SCUBE2 Suppresses Breast Tumor Cell Proliferation and Confers a Favorable Prognosis in Invasive Breast Cancer

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Abstract

Signal peptide-CUB-epidermal growth factor–like domain-containing protein 2 (SCUBE2), originally identified from the endothelium and several nonendothelial primary cell types, was recently shown to be expressed in invasive breast carcinomas. However, the protein localization and biological significance of SCUBE2 in breast cancer are unknown. In this report, we show by anti-SCUBE2 immunostaining that SCUBE2 is mainly expressed in vascular endothelial and mammary ductal epithelial cells in normal breast tissue. In addition, we observed positive staining for SCUBE2 in 55% (86 of 156) of primary breast tumors. Patients with positive SCUBE2 protein–expressing tumors had better prognosis than those with negative SCUBE2 protein–expressing tumors in terms of disease-free survival. Multivariate analysis confirmed SCUBE2 protein expression as an independent prognostic factor for disease-free survival. Furthermore, overexpression of ectopic SCUBE2 protein 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 region of SCUBE2 directly bound to and antagonized bone morphogenetic protein 2 (BMP-2) signaling. These findings support the possible involvement of SCUBE2 in breast cancer progression and SCUBE2 may serve as a useful prognostic marker. [Cancer Res 2009;69(8):3634–41]

Introduction

Invasive breast carcinoma is the most common malignant disease for women worldwide and claims over 400,000 lives per year (1). To date, treatment decisions for breast cancer mainly depend on pathologic features and clinical stage (2). However, searching for effective molecular markers is necessary to predict the disease course and guide treatment decision (3, 4) due to unpredictable clinical courses in tumors with similar pathologic features at the same clinical stage.

Microarray gene expression profiling analyses have clearly defined the molecular signature of gene sets that could predict prognosis of breast cancers (5–10). However, these studies exhibit very little gene overlap, and only a few of the breast cancer–associated genes have been validated at the protein level. A recent gene expression profiling study (11) involving cross-platform comparisons of lists of genes derived from 70 genes (6, 7) and recurrence score models (8, 9) found the breast cancer gene expression profiles overlapping by only one gene signal peptide-CUB-epidermal growth factor (EGF)–like domain-containing protein 2 (SCUBE2).

SCUBE2 was previously identified from the endothelium by an integrated genomic approach (12). However, SCUBE2 mRNA was also expressed in several nonendothelial human primary cell types, such as fibroblasts and renal mesangial cells (12). SCUBE2 belongs to a small, evolutionarily conserved SCUBE gene family (12–18). Three different members have been described and designated as SCUBE1 to SCUBE3 by the order of their discovery (12–18). These genes coding for polypeptide molecules of ~1,000 amino acids share an organized protein domain structure of at least five motifs: an NH2 terminal signal peptide sequence, nine tandem repeats of EGF-like repeats, a large N-glycosylated spacer region followed by three repeated stretches of 6-cysteine residues with unique and regular spacing and one CUB domain at the COOH terminus (12–18). When overexpressed, SCUBE2 is a secreted glycoprotein that can form oligomers and has a stable association with the cell surface (12). Yet, its protein expression in normal breast tissue and the biological significance of SCUBE2 in breast cancer remain unknown.

In this study, we investigated the protein expression/function and clinical implication of SCUBE2 in breast cancers. Our results showed that changes in SCUBE2 protein play an important role in breast cancer cell proliferation and tumor progression, and SCUBE2 is a prognostic marker for a favorable clinical outcome.

Materials and Methods

Generation of the anti–SCUBE2-specific antibody. One peptide, NH2-SHICKEAPRGSVAC-COOH (derived from the NH2 terminal of human SCUBE2 protein sequence), was used as an antigen to immunize layer chickens. IgY was purified from the yolks of eggs from immunized hens.

Patients and tumor characteristics. We investigated 156 primary breast invasive ductal carcinoma samples from patients who underwent modified radical mastectomy. Specimens were collected at the Taipei Medical University–affiliated hospitals between 1998 and 2004. All patients were examined for axillary lymph node involvement. This study was approved by the institutional review boards of our hospitals. Recurrent or metastatic disease was determined by radiographic, sonographic, bone scan, or pathologic evidence. Information on the characteristics of patients (Table 1) were collected from clinical and pathologic records. Estrogen receptor (ER), progesterone receptor (PR), and human EGF receptor 2 (HER-2) status were determined and scored on immunohistochemistry as previously described (19, 20). Staging followed the guidelines of the Cancer Staging Manual of the American Joint Committee on Cancer (21). They were
alsogradedaccordingtoamodifiedversionoftheScarff-Bloom-Richardson
system (22). Exclusion criteria included patients who received previous
partial or total resection in other hospitals, tumors that cannot be totally
removed during operation, disease at stage IV, and occurrence of secondary
malignancy during follow-up or metastatic disease within 90 dafter surgery.

