THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 30, pp. 27039–27047, July 29, 2011

Domain and Functional Analysis of a Novel Breast Tumor Suppressor Protein, SCUBE2*[S]

Received for publication, March 29, 2011, and in revised form, June 6, 2011 Published, JBC Papers in Press, June 7, 2011, DOI 10.1074/jbc.M111.244418

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Signal peptide CUB (complement proteins C1r/C1s, Uegf, and Bmp 1)-EGF domain-containing protein 2 (SCUBE2) is a secreted, membrane-associated multidomain protein composed of five recognizable motifs: an NH_2-terminal signal peptide sequence, nine copies of epidermal growth factor (EGF)-like repeats, a spacer region, three cysteine-rich repeats, and one CUB domain at the COOH terminus. Our previous clinical study showed that *SCUBE2* may act as a novel breast tumor suppressor gene and serve as a useful prognostic marker. However, the specific domain responsible for its tumor suppressor activity and the precise mechanisms of its anti-tumor effect remain unknown. Using a combination of biochemical, molecular, and cell biology techniques, we further dissected the molecular functions and signal pathways mediated by the NH_2-terminal EGF-like repeats or COOH-terminal CUB domain of SCUBE2. Independent overexpression of the NH_2-terminal EGF-like repeats or COOH-terminal CUB domain resulted in suppression of MCF-7 breast cancer cell proliferation and reduced MCF-7 xenograft tumor growth in nude mice. Molecular and biochemical analyses revealed that the COOH-terminal CUB domain could directly bind to and antagonize bone morphogenetic protein activity in an autocrine manner, whereas the NH_2-terminal EGF-like repeats could mediate cell-cell homophilic adhesions in a calcium-dependent fashion, interact with E-cadherin (a master tumor suppressor), and decrease the β-catenin signaling pathway. Together, our data demonstrate that SCUBE2 has growth inhibitory effects through a coordinated regulation of two distinct mechanisms: antagonizing bone morphogenetic protein and suppressing the β-catenin pathway in breast cancer cells.

Signal peptide CUB-EGF domain-containing protein 2 (*SCUBE2*) belongs to a small, evolutionarily conserved *SCUBE* gene family. Three isoforms have been identified and named SCUBE1 to SCUBE3 [3] by order of their discovery from zebrafish to humans (1–8). These genes, coding polypeptide molecules of about 1,000 amino acids, share a modular protein domain organization including five recognizable motifs: an NH_2-terminal signal peptide sequence, nine tandem repeats of an epidermal growth factor (EGF)-like domain, a large *N*-glycosylated spacer region, three repeated stretches of six-cysteine residues (cysteine-rich) with unique and regular spacing, and one CUB domain at the COOH terminus.

SCUBE2 is expressed in the vascular endothelium and in several nonendothelial cell types, including fibroblasts, renal mesangial cells, and mammary ductal epithelium (3, 9). The biological function of SCUBE2 in these normal cell types is currently unknown. However, SCUBE2 expression was also detected in primary invasive breast tumors (9–14). Our previous clinical study showed that patients with positive SCUBE2 protein-expressing tumors had better prognosis than those with negative SCUBE2 protein-expressing tumors in terms of disease-free survival (9). Consistently, ectopic overexpression of the full-length SCUBE2 protein resulted in suppression of MCF-7 breast cancer cell proliferation and reduced MCF-7 xenograft tumor growth in nude mice (9). Although our data suggest that *SCUBE2* can act as a novel breast tumor suppressor gene, the specific protein domain responsible for its tumor suppressor effect and the exact mechanisms of its anti-tumor activity are not well defined.

In this study, we investigated whether the NH_2-terminal EGF-like repeats or the COOH-terminal CUB domain of SCUBE2 could independently execute the growth suppression function of SCUBE2 in MCF-7 breast cancer cells. The COOH-terminal CUB domain binds and antagonizes the proliferation signaling mediated by bone morphogenetic protein (BMP), whereas the NH_2-terminal EGF-like repeats function as a Ca^{2+}-dependent homophilic adhesive module that interacts with E-cadherin and suppresses β-catenin signaling.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—HEK-293T (human embryonic kidney-293 cells stably expressing the large T-antigen of SV40 (simian virus 40)) and A2058 melanoma cells were maintained in Dulbecco's minimal essential medium (DMEM) sup-

* This work was supported by Taiwan National Science Council Grants NSC 97-2320-B-038-019-MY3 (to C. J. C.) and NSC 99-2311-B-001-022-MY3 (to R. B. Y.) and Academia Sinica Grant AS-97-FP-L16 (to R. B. Y.).

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Experimental Procedures and Figs. 1–9.

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[3] The abbreviations used are: SCUBE, signal peptide CUB-EGF-like domain-containing protein; CUB, complement proteins C1r/C1s, Uegf, and Bmp 1; BMP, bone morphogenetic protein; BMPR, BMP receptor; Dox, doxycycline; TCF, T-cell factor; LEF, lymphoid enhancer factor; FL, full-length; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

