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SCUBE2 可抑制乳癌細胞增殖與導致侵犯性乳癌病人較佳的預後

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

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本文

SCUBE2 可抑制乳癌細胞增殖與導致侵犯性乳癌病人較佳的預後

(SCUBE2 suppresses breast tumor cell proliferation and confers a favorable prognosis in invasive breast cancer)

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縮 寫 表

(Abbreviations)

BMP	**Bone morphogenetic protein**
CD	**Cluster of differentiation**
CXCR	**Chemokine receptors**
Cys-rich	**Cysteine-rich motifs**
DAB	**3,3'-diaminobenzidine**
DMEM	**Dulbecco's minimal essential medium**
Dox	**Doxycycline**
EDTA	**Ethylenediaminetetraacetic acid**
EGF	**Epidermal growth factor**
EGTA	**Ethylene glycol tetraacetic acid**
ER	**Estrogen receptor**
FBS	**Fetal bovine serum**
FL	**Full-length**
HEK	**Human embryonic kidney cells**
HepG2	**Human hepatocellular liver carcinoma cell line**
Hh	**Hedgehog**
HUVEC	**Human Umbilical Vein Endothelial Cells**
IL	**Interleukin**
LPS	**Lipopolysaccharides**
MCF-7	**Human breast cancer cell line**
MMP	**Matrix metalloproteinase**
MTT	**3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide**
PBS	**Phosphate-buffered saline**
PR	**Progesterone receptor**
RT-PCR	**Reverse transcriptase PCR**
SCUBE	**Signal peptide-CUB-EGF domain-containing protein**
SDS-PAGE	**Sodium dodecyl sulfate polyacrylamide gel electrophoresis**
SP	**Signal peptide**
TNF	**Tumor necrosis factor**
TGF	**Transforming growth factor**

目錄(Contents)

中 文 摘 要

(Abstract)

SCUBE2 (signal peptide-CUB-EGF domain-containing protein 2) 基因可產生一些可分泌而與細胞膜緊密結合的蛋白。人類 SCUBE2 蛋白最早在血管內皮細胞被發現。隨後在人類的許多正常器官，如心臟、胎盤、肺臟與胰臟也發現有 SCUBE2 蛋白的表現。到目前為止，文獻上並沒有相關研究探討 SCUBE2 蛋白在正常乳房或乳癌組織表現的情形或這類蛋白與惡性腫瘤預後的關係。

近年來微陣列研究基因表現模式的發展，使許多研究人員嘗試利用此研究利器找尋能更準確預測乳癌病人預後的相關基因，並得到一些研究成果，以作為更有效治療乳癌病人的參考。這些研究文獻報告中，許多基因在 mRNA 上的表現，對於乳癌病人預後的預測雖具有高度的一致性，但是重覆的基因卻很少，當比較這些微陣列研究模式的結果時，只有一個基因重覆出現，那就是 SCUBE2，顯示 SCUBE2 基因在乳癌腫瘤的重要性。某些個別基因微陣列研究顯示，SCUBE2 比較常在動情激素受體陽性的乳癌組織中出現。同時在具有局部侵犯的乳癌病人身上，乳癌組織中表現 SCUBE2，會明顯減低疾病復發的風險。

這些由微陣列研究產生與乳癌病人預後相關的基因雖多，但是卻很少實際在蛋白質層次去證實其相關性。過去的文獻中，極少去證實這些可預測乳癌病人預後基因所產生出來的蛋白，如何去影響腫瘤行為與其造成這些影響的分子機轉。

在這個研究中，我們藉由免疫組織化學染色技術，我們證明在非腫瘤的乳房

組織中，SCUBE2 蛋白主要位於血管內皮細胞與乳腺的管腔細胞的細胞膜上。在乳癌腫瘤組織內，SCUBE2 蛋白僅在 55 % 原發侵犯侵襲性腺管癌病人的癌症細胞上表現。經由 SCUBE2 在癌症細胞上的表現與否與乳癌病人的預後進行比對與統計分析。我們發現，乳癌細胞有表現 SCUBE2 的病人相較於乳癌細胞不表現 SCUBE2 的病人，明顯減低疾病復發的風險。經由多變量變異數分析，證明 SCUBE2 在乳癌細胞上的表現仍是減低疾病復發的一個獨立預測因子。

當大量表達外來 SCUBE2 在 MCF-7 乳癌細胞上時，會顯著降低乳癌細胞於體外的增殖速度，同時也會抑制在動物模式中的腫瘤形成。進一步經由分子生物與生化學分析探討其分子機制，我們證明 C-端的 SCUBE2 可以經由蛋白間的交互作用，拮抗骨生成蛋白(bone morphogenetic protein)的訊息傳遞。藉由這種拮抗作用，我們提供一種 SCUBE2 可以抑制乳癌細胞增殖的機轉。我們進一步發現 C-端的 SCUBE2 可藉由 matrix metallopropteinases 2 切割而產生。

總之，我們的結果首次發現乳癌細胞上 SCUBE2 蛋白的表現對於乳癌病人的病程進展具有重要的意義。藉此，SCUBE2 蛋白的表現未來也許可以當成另一個預測乳癌預後的因子。進一步，我們將探討 SCUBE2 蛋白除了 C-端外的其它部份是否具有其它生理意義。同時我們也要去了解乳癌轉移的過程中 SCUBE2 蛋白的表現是否發生改變，這種改變所代表的意義為何。再者，我們想要了解 SCUBE2 蛋白在癌化過程中如何被調控，並希望藉由動物模式更深入了解 SCUBE2 蛋白的生理意義。

英文摘要

(Abstract)

SCUBE2 (signal peptide-CUB-EGF domain-containing protein 2) encodes a secreted and surface associated proteins, originally identified from the endothelium and several non-endothelial primary cell types and organs, such as heart , placenta, lung and pancreas. No literature mentioned about the status of expressions of SCUBE2 in breast cancer tissue and their impactions of the prognosis of patients of breast cancer until now.

Several microarray studies of gene expression have identified expression profiles and gene sets that are prognostic, predictive, or both for patients with breast cancer. These evidences provide more information for effective treatment of breast cancer. Most of these models in the literatures had high rates of concordance in their outcome predictions for the individual samples. But, comparisons of the lists of genes derived from some of these apparently similar studies show that they overlap only slightly. When comparison of these gene expression models, only 1 gene overlap, which is SCUBE2.

The gene expression of SCUBE2 has been reported to be associated with estrogen receptor status in breast cancer specimens. Another microarray study found that lower expression of SCUBE2 is associated with greatest recurrent risk of locally advanced breast cancer.

Due to failure of validation of these molecule signatures of cancer cells at protein level, many associations between gene signatures and prognosis are correlative rather than mechanistic. So we could usefully investigate whether, and how, SCUBE2 might be another clinical predictor.

In this study, we demonstrate that SCUBE2 is mainly expressed in vascular endothelial and mammary ductal epithelial cells in normal breast tissue by immunohistochemistry. In addition, we observed positive staining for SCUBE2 in 55% of primary breast tumors and 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, over-expression 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 C-terminal region of SCUBE2 directly bound to and antagonized bone morphogenetic protein activity. We further to verify the matrix metalloproteinases-2 cleavage play a role in generation the C-terminal of SCUBE2.

Together, our results show for the first time that altered SCUBE2 expression is important in breast cancer progression and SCUBE2 may serve as a useful prognostic marker. In future, we would approach other possible mechanisms of tumor suppression of SCUBE2. And we also want to know the

alterations of expressions of SCUBE2 during cancer metastasis and their impaction in breast cancer metastasis biology. Furthermore, we will approach the mechanisms of regulation of SCUBE2 expression during carcinogenesis and SCUBE2 function in development through SCUBE2 knockout mice models.

緒　論

(Introduction)

SCUBE Gene Family. SCUBE (signal peptide-CUB-EGF-like domain) gene family encodes secreted proteins harboring a signal peptide at the amino terminus followed by 9 copies of EGF-like repeats and one CUB domain at the carboxyl terminus. In addition, a spacer region containing multiple potential N-linked glycosylation sites is located between the EGF-like repeats and the CUB domain in these proteins. To date, 3 distinct gene members have been identified and designated SCUBE1 to 3, in the order of their discoveries. (Yang et al., 2002; Wu et al., 2004)

SCUBE1 and Its Possible Biological Functions. The first member of SCUBE gene family, *Scube1*, is firstly isolated from a developing mouse urogenital ridge cDNA library (Grimmond et al., 2001). *Scube1* expression is firstly seen at neurectoderm of the ventral forebrain at stage of 3-7 somites mouse embryo under whole-mount *in situ* hybridization. At the later stage, *Scube1* expression could also be seen at dorsal-most region of the somites, allantois, surface ectoderm, the infundibulum, limb buds and urogenital ridge restricted to the developing gonad in males and females. But expression of

Scube1 was not detectable in heart, brain, spleen, lung, liver, skeletal muscle, kidney, or testis in adult mice. The mouse *Scube1* was mapped to the central region of chromosome 15 in mouse and the human SCUBE1 is mapped to chromosome 22q13 (Grimmond et al., 2001). During identification of all genes expressed in human vascular endothelial cells, the cDNA of SCUBE1 could be seen among the 100000 cDNA fragments derived from the endothelial cells. The expression of SCUBE1 is down-regulated in endothelial cells after IL-1β and TNF-α treatment *in vitro* and after lipopolysaccharide (LPS) injection *in vivo*. These phenomena may indicate SCUBE1 play a role during the inflammatory process (Yang et al., 2002). Using the Northern blotting and RT-PCR methods, the mRNA of SCUBE1 could be found at highly vascular tissue, such as liver, lung and kidney. We previously also found that expression of SCUBE1 mRNA and protein could be identified in human platelets. By immunohistochemistry studies, SCUBE1 is localized to the platelet- and fibrin-rich areas within the organized thrombus (Tu et al., 2006). When the CUB domains are deleted in mouse model, it resulted in brain malformation in the Scube1 (Delta cub/Delta cub) embryos (Tu et al., 2008). Together with above evidence, these results support the dual roles of SCUBE1 on brain morphogenesis and cell-cell adhesions through its distinct domain function.

SCUBE3 and Its Possible Biological Functions. Wu et al. (Wu et al., 2004) identified another gene in SCUBE family, SCUBE3, which is mapped to the human chromosome 6p21 (Figure 1). Using the real-time quantitative RT-PCR, they found that SCUBE3 mRNA is highly enriched in primary cultured osteoblasts, followed by primary HUVEC and coronary smooth muscle cells. Due to its unique expression in bones and osteoblasts, they suggested that SCUBE3 may play a critical role in bone cell biology. Haworth K et al describe mouse Scube3 gene expression pattern during embryonic development (Haworth et al., 2007). The Scube3 transcripts were initially localized to neurectoderm of the developing embryo, in the ventral rhombencephalon and caudal neuropore in mouse. As development progressed, strong expression was detected in tissue derived from triploblast, especially at the neural tube, branchial arches, fronto-nasal region, the dermomyotome of differentiating somites, the limb buds, developing tooth, hair follicle and kidney. At later stages, scube3 expression was also localized to cartilaginous primordia of the skeleton and regions of intramembranous bone formation in the developing craniofacial region (Haworth et al., 2007). Further studies revealed over-expressing SCUBE3 in the ventricular myocardium in transgenic mice models revealed significant cardiac hypertrophy in transgenic

mice animals as they aged, at 8 months by echocardiography and histopathological examinations, especially at left-ventricle under pressure overload (Yang et al., 2007) The phenotype of the SCUBE3 over-expression is mediated by physical interaction with TGF-beta1 through carboxyl-terminal portion of SCUBE3. Activation of TGF-beta1-mediated transcription results in cardiac hypertrophy. (Yang et al., 2007)

Discovery of SCUBE2 and Its Possible Biological Functions. During the sequence analysis of mouse *Scube1*, a related gene with weak match was observed. It is mapped to chromosome 7 of mouse, term *Cegp1*. The *Cegp1* in mouse corresponds to CEGP1/SCUBE2 gene in chromosome 11p15 in human (Grimmond et al., 2001). During approaching the vertebrate development of zebrafish, the *you* gene (zebrafish orthologue of mammalian SCUBE2) mutation has morphological traits in common with other related to the Hedgehog (Hh) signal pathways mutants, such as a curled tail, weak cyclopia, and U-shaped somite boundaries. Through gene analysis, knockdown experiments and injection of wild-type *scube2* mRNA into *you* mutant embryos, Kawakami A et al. identified *Scube2* is the *you* gene encodes protein (Kawakami et al., 2005). Several *in vivo* and *in vitro* studies have suggested

that bone morphogenetic protein (Bmp) signaling has an antagonistic effect on Hh signaling and that the dorsal neural tube and surface epithelium are the sources of Bmp-related molecules. Kawakami et al. therefore suspected that Bmp-dependent signaling might be affected in *you* mutants and perhaps could provide the link between *Scube2* and Hh signaling (Kawakami et al., 2005). The SCUBE2 mRNA is also identified in genes expressed in human vascular endothelial cells (Yang et al., 2002). In contrast to limited expression of SCUBE1 in highly vascular organs, the SCUBE2 transcript was identified in a broad spectrum of human tissue.

Biomarkers of Breast Cancer for Personalized Medicine. Prognostic markers have traditionally been defined as factors that predict disease outcome. Calibration of therapeutic intervention for an individual's outlook is central to effective oncological treatment. Pathologists have accurately predicted the breast cancer prognosis with considerable confidence by evaluating morphological parameters, such as tumor stage and grade, and by evaluating some molecular biomarkers, such as hormone receptor status and Her-2/*neu* gene amplification. This practice is inefficient and frequently results in inappropriate therapy and treatment-related toxicity. As a result, some

patients with aggressive disease may be under-treated, and some with indolent disease may be over-treated. In addition, for those patients who receive treatment, only a proportion of them got clinical benefit, whereas adverse side effects are common. Some techniques, including the sentinel lymph node biopsy, are example for personalized treatment and profoundly affect the clinical practice. Some of predictors currently used are not accurate enough for prediction in the individual patient. Although some target therapies are used in some malignant tumors and achieve some degree of success. To achieve personalized treatment for cancer, we need strong and independent prognostic markers that can reliably separate patients with indolent disease from those with aggressive forms. Markers prospectively predict response or resistance to specific therapies so that the right patients receive the right therapies. By the approach, we would determine prognosis and move from the traditional "trial-and-error" approach to a position involving a personalized approach, that is, giving the right drug at the right dose to the right patient.

SCUBE2 Gene Expression of Tumor Tissue Related to Breast Cancer Prognosis. Although serum is more readily available than tumor tissue, most research into cancer prognostic markers has focused on tissue rather than

serum, such as breast cancer. Some prognosis markers are reported as prognostic factors, such as Her-2/*neu* (Ross et al., 2003), urokinase plasminogen activator and plasminogen activator inhibitor 1 (Harris et al., 2007)

Genomic measures of gene expression of tissue sample of invasive breast cancer provide new informations to identify patterns of gene activity that subclassify tumours (Bhattacharjee et al., 2001; Alizadeh et al., 2000; Perou et al., 2000; Yeoh et al., 2002). These microarray studies of gene expression have identified expression profiles and gene sets that are prognostic, predictive, or both for patients with breast cancer. Fan et al. found that most models in the literatures had high rates of concordance in their outcome predictions for the individual samples (Fan et al., 2006). But, comparisons of the lists of genes derived from some of these apparently similar studies show that they overlap only slightly. The reasons for this lower-than-expected overlap are not completely known, but they probably include differences in the patient cohorts, microarray platforms, and mathematical methods of analysis. An important and unanswered question, however, is whether these predictors are actually concordant with respect to their predictions for individual patients. And whether some of them have significant predictions in prognosis. When comparison of the 70-gene (van'T Veer et al., 2002) and recurrence-score models (Paik et al., 2004), only 1 gene overlap, which is SCUBE2. These two models successfully predict the prognosis of recurrence of Tamoxifen-treated, node-negative breast cancer and stage I or II breast cancer patients which are younger than 53 years old. Another microarray study, using the gene

expression profiles in paraffin- embedded core tissue to predict response of chemotherapy in women with locally advanced breast cancer, Gianni et al. found that lower expression of SCUBE2 is associated with pathologic complete response of chemotherapy, but greatest recurrent risk of locally advanced breast cancer (Gianni et al., 2005). The expression of SCUBE2 has been reported to be associated with estrogen receptor status in breast cancer specimens (Abba et al., 2005). And intriguingly, SCUBE2 is one component of a 21 gene set that comprises a commercial breast cancer predictive test (OncotypeDX Recurrence Score from Genomic Health) currently under clinical trial (Asad et al., 2008). Based on the above evidences, we proposed that SCUBE2 protein expression has significant effect for prognosis of breast cancer patients.

Importance of Verification of SCUBE2 Expression at Breast Cancer Tumor Tissue in Protein Level. Several sets of the gene expressions using gene expression microarray and multiplex PCR and prognosis of breast cancer were done. Due to submitted whole tissue sections for gene analysis related to breast cancer patients prognosis includes breast cancer cells, endothelial cells, fibroblasts, and so on. And the endothelial cells were proved having SCUBE2 expression (Yang et al., 2002). The correlation of SCUBE2 expression at mRNA level and patient prognosis would be sum of expression of endothelial cells and breast cancer cells. The way to solve this problem is to develop the antibody specifically recognize the SCUBE2 of breast cancer. By this way, we could analyze the expression of SCUBE2 of breast cancer respect to prognosis, estrogen receptor status and response to hormone or chemotherapy.

In the genomic and proteinomic era, such molecular signatures, might improved prognostication and correlate with biological and clinical properties of the tumor. Due to failure to independently validate these molecule signature and specific measurement of the gene expression on the cancer cells, many associations between gene signatures and clinical pictures are correlative rather than mechanistic, and such associations are poor predictors of how cellular biochemical networks will behave in cancer cells. So we could usefully investigate whether, and how, some of such data might add predictive value to clinical predictors. Credible assessment of predictors is critical to establish reproducible results, and a key step towards integration of complex genomic data into outlook for individual patients (West et al., 2001; van'T Veer et al., 2002; Golub et al., 1999). Genomic data will add substantial detail to clinical information, and would provide more informations to predict breast cancer prognosis.

Significance and Novelty of the Proposed Study. The purposes of this study would approach the SCUBE2 expression of status of SCUBE2 expression in breast cancer and the expression of SCUBE2 related to the prognosis and tumorigenesis of breast cancers. Thus, in this work we would address the question of whether SCUBE2 is down-regulated during the tumorigenesis and tumor progression of breast cancer. This study also would address the possible mechanisms of SCUBE2 impaction on the breast cancer biology.

研 究 方 法 與 材 料

(Materials and Methods)

Generation of the Anti-SCUBE2 Specific Antibody. One peptide, NH_2-SHICKEAPRGSVAC-COOH, derived from the N-terminal of human SCUBE2 protein sequence, was used as an antigen to immunize layer chickens. Immunoglobulin (IgY) (COR3B) was purified from the yolks of eggs from immunized hens. The anti-CR polyclonal antibody was raised by a recombinant GST fusion protein containing human SCUBE2 resides 668 ~ 835 in the rabbit.

Antibodies. Anti-FLAG M2 (Sigma, Missouri) and anti-Myc 9E10 (Covance, New Jersey) monoclonal antibodies were purchased from Sigma and Covance, respectively. Anti-human estrogen receptor (NCL-ER-6F11 (clone: 6F11, ready to use) and anti-human progesterone receptor (NCL-L-PGR312 (clone: 16), 1:25 dilution) monoclonal antibodies were purchased from Novocastra (Newcastle upon Tyne, UK) . Anti-human c-erbB2 oncoprotein rabbit polyclonal antibody (Code A485, 1:400 dilution) were purchased from Dako (Dako, Glostrup, Demark).

Immunohistochemistry. Breast cancer specimen sections 4-μm-thick were dewaxed with xylene, rehydrated in graded concentrations of alcohol.

After rehydation, these sections are followed by pressure cooker heat-induced epitope retrieval for 10 mins with immersed within the citric acid buffer (0.01M, pH 6.0, Citric Acid Monohydrate, J.T. Baker, 0118-01, Phillipsburg, NJ) (for SCUBE2 and Flag), Target Retrieval Solution (pH 9.0, DakoCytomation, Code S 2367, Denmark) (for estrogen receptor and progesterone receptor), respectively. For the detection of Her-2/neu, antigen retrieval by boiling the slides in 0.01 M citrate buffer (pH 6.0) and 0.1 % NP40 for 3 mins was performed before incubation with antibody.