Of invasive carcinomas, 62% were associated with nodal disease. Of the 156
breast cancers, 90 (57.7%) were positive with ER, 68 (43.6%) with PR, and 35
(22.4%) with HER-2 overexpression, as seen on immunohistochemistry. At
the time of diagnosis, 16.0%, 47.4%, and 36.6% of patients had tumors at
stages I, II, and III, respectively. Median follow-up time for patients with
invasive tumors was 44.33 mo (range, 3.16–97.58 mo). In 49 cases, disease
relapsed and 19 patients died. Local and distant disease relapses that
occurred during the 90-d postsurgery period were considered part of the
primary event. Relapses after 90 days were considered new events. Relapses
were dated and reviewed through the medical record. Disease-free survival
was defined as the length of time after diagnosis to the first evidence of
clinical recurrence or metastatic disease.

Immunohistochemistry of SCUBE2 expression. Breast cancer speci-
men sections (4-μm thick) were dewaxed with xylene, rehydrated in graded
concentrations of alcohol, treated with 3% H2O2 for 30 min, washed with
PBS, blocked with normal horse serum for 30 min, and incubated at 4°C
overnight with anti-SCUBE2 antibody. Antibody binding was detected by
using biotinylated antichicken antibody and horseradish peroxidase
streptavidin (Vector) with 3,3'-diaminobenzidine as chromogen (DAKO).

Hematoxylin was used as the counterstain. The immunostaining was
considered positive when >10% of the tumor cells were immunoreactive.

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), a plasmid
encoding the FLAG-tagged full-length (FL) or D4 mutant of human SCUBE2
(FLAG.SCUBE2-FL or FLAG.SCUBE2-D4), respectively. Stable cell clones
were grown in the presence of 10 μg/mL doxycycline (to suppress SCUBE2
expression) and selected by resistance to G418 (100 μg/mL) and hygromycin
(100 μg/mL). Established cell lines were further verified by anti-FLAG
Western blot analysis to assess doxycycline-responsive FLAG.SCUBE2
protein expression.

Immunoprecipitation, Western blotting, and flow cytometric
analyses. 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 (12). Cell
surface expression of FLAG-tagged SCUBE2 was determined by anti-FLAG
antibody staining and analyzed by a FACScan machine (Becton Dickinson).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide 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. Briefly, actively growing MCF-7
Tet-off vector and MCF-7 Tet-off SCUBE2-FL or SCUBE2-D4 stable cells

Table 1. Association of SCUBE2 protein expression and clinicopathologic characteristics and other biomarkers

<table>
<thead>
<tr>
<th>Characteristics/markers</th>
<th>Total</th>
<th>SCUBE2 expression, n (%)</th>
<th>χ² test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>Age of patient, y</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>≥50</td>
<td>105</td>
<td>48 (45.7)</td>
<td>57 (54.3)</td>
</tr>
<tr>
<td>&lt;50</td>
<td>51</td>
<td>22 (43.1)</td>
<td>29 (56.9)</td>
</tr>
<tr>
<td>Histologic grade</td>
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</tr>
<tr>
<td>G1</td>
<td>24</td>
<td>10 (41.7)</td>
<td>14 (58.3)</td>
</tr>
<tr>
<td>G2</td>
<td>64</td>
<td>26 (40.6)</td>
<td>38 (59.4)</td>
</tr>
<tr>
<td>G3</td>
<td>68</td>
<td>34 (50.0)</td>
<td>34 (50.0)</td>
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<td>pT</td>
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</tr>
<tr>
<td>T1</td>
<td>38</td>
<td>14 (36.8)</td>
<td>24 (63.2)</td>
</tr>
<tr>
<td>T2</td>
<td>90</td>
<td>41 (45.6)</td>
<td>49 (54.4)</td>
</tr>
<tr>
<td>T3</td>
<td>15</td>
<td>7 (46.7)</td>
<td>8 (53.3)</td>
</tr>
<tr>
<td>T4</td>
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<td>8 (61.5)</td>
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<td>N2</td>
<td>23</td>
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<td>16 (69.6)</td>
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<tr>
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<td>10 (40.0)</td>
<td>15 (60.0)</td>
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<td>II</td>
<td>74</td>
<td>34 (45.9)</td>
<td>40 (54.1)</td>
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<td>III</td>
<td>57</td>
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<td>31 (54.4)</td>
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<td>31 (47.0)</td>
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<tr>
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<td>35 (38.9)</td>
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<td>Positive</td>
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</tr>
<tr>
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<td>107</td>
<td>35 (32.7)</td>
<td>72 (67.3)</td>
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were trypsinized and plated onto 96-well cell culture plates at 2,000 cells per well in 200 μL complete media containing doxycycline. Doxycycline was removed from the medium on the next day to induce gene expression for various times. Each data point was performed in quadruplicate, and the results are presented as relative cell growth (% mean ± SD).