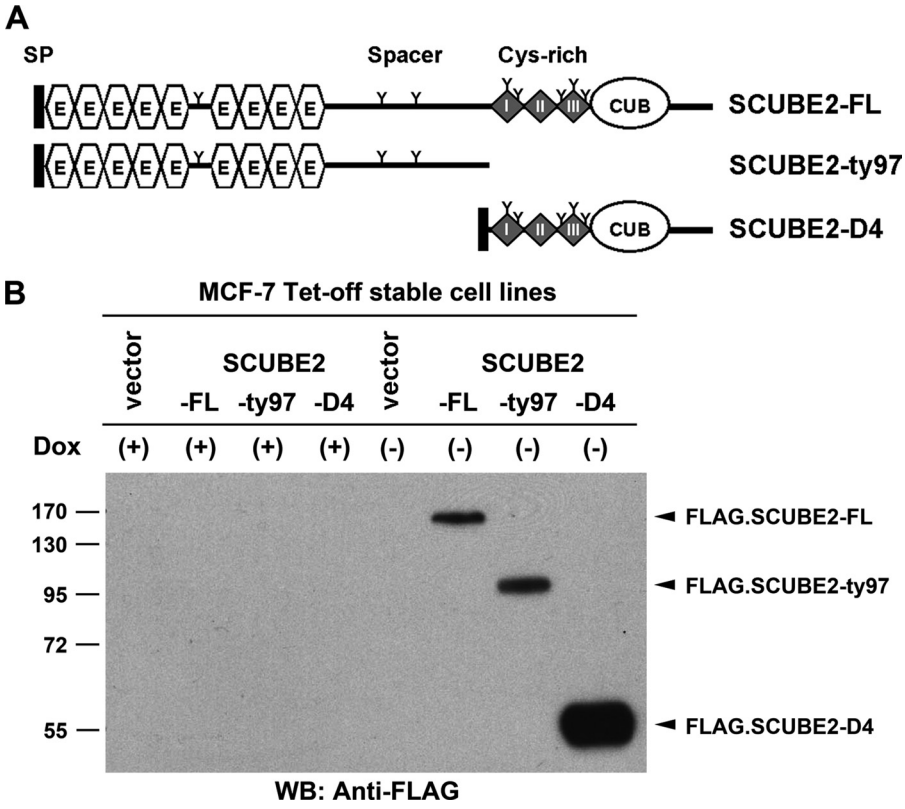

FIGURE 1. **Induction of ectopic SCUBE2 FL or deletion mutant protein (ty97 or D4) in MCF-7 Tet-Off stable clone cells.** *A*, domain organization of the SCUBE2 expression constructs used in this study. A FLAG epitope tag was added immediately after the signal peptide sequence at the NH_2 terminus for easy detection. *FL*, amino acids 1–1028; *ty97*, amino acids 1–659; *D4*, amino acids 664–1028; *SP*, signal peptide; *E*, EGF-like repeats; *Cys-rich*, cysteine-rich motifs; *CUB*, CUB domain. *B*, expression of ectopic SCUBE2 protein was confirmed by Western blot (*WB*) analysis. MCF-7 Tet-Off vector or SCUBE2-FL, -ty97, or -D4 stable cells were cultured in medium without doxycycline (*(−)Dox*) for 5 days, and the induction of ectopic NH_2-terminal FLAG-tagged SCUBE2 FL or deletion mutant protein (ty97 or D4) was determined by Western blot analysis with anti-FLAG antibody.

plemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in an atmosphere of 5% CO_2. Cells were transfected by use of Lipofectamine 2000 (Invitrogen).

Immunoprecipitation and Western Blot Analysis—Two days after transfection, cell lysates were clarified by centrifugation at 10,000 × *g* for 20 min at 4 °C. Samples underwent immunoprecipitation and then Western blot analysis as described previously (15).

Establishment of the MCF-7 Breast Cancer Cell Line Stably Expressing SCUBE2—The MCF-7 tetracycline-off (Tet-Off) vector or MCF-7 Tet-Off SCUBE2 cell lines were derived from the stable transfection of MCF-7 Tet-Off cells (Clontech) with an empty pTRE2hyg plasmid (Clontech) or a plasmid encoding the FLAG-tagged full-length (FL), ty97, or D4 mutant of human SCUBE2 (FLAG.SCUBE2-FL, FLAG.SCUBE2-ty97, or FLAG. SCUBE2-D4, respectively). Stable cell clones were selected by resistance to G418 (100 μg/ml) and hygromycin (100 μg/ml) and grown in the presence of 10 μg/ml doxycycline (Dox; to suppress SCUBE2 expression).

Tumorigenesis and Growth of Breast Tumors in Vivo—MCF-7 breast tumor growth in xenograft mouse model was performed as described previously (9). All surgical procedures followed protocols approved by the Institutional Animal Care and Utilization Committee, Academia Sinica.

Cell Proliferation Assay—The effect of SCUBE2 on the proliferation of MCF-7 breast cancer cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as described previously (9).

Tumorigenesis and Growth of Breast Tumors in Vivo—Female athymic mice (8-week-old nu/nu strain BALB/cAnN.Cg-*Foxn1*nu/CrlNarl) were implanted with 0.5 mg of 17β-estradiol 60-day release pellet (Innovative Research of America) subcutaneously on the dorsal side. Before tumor cell implantation, mice were fed Dox-containing water (200 μg/ml). The MCF-7 Tet-Off SCUBE2-ty97 or -D4 or the MCF-7 Tet-Off vector clone cells (2×10^6 cells in 50% Matrigel, BD Biosciences) were injected into the mammary fat pads of female athymic mice. After tumor growth for 12 days, the mice were randomized to receive Dox-free or Dox-containing water to induce or suppress the expression of SCUBE2, respectively.

Cell Aggregation Assay—A2058 stable cells were detached by treatment with trypsin (0.01%)-EDTA (2.5 mM) and suspended in DMEM containing 5 mM $CaCl_2$ or Ca^{2+}-free medium, at 1×10^6 cells/ml in polystyrene tubes. Then tubes were incubated on a rotating platform (10 rpm) at 37 °C for 9 h. The cell aggregation was viewed microscopically and photographed. For quantitation, cell clusters of more than four cells were considered aggregated. The data are means ± S.E. of three independent experiments performed in duplicate.

Immunofluorescence—MCF-7 cells were fixed in 4% formaldehyde, blocked with 2% fetal bovine serum for 1 h, and incubated with mouse anti-E-cadherin antibody (Invitrogen) and chicken anti-SCUBE2 antibody for 1 h. The slides were washed