After antigen retrieved and washed with water, these tissues sections were treated with 3 % H_2O_2 for 30 minutes, washed with phosphate-buffered saline (PBS), blocked with 5 % normal horse serum for 30 minutes, and incubated at 4°C overnight with anti-SCUBE2 antibody (1:2000 dilution) and Anti-Flag (1:4000 dilution). Antibody binding was detected by use of biotinylated anti-chicken antibody (Vector Laboratories, Burlingame, CA, USA for SCUBE2) and biotinylated anti-chicken antibody (Vector Laboratories, Burlingame, CA, USA for anti-Flag) and horseradish peroxidase streptavidin (Vector Laboratories, Burlingame, CA) with 3,3'-diaminobenzidine as the chromogen (Vector Laboratories, Burlingame, CA, USA). Hematoxylin was used as the counterstain. Negative controls consisted of omission of primary antibody and

blocked by antigens, which were used for generation the SCUBE2 antibody. The endothelial cells within the breast tissue were used as internal positive controls. The immunostaining was considered positive when more than 10 % of the tumor cells were immunoreactive.

For expressions of estrogen receptor (ER), progesterone receptor (PR) and Her-2/neu, the immunohistochemical staining was performed automatically using the automated Nexes IHC (Catalog No. J750-NXIHC-FS, Ventana Medical Systems, Inc., Tucson, AZ, USA) staining system. The paraffin sections were incubated with primary antibody diluted in a standard Ventana's Antibody Diluent (Ventana Medical System, Inc., Tucson, AZ, USA) for 30 minutes at 37°C. Subsequently, the slides were further processed using the Ventana STSTM Label and Ribbon Kit. The detection kit utilizes biotinylated secondary antibodies to locate the bound primary antibody, followed by the binding of streptavidin horseradish peroxidase conjugate. The complex was then visualized with hydrogen peroxidase substrate and DAB chromogen, which produces a dark brown precipitate. All incubations were performed at 37°C. There was counterstaining with haematoxylin.

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 hospital between 1998 and 2004. All patients were examined for axillary lymph node involvement. The resected specimen of primary breast carcinoma surgical specimens were fixed with 10 % formalin, embedded in paraffin, examined histopathologically. They also were graded according to a modified version of the Scarff-Bloom-Richardson system (Elston et al., 1991) Staging followed the guidelines of the Cancer Staging Manual of the American Joint Committee on Cancer (AJCC Cancer Staging Manual. 2002). This study was approved by the institutional review boards of our hospitals (TMUHIRB 20070101 and WFH F950903, please see **Appendix**). Recurrent or metastatic disease of our investigated cases was determined by radiographic, sonographic, bone scan, or pathologic evidences. Information on the characteristics of patients (Table 1) were collected from clinical and pathologic records. ER, PR, and HER-2 status were determined and scored on immunohistochemistry as previously described (Harvey et al., 1999; Carlson et al., 2006). Exclusion criteria included patients received previous partial or total resection in other hospitals, tumors could not been totally removed during operation, stage IV disease, or secondary malignancy occurred during follow-up or metastatic disease within 90 days after surgery. Of invasive

carcinomas, 62 % were associated with nodal disease. Of the 156 breast cancers, 90 (57.7 %) were positive for ER, 68 (43.6 %) for PR and 35 (22.4 %) for HER-2 overexpression as seen on immunohistochemistry. At the time of diagnosis, 16.0 %, 47.4 %, and 36.6 % of patients had stage I, II, and III tumors, respectively. Median follow-up time for patients with invasive tumors was 44.33 months (range, 3.16-97.58 months). In 50 cases, disease relapsed, and 19 patients died. Local and distant disease relapses that occurred during the 90-day post-surgery 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 following diagnosis to the first evidence of clinical recurrence or metastatic disease.

Construction of Expression Plasmids. The clone containing full-length SCUBE2 was obtained from OriGene Technologies (Rockville, MD). The SCUBE2 sequence is the same as NM_020974, except nucleotides 1287 to 1526 are spliced out in the clone. Because this commercially available cDNA for human SCUBE2 represents a splice variant lacking a portion of the EGF-like repeats and spacer region (Yang et al., 2002), Yang et al. swapped in a cDNA fragment to correct the defective region by a standard molecular

biology method. The resulting cDNA encodes a polypeptide (SCUBE2-FL, including the amino acids, 1-1028) composed of an organized protein domain structure consistent with that of its zebrafish orthologue and all other SCUBE protein members (see Figure 1) (Wu et al., 2004; Kawakami et al., 2005; Hollway et al., 2006; Woods et al., 2005). Other three deletion expression constructs of the SCUBE2 are used in this experiment. Domain organization of these expression constructs is shown in Figure 3 (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). Two of these deletion constructs, SCUBE2-ty97 and SCUBE2-rw87 mutant, were made by mimicking two null mutant alleles (ty97 or rw87) in the zebrafish Scube2 (Kawakami et al., 2005; Hollway et al., 2006; Liu et al., 2002). The sequence of SCUBE1 and SCUBE3 are based on the gene prediction and public sequence information (GenBankTM with accession number AF525689 (SCUBE1) and GenBankTM accession number AF452494 (SCUBE3), respectively). The expression plasmid for prepro BMP2 containing an open reading frame of human BMP2 cDNA (encoding amino acids 1 ~ 398). These expression plasmids were made in the pcDNA3.1 (+) vectors. The BMP-responsive promoter luciferase reporter construct I-BRE-Luc is described

previously (Benchabane et al., 2003).

The pFLAG-CMV-1 (Sigma) was used to include a FLAG tag at the amino terminus of target protein immediately after the signal peptide sequence at the NH2 terminus for easy detection. The pcDNA4/Myc-His (Invitrogen) was used to add a Myc tag to the carboxyl terminus of target protein.

Cell Culture and Transfection. HEK (human embryonic kidney)-293T, MCF-7-tet off and HepG2 were maintained in DMEM (Dulbecco's minimal essential medium) supplemented with 10 % heat-inactivated FBS (fetal bovine serum), 100 units/ml penicillin and 100 µg/ml streptomycin (GIBCO 15140-122) at 37 °C in an atmosphere of 5 % CO_2. Cells were seeded in 6-well plates overnight before transfection HEK-293T were transfected by use of Lipofectamine™ 2000 (Invitrogen, 51124). The total amount of DNA was kept constant in transfections with empty vectors, SCUBE2-FL, SCUBE2-D4, SCUBE1 and SCUBE3.

Immunoprecipitation and Western Blot Analyses. Two days after transfection, transfected cells were washed once with PBS and lysed for 15 minutes on ice in 0.5 ml of lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 % Triton X-100, 25 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 µg/ml leupeptin). Lysates were

clarified by centrifugation at 4°C for 15 minutes at 10,000 × g. Cells lysates were incubated with 1 μg of indicated antibody and 20 μl of 50 % (v/v) protein A-agarose (Pierce, Thermo Scientific, Rockford, IL) for 2 hours with gentle rocking. After three washes with lysis buffer, precipitated complexes were solubilized by boiling in Laemmli sample buffer, fractionated by SDS-PAGE, and transferred to polyvinylidene difluoride membranes. The membranes were blocked with PBS, pH 7.5, containing 0.1 % gelatin and 0.05 % Tween 20 and were blotted with the indicated antibodies. After two washes, the blots were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (The Jackson Laboratories, Maine) for 1 hour. After washing the membranes, the reactive bands were visualized with the enhanced chemiluminescence system (Amersham Biosciences).

For detections of the secretion properties of various SCUBE2 deletion constructs, we collect conditioned medium after 48 hours post-transfection. And then cells were detached with PBS/EDTA. Samples from conditioned culture medium (Medium) and cell lysates (Cell) were separated by 4-20 % SDS-PAGE and transferred to polyvinylidene difluoride membranes. Recombinant SCUBE2 proteins and various deletion constructs were detected by Western blotting with anti-Flag M2 antibody.

FACS™ Analysis. To verify the cell-surface expression of FLAG-tagged SCUBE2, transfected cells were collected and suspended in PBS/EDTA, 2 % bovine serum albumin in a volume of 0.25 ml. Either 1 µg of purified anti-FLAG M2 antibody (Sigma, F1804)or or isotype control antibody (IgG1) are added for 45 minutes on ice, Subsequently fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody (1:100 dilution, Jackson ImmunoResearch Laboratories, West Grove, PA) was added and incubated for 45 minutes on ice. FACS™ analyses were performed with a FACS™ Scan (Becton Dickinson, Mountain View, CA). Histograms were generated from measurements of 10,000 cells, and data were analyzed by use of the CellQuest software on a FACS™ caliber system.

Establishment of the MCF-7 Breast Tumor Cell Line Stably Expressing SCUBE2. The MCF-7 Tet-off Vector or MCF-7 Tet-off SCUBE2 cell lines were derived from the stable transfection of MCF-7 Tet-off cells (Clontech Laboratories, Inc.) with an empty pTRE2hyg plasmid (Clontech Laboratories, Inc.), a plasmid encoding the FLAG-tagged full-length (FL) or D4 mutant of human SCUBE2 (FLAG.SCUBE2-FL or -D4), respectively. Stable cell clones were grown in the presence of 10 µg/ml doxycycline (Dox; to suppress SCUBE2 expression) (Sigma, D9891) and selected by resistance to

G418 (100 μg/ml) (GIBCO, 11811-031) and hygromycin (100μg/ml) (Sigma H-3274). Established cell lines were further verified by anti-FLAG western blot analysis to assess Dox-responsive FLAG.SCUBE2 protein expression.

Luciferase Activity Assays. Human HepG2 cells (3 x 10^5 cells per well) were seeded into 24-well plates and transfected on the following day with 0.4 μg of the BMP-inducible luciferase reporter I-BRE-Luc (Benchabane et al., 2003) and 0.01 μg of the Renilla luciferase reporter vector used as an internal control. The transfected responding cells (HepG2) were stimulated with conditional media from signaling cells (HEK-293T) co-transfected with the BMP2 expression plasmid alone or in combination with various SCUBE2 deletion constructs, including the SCUBE2-FL, SCUBE2-D4, SCUBE2-ty97 and SCUBE2-rw87, respectively. Luciferase activity was measured following 24 hours incubation by the use of the dual reporter system (Promega, Madison, WI). Data are expressed as relative luciferase activity (firefly luciferase activity divided by Renilla luciferase activity).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) 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, MCF-7 Tet-off SCUBE2-FL or -D4 stable cells were trypsinized and plated onto 96-well cell culture plates at 2000 cells/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-week-old, nu/nu, strain BALB/cAnN.Cg-Foxn1nu/CrlNarl) were purchased from the National Laboratory Animal Center (Taipei, Taiwan). Animals were allowed to acclimate to the new environment for 1 week before being implanted with 0.5 mg of 17β-estradial 60-day release pellet (Innovative Research of America, Sarasota, FL) subcutaneously on the dorsal side 1 day 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 Dox-containing water (200 μg/ml) as described previously (Liu et al., 2002). For tumor-cell implantation, the MCF-7 Tet-off SCUBE2 or the MCF-7 Tet-off Vector clone cells were harvested, washed with PBS, and re-suspended in PBS. Then 2 x 10^6 cells in 0.2 ml of the mixture (50 % Matrigel™ (BD Biosciences)) were injected into the mammary fat pads of female athymic mice.

After tumor growth for 12 days, the mice were divided into two groups, that receive either Dox-free or -containing water to induce or suppress the expression of SCUBE2, respectively. Tumor size was measured twice a week, by use of digital calipers, and calculated as mm^3 = length x width x height x 0.5236. The experiments were terminated when the largest tumor size in these mice reached 800 mm^3. 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 (please see **Appendix**).

***In vitro* Digestion of SCUBE2 by Purified Recombinant Matrix Metalloproteinases (MMPs)**. The FLAG-tagged SCUBE2 protein produced by HEK-293T cells was incubated with various recombinant MMP, including MMP 1, 2 and 9 (500 ng; R&D Systems, Minneapolis, MN, USA) in the absence or presence of a broad-spectrum MMP inhibitor (GM6001, 20 μM, Chemicon) in buffer containing 20 mM Tris-HCl (pH 7.4), 5 mM $CaCl_2$, 150 mM NaCl, 0.05 % Brij-35 at 37 ºC for 2 hours. The cleaved fragments were analyzed by probing with anti-FLAG (N-terminus) or anti-cysteine-rich repeats (C-terminus) antibody, respectively.

Statistical Analyses. Association of positive and negative SCUBE2

protein expressions, and clinicopathologic variables of the carcinoma specimens was evaluated by chi-square 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 -negative groups in time to recurrence. The Cox proportional hazard model was used to assess the effects of several possible prognostic factors, with univariate analysis followed by multivariate analyses to identify independent prognostic factors for disease-free survival. All statistical tests were done with SPSS 10.0 (SPSS Inc., Chicago, IL).

To compare the tumor growth rates of the MCF-7 Tet-off SCUBE2 and the 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 a p <0.05 was considered statistically significant.

結　　果

(Results)

Characterization of the Anti-SCUBE2 Specific Antibody. To localize SCUBE2 protein expression in normal breast tissue or in tumors, we used the synthesize peptide (SHICKEAPRGSVAC) within the 4th EGF-like domain of SCUBE2 to immunize hen and first generated a polyclonal antibody specifically against SCUBE2. Using transient expression of SCUBE1, SCUBE2 and SCUBE3 at 293T cells, we performed western blotting to analyze the specificity of synthetic chicken antibody. They revealed chicken anti SCUBE2 antibody (clone COR 3B) only could recognize the SCUBE2, but not to SCUBE1 and SCUBE3 (Figure 4). The western blotting of anti-Flag antibody for these three transfected cells is used to verify the protein expressions of the SCUBE1-3. For further detection of the SCUBE2 protein in formalin-fixed paraffin embedded human breast cancer tissue, we performed immunocytochemistry for formalin-fixed paraffin embedded 293T cells, which are transiently expressed SCUBE1, SCUBE2 and SCUBE3 to analyze the specificity of synthetic chicken antibody in this condition. They revealed chicken anti SCUBE2 antibody (clone COR 3B) only could recognize the SCUBE2 expressed 293T cell, but not to SCUBE1 and SCUBE3 (Figure 5).

Also anti-Flag antibody is used for detection of protein expressions of the SCUBE1-3. Negative controls consisted of omission of primary antibody, using pre-immune antibody and blocked by antigens, which are used for generation the SCUBE2 antibody.

Expression of SCUBE2 Protein in Normal Breast Tissue. Using the anti-SCUBE2 polyclonal antibody, we found that endogenous SCUBE2 protein expressed on the luminal surface of ductal epithelial cells and vascular endothelial cell surface of normal breast tissue (Figure 6A). Pre-incubation of the antibody with the corresponding immunogen peptide resulted in no staining, further demonstrating the specificity of the anti-SCUBE2 staining in the breast tissues (Figure 6B).

Expression of SCUBE2 Protein is Correlated With Favorable Disease-Free Survival of Breast Cancer. To explore the role of SCUBE2 in biologic behaviors of breast cancer, 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 (Figure 6D), and 70 (44.9 %) cases were scored as negative for SCUBE2 (Figure 6C). To evaluate the potential contribution of SCUBE2 protein expression to prognosis of breast cancer, we compared clinicopathological characteristics of cases

scored as positive and negative for SCUBE2. As shown in Table 1, SCUBE2 protein expression had significant negatively association with tumor recurrence ($p < 0.0001$), PR expression ($p = 0.02$), but not associated with other characteristics.

The impact 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 (Figure 7A). 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 (Figure 7B-D).

Univariate analysis revealed a significant correlation between SCUBE2 protein expression ($p < 0.0001$), lymph node involvement ($p=0.02$), advanced clinical stage ($p = 0.0001$), PR status ($p = 0.03$) and disease-free survival with 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 [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 Cell Line. Because the clinicopatholgical association study implied that positive SCUBE2 protein expression was negatively correlated with tumor recurrence, we speculated that overexpression of SCUBE2 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-tagged SCUBE2-FL (FLAG.SCUBE2-FL) protein under the control of an inducible promoter, the tetracycline-off promoter. In addition, the MCF-7 Tet-off Vector clones containing stable integration of the empty expression vector were established as controls. Doxycyclin (Dox) was removed from the medium to induce the expression of FLAG.SCUBE2-FL protein, determined by anti-FLAG western blot analysis 1-5 days after Dox withdrawal. FLAG.SCUBE2-FL protein was readily expressed within 1 day, and expression peaked at about 4-5 days after Dox removal (Figure 8). No induction of FLAG.SCUBE2-FL was observed in the presence of Dox in the MCF-7 Tet-off SCUBE2 clones (Figure 8) or in the control MCF-7 Tet-off Vector clones (Figure 9).

To examine the effect of SCUBE2 overexpresssion on breast-cancer cell

growth, the MCF-7 Tet-off Vector, MCF-7 Tet-off SCUBE2-FL or MCF-7 Tet-off SCUBE2-D4 stable cells were cultured in the presence or absence of Dox for 12 days to suppress or induce the expression of ectopic FLAG.CUBE2 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 clone cells in the absence of Dox, whereas growth of MCF-7 Tet-off Vector stable cells was not inhibited by withdrawal of Dox (Figures 10 and 11). Furthermore, overexpression of the SCUBE2-D4 mutant protein, like the full-length protein, suppressed the growth of the MCF-7 breast-cancer cells (Figure 11). As a control, growth of MCF-7 Tet-off Vector and MCF-7 Tet-off SCUBE2 cells did not differ on culture with Dox to block the expression of ectopic SCUBE2 protein.

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 cells were injected into the mammary fat pads of nude 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 Dox-free water to induce the

expression of SCUBE2-FL. Our results showed that tumor growth from the MCF-7 Tet-off SCUBE2 cells in mice was markedly lower than that of tumors from control MCF-7 Tet-off Vector cells (Figure 12). However, mice that continued to receive Dox-containing water to suppress the SCUBE2 induction showed no difference in tumor growth rate of MCF-7 Tet-off SCUBE2 or MCF-7 Tet-off Vector cells (Figure 13). Together, these results demonstrated that overexpression of SCUBE2 suppresses MCF-7 breast-cancer cell growth both *in vitro* and *in vivo*.

SCUBE2 Antagonizes Bone Morphogenetic Protein (BMP) Activity.

Recent genetic study on zebrafish showed that BMP activity can be attenuated by the co-expression of SCUBE2 (Kawakami et al., 2005) and indicated that the C-terminal cysteine-rich repeats and the CUB domain is essential for zebrafish Scube2 function (Kawakami et al., 2005; Hollway et al., 2006; Woods et al., 2005). In addition, BMPs are multifunctional growth factors which play important roles in normal cell differentiation and proliferation (Hogan., 1996; ten Dijke et al., 2003), and have recently been implicated in promoting breast cancer cell proliferation (Pouliot et al., 2003; Clement et al., 2005). We then examined whether or not the C-terminal fragment of SCUBE2 can interact with BMP2 protein or affect BMP signaling.

A series of FLAG-tagged SCUBE2 deletion constructs was firstly generated, including the SCUBE2-D4 deletion construct encoding for only the C-terminal region (cysteine-rich repeat motif and the CUB domain) and two additional deletion mutants, SCUBE2-ty97 and -rw87, mimicking the ty97 and rw87 null mutant alleles (Kawakami et al., 2005; Hollway et al., 2006; Woods et al., 2005), respectively, in the zebrafish Scube2 gene by removing various portions of C-terminal domains (Figure 3). HEK-293T cells were transfected with a Myc-tagged BMP2 expression plasmid alone or in combination with various FLAG-tagged SCUBE2 domain deletion constructs (Figure 3). Two days after transfection, cell lysates were subjected to immunoprecipitation with the anti-Myc monoclonal antibody, and the precipitates were analyzed by immunoblotting with anti-FLAG monoclonal antibody to determine the protein interaction. Immunoprecipitation with anti-Myc antibody resulted in a specific co-precipitation of the SCUBE2 full-length and -D4 deletion protein but not -ty97 or -rw87 (Figure 14). These data suggest that SCUBE2 protein could indeed form a complex with BMP2 through its C-terminal cysteine-rich repeats and CUB domain.

To further examine whether the interaction between SCUBE2-D4 and BMP2 affected the signaling ability of BMP2, we performed a co-culture assay

in which the conditioned media derived from HEK-293T cells transfected with BMP2 alone or together with SCUBE2 deletion constructs (signaling cells) were added to the responding cells, HepG2 cells containing the BMP-responsive promoter luciferase reporter construct I-BRE-Luc (Benchabane et al., 2003). As expected, BMP2 alone produced by the signaling cells acted as a long-range signaling molecule by inducing an increase of approximately 6-fold in luciferase activity (Figure 15). Although the BMP2 protein co-expressed with SCUBE2-FL, -ty97, or -rw87 triggered the BMP-mediated transcriptional activation equally well, co-expression with the SCUBE2-D4 mutant resulted in marked attenuation of the BMP response (Figure 15).