**Tumorigenesis and growth of breast tumors in vivo.** Female athymic mice (8-wk-old nu/nu strain BALB/cAnNCg-Foxn1nu/CrlNarl) were purchased from the National Laboratory Animal Center. Animals were allowed to acclimate to the new environment for 1 wk before being implanted with 0.5 mg of 17β-estradiol 60-d release pellet (Innovative Research of America) s.c. on the dorsal side 1 d before tumor cell implantation to support the growth of the estrogen-dependent MCF-7 Tet-off cell-derived tumors. Before tumor cell implantation, mice were fed doxycycline-containing water (200 μg/mL), as described previously (23). For tumor cell implantation, the MCF-7 Tet-off SCUBE2-FL or the MCF-7 Tet-off vector clone cells were harvested, washed with PBS, and resuspended in PBS. Then, 2 × 10^6 cells in 0.2 mL of the mixture (50% Matrigel, BD Bioscience) were injected into the mammary fat pads of female athymic mice. After tumor growth for 12 d, the mice were randomized to receive doxycycline-free or doxycycline-containing water to induce or suppress the expression of SCUBE2, respectively. Tumor size was measured twice a week by using digital calipers and calculated by length × width × height × 0.5236 (in mm³). The experiments were terminated when the tumor size reached 800 mm³. Tumor growth in vivo was approximately exponential but varied slightly between animals. All surgical procedures followed protocols approved by the Institute Animal Care and Utilization Committee, Academia Sinica.

**Luciferase activity assays.** The bone morphogenetic protein (BMP)-responsive luciferase reporter assay was performed as described previously (16).

**In vitro digestion of SCUBE2 by purified recombinant matrix metalloproteinases.** The FLAG-tagged SCUBE2 protein produced by HEK-293T cells was incubated with various recombinant matrix metalloproteinase (MMP; 500 ng; R&D Systems) in the absence or presence of a broad-spectrum MMP inhibitor (GM6001, 20 μmol/L, Merck) in a buffer containing 20 mmol/L Tris-HCl (pH 7.4)/5 mmol/L CaCl₂/150 mmol/L NaCl/0.05%Brij-35 at 37°C for 2 h. The cleaved fragments were analyzed by probing with anti-FLAG (NH₂ terminus) or anti–cysteine-rich repeats (COOH terminus) antibody, respectively.

**Statistical analyses.** Association of positive and negative SCUBE2 protein expressions and clinicopathologic variables of the carcinoma specimens was evaluated by χ² test. Kaplan-Meier survival curves were calculated with tumor recurrence/metastasis or death due to breast cancer used as the end point. A log-rank test was used to calculate the disease-free survival, defined as the difference between SCUBE2-positive and SCUBE2-negative groups in time to recurrence. The Cox proportional hazard model was used to assess the effects of several possible prognostic factors with univariate analyses to identify independent prognostic factors for disease-free survival. All statistical tests were done with SPSS 10.0 (SPSS, Inc.). To compare the tumor growth rates of MCF-7 Tet-off SCUBE2-FL and MCF-7 Tet-off vector cells in animals, we estimated individual tumor volume at various times and then compared the growth rates by Student’s t test. A two-tailed P test was used in all analyses, and P < 0.05 was considered statistically significant.
Results

Characterization of the anti–SCUBE2-specific antibody. To localize SCUBE2 protein expression in normal breast tissue or tumors, we first generated a polyclonal antibody specifically against SCUBE2. As shown in Supplementary Fig. S1A, this antibody recognized only SCUBE2 and did not cross-react with SCUBE1 or SCUBE3. Furthermore, we used this antibody for immunocytochemistry to detect SCUBE2 protein overexpressed in HEK-293T cells under formalin-fixed, paraffin-embedded conditions. As shown in Supplementary Fig. S1B, cells expressing SCUBE2 protein were positive for anti-SCUBE2 staining, whereas cells expressing SCUBE1 or SCUBE3 proteins were negative.