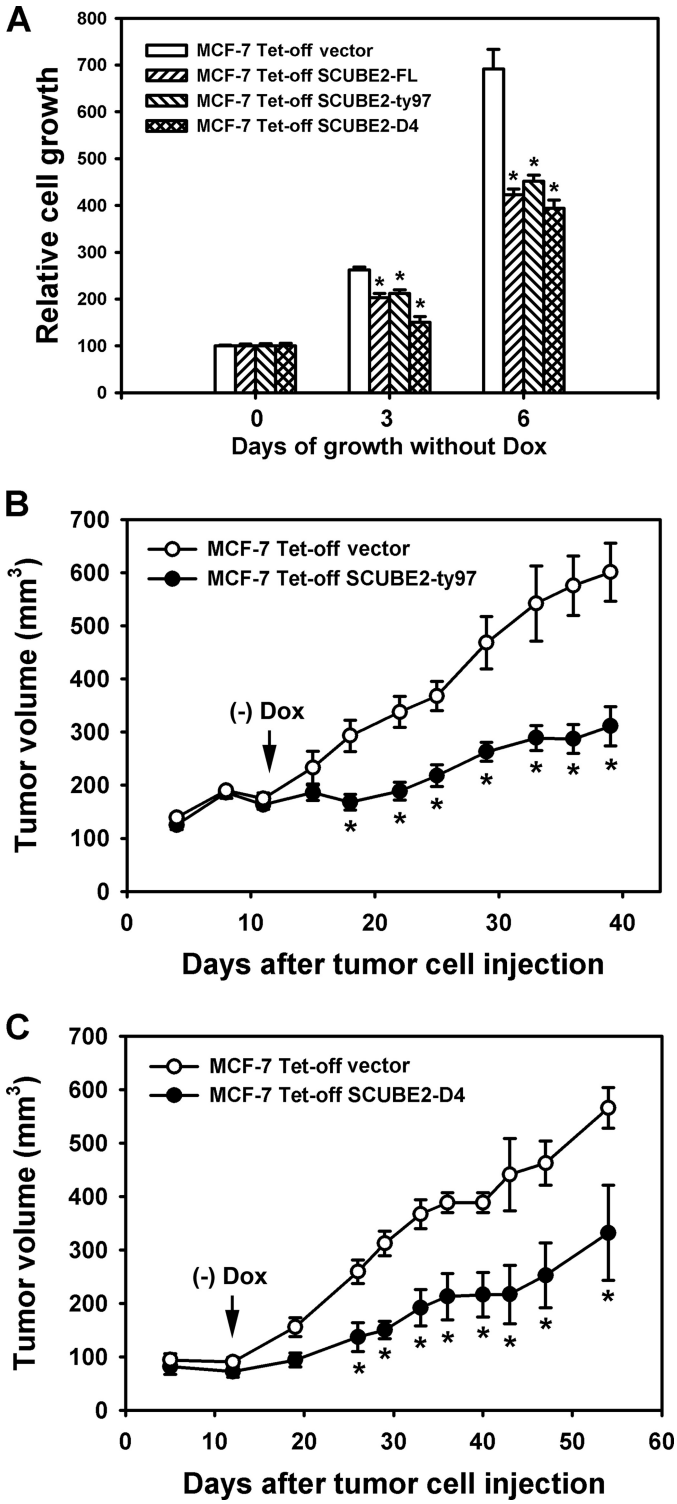

FIGURE 2. **Independent overexpression of NH_2- (ty97) or COOH- (D4) terminal fragment of SCUBE2 suppresses MCF-7 breast cancer cell proliferation *in vitro* and breast tumor growth *in vivo*.** *A*, overexpression of SCUBE2-FL, -ty97, or -D4 deletion protein suppresses MCF-7 breast cancer cell proliferation *in vitro*. The MCF-7 Tet-Off vector or SCUBE2-FL, -ty97, or -D4 stable cells were cultured in medium without doxycycline (*(−)Dox*) to induce the expression of SCUBE2 deletion protein. Cell proliferation was measured over the next 6 days by MTT assay. *, $p < 0.01$ (SCUBE2-FL, -ty97, or -D4 *versus* vector) ($n = 4$). *B* and *C*, induction of SCUBE2-ty97 and -D4 deletion protein reduces MCF-7 breast tumor growth in xenograft mouse model. The MCF-7 Tet-Off vector or SCUBE2-ty97 or -D4 stable cells were injected into nude mice to induce tumor formation. After tumor growth for 12 days and tumor development, the mice were divided into two groups to continue to receive doxycycline (*(+)Dox*) or not (*(−)Dox*). Growth of the MCF-7 Tet-Off vector or SCUBE2-ty97 or -D4 cells was measured as a function of time in the absence of doxycycline. Data points, mean tumor volumes (length × width × height × 0.5236 (in mm^3)); *error bars*, S.E. *, $p < 0.01$ ($n = 10$ or 7 for the MCF-7 Tet-Off vector tumors, $n = 10$ for the SCUBE2-ty97 tumors, and $n = 7$ for the SCUBE2-D4 tumors).

three times with PBS and stained with Alexa Fluor 488-labeled anti-mouse IgG antibody and Alexa Fluor 594-labeled anti-chicken IgG antibody for 1 h. The cells were washed three times in PBS, and slides were mounted by using VECTASHIELD mounting medium with DAPI (Vector Laboratories). Fluorescence images were captured at room temperature under a confocal microscope (model LSM 510; Carl Zeiss MicroImaging).

Luciferase Reporter Assays—MCF-7 Tet-Off stable cells (1.5×10^5 cells/well) were plated in 24-well plates and incubated overnight at 37 °C. The following day, cells were transfected with 0.5 μg of T-cell factor/lymphoid enhancer factor (TCF/LEF) luciferase constructs (containing the wild-type pTOP-FLASH or mutant pFOP-FLASH) (16) and internal control (0.05 μg of pRL-TK *Renilla* luciferase plasmid) (Promega) with FuGENE HD (Invitrogen) according to the manufacturer's instructions. The cells were cultured for an additional 2 days after the transfection and then harvested and prepared for TCF/LEF reporter assay. Reporter assays involved the Dual-Luciferase reporter assay system (Promega).

RESULTS

Establishing Inducible MCF-7 Breast Cancer Cell Lines Expressing SCUBE2 Full-length, NH_2-, or COOH-terminal Mutant Protein—To further investigate the specific domain contributing to the breast tumor suppressor activity, we engineered three stable MCF-7 breast cancer cell lines under the control of an inducible promoter, the Tet-Off promoter, with the expression of: 1) the FL protein, 2) the NH_2-terminal mutant (ty97) encoding nine EGF-like repeats and the spacer region mimicking the *ty97* allele in zebrafish (4, 5, 7), or 3) the COOH-terminal mutant (D4) containing the COOH-terminal fragment of three cysteine-rich motif repeats and the CUB domain. The FLAG epitope tag was added for easy detection of these constructs, and the lines were designated FLAG. SCUBE2-FL, -ty97, or -D4 in accordance with our previous study (8, 9) (Fig. 1*A*). The MCF-7 Tet-Off vector clone containing stable integration of the empty expression vector was established as the control. Removal of Dox in the medium induced the expression of FLAG.SCUBE2-FL, -ty97, and -D4 protein, as confirmed by anti-FLAG Western blot analysis (Fig. 1*B*). However, SCUBE2-FL or mutant protein was not induced in the presence of Dox in these stable MCF-7 cell lines or in the control MCF-7 Tet-Off vector clone (Fig. 1*B*).

Overexpression of SCUBE2 or Its Mutant Proteins Suppresses Proliferation, but Does Not Increase Apoptosis, of MCF-7 Breast Cancer Cells Both in Vitro and in Vivo—To investigate the effects of SCUBE2 expression on breast cancer cell growth, the MCF-7 Tet-Off vector and SCUBE2-FL, ty97, and -D4 stable cells were cultured in the presence or absence of Dox for up to 6 days to suppress or induce the expression of ectopic FLAG. SCUBE2-FL, -ty97, or -D4 protein. Cell growth was then measured by MTT assay. Induction of ectopic SCUBE2-FL protein,