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 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 (Figure 16); only the SCUBE2-D4 mutant potently suppressed the secretion of mature BMP2 into the culture medium.

To further verification of possible mechanism of suppression of secretion of mature BMP2, we determined subcellular distribution of the N- or C-terminal fragment of SCUBE2. As Figure 16, the C-terminal D4 mutant is mainly resided within the cells and defective in secretion. HEK-293T cells were transfected with the SCUBE2-FL, SCUBE2-ty97 and SCUBE2-D4 expression plasmids. Two days after transfection, samples from conditioned medium or cell lysates were immunoprecipitated with anti-FLAG antibody and followed by western blot analysis. Consistently, while overexpressed SCUBE2-FL or -ty97 proteins were secreted into the conditioned medium and properly targeted on the cell surface, the SCUBE2-D4 mutant was defective in secretion (Figure 16). When we determined the surface expression by FACS™ analysis 24 hours after transfection of SCUBE2-FL, SCUBE2-ty97 and SCUBE2-D4 expression plasmids, the results also revealed that SCUBE2-D4 protein was incapable of targeting to the cell surface stained with anti-FLAG antibody, but the SCUBE2-FL or -ty97 proteins could be detected by anti-FLAG antibody. Therefore, the C-terminal fragment represented by the SCUBE2-D4 that binds BMP protein, but without the N-terminal region for membrane binding or

secretion, acts to confine BMP protein within the cells thus preventing its secretion and function (Figure 17).

***In vitro* Cleavage of SCUBE2 by Purified Recombinant Matrix Metalloprotease 2 (MMP2).** To further clarify the physiological 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 matrix metallopropteinases (MMP1, 2, or 9) on the cleavage of SCUBE2, because these MMPs have been implicated in the proteolytic processes associated with breast cancer biology (Abba et al., 2005; Bertucci et al., 2004). Recombinant N-terminal FLAG-tagged SCUBE2 protein (FLAG.SCUBE2) produced by HEK-293T cells was prepared and used as a substrate for in vitro reaction with purified recombinant MMP proteases. Recombinant SCUBE2 and its cleaved product were analyzed by SDS-PAGE and western blot analysis using an anti-FLAG antibody or anti-CR polyclonal antibody, respectively. As shown in Figure 18, *in vitr*o digestion of FLAG.SCUBE2 protein by MMP2, but not MMP1 or MMP9.

討　論

(Discussion)

In the present study, we produced an anti-SCUBE2-specific antibody and used a variety of experimental approaches to examine the 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 (van de Vijver et al., 2002; van 't Veer et al., 2002; Paik et al., 2004; Sorlie et al., 2003), the precise cell types expressing the SCUBE2 protein in malignant breast tissues were virtually uninvestigated. Consistent with its endothelial origin (Yang et al. 2002), SCUBE2 immunoreactive staining was localized in vascular endothelial cells. In addition, SCUBE2 protein was found in mammary ductal epithelial cells in normal breast tissue (Figure 6). Using immunohistochemistry study, we had showed that SCUBE2 may be used as a prognostic marker for tumor recurrence in invasive ductal carcinoma breast cancer. Down-regulation of SCUBE2 in breast cancer with invasive ductal carcinoma type was significantly associated with recurrence of breast cancer in entire cohort. When stratified by tumor stage, the expression of SCUBE2 still had significantly increased disease-free survivals compared to SCUBE2 -negative cases in each stage.

We further investigated the functional role of SCUBE2 in breast cancer cells and showed that constitutive expression of SCUBE2 in MCF-7 suppressed proliferation and invasiveness *in vitro* and MCF-7 tumor development *in vivo* in an orthotopic breast cancer model. Molecular and biochemical analyses revealed that the COOH terminal region of SCUBE2 directly bound to and antagonized bone morphogenetic protein activity.

Our clinical association study suggested that patients expressing SCUBE2 protein might have better disease-free survival, but not overall survival, than those without SCUBE2 expression. This might be due to by the relatively small number of death events that occurred during the follow-up period, or caused by difference of treatment modality, including local control and adjuvant chemotherapy. Therefore, the findings of this study required further validation in a larger cohort of patients from multicenter trials. Regardless, our data suggested that alterations in SCUBE2 expression are important in breast cancer progression.

Verification of SCUBE2 Expression at Protein Level Predict the Prognosis of Breast Cancer Patients. In past decades, genomic measures of gene expression provided a lot of new information to identify patterns of

gene expression within different breast cancer, that subclassify tumors (Bhattacharjee et al., 2001; Alizadeh et al., 2000; Perou et al., 2000; Yeoh et al., 2002). The prediction of tumor recurrence by evaluating primary breast cancer mRNA expression of tumor-related genes was potentially promising, because the primary tumor was readily assessed after resection, radical surgery or core needle biopsy. And some studies had demonstrated that the potential of recurrence and distant metastasis may be attributable to gene expression of primary tumor at the time of diagnosis (van de Vijver et al., 2002; van 't Veer et al., 2002; Huang et al., 2003). Such gene expression patterns might correlate well with biological and clinical properties of the tumor, so they were worth to further investigate whether, and how, these gene products might be rationally added as another biomarker to predict the prognosis of breast cancer patients for changing current therapeutic modality. Critical and credible assessments of these gene products were key steps towards integration of complex genomic data into clinical applications (West et al., 2001; van 't Veer et al., 2002; Golub et al., 1999).

Gene expression analysis using total RNA of bulk tissue usually could not assign specific messages to particular cell types. Due to endothelial cells proved having SCUBE2 mRNA expression (Yang et al., 2002), correlation of

SCUBE2 mRNA expression of whole tissue fragments and patient prognosis would be sum of expression of endothelial cells, breast cancer cells and other cell types. Given detection of gene expression at the protein level, unlike measurement of mRNA copy number, indicated a true functional state. We developed the antibody specifically recognize the SCUBE2 protein in human breast cancer tissue to investigate whether SCUBE2 in breast cancer cells indeed had predictive value for patient outcome. By this way, we could analyze the expression of SCUBE2 of breast cancer in protein level respect to prognosis, especially focusing on the recurrence and survivals. To make the study cohort as homogeneous as possible, we included only cases of invasive ductal carcinoma histology. We also included only patients who had adequate staging. Significant association between expression of SCUBE2 and better disease-free survivals was seen in entire cohort. When stratified by tumor stage, the expression of SCUBE2 still had significantly increased disease-free survivals compared to SCUBE2 -negative cases.

SCUBE2 Down-regulation in Breast Cancer and In Situ Lesions. In this report, we demonstrated strong SCUBE2 expression on the luminal surface of normal breast ducts by immunohistochemistry. In agreement with

the behavior of tumor suppressor genes, we observed down-regulation of SCUBE2 in 44.9 percent of invasive ductal carcinoma breast cancer expression of SCUBE2. In addition, 37 % of carcinoma in situ lesions around the SCUBE2 negative breast cancer also revealed down regulation of SCUBE2. Together above, that indicated that down-regulation of SCUBE2 was an early event of breast cancer carcinogenesis.

Breast Tumor Cell Proliferation Suppressed by SCUBE2 Through Attenuation of Bone Morphogenetic Protein-2 Signaling Pathway. In our study, we demonstrated that constitutive expression of SCUBE2 play a role in the control of cell growth *in vitro*. Our *in vivo* tumorigenicity study also showed that elevated SCUBE2 expression reduced the tumor size induced by MCF-7 cell in nude mice. Previous genetic studies on zebrafish showed that BMP activity can be attenuated by the co-expression of SCUBE2 (Kawakami et al., 2005) and indicated that the C-terminal cysteine-rich repeats and the CUB domain is essential for zebrafish *Scube2* function (Kawakami et al., 2005; Hollway et al., 2006; Woods et al., 2005). Most interestingly, our molecular and biochemical experiments revealed that SCUBE2 protein was subjected to limited proteolysis in releasing an active C-terminal fragment for its anti-BMP

activity (Figures. 8, 14, 15 and 16), which might, at least in part, account for the anti-proliferative effect of SCUBE2 on breast-cancer cells and contribute to favorable disease outcome in breast cancer patients.

Recently, BMPs had been implicated in promoting breast cancer cell proliferation (Pouliot et al., 2003; Clement et al., 2005). Some *in vitro* studies revealed BMP-2 signal pathway is important for breast cancer progression. BMP-2 had been found to suppress apoptosis induced by tumor necrosis factor-alpha (Chen et al., 2001) or by serum deprivation (Izumi et al., 2001). BMP2 also enhanced migration and invasion of a MCF-7 breast cancer cell line, and its over-expression supported tumor formation in a breast cancer xenograft model (Clement et al., 2005). Furthermore, inhibition of the BMP signaling pathway by over- expression of a dominant-negative form of BMP type II receptor repressed proliferation of T-47D breast cancer cells (Pouliot et al., 2003). Raida et al. found that BMP-2 confers resistance to hypoxic cell death in an autocrine/paracrine manner, possibly by activating the MAPK and Id-1 pathway (Raida et al., 2005). BMP-2 also played a role as a chemoattractant *in vitro* and BMP-2 over-expression enhanced the *in vitro* migratory and invasive properties. And BMP-2- over-expressing MCF-7 cells induced tumor growth in nude mice (Clement et al., 2005).

SCUBE2-D4 is a Critical Domain for Bone Morphogenetic Protein-2 Signaling Pathway Attenuation. Over-expression of the SCUBE2-D4 mutant protein, as for the SCUBE2 full-length (FL) protein, suppressed the growth of the MCF-7 breast-cancer cells (Figure 11). These results further supported the notion that the proteolytic fragment of D4 constitutes a critical and essential component for the function of the SCUBE2-FL protein. Furthermore, while over-expressed SCUBE2-FL or -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 (Figure 17). Again, these results implied that a potential limited proteolysis may be of biological relevance to produce functional distinct SCUBE2 fragment. As such, the C-terminal fragment represented by the SCUBE2-D4 that bound BMP protein, but without the N-terminal region for membrane binding or secretion, acted to confine BMP protein within the cells thus preventing its secretion and function (Figures 14, 15 and16). In agreement with this notion, our results suggested that SCUBE2 functions as a BMP antagonist, and over-expression of SCUBE2 protein suppressed MCF-7 breast cancer cell proliferation *in vitro* and reduced MCF-7 tumor growth *in vivo* in an orthotopic

mouse model (Figure. 12). However, further evaluating the clinical impact of SCUBE2 protein over-expression on BMP signaling and its effect on breast cancer progression was needed.

Matrix Metalloproteinases-2 Cleavage of SCUBE2 Provides the Mechanism of Production of SCUBE2-D4. Due to C-terminal domain of SCUBE2 play a role in attenuation of BMPs signal pathway, we further approached whether some MMPs, reported previously in breast cancer tissue, played a role in generation the C-terminal of SCUBE2. By this way, we could clarify the physiological 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 matrix metallopropteinases (MMP1, 2, or 9) on the cleavage of SCUBE2, because these MMPs have been implicated in the proteolytic processes associated with breast cancer biology (Park et al., 2008; Das et al., 2008). Recombinant N-terminal FLAG-tagged SCUBE2 protein (FLAG.SCUBE2) produced by HEK-293T cells was prepared and used as a substrate for *in vitro* reaction with purified recombinant MMP proteases. As shown in Figure 18, *in vitro* digestion

of FLAG.SCUBE2 protein by MMP2, but not MMP1 or MMP9, induced the appearance of N-terminal 72 kDa and C-terminal 55 kDa fragments, respectively. Together, these data identified that SCUBE2 is an *in vitro* substrate for a breast cancer-associated MMP2, and MMP2 digestion may liberate the C-terminal fragment to bind and antagonize BMP activity. Although the precise identity of the tumor-associated protease(s) for SCUBE2 cleavage *in vivo* had yet to be revealed, such proteolytic process may represent a regulatory mechanism whereby SCUBE2 was activated in a cellular context-dependent manner to execute its distinct functions in cancer versus normal cells.

The tumor microenvironment is critical for signaling information that regulates tumor progression and tumorigenesis. In past decades, matrix metalloproteinases (MMPs) were considered to play a role in metastasis through degradation of connective tissue stroma and basement membrane. (Nagase et al., 1999; Bergers et al.,1999). In addition to promote the tumor spread through extracellualr matrix (ECM) degradation, the degraded proteins also mediated the angiogenesis and metastasis. MMPs also initiated epithelial-mesenchymal transition and genomic instability. Due to strong correlation between MMPs expression and tumour progression or metastasis in human

tumour samples, (Egeblad and Werb., 2002; Overall and Kleifeld., 2006; Overall and Lopez-Otin., 2002), it was naturally assumed that such over-expression was causal or at least important in the progression of the disease. By the conception, several clinical trails were performed, but unexpectedly, there was a global failure in meeting the predicted endpoints of increased life expectation.

Recent literatures revealed that many MMPs are in fact protective in cancer, performing part of host functions against the carcinoma. This concept of elevated expression of these MMPs being always associated with worsened disease outcome is challenged (Overall and Kleifeld., 2006). Hence, in some types of cancer, MMPs activities were probably more relevant during the early stages of tumour development, with MMP-dependent signaling more relevant biologically than ECM degradation. One of these examples was monocyte chemoattractant protein-3 (MCP-3) was cleaved by matrix metalloproteinase gelatinase A (MMP-2) and the conversion of chemokine agonists to antagonists by MMPs (McQuibban et al., 2000). Another example was that MMPs might play an anti-inflammatory role that could be beneficial in tumor degradation by releasing antiangiogenic epitopes from ECM, that also would be blocked by the use of broad-spectrum MMPIs (Overall and Kleifeld., 2006).

Moreover, following the identification of MMP-2 as a cancer anti-target, MMPs-3, -8, -9, -11, -12 and -19 had also been shown to play protective roles (Balbin et al., 2003; Lopez-Otin et al., 2007; Martin and Matrisian., 2007).

Adhesion Function of SCUBE2 Maybe Another Mechanism for Suppression of Breast Cancer Progression. In addition to C-terminal portion of SCUBE2 could attenuate the breast cancer cells proliferation through attenuation of the BMP signaling pathway, SCUBE2 might suppress the breast cancer progression through other mechanisms.

Previously, literature had demonstrated that SCUBE2 could behave as an adhesion molecule. When co-expression of SCUBE2 with either closely related SCUBE1 or SCUBE2, SCUBE2 could form homo- and heteromeric complex (Yang et al., 2002). Cell adhesion is a functional process required for the correct functioning of multicellular organisms. It has been clear that cell adhesion molecules are involved in a broad range of processes, including cell-cell and cell-matrix interactions, cell migration, cell cycle and signaling as well as morphogenesis during development and tissue regeneration (Bissell and Radisky., 2001). In addition, adhesion molecules have a number of diverse roles, amongst which is the control of tissue architecture and the

maintenance of tissue integrity of benign or malignant tumor. Most tumors have an abnormal architecture and loss of tissue integrity is thought to be an important step in the development of local invasion. Thus, alterations in adhesion molecules may have a role in both tumor development and tumor invasion. In breast cancer, some adhesion molecules seem to play a crucial role in tumor invasion and metastasis (Hazan et al., 2000; Knudsen and, Wheelock., 2005; Derksen et al., 2006). Loss or reduction of intercellular adhesion molecules had been postulated to facilitate tumor cell detachment form a primary tumor, ultimately leading to metastasis.

Tumor suppression may require signal transduction in additional to the initial adhesion event. Previous evidences are emerging that some adhesion molecules could serve as transducers of outside-in signaling to control proliferation (Pece and Gutkind., 2000; Perrais et al., 2007; Xie and Bikle., 2007). And these adhesion molecules associate and control the action of some kinds of receptor thyrosine kinase (ce et al., 2000; Perrais et al., 2007; Qian et al., 2004). We had demonstrated the C-terminal of SCUBE2 could function as an inhibitor of BMP-2 signaling pathways. The function of N-terminal domain was still unknown. Based on the previous studies about the SCUBE1, it could act as a cell adhesion molecule through Ca^{2+}-dependent interactions mediated

by EGF-like repeat modules based on the *In vitro* cell-based assays. Previous study revealed expression of EGF-like repeats 7–9 of SCUBE1 appear to function as an independent molecular unit that is sufficient for the formation of SCUBE1- mediated homophilic adhesions between adjacent cells (Tu et al., 2008).

To approach the role of SCUBE2 EGF-like repeats in intercellular adhesion, we would evaluate the aggregating properties of the SCUBE2 EGF-like repeat mutant stable transfectant cell lines in the future by cell aggregation assay. And we could assess the tumor formation *in vivo* by xenograft model.

Re-expression of SCUBE2 May Mediate Lymph Node Metastasis of Breast Cancer. The role of adhesion molecules in the establishment of tumor metastases was much less certain (Ilyas., 2000). The previously *In vitro* studies have shown that E-cadherin is down-regulated in a transient way (Mareel et al., 1995). They had previously reported reduced or absent expression of E-cadherin (50 %) in primary breast carcinomas. Reduced expression of the adhesion protein was significantly associated with the development of metastases (Bukholm et al., 1998). They also found strong

immunoreactivity for E-cadherin in the metastatic lesions from patients with primary tumors which lacked expression of these proteins. The mechanism behind this re-expression remained unclear. Mareel et al. (Mareel et al., 1995) had previously shown that tumor cells re-express E-cadherin at the metastatic site.

We found that the expression of SCUBE2 in primary tumors was reduced. In human breast tumor without SCUBE2 expression at primary sites, SCUBE2 were re-expressed at the metastatic tissue in the most of cases (unpublished data). In our primitive data, 23 node-positive breast cancer cases, 17 of 23 cases revealed loss of expression of SCUBE2 in primary sites. Within these 17 cases, 13 cases revealed re-expression of SCUBE2 in the metastatic sites of lymph nodes.

Re-expression of these adhesion molecules by tumor cells after release from the primary site may be important and perhaps necessary for tumor cells to adhere in remote organs. Metastasis development is a complex process involving cell dissociation, cell migration, matrix invasion, and transportation of tumor cells to remote sites with adhesion between tumor cells (Woodhouse et al., 1997). For tumor cells to break loose from a primary mass, enter blood vessels or lymphatics, and produce a secondary growth at a distant site, they

must go through a series of steps. Normal cells were neatly glued to each other and their surroundings by a variety of adhesion molecules (Bissell et al., 2001). E-cadherins was an example that mediates homotypic adhesions in epithelial tissue, thus serving to keep the epithelial cells together and to relay signals between the cells. In breast cancer, there was a transiently down-regulation of E-cadherin expression and re-expression at metastatic site. The expression of SCUBE2 also revealed similar manners. Presumably, this down-regulation reduced the ability of cells to adhere to each other and facilitated their detachment from the primary tumor and their advance into the surrounding tissues.

Arrest and extravasation of tumor emboli at distant sites involved adhesion to the endothelium, followed by egress through the basement membrane. Involved in these processes were also through adhesion molecules. Of particular interest was the CD44 adhesion molecule, which was expressed on normal T lymphocytes and was used by these cells to migrate to selective sites in the lymphoid tissue. Such migration is accomplished by the binding of CD44 to hyaluronate on high endothelial venules, and over-expression of this molecule might favor metastatic spread. Because the first step in extravasation was adhesion to the endothelium, tumor cells might

have adhesion molecules whose ligands or interaction proteins are expressed preferentially on the endothelial cells of the target organ. Indeed, it had been shown that the endothelial cells of the vascular beds of various tissues differ in their expression of these ligands or proteins for adhesion molecules (Ruoslahti., 2002). We also demonstrated that SCUBE2 differs in expression of endothelial cells on variable non- neoplastic organs. SCUBE2 expressions on the endothelial cells were seen at lymph nodes (axillary or pelvic), bone and lung, but not expressed on the endothelial cells at cartilage and spleen (unpublished data). These patterns were roughly correlated the metastasis sites of advanced breast cancer. So we proposed that homotypic protein-protein interaction between re-expressing SCUBE2 breast cancer cells and SCUBE2 expressing endothelial cells maybe a factor mediating the breast cancer metastasis. We would approach the hypothesis in the future.

SCUBE2 Enhancing Hedgehog Signaling Pathway in Lymph Node Metastasis. Hedgehog signaling pathway also played a role in lymph node metastasis of cancer. The site at which circulating tumor cells left the capillaries to form secondary deposits is not only related to the anatomic location of the primary tumor, but many observations suggested that natural

pathways of drainage did not wholly explain the distribution of metastases. Chemokines could play a very important role in determining the target tissues for metastasis. For example, some breast cancer cells express the chemokine receptors CXCR4 and CCR7 (Müller et al., 2001). The chemokines that bind to these receptors werw highly expressed in tissues to which breast cancers commonly metastasize. Blockage of the interaction between CXCR4 and its receptor decreased breast cancer metastasis to lymph nodes and lungs.