Expression of SCUBE2 protein in normal breast tissue. Using the anti-SCUBE2 polyclonal antibody, we found that endogenous SCUBE2 protein was expressed on the ductal epithelial or vascular endothelial cell surface of normal breast tissue (Supplementary Fig. S2A). Preincubation of the antibody with the corresponding immunogen peptide resulted in no staining, further showing the specificity of the anti-SCUBE2 staining in the breast tissues (Supplementary Fig. S2B).

Expression of SCUBE2 protein is correlated with favorable disease-free survival of breast cancer. To explore the role of SCUBE2 in breast tumor progression, we conducted a retrospective study of SCUBE2 expression in 156 breast carcinoma biopsy samples. There are 86 (55.1%) of the primary tumors scored as positive for SCUBE2 (Supplementary Fig. S2D) and 70 cases (44.9%) were scored as negative for SCUBE2 (Supplementary Fig. S2C). To evaluate the potential contribution of SCUBE2 protein expression to prognosis of breast cancer, we compared clinicopathologic characteristics of cases scored as positive and negative for SCUBE2. As shown in Table 1, SCUBE2 protein expression had a significant negative association with tumor recurrence ($P < 0.0001$) and PR expression ($P = 0.02$) but is not associated with other characteristics.

The effect of SCUBE2 expression on disease-free survival was further analyzed by the Kaplan-Meier method. Survival analysis revealed a statistically significant relation between positive SCUBE2 protein expression and favorable disease-free survival in breast cancer patients (Fig. 1A). Patients were further stratified by initial tumor stage, but SCUBE2 protein expression remained significantly associated with favorable disease-free survival for patients with stage I ($P = 0.0183$), stage II ($P = 0.0165$), or stage III ($P = 0.0007$) disease (Fig. 1B–D).

Univariate analysis revealed a significant correlation between disease-free survival and SCUBE2 protein expression ($P < 0.0001$), lymph node involvement ($P = 0.02$), advanced clinical stage ($P = 0.0001$), or PR status ($P = 0.03$) of breast cancer (Table 2). Further multivariate analysis based on Cox proportional hazards models showed that clinical stage [hazard ratio (HR) 2.86, 95% confidence interval (95% CI) 1.25–6.51] and positive SCUBE2 protein expression (HR 0.26, 95% CI 0.13–0.49) remained independent prognostic factors for disease-free survival (Table 2).

Overexpression of SCUBE2 protein suppresses proliferation of MCF-7 breast cancer cell line. Because the clinicopathologic association study implied that positive SCUBE2 protein expression was negatively correlated with tumor recurrence, we speculated that overexpression of SCUBE2 protein may lead to suppression of growth of breast tumors. To test the hypothesis, we first engineered stable MCF-7 breast cancer cell lines (MCF-7 Tet-off SCUBE2-FL clones) with the expression of FLAG-SCUBE2-FL protein under the control of an inducible promoter, the Tet-off promoter. In addition, the MCF-7 Tet-off vector clones containing stable integration of the empty expression vector were established as controls. Doxycyclin was removed from the medium to induce the expression of FLAG-SCUBE2-FL protein, determined by anti-FLAG Western blot analysis 1 to 5 days after doxycycline withdrawal. FLAG-SCUBE2-FL protein was readily expressed within 1 day, and expression peaked at ~4 to 5 days after doxycycline removal (Fig. 2A). No induction of FLAG-SCUBE2-FL was observed in the presence of doxycycline in the MCF-7 Tet-off SCUBE2-FL clones (Fig. 2A) or in the control MCF-7 Tet-off Vector clones (data not shown).