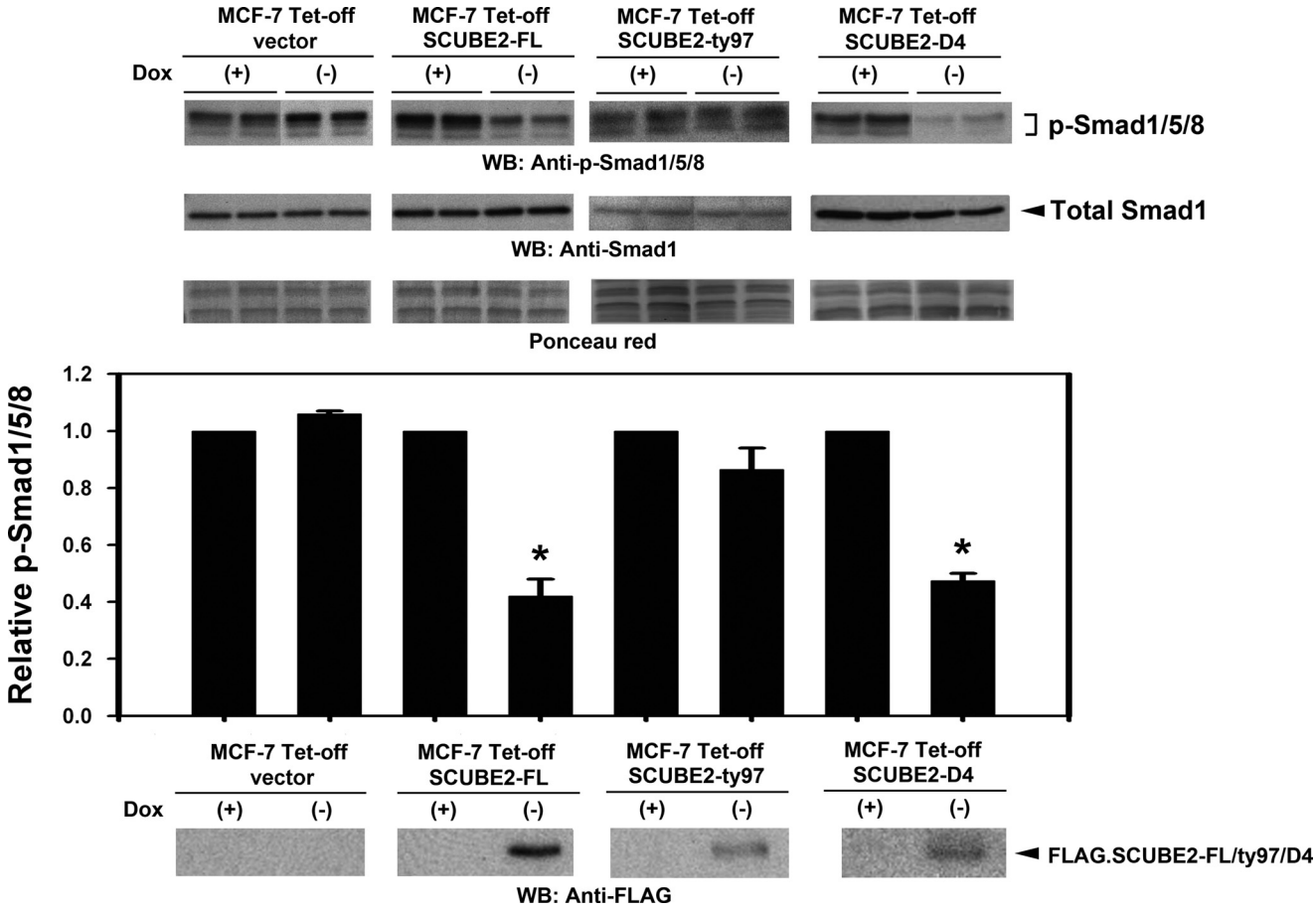

FIGURE 3. **SCUBE2 antagonizes autocrine BMP-mediated signaling through its CUB domain.** MCF-7 Tet-Off vector and SCUBE2-FL, -ty97, and -D4 stable cells were cultured in medium without doxycycline (*(−)Dox*) for 5 days, and the induction of ectopic NH_2-terminal FLAG-tagged SCUBE2-FL or deletion mutant protein (ty97 or D4) was determined by Western blot (*WB*) analysis with anti-FLAG antibody (*bottom panel*). Total protein lysates (20 μg) from each cell line in duplicate were subjected to Western blot analysis with an antibody specific against the phosphorylated Smad1/5/8 (*p-Smad1/5/8*) or total Smad1 protein (*top panel* and *second panel*). Ponceau red staining of the PVDF membrane confirmed the relative loading of total proteins for each sample (*third panel*). Shown is a histographic representation of quantified data of the phosphorylated Smad1/5/8 and total Smad1 protein concentrations in respective groups (quantified by densitometric scanning and normalized by the total amount of protein loading). Data are means ± S.E. of two experiments. *, $p < 0.01$ compare with *(+)Dox*.

as well as SCUBE2-ty97 and -D4, suppressed the proliferation of these MCF-7 cell lines in the absence of Dox (Fig. 2*A*). The control MCF-7 Tet-Off vector clone and SCUBE-FL, -ty97, and -D4 cells did not differ in growth on culture with Dox to block the expression of ectopic SCUBE2 proteins (data not shown).

Because overexpression of SCUBE2-ty97 or -D4 protein inhibited MCF-7 breast cancer cell growth *in vitro*, we next examined breast tumor growth *in vivo* in nude mice. MCF-7 Tet-Off vector or SCUBE2-ty97 or -D4 cells were injected into the mammary fat pads of nude mice that received estrogen pellets to promote the growth and development of breast tumors. After tumor growth for 12 days, the mice were fed Dox-free water to induce the expression of SCUBE2-ty97 or -D4 protein. Sustained expression of ectopic SCUBE2 or its mutant proteins in MCF-7 breast cancer cells in long term cultured conditions or in xenograft tumors was confirmed by Western blotting or immunohistochemistry, respectively (supplemental Fig. 1). In agreement with the tumor suppressor activity of SCUBE2-FL in our previous study (9), tumor growth from MCF-7 Tet-Off SCUBE2-ty97 or -D4 cells in mice was significantly lower than that of tumors from control MCF-7 Tet-Off vector cells (Fig. 2, *B* and *C*). Consistently, the proliferation index as evidenced by the number of nuclei stained positive for Ki-67 (a cell proliferation marker) was significantly lower in tumors expressing SCUBE2-FL, -ty97, or -D4 protein than in tumor tissues not expressing these proteins (supplemental Fig. 2).

To further clarify whether overexpression of SCUBE2-FL or its mutant proteins also increased cell death in MCF-7 breast cancer cells, we then performed the terminal deoxynucleotidyl end labeling (TUNEL) assay to evaluate the apoptotic status of different MCF-7 breast cancer cells or tumors. However, we did not reveal any discernable difference in the number of apoptotic (TUNEL-positive) cells in cultured conditions or in tumor tissues excised from mice injected with MCF-7 Tet-Off vector clone or MCF-7 Tet-Off SCUBE2-FL, -ty97, or -D4 cells grown in the absence of Dox (supplemental Fig. 3). Together, these results demonstrated that both the NH_2-terminal SCUBE2-ty97 and the COOH-terminal SCUBE2-D4 fragment, like the FL protein, are capable of breast tumor suppressor activity, but not inducing cell death, both *in vitro* and *in vivo*.

The COOH-terminal SCUBE2-D4 Protein, but Not the NH_2-terminal SCUBE2-ty97 Protein, Can Antagonize BMP Signaling in MCF-7 Breast Cancer Cells—BMPs are multifunctional growth factors involved in controlling cell differentiation and proliferation (17, 18) and have recently been implicated in pro-