Some target organs may liberate chemoattractants that tended to recruit tumor cells to the site. Through this mechanism, tumor cells could proliferate at the metastatic site (Fidler., 2003). Recent studies had revealed that Hh proteins not only determine patterning and cell fate during embryonic development, but also function in cell fate determination of self-renewing tissues in the adult, such as the hemopoietic and immune systems. In the thymus, Hh signaling could play an important role regulating thymic cellularity as well as the development of $CD4^{-}CD8^{-}$ thymocytes (Outram et al., 2000; Sacedon et al., 2003; Varas et al., 2003; Shah et al., 2004). In addition, the effector function of peripheral $CD4^{+}$ T cells was modulated by Shh (Lowrey et al., 2002; Stewart et al., 2002). In Sacedón et al study (Sacedón et al., 2005), they analyzed the expression of Shh in peripheral lymphoid organs and

showed that Shh was produced by follicular dendritic cells (FDCs), mainly in germinal centers (GCs). GCs were specialized microenvironments and SHH could protect GC B cells from Fas-induced apoptosis and involved in the FDC-mediated rescue of GC B cells from apoptosis. Recently we found a novel function for SCUBE2 in the SHH pathway by acting as a signalling component through interactions with SHH ligand and PTCH1 to promote its signal transduction (Tsai et al., 2009). Our data suggested that the caveolin-1- or raft-associated SCUBE2 protein may concentrate the Hh ligand within membrane raft microdomains and facilitate the presentation of Hh ligand to the PTCH1 receptor in the responding cells. In this situation, SHH could play a role of chemoattractants in breast cancer lymph node metastasis due to SHH also rich in lymph node (Sacedón et al., 2005). In esophageal carcinoma, it was proved that Gli-1 expression is associated with lymph node metastasis and tumor progression (Mori et al., 2006). Due to the evidences of interaction between the SCUBE2 and Hh pathway was important to breast cancer tumorgenesis, it was worth to approach the relationship between the SCUBE2 expression and Hh pathway signaling within the human breast cancer tissue at primary site and lymph node metastasis sites in the future. By this way, we would develop new therapeutic modality to treat the metastatic breast cancer

patients.

Expression of SCUBE2 Might Serve as a Hormone Responsive Element. Abba et al. (Abba et al, 2005) indicated a possible association between SCUBE2 expression and estrogen receptor (ER) status based on integrated the breast cancer comparative transcriptome analysis. They reported SCUBE2 mRNA expression associated with ER status of breast cancer specimens, and the ER-associated SCUBE2 expression was further validated by real-time quantitative RT-PCR analysis (Abba et al, 2005; Bertucci et al. 2004). We found that SCUBE2 expression is significantly associated with progesterone receptor (PR) (p=0.037). Although no significant association between estrogen receptor and SCUBE2 expression (p=0.09) in our studies, but we found a tendency for higher number of SCUBE2 expression (61.1 %) in ER-expressing group when compared to non-ER-expressing counterparts (47.0 %). This could be explained by differences of the cutoff criteria for estrogen receptors positivity. More than 5 % of tumor cells staining positive for ER was determined as positive in Abba et al study, instead of 10 % in our study, which was recommended in recent consensus in breast cancer (Abba et al, 2005;). The potential positive association of SCUBE2 expression and ER or

PR status suggested that expression of SCUBE2 might serve as a hormone responsive element and may be regulated by estrogen and progesterone. At the same time, the SCUBE2 may be an indicator of responsiveness to endocrine therapy. However, this suggestion requires further verification in a large cohort of patients responding and not responding to endocrine therapy.

Possible Gene Regulations of SCUBE2 in Breast Cancer

Carcinogenesis. DNA Methylation of the DNA is an important epigenetic (i.e., not associated with alteration in the primary structure of the DNA) phenomenon, which plays important roles in regulation of gene expression, maintenance of genome integrity, and genomic imprinting. DNA methylation of cytosine of CpG dinucleotides plays an important role in the regulation of gene expression and differentiation in mammals (Bird., 1992; Eden and Cedar., 1994). Several examples had been described in which the transcriptional expression of a particular gene in inversely correlated with level of methylation of its CpG island. Complex changes in the methylation status of a number of genes, including HLA class I genes, could be observe during development (Monk et al. 1987). DNA methylation also plays an important role in carcinogenesis, which had been a topic of considerable interest in the last few

years. Compared the methylation status in genomic DNA from expressing and non-expressing tissue, reproducible differences at several CpG islands correlated the expressions of certain protein (Guillaudeux et al. 1993). We would test whether the status of methylation in SCUBE2 promotor significantly correlate with protein expression in breast cancer cells in human tissue. We also could test whether 5' azacytidine demethylating agent could induce SCUBE2 expression in lower expressing breast cancer cells *in vitro*.

We also found putative binding sites of transcription repressors, Snail, Slug, SIP1 (smad interacting protein-1) and Twist on the promoter area of SCUBE2. These transcription repressors were novel parameters of disease aggressiveness in metastatic ovarian and breast carcinoma (Elloul S et al., 2005). It was demonstrated previously that the Snail family of transcription factors and Smad-interacting protein 1 (Sip1) regulate E-cadherin and matrix metalloproteinase 2 (MMP-2) expression, cellular morphology, and invasion in carcinoma. They functionally linked to human breast cancer progression and metastasis (Come et al. 2004). We also found that there was an E-box on the promoter region of SCUBE2. Thus, we would undertake to clone the SCUBE2 gene promoter in human breast cancer cell lines to get insights into the mechanisms of the regulation of SCUBE2 expression. By this way, we would

try to find some critical transcription factors present to activate SCUBE2 expression

Generation Scube2 Knockout Mice to Approach the Scube2 Functions. Genetic manipulation of mouse genes *in vivo* is a powerful approach to understanding the function of a gene, both during embryonic development and in adult tissues. Based on the previous data and our findings through the immunohistochemistry, the ducts of normal breast could express the SCUBE2. We have shown that SCUBE2 plays a critical role in breast cancer and demonstrate that C- terminal of SCUBE2 is important in BMP-2 and SCUBE2. Towards unraveling the roles of SCUBE2's growth suppressive activity in normal breast development, breast homeostasis and tumorigenesis, it is necessary to use gene targeting and embryonic stem cell technologies to generate Scube2 knockout mice. The Scube2 knockout mice could let us to determine the roles of Scube2's growth suppressive function *in vivo* by generating mice with a targeted deletion of the C-terminal of SCUBE2. We would design a gene targeting strategy that is specific to C-terminal of Scube2 according the MICR targeting strategy (Adams et al. 2004).

結 論 與 展 望

(Conclusion and Perspective)

Our study provides strong evidence that elevated SCUBE2 protein expression has a role in suppressing breast-cancer cell proliferation, possibly through its anti-BMP activity. The protein could serve as an independent prognostic biomarker for breast cancer. Our data is the first time to identify that SCUBE2 may be an additional tumor recurrence marker that can supplement the current clinicopathologic standards to improve treatment paradigms for women diagnosed with invasive ductal carcinoma. Reasoning that these findings might be extended to the identification of some agents, that can activate SCUBE2 expression for development, as a new tumor-suppression therapeutic modality in breast cancer.

Further studies are needed to define the regulatory mechanisms of SCUBE2 both at gene and protein levels during breast cancer progression and to explore its potential clinical utility for breast tumors. By the proposed mechanisms, we hoped we can obtain in-depth knowledge of how SCUBE2 performs its actions in breast cancer cells, especially by Hh dependent mechanisms in the future. Through verify the function of N-terminal domain of SCUBE2, we could further realize the function of SCUBE2. Ongoing studies in

understanding the impaction of re-expression of breast cancer cells in metastatic site would provide knowledge SCUBE2 mediated lymph node metastasis in breast cancer. This will also provide evidences to evaluate if SCUBE2 is another factor for potential therapeutic application in breast cancer with metastasis. These ongoing researches could provide the breast cancer community with new insight into breast cancer tumorigenesis, and lead to the development of more effective treatment strategy against breast cancer.

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Table 1. Association of SCUBE2 Protein Expression and Clinico-pathological Characteristics and Other Biomarkers.

		SCUBE2 expression, n (%)		χ^2 test
Characteristics/markers	Total	Negative	Positive	*p*
Age of patient, yrs				0.76
≧ 50	105	48 (45.7)	57 (54.3)	
< 50	51	22 (43.1)	29 (56.9)	
Histological grade				0.53
G1	24	10 (14.7)	14 (58.3)	
G2	64	26 (40.6)	38 (59.4)	
G3	68	34 (50.0)	34 (50.0)	
pT				0.48
T1	38	14 (36.8)	24 (63.2)	
T2	90	41 (45.6)	49 (54.4)	
T3	15	7 (46.7)	8 (53.3)	
T4	13	8 (61.5)	5 (38.5)	
Lymph node status				0.39
N0	60	26 (43.3)	34 (57.7)	
N1	47	24 (51.1)	23 (48.9)	
N2	23	7 (30.4)	16 (69.6)	
N3	26	13 (50.0)	13 (50.0)	
Cancer stage				0.86
I	25	10 (40.0)	15 (60.0)	
II	74	34 (45.9)	40 (54.1)	
III	57	26 (45.6)	31 (54.4)	
ER status				0.08
Negative	66	35 (53.0)	31 (47.0)	
Positive	90	35 (38.9)	55 (61.1)	
PR status				0.02
Negative	88	47 (53.4)	41 (46.6)	
Positive	68	23 (33.8)	45 (66.2)	
HER-2/neu status				0.62
Negative	121	53 (43.8)	68 (56.2)	
Positive	35	17 (48.6)	18 (51.4)	
Recurrence				<0.0001
Yes	49	35 (71.4)	14 (28.6)	
No	107	35 (32.7)	72 (67.3)	

ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

Table 2. Univariate and Multivariate Survival Analysis by Cox Proportional Hazards Models

Variables	Univariate		Multivariate	
	HR (95% CI)	*p*	HR (95% CI)	*p*
SCUBE2 (Positive vs. Negative)	0.25 (0.13-0.47)	<0.0001	0.26 (0.13-0.49)	<0.0001
Grade (1 & 2 vs. 3)	1.39 (0.79-2.43)	0.26	1.12 (0.58-2.16)	0.74
Lymph node status (Positive vs. Negative)	2.05 (1.10-3.82)	0.02	1.11 (0.46-2.68)	0.82
Stage (I & II vs. III)	3.01 (1.71-5.30)	0.0001	2.86 (1.25-6.51)	0.01
ER (Positive vs. Negative)	0.82 (0.46-1.44)	0.48	1.17 (0.53-2.56)	0.70
PR (Positive vs. Negative)	0.51 (0.28-0.93)	0.03	0.73 (0.34-1.59)	0.43
Her-2/neu (Positive vs. Negative)	1.39 (0.79-2.46)	0.26	1.02 (0.56-1.85)	0.95

HR, hazard ratio; 95% CI, 95% confidence interval.

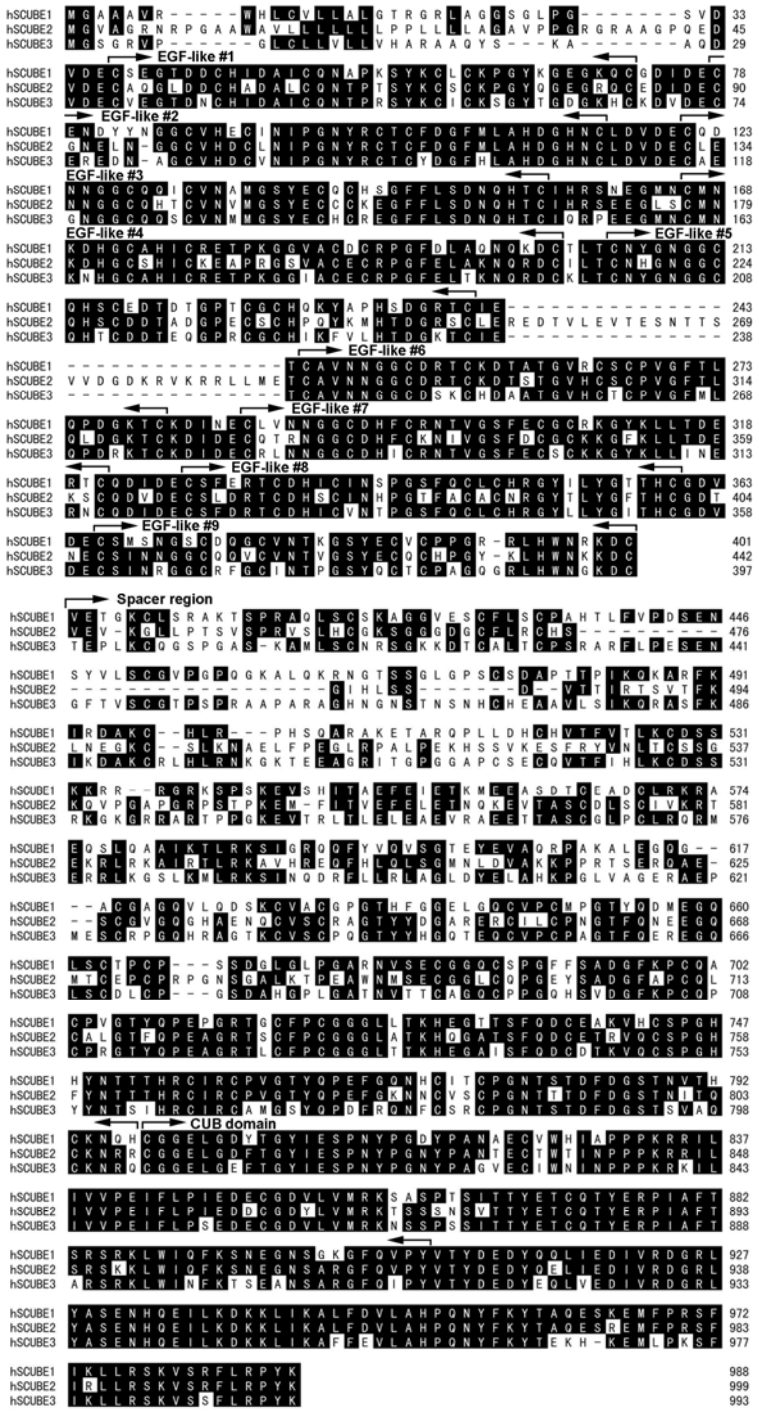

Figure 1. Alignment of human SCUBE1~3.

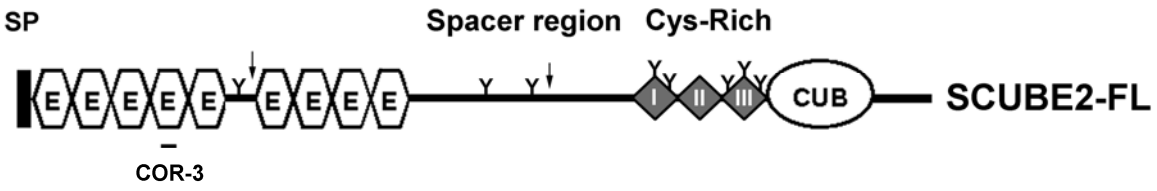

Figure 2. Domains structures of SCUBE2 and antigen location of chicken anti-SCUBE2 antibody.

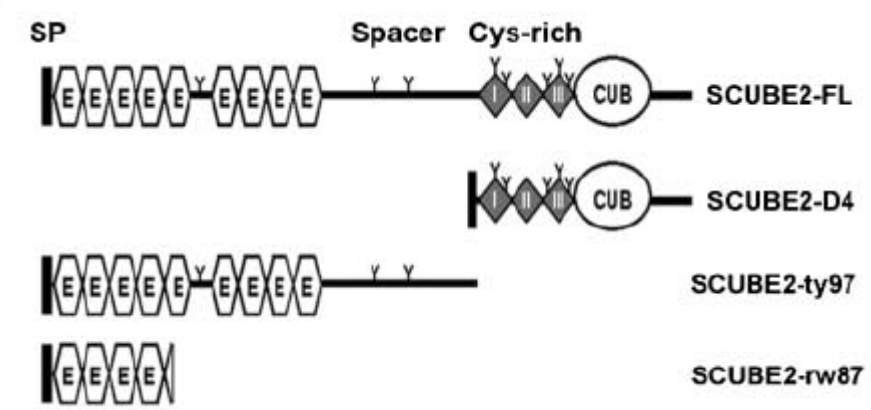

Figure 3. Domain organization of the SCUBE2 expression constructs used in this experiment. A FLAG epitope tag was added immediately after the signal peptide sequence, thus at the N-terminus, for easy detection. Two deletion constructs, SCUBE2-ty97 and -rw87 mutant, were made by mimicking two null mutant alleles (*ty97* or *rw87)* in the zebrafish *Scube2*. 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.

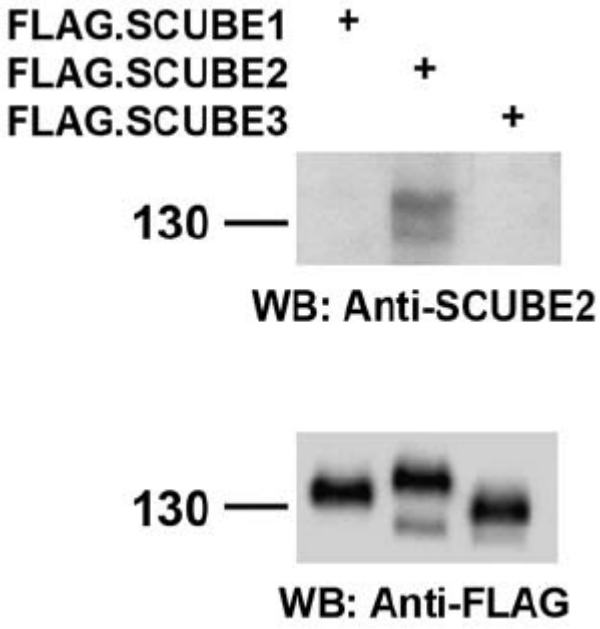

Figure 4. Production and characterization of the anti-SCUBE2 specific antibody. Specificity of anti-SCUBE2 antibody by western blot analysis. The anti-SCUBE2 antibody could specifically recognize the recombinant SCUBE2 but not SCUBE1 or SCUBE3 protein expressed in HEK-293T cells (top panel). As a control for the protein loading, expression of the FLAG-tagged SCUBE protein was confirmed by anti-FLAG antibody (bottom panel).

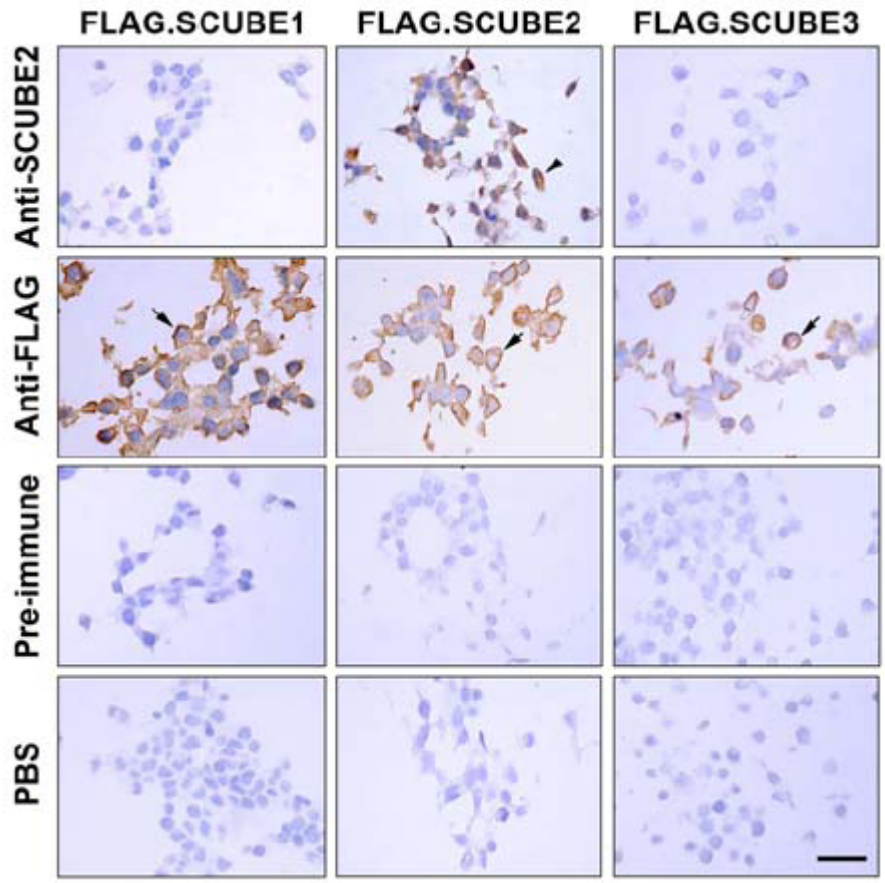

Figure 5. The anti-SCUBE2 antibody specifically detects SCUBE2 protein in formalin-fixed, paraffin-embedded HEK-293T cells. The anti-SCUBE2 antibody stained HEK-293T cells expressing SCUBE2 but not SCUBE1 or 3 (brown color, arrowhead, top panel). Immunostaining with anti-FLAG antibody verified the protein expression of the respective FLAG-tagged SCUBE protein (brown color, arrow, second panel). Staining specificity was confirmed by use of pre-immune serum or omission of the anti-SCUBE2 antibody (PBS) showing no specific staining (third and bottom panels, respectively). Bar = 50 μm.