To examine the effect of SCUBE2 overexpression on breast cancer cell growth, the MCF-7 Tet-off vector or MCF-7 Tet-off SCUBE2-FL stable cells were cultured in the presence or absence of doxycycline for 12 days to suppress or induce the expression of ectopic FLAG-SCUBE2-FL protein, respectively. Cell proliferation was then measured by MTT assay. Induction of ectopic SCUBE2-FL protein suppressed the growth of the MCF-7 Tet-off SCUBE2-FL clone cells in the absence of doxycycline (Fig. 2B). Furthermore, overexpression of the SCUBE2-D4 mutant protein, like the FL protein, suppressed the growth of the MCF-7 breast cancer cells (Supplementary Fig. S3). As a control, growth of MCF-7 Tet-off vector and MCF-7 Tet-off SCUBE2 cells did not differ on culture with doxycycline to block the expression of ectopic SCUBE2 protein (data not shown).

SCUBE2 represses tumor growth of MCF-7 cells in vivo. Because SCUBE2 overexpression inhibited MCF-7 breast cancer cell growth in vitro, we next investigated breast tumor growth in vivo in nude mice. MCF-7 Tet-off vector or MCF-7 Tet-off SCUBE2-FL cells were injected into the mammary fat pads of nude mice. A

### Table 2. Univariate and multivariate survival analysis by Cox proportional hazards models

<table>
<thead>
<tr>
<th>Variables</th>
<th>Univariate HR (95% CI)</th>
<th>P</th>
<th>Multivariate HR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCUBE2 (positive versus negative)</td>
<td>0.25 (0.13–0.47)</td>
<td>&lt;0.0001</td>
<td>0.26 (0.13–0.49)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Grade (1 and 2 versus 3)</td>
<td>1.39 (0.79–2.43)</td>
<td>0.26</td>
<td>1.12 (0.58–2.16)</td>
<td>0.74</td>
</tr>
<tr>
<td>Lymph node status (positive versus negative)</td>
<td>2.05 (1.10–3.82)</td>
<td>0.02</td>
<td>1.11 (0.46–2.68)</td>
<td>0.82</td>
</tr>
<tr>
<td>Stage (I and II versus III)</td>
<td>3.01 (1.71–5.30)</td>
<td>0.0001</td>
<td>2.86 (1.25–6.51)</td>
<td>0.01</td>
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<tr>
<td>ER (positive versus negative)</td>
<td>0.82 (0.46–1.44)</td>
<td>0.48</td>
<td>1.17 (0.53–2.56)</td>
<td>0.70</td>
</tr>
<tr>
<td>PR (positive versus negative)</td>
<td>0.51 (0.28–0.93)</td>
<td>0.03</td>
<td>0.73 (0.34–1.59)</td>
<td>0.43</td>
</tr>
<tr>
<td>HER-2/neu (positive versus negative)</td>
<td>1.39 (0.79–2.46)</td>
<td>0.26</td>
<td>1.02 (0.56–1.85)</td>
<td>0.95</td>
</tr>
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SCUBE2 antagonizes BMP activity. Recent genetic study on zebrafish showed that BMP activity can be attenuated by the coexpression of SCUBE2 (24) and indicated that the COOH terminal cysteine-rich repeats and the CUB domain are essential for zebrafish Scube2 function (24–26). In addition, BMPs are multifunctional growth factors that play important roles in normal cell differentiation and proliferation (27, 28) and have recently been implicated in promoting breast cancer cell proliferation (29, 30). We then examined whether or not the COOH terminal fragment of SCUBE2 can interact with BMP2 protein or affect BMP signaling.

A series of FLAG-tagged SCUBE2 deletion constructs was first generated, including the SCUBE2-D4 deletion construct encoding only the CUB domain (coexpression with BMP2 alone produced by the signaling cells acted as a long-range signaling molecule by inducing an increase of ~6-fold in luciferase activity (Fig. 3C)). Although the BMP2 protein coexpressed with SCUBE2-FL, SCUBE2-ty97, or SCUBE2-rw87 triggered the BMP-mediated transcriptional activation equally well, coexpression with the SCUBE2-D4 mutant resulted in marked attenuation of the BMP response (Fig. 3C).