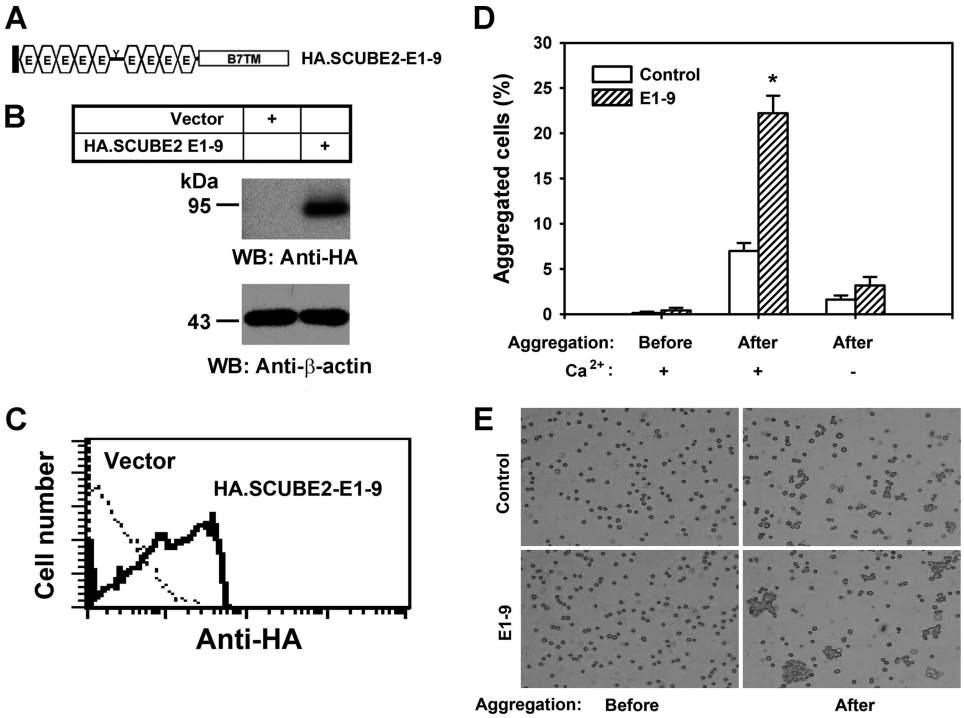

FIGURE 4. **The EGF-like repeats 1–9 are sufficient for homophilic reciprocal interactions of SCUBE2 in a Ca^{2+}-dependent manner.** *A*, domain composition of the SCUBE2 EGF-like repeats 1–9 mutant. The EGF-like repeats 1–9 deletion (*E1–9*, amino acids 47–444) were fused to the B7 transmembrane domain (*B7TM*) to target the chimeric protein on the plasma membrane. An HA epitope tag was added to monitor the expression of this mutant protein construct (HA.SCUBE2-E1–9). *B*, Western blot (*WB*) analysis of the HA.SCUBE2-E1–9 mutant protein. The A2058 melanoma stable line expressing the HA.SCUBE2-E1–9 mutant construct underwent Western blot analysis with anti-HA antibody to verify the expression of the recombinant protein. *C*, surface expression of the HA.SCUBE2-E1–9 mutant protein. The A2058 stable cell line was analyzed by flow cytometry with anti-HA antibody to confirm the surface targeting of this mutant protein. *D*, degree of aggregation in suspension of parental and HA.SCUBE2-E1–9 stable lines. Parental (*Control*) or HA.SCUBE2-E1–9 (*E1–9*) mutant stable cells were detached and allowed to aggregate in suspension culture in the presence (+) or absence (−) of Ca^{2+} in aggregation medium for 9 h. Aggregates were defined as clusters of four or more cells. *, $p < 0.01$ *versus* control cells. *E*, representative micrographs of aggregates formed by parental (control) or HA.SCUBE2-E1–9 mutant stable line. The experiments were performed three times in duplicate with similar results. Original magnification, ×100.

moting breast cancer cell growth (19, 20). Because a previous genetic study showed that zebrafish SCUBE2 could inhibit the BMP signaling when co-injected with BMP mRNA into the embryos (4), we thus investigated whether the breast cancer suppressor effect of SCUBE2 is attributed to its anti-BMP activity in MCF-7 breast cancer cells.

The BMP ligands transduce signaling by binding to two distinct transmembrane serine/threonine kinase type I and type II receptors (BMPR-I or BMPR-II) (17, 18). We examined whether these signaling components are expressed in MCF-7 breast cancer cells by RT-PCR analysis. As shown in supplemental Fig. 4, the mRNA for BMP ligands (BMP2 and BMP4) and receptors (BMPR-IA, BMPR-IB, and BMPR-II) are indeed expressed in MCF-7 breast cancer cells, which suggests that BMP signaling may act in an autocrine manner in these breast cancer cells. Because phosphorylation of Smad1/5/8 (phospho-Smad1/5/8) is a hallmark of the BMP pathway activation, we then compared the phosphorylation status of Smad1/5/8 among MCF-7 Tet-Off vector control cells or MCF-7 cells expressing SCUBE2-FL, ty97, or D4 protein. As shown in Fig. 3, immunoblotting with an anti-phospho-Smad1/5/8 antibody revealed a significant reduction (>50%) in basal phospho-Smad1/5/8 level by induced expression of SCUBE2-FL protein in MCF-7 cells. Furthermore, this inhibitory effect on BMP signaling can be attributed to the COOH-terminal SCUBE2-D4 protein and not to the NH_2-terminal protein region (Fig. 3). Furthermore, the BMP2-induced phospho-Smad1/5/8 level was also markedly suppressed by overexpression of SCUBE2-FL or SCUBE2-D4 mutant protein in MCF-7 breast cancer cells (supplemental Fig. 5). Together, our results revealed that reduction of proliferative BMP signaling through the COOH-terminal portion of SCUBE2 may be responsible for SCUBE2-mediated tumor suppressor activity in breast cancer cells.

The NH_2-terminal EGF-like Repeat Motif of SCUBE2 Acts as a Homophilic Adhesive Module in a Calcium-dependent Fashion—To further elucidate the function and signaling mediated by the NH_2-terminal region of SCUBE2, we focused on the nine tandem copies of EGF-like repeats because these motifs are involved in homophilic/heterophilic protein-protein interactions and intracellular signal transduction (21, 22). A new deletion mutant (HA.SCUBE2-E1–9) containing an HA tag at the NH_2 terminus (for easy detection) and the EGF-like repeats 1–9 fused to the B7 extracellular and transmembrane domain (B7TM) (for cell surface expression) was constructed and stably expressed in A2058 melanoma cells lacking endogenous SCUBE2 expression (Fig. 4*A*). Western blot analysis and flow cytometry with anti-HA antibody confirmed that ectopic HA.SCUBE2-E1–9 protein was expressed by A2058 cells and transported to the cell surface (Fig. 4, *B* and *C*).

To examine the involvement of SCUBE2 EGF-like repeats in intercellular adhesion, we evaluated the aggregating properties of the SCUBE2 EGF-like repeats 1–9 mutant (E1–9) stable