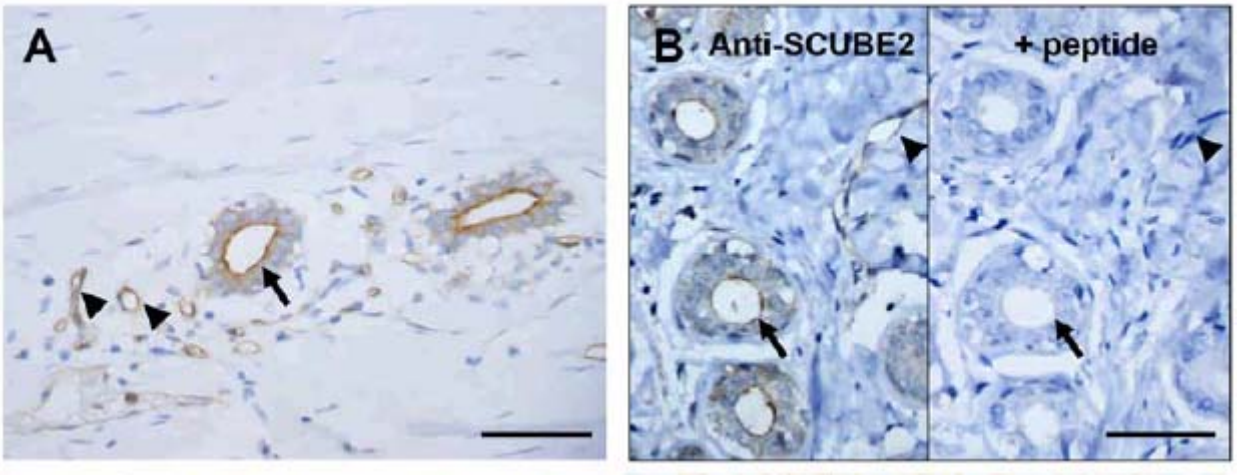

Figure 6 (A and B). A, Expression of SCUBE2 protein in normal breast tissue. Anti-SCUBE2 immunoreactive staining was found on the luminal surface of normal breast ducts (arrow) and vascular endothelial cells (arrowheads). B, Specificity of the anti-SCUBE2 staining. The luminal membrane of ductal epithelial and endothelial cells was immunoreactive (arrow and arrowhead, left panel). Consecutive sections were stained with anti-SCUBE2 antibody pre-absorbed with the respective peptide antigen (+ peptide, right panel). Pre-absorption of the anti-SCUBE2 antibody with the corresponding peptide immunogen resulted in no immunostaining, which confirmed the specificity of the anti-SCUBE2 immunoreactive signal in the breast tissue. Bar = 50 μM.

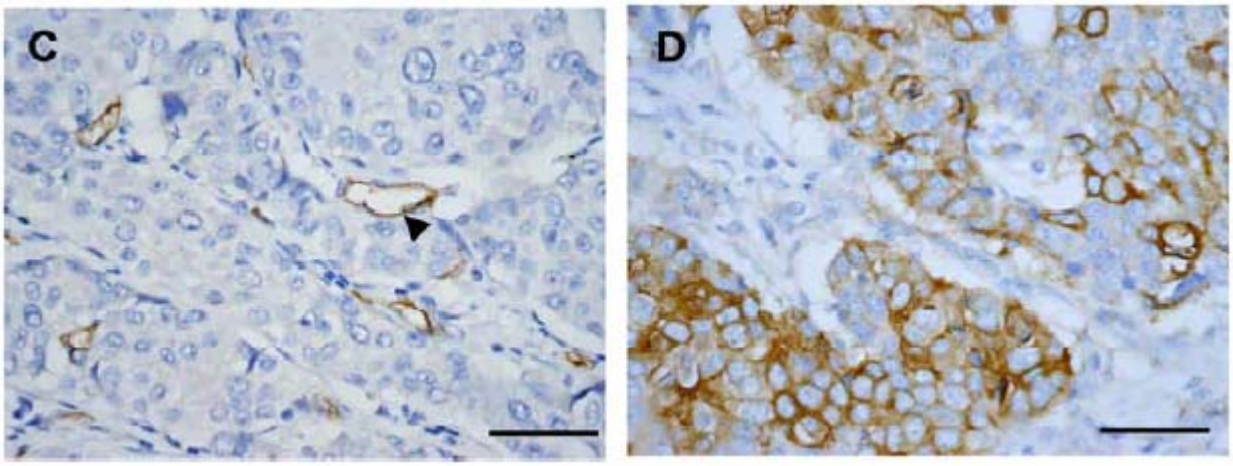

Figure 6 (C and D). Expression of SCUBE2 protein in breast tumors by immunohistochemistry. Representative images of negative (C) and positive tumor staining (D) for SCUBE2. Despite no tumor staining in Panel C, intratumor vascular endothelial cells remained positive for anti-SCUBE2 antibody (arrowhead), which serves as an internal control for immunohistochemistry. Bar = 50 μM.

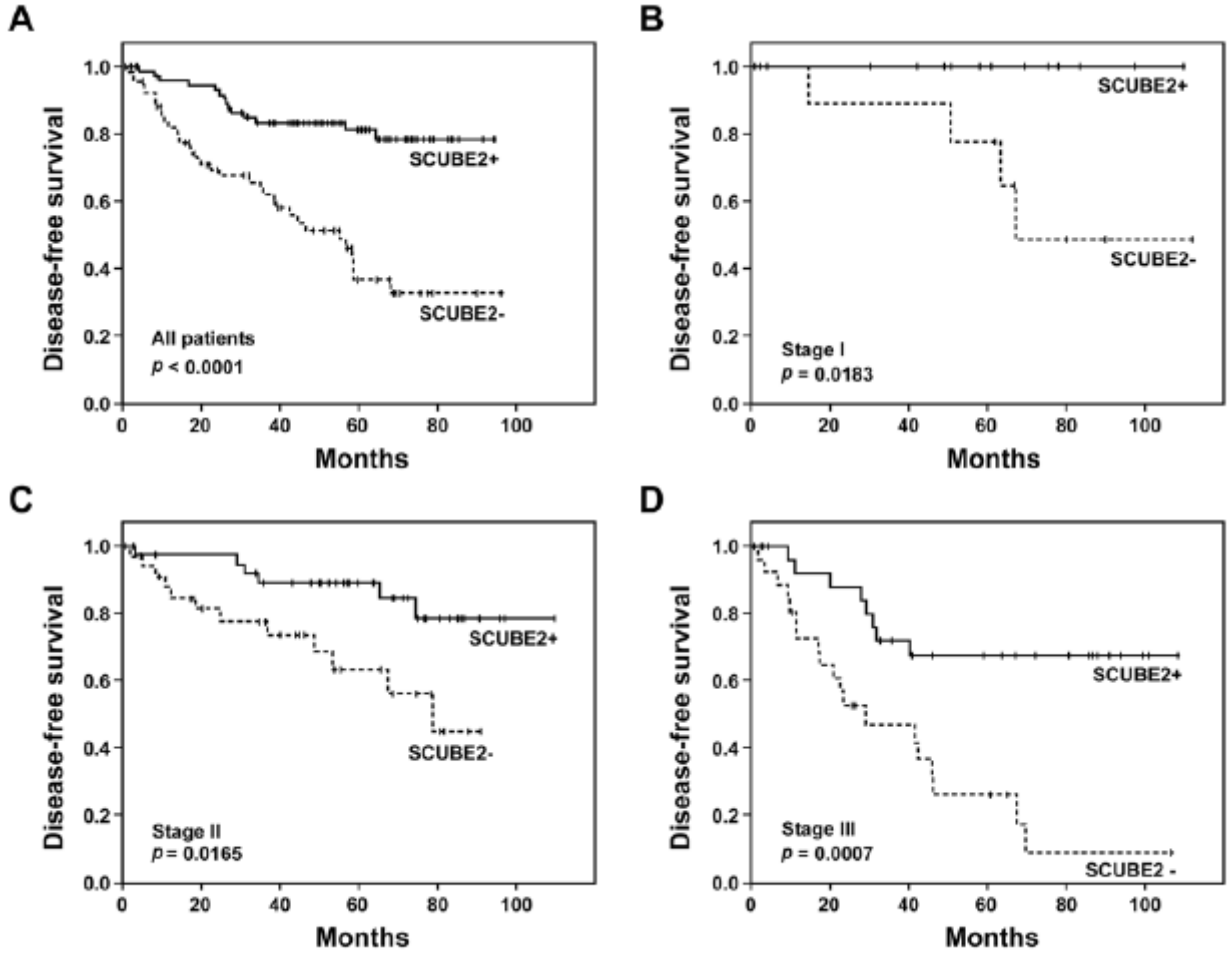

Figure 7. Kaplan-Meier disease-free survival curves for patients with breast cancer in terms of anti-SCUBE2 negative or positive status. A, All patients by negative (n = 70) or positive (n = 86) SCUBE2 status. B, Patients with stage I disease by negative (n = 10) or positive (n = 15) SCUBE2 status. C, Patients with stage II disease by negative (n = 34) or positive (n = 40) SCUBE2 status. D, Patients with stage III disease by negative (n = 26) or positive (n = 31) SCUBE2 status.

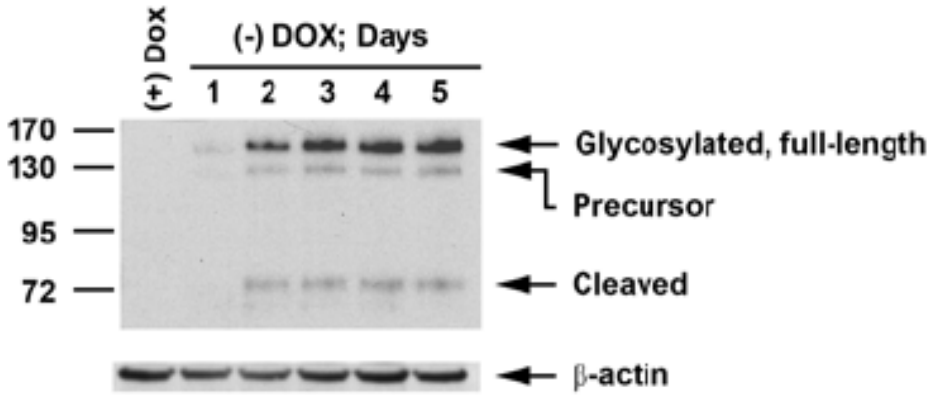

Figure 8. SCUBE2 overexpression suppresses MCF-7 breast-cancer cell proliferation *in vitro* and breast tumor growth *in vivo*. Induction of ectopic SCUBE2 protein in MCF-7 Tet-off SCUBE2 clone cells. MCF-7 Tet-off SCUBE2 cells were cultured in the medium without doxycycline, (-) Dox, for 5 days, and the induction of ectopic N-terminal FLAG-tagged SCUBE2 protein expression was determined by western blot analysis with anti-FLAG antibody. Anti-β-actin expression was used as a loading control. Double bands for the FLAG.SCUBE2 are due to the glycosylation of this protein (precursor and glycosylated, full-length form). A limited proteoytic fragment is also observed after induction (cleaved).

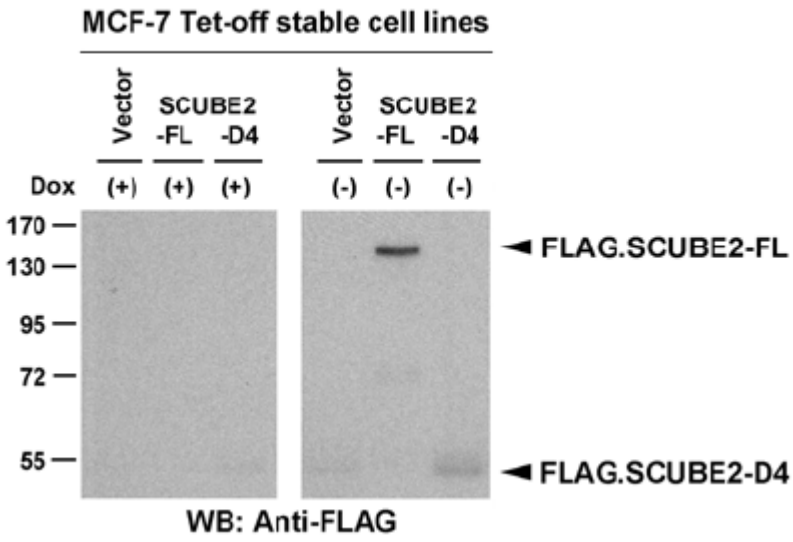

Figure 9. Overexpression of SCUBE2-FL (full-length) and D4 mutant protein suppresses MCF-7 breast-cancer cell proliferation *in vitro*. A, Induction of ectopic SCUBE2-FL or -D4 protein in MCF-7 Tet-off stable clone cells. MCF-7 Tet-off SCUBE2-FL or -D4 cells were cultured in the medium with or without doxycycline, (+) or (-) Dox, for 5 days. The induction of ectopic FLAG-tagged SCUBE2-FL or -D4 protein expression was determined by western blot analysis using anti-FLAG antibody.

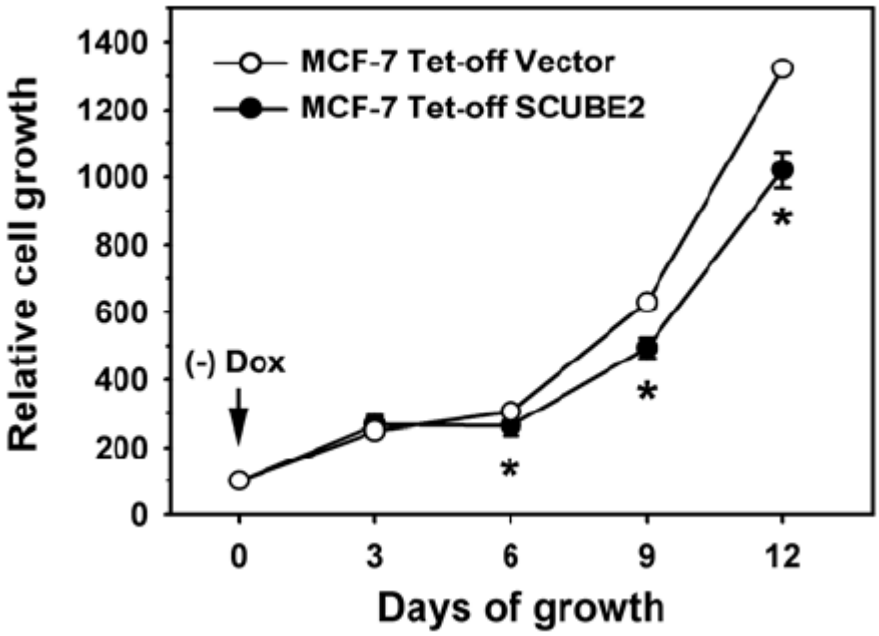

Figure 10. Effect of SCUBE2 protein expression on MCF-7 breast-cancer cell proliferation. The MCF-7 Tet-off Vector and the MCF-7 Tet-off SCUBE2 stable cells were cultured in medium without doxycycline, (-) Dox, to induce the expression of SCUBE2 protein. Cell proliferation was measured over the next 12 days by MTT assay. *, $p < 0.01$.

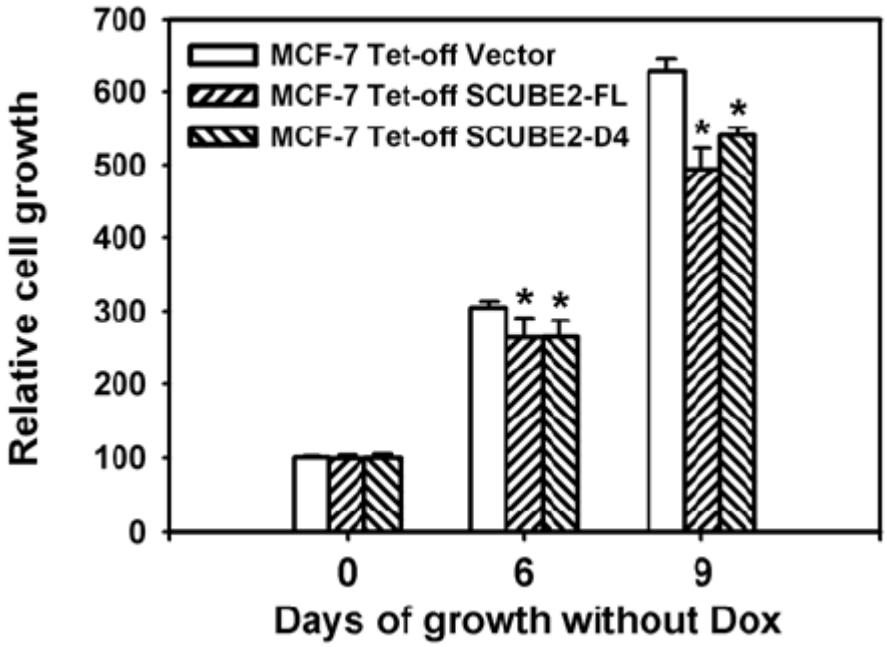

Figure 11. Effect of SCUBE2 protein overexpression on MCF-7 breast-cancer cell proliferation. The MCF-7 Tet-off Vector, SCUBE2-FL, or SCUBE2-D4 stable cells were cultured in medium without doxcycline, (-) Dox, to induce the expression of SCUBE2 protein. Cell proliferation was measured over the next 6 days by MTT assays. *, $p < 0.01$ (Vector *vs.* SCUBE2-FL or -D4).

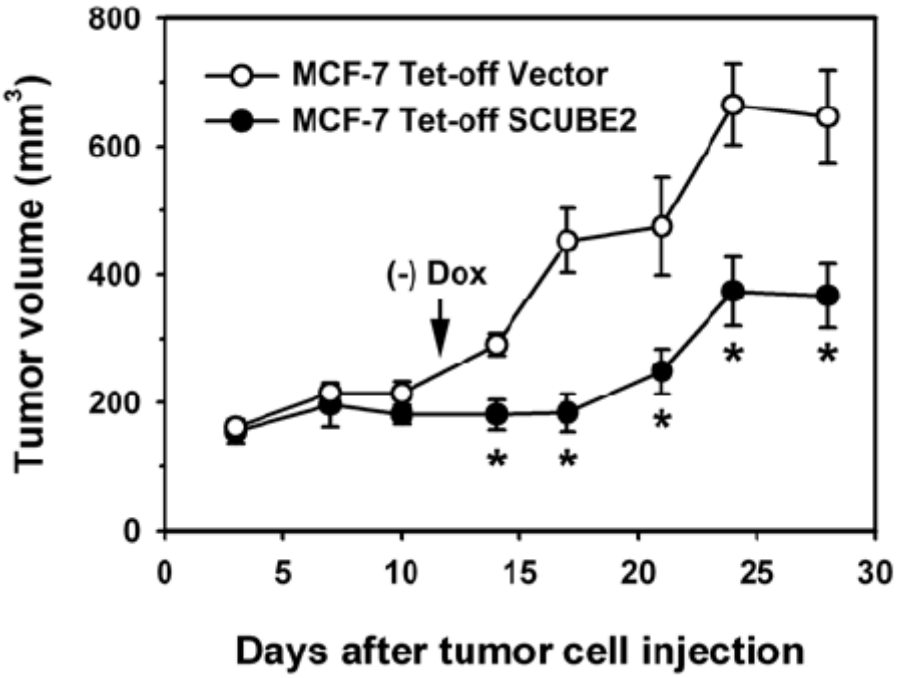

Figure 12. Induction of ectopic SCUBE2 reduces MCF-7 breast tumor growth in xenograft mouse model. The MCF-7 Tet-off Vector or the MCF-7 Tet-off SCUBE2 stable cells were injected into nude mice to induce tumor formation. After tumor growth for 12 days and tumor development, the mice were divided into groups to continue to receive doxycycline or not, (-) Dox. Growth of the MCF-7 Tet-off Vector or the MCF-7 Tet-off SCUBE2 cells was measured as a function of time in the absence of doxycycline (mean tumor volumes ± SEM). *, $p < 0.01$ (n=13 for the MCF-7 Tet-off Vector tumors and n=14 for the MCF-7 Tet-off SCUBE2 tumors).