Because the proteolytic processing of the large prepro precursor of BMP (proBMP) and its subsequent secretion into the extracellular space are the essential steps in the production of the biologically active form of BMP ligands, we then investigated whether the inhibition of BMP2 signaling by SCUBE2-D4 occurs in mice that received estrogen pellets to promote the growth and development of breast tumors as described in Materials and Methods. After tumor growth for 12 days, the mice were fed doxycycline-free water to induce the expression of SCUBE2-FL. Tumor growth from the MCF-7 Tet-off SCUBE2-FL cells in mice was markedly lower than that of tumors from control MCF-7 Tet-off vector cells (Fig. 2C). However, mice that continued to receive doxycycline-containing water to suppress the SCUBE2 induction showed no difference in tumor growth rate of MCF-7 Tet-off SCUBE2-FL or MCF-7 Tet-off vector cells (Supplementary Fig. S4). Together, these results showed that overexpression of SCUBE2 suppresses MCF-7 breast cancer cell growth both in vitro and in vivo.

**Figure 2.** SCUBE2 overexpression suppresses MCF-7 breast cancer cell proliferation in vitro and breast tumor growth in vivo. A, induction of ectopic SCUBE2-FL protein in MCF-7 Tet-off SCUBE2-FL clone cells. MCF-7 Tet-off SCUBE2-FL cells were cultured in the medium without doxycycline [(+)Dox] for 5 d, and the induction of ectopic NH2 terminal FLAG-tagged SCUBE2-FL protein expression was determined by Western blot analysis with anti-FLAG antibody. Anti-FLAG expression was used as a loading control. Double bands for the FLAG.SCUBE2-FL are due to the glycosylation of this protein (precursor and glycosylated, FL form). A limited proteolytic fragment is also observed after induction (cleaved). B, effect of SCUBE2 protein expression on MCF-7 breast cancer cell proliferation. The MCF-7 Tet-off vector and the MCF-7 Tet-off SCUBE2-FL stable cells were cultured in a medium without doxycycline [(+)Dox] to induce the expression of SCUBE2-FL protein. Cell proliferation was measured over the next 12 d by MTT assay. *, P < 0.01. C, induction of ectopic SCUBE2-FL protein reduces MCF-7 breast tumor growth in xenograft mouse model. The MCF-7 Tet-off vector or the MCF-7 Tet-off SCUBE2-FL stable cells were injected into nude mice to induce tumor formation. After tumor growth for 12 d and tumor development, the mice were divided into groups to continue to receive doxycycline or not [(+)Dox]. Growth of the MCF-7 Tet-off vector or the MCF-7 Tet-off SCUBE2-FL cells was measured as a function of time in the absence of doxycycline. Points, mean tumor volumes; bars, SE. *, P < 0.01.
the intracellular or extracellular environment. HEK-293T cells were transfected with plasmids expressing proBMP2 alone or in combination with various SCUBE2 deletion constructs. Western blot analysis of the cell lysates and conditioned media from these cultures revealed all deletion mutants with no effect on total proBMP2 synthesis (Fig. 3D); only the SCUBE2-D4 mutant potently suppressed the secretion of mature BMP2 into the culture medium. Consistently, whereas overexpressed SCUBE2-FL, or SCUBE2-ty97 proteins were secreted into the conditioned medium and properly targeted on the cell surface, the SCUBE2-D4 mutant was defective in membrane association and secretion (Fig. 4). Therefore, the COOH terminal fragment represented by SCUBE2-D4, which binds BMP protein but without the NH2 terminal region for membrane binding or secretion, acts to confine BMP protein within the cells, thus preventing its secretion and function (Fig. 3).

**In vitro cleavage of SCUBE2 by purified recombinant MMP2.** To further clarify the physiologic significance of proteolytic processing of SCUBE2 in human breast cancers, we sought to identify the potential breast cancer–associated proteases that can cleave SCUBE2 protein *in vitro*. We initially undertook the candidate approach by examining the effect of MMPs (MMP1, MMP2, or MMP9) on the cleavage of SCUBE2, because these MMPs