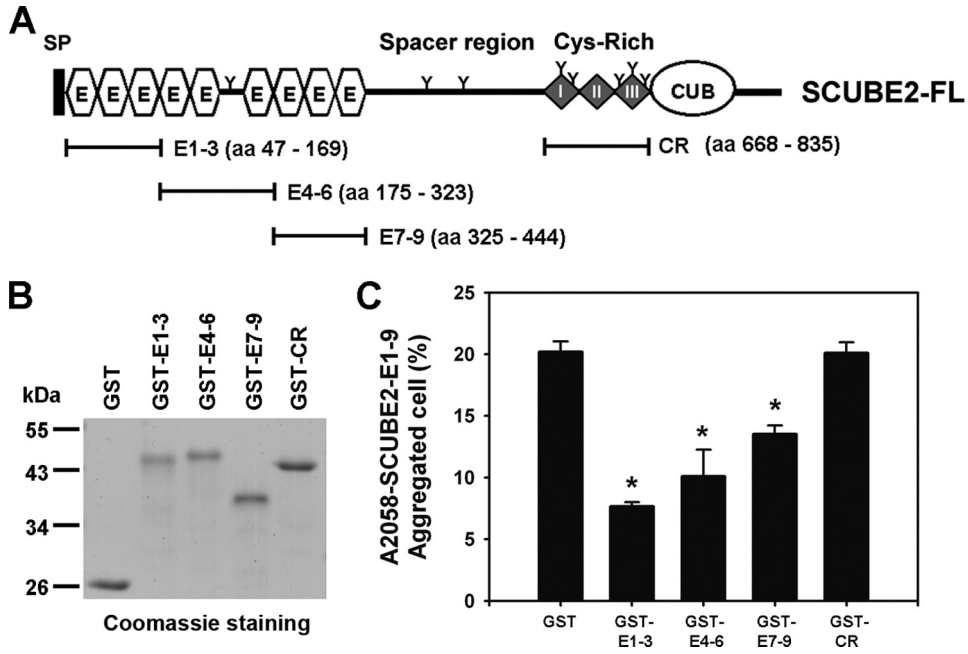

FIGURE 5. **Recombinant EGF-like repeat fragment inhibits SCUBE2-mediated cell aggregation.** *A*, domain structure of SCUBE2 with recombinant fragment. Diagram of the SCUBE2 protein shows the location of the recombinant GST fusion protein: GST-E1–3 (amino acids 47–169), GST-E4–6 (amino acids 175–323), GST-E7–9 (amino acids 325–444), and GST-CR (cysteine-rich repeat motifs) (amino acids 668–835). *SP*, signal peptide; *aa*, amino acid number. *B*, purification of recombinant SCUBE2 protein fragments. GST-SCUBE2 fusion proteins purified from the soluble fraction of bacterial lysates with glutathione-Sepharose beads were analyzed by SDS-PAGE and stained with Coomassie Brilliant Blue. *C*, specific blocking of SCUBE2-mediated cell aggregation by fragments of recombinant EGF-like repeats 1–9. The 9-h aggregation of HA.SCUBE2-E1–9 stable cells was performed in the presence of GST alone, GST-E1–3, GST-E4–6, GST-E7–9, or GST-CR recombinant protein (10 μg/ml) and expressed as the degree of aggregation (%). *, $p < 0.01$ *versus* GST control.

transfectant cell line by cell aggregation assay. Monolayer cultures of the parental (control) or transfected stable cells were dissociated into single cells and allowed to aggregate in suspension culture. When the suspensions were gently shaken, A2058 cells expressing the SCUBE2 E1–9 mutant tended to aggregate (Fig. 4, *D* and *E*). The parental cells aggregated poorly in suspension (5%), and a good proportion (~22%) of the SCUBE2 E1–9 mutant-transfected clone aggregated after 9 h of shaking (Fig. 4, *D* and *E*).

The chelating of calcium by Ca^{2+}-binding EGF-like repeats is important for the structural integrity and function of several other EGF-like domain-containing proteins (23, 24). Because 6 of 9 EGF-like repeats (*i.e.* repeats 1–3 and 7–9) contain Ca^{2+}-binding consensus sequences (3, 8), we then tested whether SCUBE2 EGF-like repeats 1–9 could function in a Ca^{2+}-dependent manner. When the same cell lines were dissociated with gentle pipetting in the presence of 5 mM EDTA or allowed to aggregate in the absence of Ca^{2+}, no apparent aggregation (<5%) was observed during the incubation period for either the parental or the EGF-like repeats 1–9 mutant transfectants (Fig. 4*D*). Consistent with these findings, the NH_2-terminal SCUBE2-ty97 mutant protein, but not the COOH-terminal SCUBE2-D4, could promote the homophilic reciprocal interactions of SCUBE2 in MCF-7 breast cancer cells in a calcium-dependent manner (supplemental Fig. 6).

To further determine the specificity and the relative contribution of each EGF-like repeat participating in homophilic, intercellular adhesion of SCUBE2, we added the recombinant GST fusion protein fragment containing the EGF-like repeats 1–3 (GST-E1–3), 4–6 (GST-E4–6), or 7–9 (GST-E7–9) or cysteine-rich motif (GST-CR) into the cell aggregation assay to block the cell-cell interactions. GST alone or GST-CR could not block SCUBE2-induced aggregation, but each soluble EGF-like repeat protein GST-E1–3, E4–7, or E7–9 markedly inhibited the A2058 SCUBE2-E1–9-mediated cell aggregation assay (Fig. 5). Together, these data suggest that each EGF-like repeat, not a specific one, participates in trans-interactions between SCUBE2 molecules on adjacent cells in a Ca^{2+}-dependent fashion.

The NH_2-terminal EGF-like Repeats of SCUBE2 Could Interact with E-cadherin—Because the anti-breast tumor activity and adhesive property of the NH_2-terminal EGF-like repeats are reminiscent of E-cadherin (a master tumor suppressor gene) that also mediates cell-cell homophilic adhesion in a calcium-dependent manner (25, 26), we next determined whether the EGF-like repeats of SCUBE2 could interact with E-cadherin. HEK-293T cells were transfected with HA.SCUBE2-E1–9 expression plasmid alone or together with a Myc-tagged E-cadherin (E-cadherin.Myc). Two days later, cell lysates were immunoprecipitated with anti-HA antibody, and the precipitates were analyzed by anti-Myc immunoblotting to determine the protein interaction. As shown in Fig. 6*A*, immunoprecipitation with anti-HA antibody for HA.SCUBE2-E1–9 resulted in a specific co-precipitation of the E-cadherin.Myc protein. Similar pulldown experiments in HEK-293T cells also showed that the FLAG-tagged SCUBE2 expression constructs encoding FL or ty97 but not D4 could specifically interact with E-cadherin (Fig. 6*B*). Likewise, immunoprecipitation of endogenous E-cadherin resulted in a pulldown of SCUBE2 protein in MCF-7 Tet-Off SCUBE2-FL cells (supplemental Fig. 7). Together, these data suggest that SCUBE2 protein could form a complex with E-cadherin mainly through its NH_2-terminal EGF-like repeats.