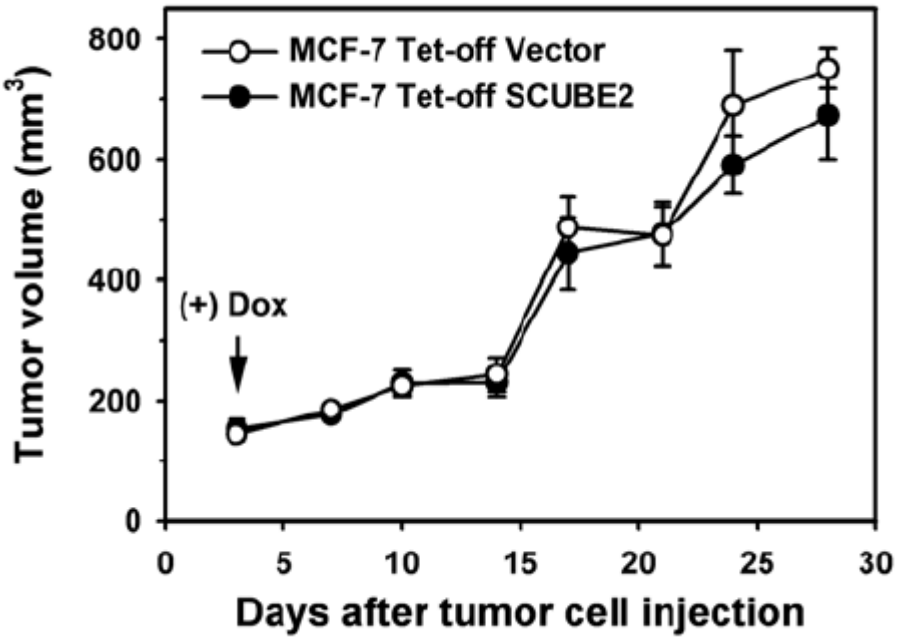

Figure 13. Growth of the MCF-7 Tet-off Vector or the MCF-7 Tet-off SCUBE2-FL tumors in nude mice in the presence of doxycycline. The MCF-7 Tet-off Vector or MCF-7 Tet-off SCUBE2-FL clone cells were injected into nude mice. The mice continued to receive doxycycline in drinking water. Tumor growth was compared and plotted as a function of time until the termination of experiments (n=5 in each group).

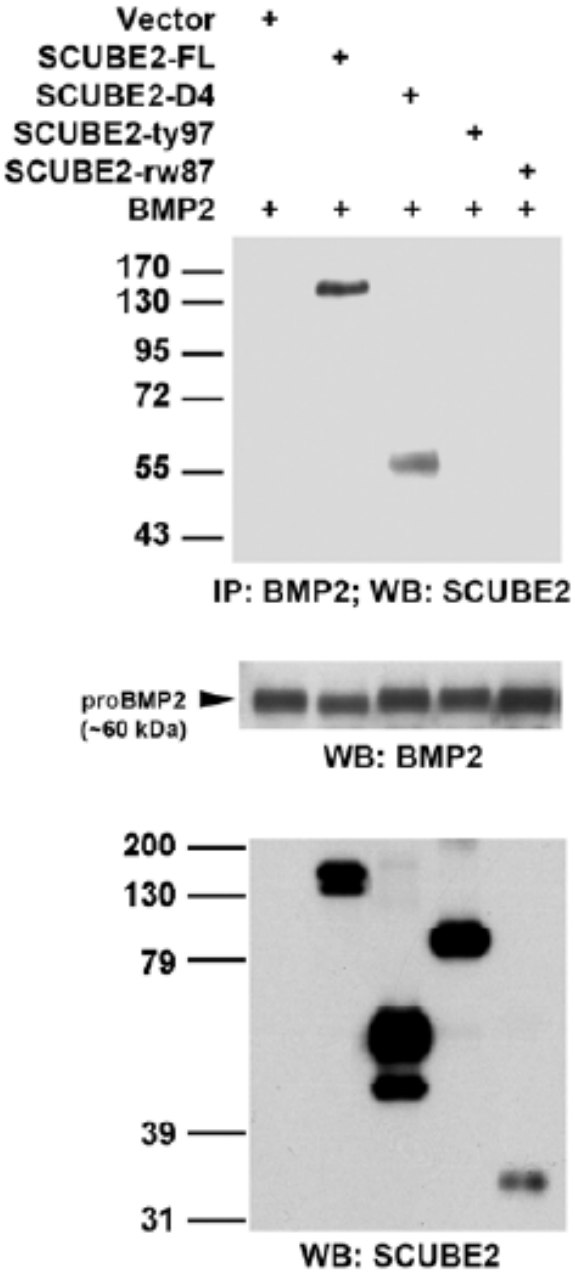

Figure 14. Interaction between SCUBE2 and BMP2. The BMP2 expression construct (Myc-tagged) was transfected alone or together with the expression plasmids encoding indicated FLAG-tagged SCUBE2 proteins in HEK-293T cells. Two days post-transfection, cell lysates underwent immunoprecipitation (IP) and western blot analysis (WB) with antibodies as indicated to determine the protein interactions. proBMP2, precursor BMP2 .

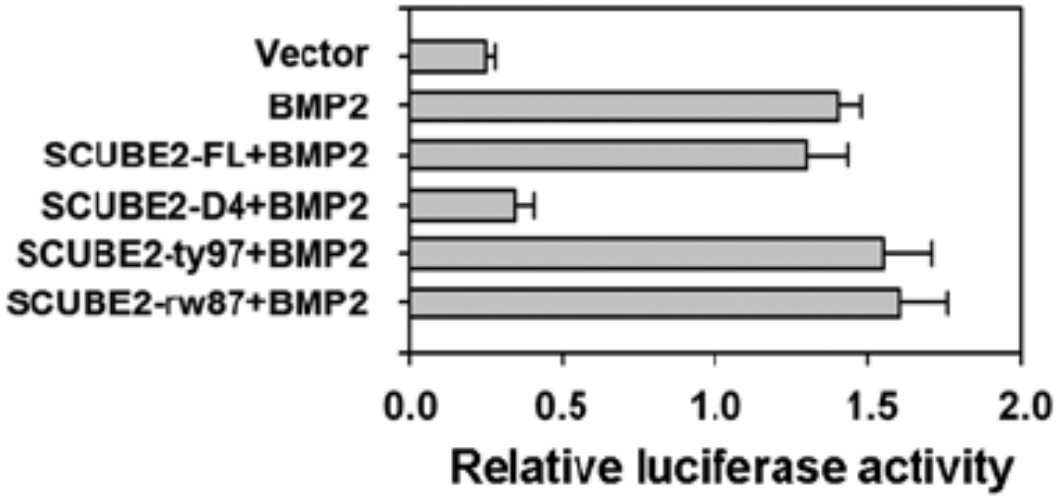

Figure 15. The C-terminal region of SCUBE2 binds BMP protein and acts as a BMP antagonist. Co-expression of SCUBE2-D4 mutant attenuated the BMP2 signaling activity. Conditional media from signaling cells (HEK-293T) co-transfected with the BMP2 expression plasmid alone or in combination with various SCUBE2 deletion constructs as indicated were added to the responding cells (HepG2) that contained the BMP-responsive luciferase reporter plasmid I-BRE-luc.

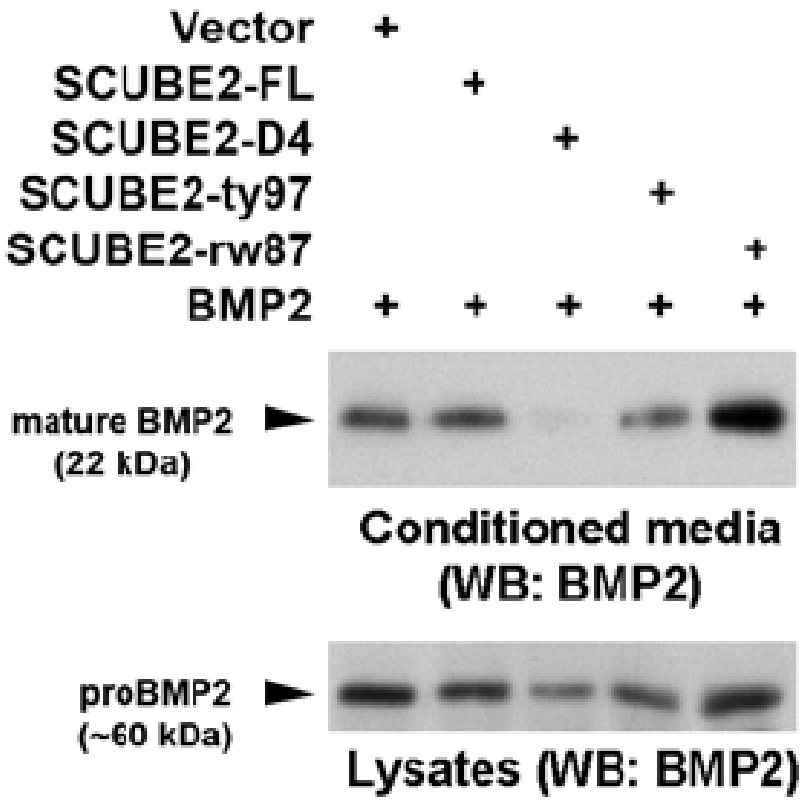

Figure 16. Western blot analysis of SCUBE2-D4 mutant inhibits BMP2 precursor processing into active, secreted molecule. HEK-293T cells were transfected with indicated expression plasmids for BMP2 and SCUBE2 mutants.

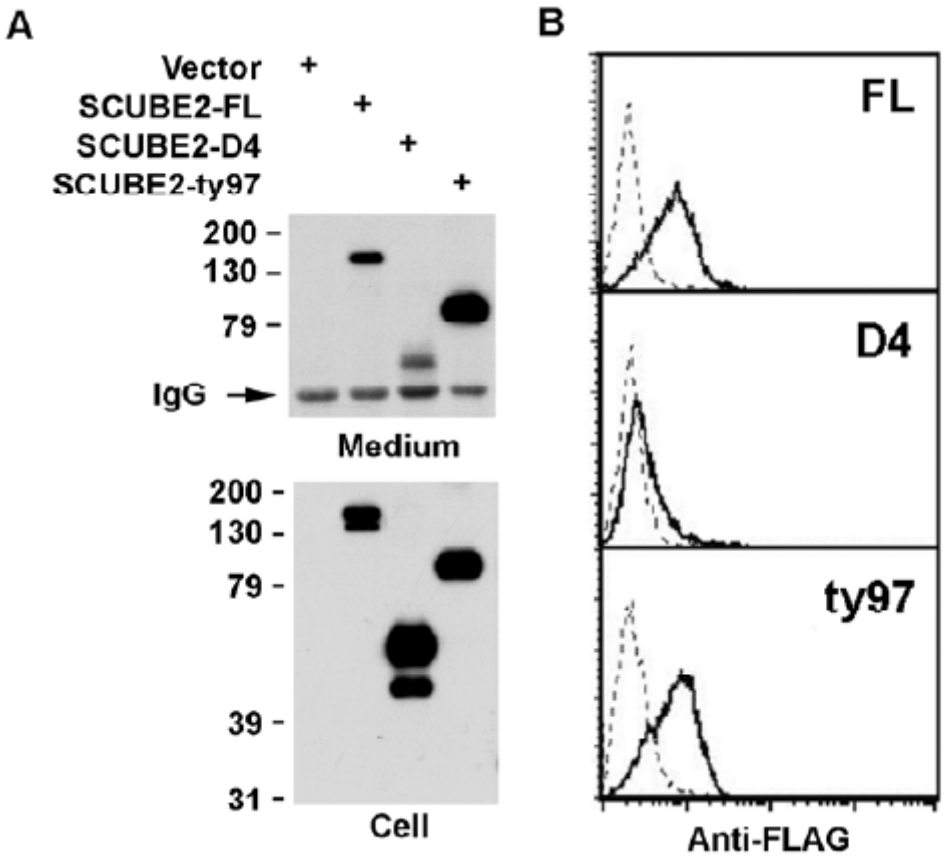

Figure 17. A. The C-terminal D4 mutant is defective in secretion and mainly resided within the cells. HEK-293T cells were transfected with the indicated expression plasmid. Two days after transfection, samples from conditioned medium or cell lysates were immunoprecipitated with anti-FLAG antibody and followed by western blot analysis. B, The SCUBE2-D4 protein is incapable of targeting to the cell surface. Twenty-four hours after transfection, a set of transfected cells as above was detached and stained with anti-FLAG antibody to determine the cell surface expression by FACS analysis.

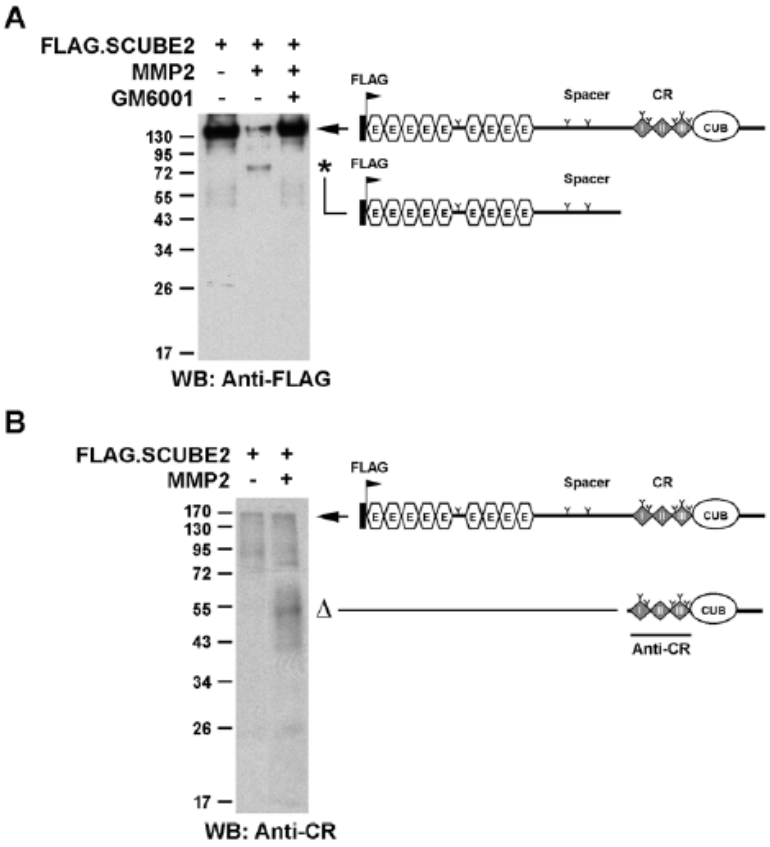

Figure 18. *In vitro* cleavage of SCUBE2 by purified recombinant matrix metalloprotease 2 (MMP2). Cell lysates from HEK-293T cells transfected with a expression plasmid producing the N-terminal FLAG-tagged SCUBE2 full-length protein (FLAG.SCUBE2) were prepared and incubated with purified recombinant human MMP2 (500 ng) at 37 ºC for 2 h in the absence of presence of a broad-spectrum MMP inhibitor (GM6001, 20 μM). Recombinant SCUBE2 and its cleaved product were analyzed by SDS-PAGE and western blot analysis using an anti-FLAG antibody (A) or anti-CR polyclonal antibody (B), respectively. Bands corresponding to uncleaved, full-length product (arrows in panel A and B), N-terminal fragment (asterisk in panel A), or C-terminal fragment (triangle in panel B) are indicated to the right of each gel. The processing of SCUBE2 by MMP2 appears to be specific since addition of an inhibitor for MMP (GM6001) completely blocked the cleavage of recombinant SCUBE2 protein (in panel A).

附　錄

(Appendix):

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Vascular Matrix Biology and Bioengineering Workshop

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Certificate of IRB Approval

(TMUHIRB 20070101 and WFH F950903)

Animal protocol (RMiIBMYR2005098)

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 activity. Together, our results show for the first time that altered SCUBE2 expression is important 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 NH_2 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, NH_2-SHICKEAPRGSVAC-COOH (derived from the NH_2 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

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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also graded according to a modified version of the Scarff-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 d after 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 specimen sections (4-μm thick) were dewaxed with xylene, rehydrated in graded concentrations of alcohol, treated with 3% H_2O_2 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

Characteristics/markers	Total	SCUBE2 expression, *n* (%)		χ^2 test
		Negative	Positive	*P*
Age of patient, y				0.76
≥50	105	48 (45.7)	57 (54.3)	
<50	51	22 (43.1)	29 (56.9)	
Histologic grade				0.53
G_1	24	10 (14.7)	14 (58.3)	
G_2	64	26 (40.6)	38 (59.4)	
G3	68	34 (50.0)	34 (50.0)	
pT				0.48
T1	38	14 (36.8)	24 (63.2)	
T2	90	41 (45.6)	49 (54.4)	
T3	15	7 (46.7)	8 (53.3)	
T4	13	8 (61.5)	5 (38.5)	
Lymph node status				0.39
N0	60	26 (43.3)	34 (57.7)	
N1	47	24 (51.1)	23 (48.9)	
N2	23	7 (30.4)	16 (69.6)	
N3	26	13 (50.0)	13 (50.0)	
Cancer stage				0.86
I	25	10 (40.0)	15 (60.0)	
II	74	34 (45.9)	40 (54.1)	
III	57	26 (45.6)	31 (54.4)	
ER status				0.08
Negative	66	35 (53.0)	31 (47.0)	
Positive	90	35 (38.9)	55 (61.1)	
PR status				0.02
Negative	88	47 (53.4)	41 (46.6)	
Positive	68	23 (33.8)	45 (66.2)	
HER-2/neu status				0.62
Negative	121	53 (43.8)	68 (56.2)	
Positive	35	17 (48.6)	18 (51.4)	
Recurrence				<0.0001
Yes	49	35 (71.4)	14 (28.6)	
No	107	35 (32.7)	72 (67.3)	

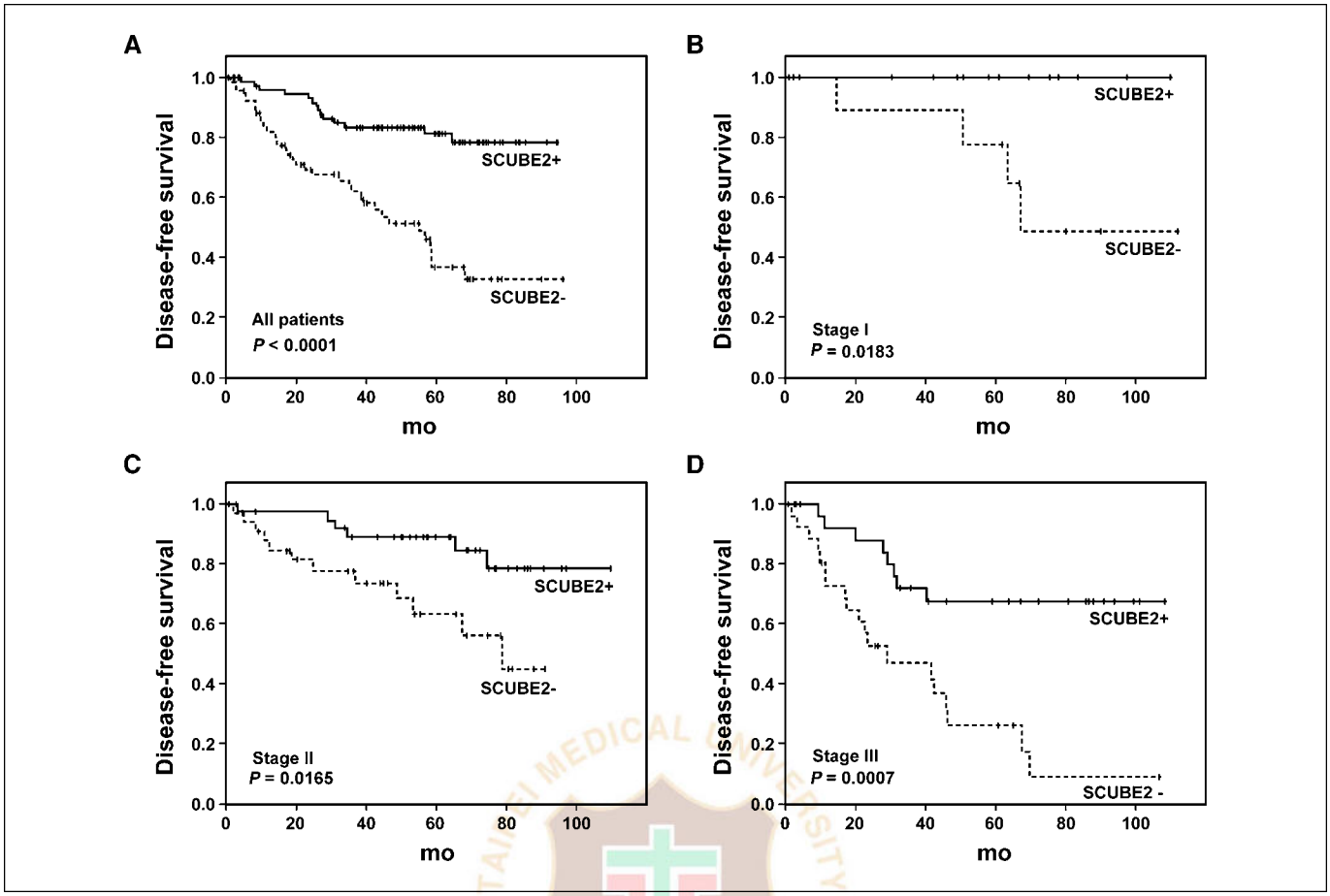

Figure 1. Kaplan-Meier disease-free survival curves for patients with breast cancer in terms of anti-SCUBE2–negative or anti-SCUBE2–positive status. *A,* all patients by negative (n = 70) or positive (n = 86) SCUBE2 status. *B,* patients with stage I disease by negative (n = 10) or positive (n = 15) SCUBE2 status. *C,* patients with stage II disease by negative (n = 34) or positive (n = 40) SCUBE2 status. *D,* patients with stage III disease by negative (n = 26) or positive (n = 31) SCUBE2 status.