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**Figure 3.** The COOH terminal region of SCUBE2 binds BMP protein and acts as a BMP antagonist. **A,** domain organization of the SCUBE2 expression constructs used in this experiment. A FLAG epitope tag was added immediately after the signal peptide sequence at the NH2 terminus for easy detection. Two deletion constructs, SCUBE2-ty97 and SCUBE2-rw87 mutant, were made by mimicking two null mutant alleles (ty97 or rw87) in the zebrafish Scube2 (24–26). FL, amino acids 1–1028; D4, amino acids 664–1028; ty97, amino acids 1–659; rw87, amino acids 1–223; SP, signal peptide; E, EGF-like repeats; Cys-rich, cysteine-rich motifs; CUB, CUB domain. **B,** interaction between SCUBE2 and BMP2. The BMP2 expression construct (Myc-tagged) was transfected alone or together with the expression plasmids encoding indicated FLAG-tagged SCUBE2 proteins in HEK-293T cells. Two days posttransfection, cell lysates underwent immunoprecipitation (IP) and Western blot analysis (WB) with antibodies as indicated to determine the protein interactions. proBMP2, precursor BMP2. **C,** coexpression of SCUBE2-D4 mutant attenuated the BMP2 signaling activity. Conditional media from signaling cells (HEK-293T) cotransfected with the BMP2 expression plasmid alone or in combination with various SCUBE2 deletion constructs as indicated were added to the responding cells (HepG2) that contained the BMP-responsive luciferase reporter plasmid I-BRE-luc (31). **D,** Western blot analysis of SCUBE2-D4 mutant inhibits BMP2 precursor processing into active, secreted molecule. HEK-293T cells were transfected with indicated expression plasmids for BMP2 and SCUBE2 mutants.
have been implicated in the proteolytic processes associated with breast cancer biology (32, 33). Recombinant NH2 terminal FLAG-tagged SCUBE2-FL protein (FLAG.SCUBE2-FL) produced by HEK-293T cells was prepared and used as a substrate for \textit{in vitro} reaction with purified recombinant MMP proteases. As shown in Supplementary Fig. S5, \textit{in vitro} digestion of FLAG.SCUBE2-FL protein by MMP2, but not MMP1 or MMP9 (data not shown), induced the appearance of NH2 terminal 72-kDa fragment and COOH terminal 55-kDa fragment, respectively.

**Discussion**

In the present study, we produced an anti–SCUBE2-specific antibody and used a variety of experimental approaches to examine protein localization, function, and clinical implications of a newly described human gene, SCUBE2, with a role in breast carcinoma. Despite repeated observations of elevated SCUBE2 mRNA expression in breast cancer tissues (6–8, 34), the precise cell types expressing the SCUBE2 protein in malignant breast tissues were virtually uninvestigated. Consistent with its endothelial origin (12), SCUBE2 immunoreactive staining was localized in vascular endothelial cells. In addition, SCUBE2 protein was found in mammary ductal epithelial cells in normal breast tissue (Supplementary Fig. S2A). However, the biological significance of SCUBE2 protein in these two normal cell types is currently unknown and remains to be further studied.

Our clinical association study suggests that patients expressing SCUBE2 protein may have better disease-free survival, but not overall survival, than those without SCUBE2 expression. This may be due to the relatively small number of death events that occurred during the follow-up period or caused by the difference of treatment modality, including local control and adjuvant chemotherapy. However, it is of interest to note that two independent breast tumor gene expression profiling analyses deposited in a public microarray database\(^7\) are broadly in line with our findings (6, 7), confirming the association of SCUBE2 mRNA expression with a better clinical outcome. Regardless, our findings require further validation in a larger cohort of patients from multicenter trials.

Our molecular and biochemical experiments revealed that SCUBE2 protein was subjected to limited proteolysis in releasing an active COOH terminal fragment for its anti-BMP activity (Fig. 3), which may, at least in part, account for the antiproliferative effect of SCUBE2 on breast cancer cells and contribute to favorable disease outcome in breast cancer patients. In support of this, overexpression of the COOH terminal SCUBE2-D4 fragment, as for the SCUBE2-FL protein, suppressed the growth of the MCF-7 breast cancer cells (Supplementary Fig. S3). Yet, it is likely that other potential mechanisms may be responsible for the breast tumor suppressor activity of SCUBE2 and need to be resolved in future studies.

Our data showed that SCUBE2 is an \textit{in vitro} substrate for a breast cancer–associated MMP2 (Supplementary Fig. S5); however, the precise identity of the tumor-associated protease(s) for SCUBE2 cleavage \textit{in vivo} has yet to be revealed. It is tempting to speculate that such proteolytic process may represent a regulatory mechanism whereby SCUBE2 is activated in a cellular context–dependent manner to execute its distinct functions in cancer versus normal cells.

BMPs are multifunctional signaling molecules that belong to the transforming growth factor-β superfamily (35, 36). BMPs play critical roles during development and control diverse cellular

\(^7\) [http://www.oncomine.org](http://www.oncomine.org)
SCUBE2, a Novel Prognostic Marker for Breast Cancer

1. References


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Chien-Jui Cheng, Yuh-Charn Lin, Ming-Tzu Tsai, et al.


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