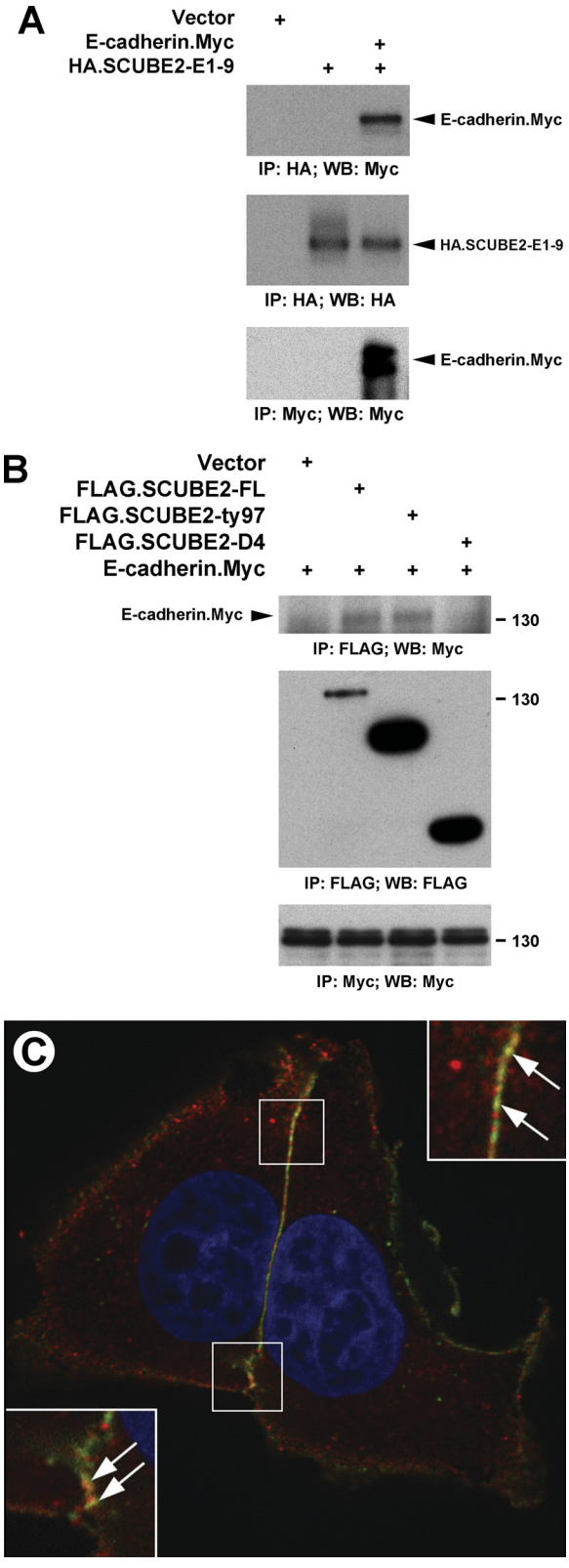

FIGURE 6. **SCUBE2 interacts and co-localizes with E-cadherin.** *A*, SCUBE2 interacts with E-cadherin through its NH_2 EGF-like repeats. Lysates of HEK-293T cells transiently transfected with HA-tagged SCUBE2 EGF-like repeats 1–9 (HA.SCUBE2-E1–9) alone or together with Myc-tagged E-cadherin (E-cadherin.Myc) constructs were immunoprecipitated (*IP*) with anti-HA antibody, and associated E-cadherin protein was determined by Western blot analysis with anti-Myc antibody (*top panel*). The protein expression of HA.SCUBE2-E1–9 and E-cadherin.Myc protein was verified by anti-HA and anti-Myc Western blot (*WB*) analysis, respectively (*middle* and *bottom panels*). *B*, the SCUBE2-FL or -ty97 forms a complex with E-cadherin. The E-cadherin expression construct (Myc-tagged) transfected alone or together with the expression plasmid encoded the indicated FLAG-tagged SCUBE2 proteins in HEK-293T cells. Two days after transfection, cell lysates underwent immunoprecipitation and Western blot with antibodies as indicated to determine the protein interactions. *C*, SCUBE2 co-localizes with E-cadherin at MCF-7 breast cancer cell contact sites. Co-localization of SCUBE2 and E-cadherin protein was visualized by confocal microscopy. SCUBE2 localization is detected by chicken anti-SCUBE2 antibody (9) and Alexa Fluor 594-conjugated goat anti-chicken IgG (*red*). E-cadherin is seen with mouse anti-E-cadherin antibody and Alexa Fluor 488-conjugated goat anti-mouse IgG (*green*). The overlay demonstrates co-localization of SCUBE2 with E-cadherin at cell-cell contact sites (*arrows*).

In agreement with these findings, SCUBE2 co-localized with endogenous E-cadherin at cell-cell contact sites in MCF-7 breast cancer cells on confocal immunofluorescence microscopy (Fig. 6*C*).

Expression of the SCUBE2 NH_2-terminal EGF-like Repeats Decreases the β-Catenin Protein Level and Suppresses the Transcriptional Activity of β-Catenin in Breast Cancer Cells—Because the cadherin-associated protein β-catenin can link cadherins to the actin cytoskeleton (26, 27) or translocate into the nucleus and up-regulate genes such as cyclin D1, cyclin-dependent kinase 4 (CDK4), and c-Myc (28–30) to modulate tumor development and progression, we further asked whether altered SCUBE2 expression affects β-catenin protein expression and transcriptional activity. Western blot analysis revealed that protein expression of β-catenin was significantly down-regulated in the MCF-7 Tet-Off SCUBE2-FL or -ty97 but not -D4 stable cells as compared with MCF-7 Tet-Off vector cells (Fig. 7). Furthermore, we investigated the transcriptional activity of β-catenin/TCF transcriptional factors by using a TCF reporter assay (TOP-FLASH) (16). As shown in Fig. 7, in the SCUBE2-FL- or -ty97-overexpressing MCF-7 cells, the relative TOP-FLASH activity was markedly reduced as compared with the vector control cells but not changed in SCUBE2-D4 cells. Therefore, suppression of breast cancer cell growth mediated by the NH_2-terminal EGF-like repeats was associated with E-cadherin interaction and related to the decreased β-catenin protein expression and transcriptional activity.

Because β-catenin plays critical roles not only in cadherin-mediated cell adhesion but also in canonical Wnt signaling, we also examined whether SCUBE2 affects the Wnt-induced β-catenin/TCF activity. However, expression of SCUBE2 or its mutant proteins produces no effect on the Wnt-induced β-catenin/TCF transcriptional reporter activity (supplemental Fig. 8). Furthermore, we also analyzed the phosphorylation of glycogen synthase kinase 3β, which phosphorylates and targets β-catenin for proteasomal degradation. Again, the overall phosphorylation status of glycogen synthase kinase 3β did not differ between the control cells and the SCUBE2-expressing MCF-7 breast cancer cells (supplemental Fig. 9). Together, these results suggested that SCUBE2-mediated decrease in β-catenin protein level is not directly regulated by canonical Wnt-glycogen synthase kinase 3β proteasomal degradation pathway.

DISCUSSION

Microarray gene expression profiling analyses have recently been used to uncover the signature genes associated with breast