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/cAnN.Cg-*Foxn1*nu/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-estradial 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 mm^3). The experiments were terminated when the tumor size reached 800 mm^3. 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 $CaCl_2$/150 mmol/L NaCl/0.05% Brij-35 at 37°C for 2 h. The cleaved fragments were analyzed by probing with anti-FLAG (NH_2 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 χ^2 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 analysis followed by multivariate 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. S1*A*, 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. S1*B*, 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. S2*A*). 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. S2*B*).

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. S2*D*) and 70 cases (44.9%) were scored as negative for SCUBE2 (Supplementary Fig. S2*C*). 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. 1*A*). 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. 1*B–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. 2*A*). No induction of FLAG.SCUBE2-FL was observed in the presence of doxycycline in the MCF-7 Tet-off SCUBE2-FL clones (Fig. 2*A*) or in the control MCF-7 Tet-off Vector clones (data not shown).

To examine the effect of SCUBE2 overexpresssion 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. 2*B*). 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

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

Variables	Univariate		Multivariate	
	HR (95% CI)	*P*	HR (95% CI)	*P*
SCUBE2 (positive versus negative)	0.25 (0.13–0.47)	<0.0001	0.26 (0.13–0.49)	<0.0001
Grade (1 and 2 versus 3)	1.39 (0.79–2.43)	0.26	1.12 (0.58–2.16)	0.74
Lymph node status (positive versus negative)	2.05 (1.10–3.82)	0.02	1.11 (0.46–2.68)	0.82
Stage (I and II versus III)	3.01 (1.71–5.30)	0.0001	2.86 (1.25–6.51)	0.01
ER (positive versus negative)	0.82 (0.46–1.44)	0.48	1.17 (0.53–2.56)	0.70
PR (positive versus negative)	0.51 (0.28–0.93)	0.03	0.73 (0.34–1.59)	0.43
HER-2/neu (positive versus negative)	1.39 (0.79–2.46)	0.26	1.02 (0.56–1.85)	0.95

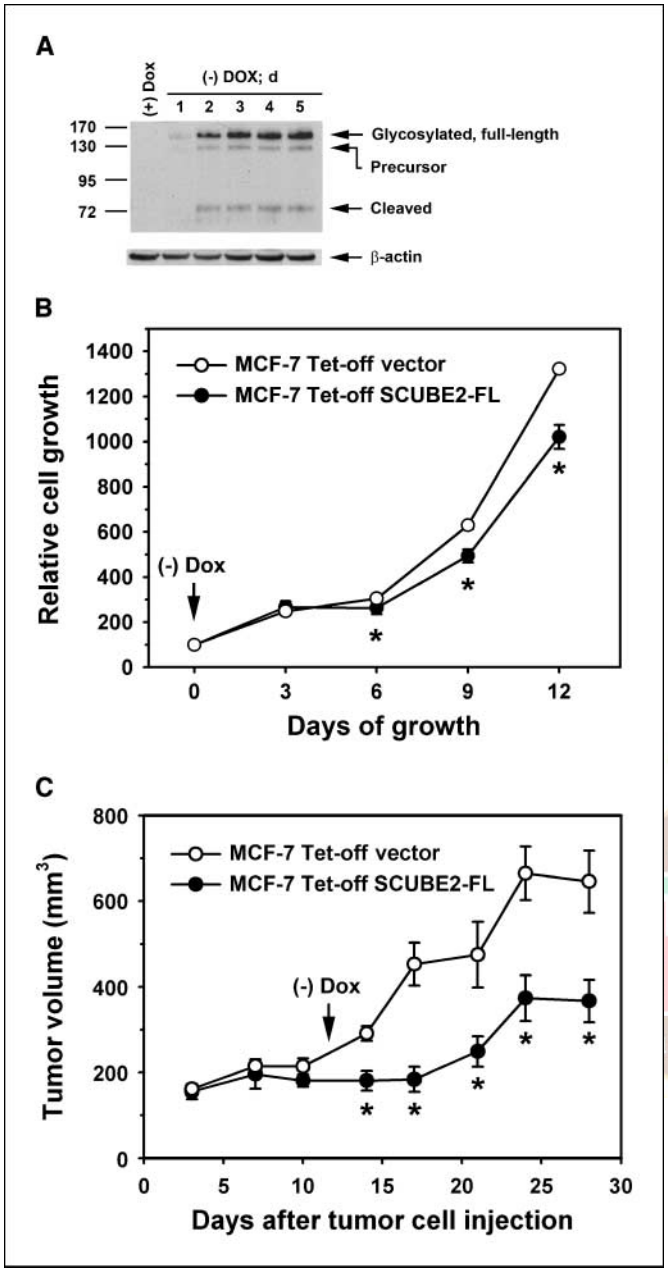

Figure 2. SCUBE2 overexpression suppresses MCF-7 breast cancer cell proliferation *in vitro* and breast tumor growth *in vivo*. *A,* induction of ectopic SCUBE2-FL protein in MCF-7 Tet-off SCUBE2-FL clone cells. MCF-7 Tet-off SCUBE2-FL cells were cultured in the medium without doxycycline [*(−)Dox*] for 5 d, and the induction of ectopic NH_2 terminal FLAG-tagged SCUBE2-FL protein expression was determined by Western blot analysis with anti-FLAG antibody. Anti–β-actin 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 proteoytic 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. *, $P < 0.01$ ($n = 13$ for the MCF-7 Tet-off vector tumors and $n = 14$ for the MCF-7 Tet-off SCUBE2-FL tumors).

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. 2*C*). 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*.

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 for only the COOH terminal region (cysteine-rich repeat motif and CUB domain) and two additional deletion mutants, SCUBE2-ty97 and SCUBE2-rw87, mimicking the *ty97* and *rw87* null mutant alleles (24–26), respectively, in the zebrafish *Scube2* gene by removing various portions of COOH terminal domains (Fig. 3*A*). HEK-293T cells were transfected with a Myc-tagged BMP2 expression plasmid alone or in combination with various FLAG-tagged SCUBE2 domain deletion constructs (Fig. 3*B*). Two days after transfection, cell lysates were subjected to immunoprecipitation with the anti-Myc monoclonal antibody, and the precipitates were analyzed by immunoblotting with anti-FLAG monoclonal antibody to determine protein interaction. Immunoprecipitation with anti-Myc antibody resulted in a specific coprecipitation of the SCUBE2-FL and SCUBE2-D4 deletion protein, but not SCUBE2-ty97 or SCUBE2-rw87 (Fig. 3*B*). These data suggest that SCUBE2 protein could indeed form a complex with BMP2 through its COOH terminal cysteine-rich repeats and CUB domain.

To further examine whether the interaction between SCUBE2-D4 and BMP2 affected the signaling ability of BMP2, we performed a coculture assay in which the conditioned media derived from HEK-293T cells transfected with BMP2 alone or together with SCUBE2 deletion constructs (signaling cells) were added to the responding cells, HepG2 cells containing the BMP-responsive promoter luciferase reporter construct I-BRE-Luc (31). As expected, 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. 3*C*). 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. 3*C*).

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

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. 3*D*); 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 NH_2 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

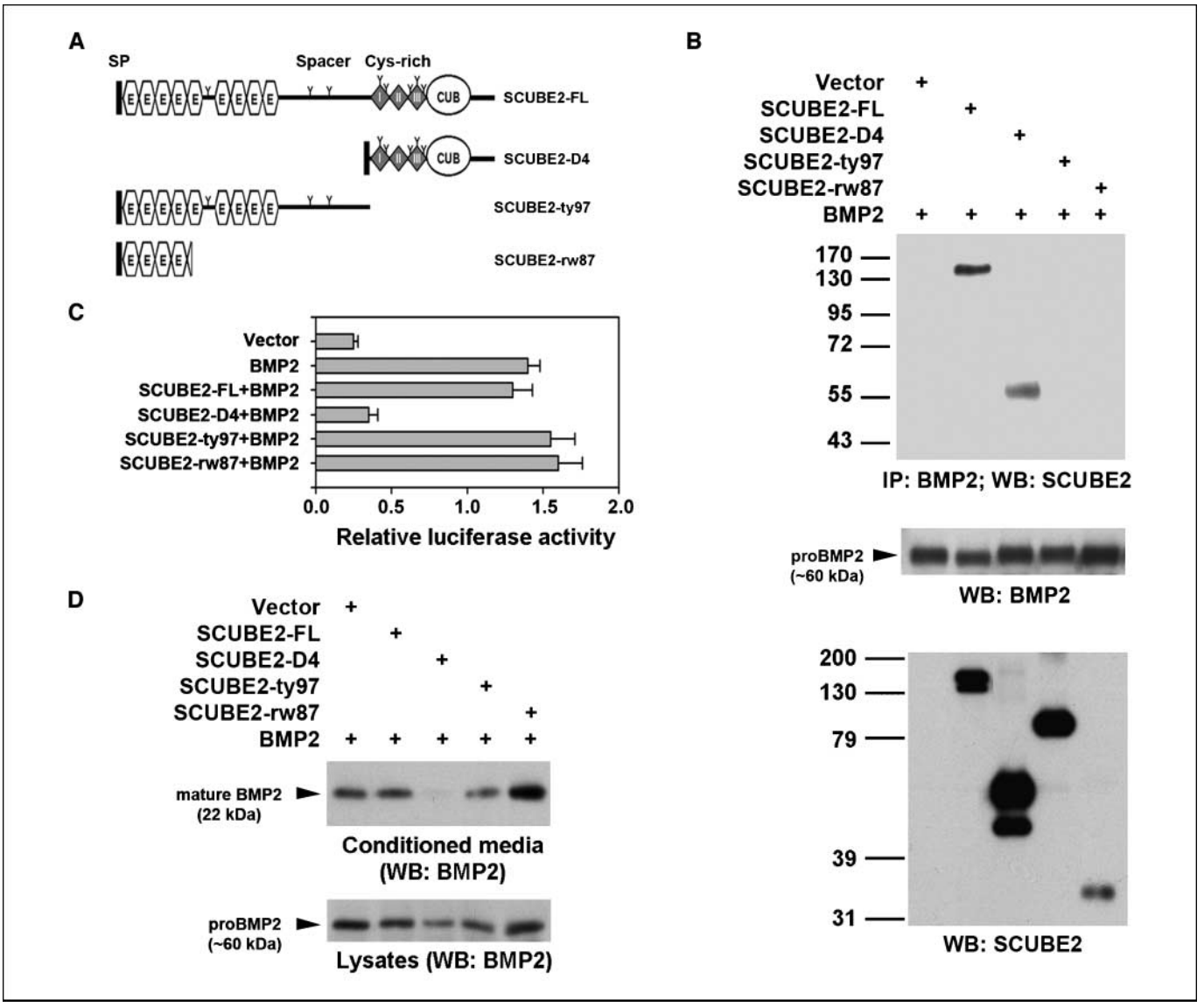

Figure 3. The COOH terminal region of SCUBE2 binds BMP protein and acts as a BMP antagonist. *A,* domain organization of the SCUBE2 expression constructs used in this experiment. A FLAG epitope tag was added immediately after the signal peptide sequence at the NH_2 terminus for easy detection. Two deletion 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.

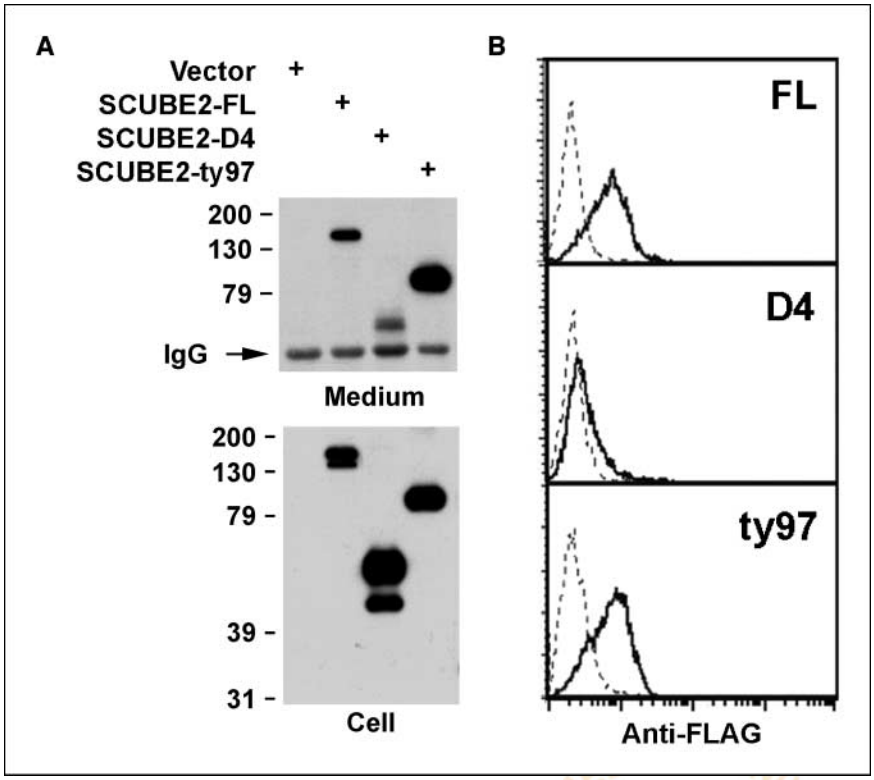

Figure 4. Subcellular distribution of the NH_2 or COOH terminal fragments of SCUBE2. *A,* the COOH terminal D4 mutant is mainly resided within the cells and defective in secretion. HEK-293T cells were transfected with the indicated expression plasmid. Two days after transfection, samples from conditioned medium or cell lysates were immunoprecipitated with anti-FLAG antibody and followed by Western blot analysis. *B,* the SCUBE2-D4 protein is incapable of targeting the cell surface. Twenty-four hours after transfection, a set of transfected cells as above was detached and stained with anti-FLAG antibody to determine the cell surface expression by fluorescence-activated cell sorting analysis.

have been implicated in the proteolytic processes associated with breast cancer biology (32, 33). Recombinant NH_2 terminal FLAG-tagged SCUBE2-FL protein (FLAG.SCUBE2-FL) produced by HEK-293T cells was prepared and used as a substrate for *in vitro* reaction with purified recombinant MMP proteases. As shown in Supplementary Fig. S5, *in vitro* digestion of FLAG.SCUBE2-FL protein by MMP2, but not MMP1 or MMP9 (data not shown), induced the appearance of NH_2 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. S2*A*). 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 *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 *in vivo* has yet to be revealed. It is temping 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

processes, including proliferation, differentiation, and apoptosis, in various cell types (27, 28). Recent studies showed that BMP ligands and their receptor components are expressed and activated in breast cancer and may contribute to breast cancer progression in ER-positive breast cancer (37–39). Likewise, BMP2 enhanced migration and invasion of a MCF-7 breast cancer cell line, as well as its overexpression, supported tumor formation in a breast cancer xenograft model (29). Furthermore, inhibition of the BMP signaling pathway by overexpression of a dominant-negative form of BMP type II receptor repressed proliferation of T-47D breast cancer cells (30). Together, these findings strongly suggest that BMPs may promote breast cancer cell proliferation. In agreement with this notion, our results suggest that SCUBE2 functions as a BMP antagonist (Fig. 3), and overexpression of SCUBE2 protein suppressed MCF-7 breast cancer cell proliferation *in vitro* and reduced MCF-7 tumor growth *in vivo* in an orthotopic mouse model (Fig. 2). However, further evaluation of the clinical effect of SCUBE2 protein overexpression on BMP signaling and its effect on breast cancer progression is needed.

In summary, our study provides evidence that elevated SCUBE2 protein expression has a role in suppressing breast cancer cell proliferation, at least through its anti-BMP activity. The protein could serve as an independent prognostic biomarker for breast cancer. Further studies are needed to define the regulatory mechanisms of SCUBE2 at both gene and protein levels during breast cancer progression and explore its potential clinical utility for breast tumors.

Disclosure of Potential Conflicts of Interest

A patent on the utility of SCUBE2 as a breast cancer biomarker may be filed by the Academia Sinica, but there is no current financial interest. Otherwise, the authors declare no competing financial interests.

Acknowledgments

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SCUBE2 suppresses breast tumor cell proliferation and confers a favorable prognosis in invasive breast cancer

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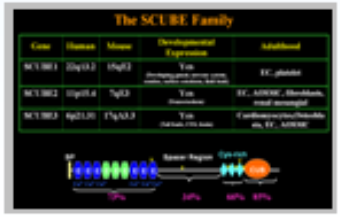
Abstract

SCUBE2 (signal peptide-CUB-EGF domain-containing protein 2), originally identified from the endothelium and several non-endothelial primary cell types, was recently shown to be expressed in invasive breast carcinomas. In this report, we demonstrate 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 58% (98/168) 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. 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 C-terminal region of SCUBE2 directly bound to and antagonized bone morphogenetic protein activity. Together, our results show for the first time that altered SCUBE2 expression is important in breast cancer progression and SCUBE2 may serve as a useful prognostic marker.

Introduction

- Invasive breast carcinoma is the most common malignant disease for women worldwide and claims over 400,000 lives per year. To date, treatment decisions for breast cancer mainly depend on the pathological features and clinical stage. Therefore, searching for effective molecular markers is necessary to predict the disease course and guide treatment decision.
- SCUBE2 (signal peptide-CUB-EGF-like domain-containing protein 2) belongs to an evolutionarily conserved SCUBE gene family. To date, three distinct isoforms have been cloned and named SCUBE1 to SCUBE3. These genes coding for polypeptide molecules of about 1000 amino acids share an organized protein domain structure of at least 5 motif:
  1) an N-terminal signal peptide sequence
  2) 9 tandem repeats of epidermal growth factor (EGF)-like repeats
  3) a large N-glycosylated spacer region
  4) three repeated stretches of 6-cysteine residues with unique and regular spacing
  5) one CUB domain at the C terminus
- SCUBE2 originally identified from the endothelium and several non-endothelial primary cell types. When overexpressed, SCUBE2 is a secreted glycoprotein that can form oligomers and has a stable association with the cell surface. Yet, its protein expression in normal breast tissue and the biological significance of SCUBE2 in breast cancer remain unknown.

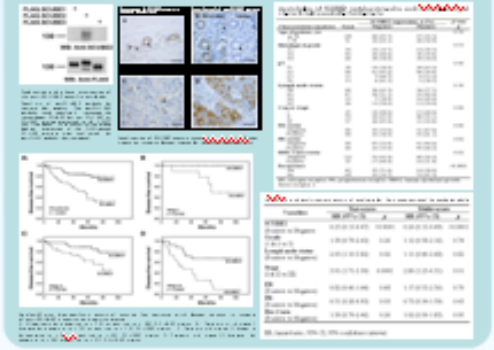


Aim

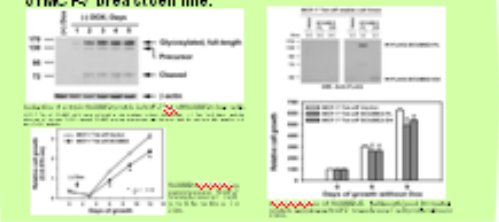
In this study, we investigated the protein expression / function and clinical implication of SCUBE2 in breast cancers.

