immunoprecipitation (IP) using normal goat IgG and anti-Smurf1 antibody was performed using cell lysate of MDA-MB-231, and co-precipitated Smurf2 (upper panel) and precipitated Smurf1 (lower panel) were detected by immunoblotting using anti-Smurf2 antibody and anti-Smurf1 antibody, respectively. D, ubiquitination of Smurf1 by Smurfs. 293T cells were transfected with the indicated plasmids and treated with 10 μM MG132 for 6 h. Cell lysates were subjected to immunoprecipitation with anti-FLAG antibody (FLAG-Ub, FLAG-tagged ubiquitin) followed by anti-Myc immunoblotting. The upper panel shows ubiquitination of Smurf1, and the lower two panels show the expression of each protein. E, degradation of Smurf1 by Smurf2(WT) but not by Smurf2(CA). 293T cells were transfected as indicated, and steady-state levels of Smurf1 protein in the presence or absence of Smurf2(WT) or Smurf2(CA) were determined by immunoblotting as shown in the upper panel; the lower two panels show the expression of Smurf2 and β-actin. F, Smurf2(WT), but not Smurf2(CA), accelerated Smurf1 protein turnover. 293T cells were transiently transfected with the indicated constructs. Twenty-four hours after transfection, the cells were treated with 10 μg/ml of cycloheximide (CHX) for the indicated periods and harvested for immunoblotting with anti-Myc, anti-FLAG, and anti-β-actin antibodies.
substrates were only marginally affected (supplemental Fig. S1). These results, together with the results shown in Fig. 1B, indicate that cell motility of Smurf2-knocked down cells was promoted through the mechanism independent on TGF-β-signaling.

Knockdown of Smurf2 Enhances Bone Metastasis of MDA-MB-231 Cells in Vivo—To examine the effect of endogenous Smurf2 on bone metastasis of breast cancer cells in vivo, MDA-control cells, MDA-S1KD cells, and MDA-S2KD cells were inoculated into the left cardiac ventricle of immunodeficient mice to allow the formation of bone metastasis. Progression of bone metastasis was assessed by whole-body x-ray radiography. As shown in Fig. 4, the frequency of bone metastasis in the whole body (Fig. 4A) as well as tibiae (Fig. 4B) of mice inoculated with MDA-S2KD cells was higher than that in mice inoculated with MDA-control cells. On the other hand, knockdown of Smurf1 did not affect bone metastasis of MDA-MB-231 cells in vivo. Interestingly, the area of bone metastasis in the tibiae of mice inoculated with MDA-S2KD cells was larger than that in mice inoculated with MDA-control cells or MDA-S1KD cells (Fig. 4C). These results suggest the possibility that Smurf2 prevents bone metastasis of breast cancer cells in vivo.

DISCUSSION

Because ubiquitin-dependent protein degradation plays key roles in various biological processes, the activities of E3 ubiquitin ligases are frequently regulated by several modifications including phosphorylation, acetylation, and ubiquitination. The Nedd4-like family of E3 ubiquitin ligases includes nine members, e.g. Smurf1, Smurf2, AIP/Itch, Nedd4-2, and WWP1, and shares a similar structure, i.e. a C2 domain at the N terminus, two to four WW domains in the middle of the protein, and a HECT domain at the C terminus (29). Among them, AIP/Itch has been reported to be phosphorylated in response to epidermal growth factor (30) or insulin-like growth factor (31). Nedd4-2 is phosphorylated by serum and glucocorticoid-inducible kinase (SGK1), a target of the phosphatidylinositol 3-kinase (PI3K)/PDK1 pathway (32). Moreover, AIP/Itch and WWP1 have been reported to be regulated by ubiquitin-dependent degradation (29, 33). However, little is known regarding regulation of Smurf1 and Smurf2 by post-translational modifications.

In the present study, we demonstrated that Smurf2 posttranslationally regulates Smurf1, whereas the reverse is not true. Knockdown of Smurf2 in breast cancer MDA-MB-231 cells increased expression of Smurf1 protein and enhanced cell migration in vitro and bone metastasis in vivo, whereas Smurf1 knockdown in the cells reduced cell migration in vitro. Knockdown of Smurf1, however, did not affect bone metastasis of MDA-MB-231 cells in vivo, presumably because Smurf1 knockdown reduced TGF-β-independent cell migration but

FIGURE 3. The effects of Smurf1 knockdown on migration of MDA-S2KD cells. A, mRNA expression levels of Smurf1 (upper panel) and Smurf2 (lower panel) with or without Smurf1 siRNA transfection in MDA-control cells and MDA-S2KD were measured by quantitative real-time RT-PCR. B, the effects of Smurf1 knockdown on migration of MDA-S2KD cells were determined by a chamber migration assay. MDA-control cells and MDA-S2KD cells were seeded in the upper chambers of 8-μm pore culture inserts. Twenty hours later, cells that migrated through the membranes were fixed, stained, and then counted. Representative pictures of staining of migrated cells are shown (upper panel). The graph shows the number of cells that invaded through the membrane (lower panel).
In summary, we have shown that Smurf2 represses the migration of MDA-MB-231 cells through the degradation of Smurf1. We also have shown the difference of function between Smurf1 and Smurf2 in human breast cancer cells. The regulation and biological functions of E3 ubiquitin ligases are very complex processes. However, understanding the oncogenic or tumor suppressive potential of E3 ubiquitin ligases may facilitate the identification and development of biomarkers and drug targets in human cancer.

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