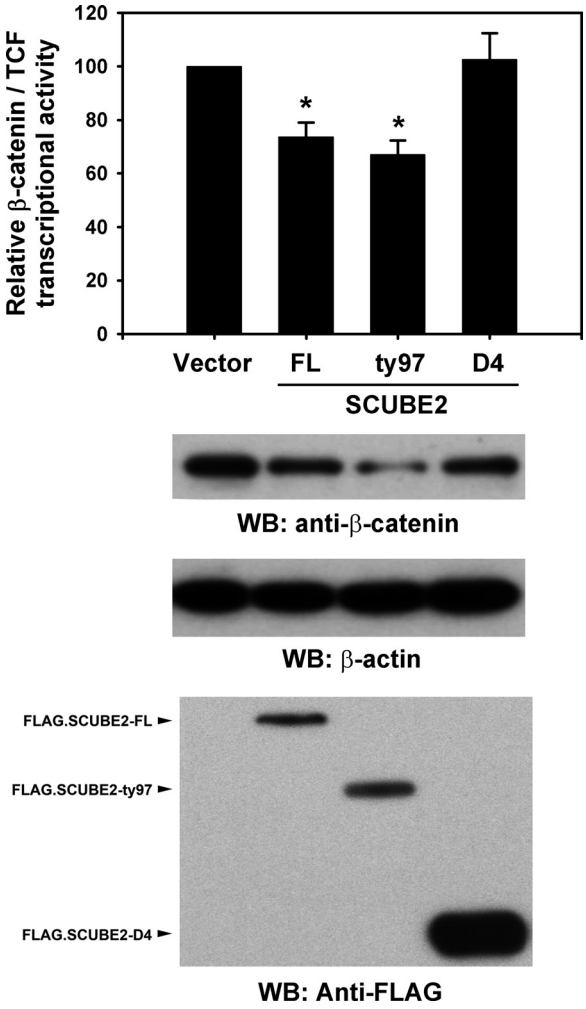

FIGURE 7. **Expression of NH_2-terminal SCUBE2 EGF-like repeats decreases β-catenin protein expression and reduces transcriptional activity of β-catenin.** MCF-7 Tet-Off vector or SCUBE2-FL, -ty97, or -D4 stable cells were cultured in medium without doxycycline (*(−)Dox*) for 5 days, and the induction of ectopic NH_2-terminal FLAG-tagged SCUBE2-FL or deletion mutant protein (ty97 or D4) was determined by Western blot (*WB*) analysis with anti-FLAG antibody (*bottom panel*). Total protein lysates (20 μg) derived from each cell line were subjected to Western blot analysis with antibody specific against β-catenin or anti-β-actin as a loading control, respectively. TCF luciferase constructs (0.5 μg) containing the wild-type (pTOP-FLASH) or mutant (pFOP-FLASH) TCF-binding sites (16) were transfected into MCF-7 Tet-Off vector or SCUBE2-FL, -ty97, or -D4 cells (1.5×10^5 cells/well). After 2 days, cells were harvested and prepared for the TCF/LEF reporter assay. The firefly (TOP-FLASH or FOP-FLASH) luciferase activity was corrected for *Renilla* luciferase activity (pRL-TK) to control for transfection efficiency. Relative luciferase activity was normalized by calculating the pTOP-FLASH/pFOP-FLASH activity ratio for each condition. Data were further normalized to values obtained in MCF-7 Tet-Off vector cells (100%). Results from three independent experiments were averaged (means ± S.E.). *, $p < 0.01$ *versus* vector.

cancer progression or even gene sets that can predict prognosis of breast cancers (11–14, 31, 32). However, comparison of these microarray data revealed very little gene overlap among these profiling studies, and virtually no novel breast tumor-associated genes are validated at the protein level. Intriguingly, a recent cross-platform microarray data analysis identified *SCUBE2* as the only gene shared among these gene expression profiling studies (10). In light of this finding, we demonstrated that alteration in SCUBE2 protein plays an important role in breast cancer cell growth and tumor progression and that SCUBE2 is a prognostic marker for a favorable clinical outcome (9). These studies indicate that *SCUBE2* appears to function as a novel breast tumor suppressor gene. However, the specific domain and exact mechanisms of its tumor suppressor function are not well defined.

In the present study, we further characterized the breast tumor suppressor effect of the *SCUBE2* gene in SCUBE2-overexpressing MCF-7 breast cancer cells. Our data suggest that the multidomain SCUBE2 protein is capable of executing its anti-breast tumor activity through a coordinated regulation of at least two distinct signaling mechanisms via its NH_2- or COOH-terminal region. The NH_2-terminal EGF-like repeats can interact with the master tumor suppressor E-cadherin and reduce β-catenin transcriptional activity, and the COOH-terminal CUB domain acts as an anti-BMP module in diminishing the proliferation-stimulating BMP autocrine signaling.

BMPs are multifunctional signaling molecules involved in modulation of proliferation, differentiation, and apoptosis in a variety of cell types (17, 18). Recent studies also showed that the BMP signal pathway is expressed and activated in breast tumors and contributes to breast cancer progression. For example, BMP2 enhanced the migration and invasion of a MCF-7 breast cancer cell line and supported tumor formation in a mouse model (20). In addition, inhibition of the BMP signal pathway by a dominant-negative form of BMPR-II reduced proliferation of T-47D breast cancer cells (19). Together, these data strongly support that BMP signaling plays an important role in breast cancer cell proliferation. Therefore, our results demonstrating that SCUBE2 via its COOH CUB domain could antagonize BMP signaling and suppress proliferation of MCF-7 breast cancer cells (Figs. 2 and 4) is of biological relevance and is consistent with anti-BMP activity of its orthologue in zebrafish model (4).

One important finding derived from this study is that the breast cancer suppressor SCUBE2 resides in close proximity with E-cadherin at cell-cell contact sites in breast cancer cells and can form a complex with E-cadherin (Fig. 6). Of note, E-cadherin associates strongly with SCUBE2 but weakly with SCUBE1 or SCUBE3 when overexpressed in HEK-293T cells and examined by a co-immunoprecipitation assay (Fig. 6 and data not shown). Although our data validated the specific interaction of SCUBE2 with E-cadherin through its NH_2-terminal EGF-like repeats (Fig. 6, *A* and *B*), the biochemical and structural basis responsible for such unique interaction requires further investigation. In addition, we were unable to demonstrate a "direct" physical interaction between recombinant FLAG-tagged SCUBE2 protein and *in vitro*-translated E-cadherin protein in a pulldown assay (not shown), so a yet unidentified cellular factor is essential to stabilize the SCUBE2-E-cadherin complex in breast cancer cells.

Another interesting observation is that SCUBE2 down-regulates β-catenin protein expression and reduces β-catenin transcriptional activity. A simple explanation is that SCUBE2 stabilizes the E-cadherin-adherens complex by recruiting β-catenin at the plasma membrane, thus lowering the total protein level of β-catenin within the cytoplasm and nucleus in breast cancer cells to result in reduced β-catenin-mediated transcription. However, SCUBE2 may be involved in the regulation of β-catenin stability and/or its degradation via other signal pathways. Regardless, further investigation is needed to shed light

on the mechanisms by which SCUBE2 decreases β-catenin protein expression and signaling in breast cancer cells.

Because β-catenin target genes, such as cyclin D1, CDK4, and c-Myc, play important roles in tumor development and progression, we analyzed the protein levels of these β-catenin targets by Western blot analysis. However, the protein expression of cyclin D1, CDK4, and c-Myc remained basically unchanged in the SCUBE2-overexpressing MCF-7 breast cancer cells as compared with vector control cells (data not shown). Therefore, genes other than these molecules must participate in SCUBE2-mediated suppression of breast cancer cell growth. Further studies are needed to elucidate the molecular identity of β-catenin targets involved in the SCUBE2-mediated tumor suppressor effect.

In human tumors, the loss or reduction in level of classical tumor suppressor genes such as E-cadherin can be caused by mutational inactivation or silencing of the promoter (33, 34). Such silencing takes place by an epigenetic mechanism (DNA methylation) (35) or through the action of the E-box-binding transcriptional repressors, including SNAIL (36, 37), SLUG (38), or TWIST (39). Because SCUBE2 mRNA is co-regulated with E-cadherin in a number of human breast cancer cell lines[4] and because CpG islands and multiple E-box elements (5′-CANNTG-3′) are present within the promoter region of the *SCUBE2* gene, this novel tumor suppressor gene *SCUBE2* may be silenced or inactivated by similar regulatory mechanisms during breast cancer progression, which we will address in our future studies.

In summary, our data unraveled the molecular basis for a novel breast tumor suppressor gene *SCUBE2* and demonstrated that SCUBE2 mediates its tumor suppressor effect via at least two distinct mechanisms in a coordinated suppression of the BMP and β-catenin signaling pathways.

Acknowledgments—We thank Konan Peck for critically reading the manuscript and Cheng-Fen Tu for technical assistance.

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