Results

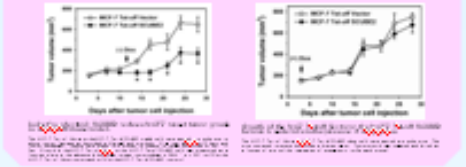
1. Expression of SCUBE2 protein is correlated with favorable disease-free survival of breast cancer.



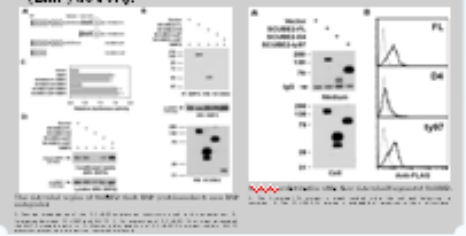
2. Overexpression of SCUBE2 protein suppresses proliferation of MCF-7 breast cell line.



3. SCUBE2 represses tumor growth of MCF-7 cells *in vivo*.



4. SCUBE2 antagonizes bone morphogenetic protein (BMP) activity.



5. *In vitro* cleavage of SCUBE2 by purified recombinant matrix metalloproteinase 2 (MMP2).

Summary

- Our clinical association study suggests that patients expressing SCUBE2 protein may have better disease-free survival than those without SCUBE2 expression. Thus, SCUBE2 can serve as an independent prognostic biomarker for breast cancer.
- Biochemical analyses reveal that SCUBE2 protein is subjected to limited proteolysis in releasing an active C-terminal fragment for its anti-BMP activity, which may, at least in part, account for the anti-proliferative effect of SCUBE2 on breast-cancer cells and contribute to favorable disease outcome in breast cancer patients.

證 明 書

茲證明本院臨床試驗(人體試驗)委員會通過計劃案如下：

研究計劃名稱：#TMUHIRB 20070101
SCUBE2 表現在乳癌的癌化上角色與預後的相關性探討

主 持 人：鄭建睿醫師(臺北醫學大學附設醫院 病理科)

執行期限：民國九十六年至民國九十七年

臺北醫學大學附設醫院 院 長

中 華 民 國 九 十 六 年 一 月 二 十 三 日

台北市立萬芳醫院
委託財團法人私立臺北醫學大學辦理
人體試驗委員會

中華民國九十六年二月八日

計畫名稱：SCUBE2 表現在乳癌的癌化上的角色與預後的相關性探討
計畫主持人：台北市立萬芳醫院病理科 鄭建睿 醫師
案　　號：F950903

上述計畫已於九十六年二月七日經本院人體試驗委員會審查通過，特此證明。

主任委員

連吉時

連吉時副院長

Certificate of IRB Approval
Taipei Medical University • Municipal Wanfang Hospital

Date: February 8, 2007

The project entitled: "The role of expression of SCUBE2 in tumorigenesis and prognosis of breast cancer." , submitted by investigator Chien-Jui Cheng, Attending Physician of Pathology Department, TMU-Wan Fang Hospital, has been approved by the Institutional Review Board on February 7, 2007 with the approval number F950903.

Gi-Shih Lien

Gi-Shih Lien, M.D., Ph.D.
Chairman
IRB

委託財團法人私立臺北醫學大學辦理
經人體試驗委員會通過
Approved by Institutional Review Board
FEB - 8, 2007
Taipei Medical University-
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DEC 12 2003

中央研究院實驗動物使用同意書

核定 Protocol#：RMiIBMYR2005098 審查通過日期：2003 年 12 月 12 日
效期至 2007 年 12 月 31 日(依計畫核定之期限)

計畫主持人基本資料

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計畫相關資料 (流水號#030098)

	計畫名稱
中文	探討基因剔除小老鼠之生理變異
英文	Functional studies of gene knockout mice

經費來源： ☑ 中研院 ☐ 國科會 ☐ 國衛院 ☐ 農委會
☐ 其他

核定執行期限：2005 年 1 月 1 日 至 2007 年 12 月 31 日
(如計畫執行期限有更動，請主動知會本小組)

計畫核定編號：
(如：國科會編號 NSC89-2000-A-002-001)

使用動物

種名	品系名		
mouse	Balb/c	C57BL6	129Sv

中央研究院實驗動物申請表(哺乳類適用)

(請以與 Word 相容之電腦軟體填寫，表格填寫空間可依需求自行擴張)

本欄限由動物實驗管理小組填寫
流水號#_____
PROTOCOL#_____

一、計畫主持人基本資料

姓名	單位	職稱
楊瑞彬	生醫所	副研究員
電話 (02)26523943	傳真 (02)27858847	手機 0932658609
電子郵件 rbyang@ibms.sinina.edu.tw		

二、計畫相關資料

	計畫名稱
中文	探討基因剃除小老鼠之生理變異
英文	Functional studies of gene knockout mice

經費來源：■中研院 □ 國科會 □ 國衛院 □ 農委會
□ 其他_____

擬執行期限*：__94__年__1__月__1__日 至__96__年__12__月__31__日

核定執行期限*：____年____月____日 至____年____月____日

(* 申請時，計畫如已獲核准者，填寫核定清單上之執行期限；尚未核定者，填寫擬執行期限。待核准後，須主動知會正式核准之執行期限。)

計畫核定編號：_____

(如：國科會編號 NSC89-2000-A-002-001)

三、本所同意此動物實驗使用許可之申請以及同意提供此申請案所需之動物飼養之管理與空間。

_____(所長簽署)_____(動物房負責人簽署)

四、動物來源及使用量相關資料(請依不同物種分別計量置於不同表格)：

請圈選執行計畫第 ■第一年 ■第二年 ■第三年 □第四年 □第五年 □其他第___年

(如每年使用量有不同者，請分年分表格分物種分別填寫，可自行延伸所需之表格；相同者，可複選)

動物物種	品系	動物來源	動物來源有無SPF規範**	動物飼養空間有無SPF 規範**	全年使用累計總隻數(單位)	每月平均使用隻數	每月平均寄養隻數
mouse	129Sv	分生所	有	有	250	25	25
mouse	C57BL6	國家動物中心	有	有	350	35	35
mouse	BALB/C	國家動物中心	有	有	50	5	5
					總計_650_	__65__	__65__

動物物種	品系	動物來源	動物來源有無SPF規範**	動物飼養空間有無SPF 規範**	全年使用累計總隻數(單位)	每月平均使用隻數	每月平均寄養隻數
				總計	_____	_____	_____

**動物來源之 SPF 規範必須相當或超過動物飼養空間之 SPF 規範。

五、執行動物實驗人員之相關資料(未在本申請表中列出人員，不得執行此計畫相關實驗)：

姓名	雇用期限	動物實驗經驗
（一）楊瑞彬	92.8.27 至今	3 年
（二）蔡明孜	92.8.27 至今	無
（三）蘇岳行	92.10.01 至今	無
（四）吳柏宗	92.10.06 至今	無

六、動物飼養場所 (限於本院各所指定之動物房)：

□ **植物所** □ **動物所** □ **分生所■生醫所** □**生化所** □ **生農所**

____6___ 樓 _____ 區 _____ 室

七、動物有否定期健康檢測：

(一) ■有(回答問題八，省略問題九)

(二) □ 無(回答問題九，省略問題八)

八、動物健康檢測相關資料：

(一) 檢測項目(如：MHC、Ectromelia、LCMV、Sendai、Mycoplasma、Theiler's (GDVII)等)：
MHV, MVM, Mycoplasma, Sendai virus,

(二) 檢測動物：

□ 如本申請表第四項所列之動物

■衛兵鼠(sentinel mice)與飼養表所列動物同房

□ 衛兵鼠(sentinel mice)與飼養表所列動物不同房

□ 其他 _____

(三) 檢測方式：

■ necropsy

■ PCR

■ Elisa

☐ microbiology
☐ 其他 _____

(四) 檢測頻率：每__3_個月檢測一次；每年共檢測___4___次

(五) 檢測報告記錄保管處：_生醫所動物房_____

(六) 負責檢測單位及負責人：_高志誠_____

九、使用動物無健康檢測者：

(一) 是否計畫在近期內開始實驗動物之健康檢測？ ☐ 是 ☐ 否

(二) 若是，預計何時開始？請列出擬檢測項目。

十、說明擬使用活體動物(而非其他非活體模擬)模式之必要性。

須建立基因剃除小老鼠,來進一步研究一個新基因之生理功能

十一、說明擬使用動物數量之依據。

本計畫主要是要比較野生種與基因剃除小老鼠其病理組織上之差異,預計收集不同年齡之動物來比較。

十二、請勾選動物實驗之性質：

(一) ■一般性 (回答問題十三，省略問題十四~十八)
(如，取細胞組織、靜脈注射、皮下注射、腹腔注射等一般性不涉及或僅短暫涉及動物疼痛或不適。)

(二) ☐ 特殊性 (造成動物長期疼痛或不適。)

☐ **1. 存活手術(survival surgery)**(回答問題十四，省略問題十三、十五~十八)
☐ **2. 癌症研究(cancer study)引起之病痛** (回答問題十五，省略問題十三、十四、十六~十八)
☐ **3. 非癌症研究(cancer study)所引起之病痛**(回答問題十六，省略問題十三~十五、十七、十八)

☐ **4. 生產 hybridoma ascites 所引起之病痛** (回答問題十七，省略問題十三~十五、十六、十八)

☐ **5. 其他** (請逕行回答問題十八，省略問題十三~十七)

十三、動物實驗屬一般性者，請說明實驗內容、方法。使用藥物者，請說明劑量。

以二氧化碳安樂死,取細胞組織

若本計畫涉及使用動物生產抗體者，請註明下列事項：

N/A

使用之抗原之名稱為：_____

性質為 ☐ 蛋白質 ☐ DNA 或☐ 其他 _____

抗原來源：☐ 自製 ☐ 廠商

抗原之注射方法、劑量、及途徑

抗原是否有添加佐劑：☐ 有 ☐ 無；若有，佐劑為 _____

採血(血清)方式及頻率_____

所注射之抗原是否具有

生物危險性質? ☐ 有 ☐ 無

放射線危險性質? ☐ 有 ☐ 無

化學危險性質? ☐ 有 ☐ 無

若有，請說明危險物品之廢棄物處理、安全防護措施及屍體處置方式：

十四、動物實驗屬存活手術(survival surgery)者，請說明 1. 手術房之地點及相關設備；2. 實驗內容、方法、劑量與步驟 (含動物固定、注射麻醉、手術、及術後照顧等)。

N/A

十五、動物實驗因癌症等研究造成實驗動物病痛者，請說明 1. 實驗終結點(study endpoint)；或 2.病危基準(moribund criteria)。

N/A

十六、動物實驗因癌症以外研究所引起動物病痛者，請說明實驗步驟及減輕病痛之具體措施。

N/A

十七、動物實驗因生產 hybridoma ascites 引起病痛者，請說明 1. 實驗終結點(study endpoint)；或 2. 病危基準(moribund criteria)；3. 抽取 ascites 之方式及頻率。

N/A

十八、動物實驗屬其他類型者，請說明實驗內容、方法；使用藥物者，請說明劑量。

N/A

十九、動物飼養　■由動物中心專人負責　□ 由實驗室人員負責；

二十、如動物需安樂死，請說明安樂死的方法。

以動物室提供之二氧化碳進行動物之安樂死。

二十一、動物屍體處理方式。

集中冷凍後,交由合格廠商焚化處理。

二十二、如進行危險性實驗(含生物危險、放射線及化學危險性實驗等)，請說明危險物品種類及實驗步驟、廢棄物處理、安全防護措施及屍體處置。

N/A

（一）如果實驗需使用有潛在危險之物質(如感染性物質、致癌藥物、毒物或放射線物質)，請具體指出使用物質及是否經過相關單位認可。

1. 放射線物質是否經過行政院原子能委員會認可

□ 是

放射線物質執照證號：_____

放射線物質操作執照證號：_____

□ 否

□ 待審中

2. 毒性化學物質是否經過行政院環境保護署認可

□ 是

許可證、登記備查或核可之證號 _____

毒性化學物質專業技術管理人員設置核定文件證號_____

□ 否

□ 待審中

3. 請具體指出使用感染性物質為何_____

是否經過相關單位認可

□ 是

已取得中研院生物實驗安全委員會基因重組實驗申請同意書

同意書編號_____

□ 否

□ 待審中

（二）請陳述進行危險物品實驗施用之方法、途徑及場所、牽涉人員、對人員及動物採取之保護措施及屍體處理方法。

我保證以上所填資料完全屬實

計畫主持人簽名 _____

填報日期 _____

我保證以上所填資料完全屬實

計畫主持人簽名 楊瑞彬

填報日期 92.10.9

中央研究院實驗動物使用同意書

核定 Protocol#：RMiIBMYR2005098 審查通過日期：2003 年 12 月 12 日
增補核可日期：2007 年 7 月 31 日 效期至 2007 年 12 月 31 日
增補項目：增加動物品系

計畫主持人基本資料

姓名	單位	職稱
楊瑞彬	生醫所	副研究員
電話 2652-3943	傳真 2785-8847	手機 0932-658-609
電子郵件 rbyang@ibms.sinica.edu.tw		

計畫相關資料 (流水號#030098)

	計畫名稱
中文	探討基因剔除小老鼠之生理變異
英文	Functional studies of gene knockout mice

經費來源：☑ 中研院 ☐ 國科會 ☐ 國衛院 ☐ 農委會
☐ 其他

核定執行期限：2005 年 1 月 1 日 至 2007 年 12 月 31 日
(如計畫執行期限有更動，請主動知會本小組)

計畫核定編號：
(如：國科會編號 NSC89-2000-A-002-001)

使用動物

種名	品系名		
mouse	Balb/c	C57BL6	129Sv
	ICR	FVB	FVB/n
	Athymic Balb/c nude		

■增加動物品系

動物物種	品系	動物來源	動物來源有無SPF規範**	動物飼養空間有無SPF規範**	全年使用累計總隻數(單位)	每月平均使用隻數	每月平均寄養隻數
Mice	Athymic Balb/c nude mice	NTU hospital animal facility sell nude or scid mice	有	有	160	14	14

本實驗共需 160 隻老鼠。

我們將 control vectors, SCUBE2 和 SCUBE2 的兩段 fragments (ty97 與 D4) stably transfection 到具有 Tet-off system 的 MCF-7 與 MDA-231 細胞中 (共有 8 種細胞)。

根據文獻，每種細胞需有 5-7 隻老鼠長出腫瘤，才能進行統計。由於實驗進行需 7 至 8 週以上，為避免動物因實驗操作與期間之耗損而造成死亡，故每組以使用 10 隻為宜。由於有 8 種細胞，每種細胞均需有兩組(doxycycline-free and doxycycline-containing water)，所以需使用 160 隻動物。

☐ 增加一般性實驗步驟

增加之實驗步驟：_____

☐ 計畫核定期限與申請時不同者

核定期限：_____

我保證以上所填資料完全屬實

計畫主持人簽名 楊瑞林

填報日期 2007-7-27

中央研究院實驗動物使用同意書

核定 Protocol#：**RMiIBMYR2005098** 審查通過日期：2003 年 12 月 12 日
增補核可日期：2007 年 12 月 31 日 效期至 2008 年 12 月 31 日
增補項目：增加動物實驗步驟

計畫主持人基本資料

姓名	單位	職稱
楊瑞彬	生醫所	副研究員
電話 2652-3943	傳真 2785-8847	手機 0932-658-609
電子郵件 rbyang@ibms.sinica.edu.tw		

計畫相關資料 (流水號#030098)

	計畫名稱
中文	探討基因剔除小老鼠之生理變異(延期)
英文	Functional studies of gene knockout mice

經費來源： ☑ 中研院 ☐ 國科會 ☐ 國衛院 ☐ 農委會
☐ 其他

核定執行期限：2005 年 1 月 1 日 至 2008 年 12 月 31 日
(如計畫執行期限有更動，請主動知會本小組)

計畫核定編號：
(如：國科會編號 NSC89-2000-A-002-001)

使用動物

種名	品系名		
mouse	Balb/c	C57BL6	129Sv
	ICR	FVB	FVB/n
	Athymic Balb/c nude		

■ 增加一般性實驗步驟

增加之實驗步驟： 小鼠麻醉

8-week-old nude mice, 100 μl mixed 麻醉劑 / 30-40 g (nude mice)

Start at 50 μl, IM (打入屁股肌肉), 老鼠約一小時清醒。

廠商:

Imalgene 1000 易眠靜	管制藥品,需申請	臺灣龍馬 **0800050599**
Rompum (25ml)		耐吉斯 **26621977**
Atropin		易林藥品 **28816581**

Mixed 麻醉劑:

Atropin (1mg/ml)	**4ml**
Ketamine(100mg/ml)	**2ml**
Rompine(2%)	**2ml**
Saline	**2ml**

Mix 均勻,(2:1:1:1) ,store at RT

有關管制藥品已遞送醫藥教育研究試驗計畫使用管制藥品申請書

□ 計畫核定期限與申請時不同者

我保證以上所填資料完全屬實

計畫主持人簽名 楊瑞林

填報日期 96-12-31

醫藥教育研究試驗計畫使用管制藥品申請書

計畫主持人姓名	楊　瑞　彬	身分證統一編號	B120750601	申請日期	97 年 02 月 14 日
醫藥教育研究試驗計畫名稱	探討基因剔除小老鼠之生理變異			請選乙項 ■初次使用申請 □增加使用量 □延長使用期限	
執行計畫期間 （請選乙項）	■初次使用申請自 97 年 02 月 14 日至 97 年 12 月 31 日 □延長使用期限自　年　月　日延至　年　月　日			聯絡電話	（ 02 ） 2789-9063
應檢附資料 （詳閱背面說明） （請選乙項）	■本計畫為初次使用申請 ■計劃書相關文件影本 ■管制藥品之用法、用量及需用數量之估算說明 ■計畫主持人身分證明文件影本 □業經核准使用，本次申請變更： □增加管制藥品使用量，應請檢附原核准函影本，及增加使用量之理由、估算說明 □延長使用期間，應請檢附原核准函影本，及延長之理由、使用期限 □原核准函日期文號：			傳真號碼	（）

申請使用管制藥品品項數量 （請選乙項） ■初次使用申請 □增加使用量	項次	藥品名稱	管制藥品成分及含量	製造廠名稱及國別	執行期間需用量
		Ketamine (以下空白) 共計 1 種藥品	Ketamine-HCl 100 mg/ml (每瓶 10 ml) (以下空白)	龍馬躍股份有限公司(台灣)	1000 mg = 10 ml = 1 瓶

醫藥教育研究試驗計畫執行地址 (倘不同於登記證地址)	郵遞區號□□□　臺北（市）　南港（區） 研究院 路 二 段 巷 弄 128 號 樓		
申請機構業者名稱	中央研究院生物醫學科學研究所	管制藥品登記證字號	ARR089000002902
機構業者地址 （登記證）	郵遞區號115　臺北（市）　南港（區） 研究院 路 二 段 巷 弄 128 號 生医所 樓		
機構負責人簽章	陳垣崇	機構印信戳記	請蓋機構業者印章
管制藥品管理人簽章	李婷婷		
計畫主持人簽章	楊瑞彬		
備註			

格式 111

中央研究院生物醫學科學研究所
申請管制藥品從事研究試驗
用法／用量／需用數量之估算說明

研究試驗計畫名稱（中文）	探討基因剔除小老鼠之生理變異		
研究試驗主持人姓名	楊　瑞　彬	身份證號碼	B120750601
使用之管制藥品名稱	Ketamine		
管制藥品用法說明	每隻小鼠手術前以肌肉注射 ketamine (70 mg/kg) 進行深度麻醉，再進行手術。手術後將小鼠以墊料覆蓋，並給予保溫燈照射以免失溫，20-30 分鐘後，待小鼠甦醒後，確定進食與活動無礙，再放回鼠盒內，隔日再一次觀察小鼠恢復狀況。		
管制藥品用量說明	Ketamine 注射劑量為 70 mg/kg。小鼠重量為 20~40 g，視品種及年紀而定，故每隻小鼠約注射 1.4~2.8 mg 不定。		
研究期間：自　97 年　02 月　14 日至　97 年　12 月　31 日			
管制藥品需用數量之估算說明	小鼠平均重量為 30 g，故每隻小鼠約注射 2.1 mg 不等，已核准之實驗動物申請表中預定進行 160 隻小鼠手術，但唯恐實驗中，因腫瘤注射而造成小鼠死亡，所以預先採購 3 次實驗所需之量。 所以估算如下： 160 mice × 2.1 mg/mice X 3 = 1008 mg (約 1000 mg)		
研究試驗主持人簽章	楊瑞彬	管制藥品管理人簽章	李