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Report

Isolation and Characterization of a novel secreted protein, SCUBE2 in Vascular Neointimal Hyperplasia Formation

Objective : The aim of the study is to investigate the protein expression and function of a novel secreted protein, SCUBE2 in vascular neointimal hyperplasia formation

Background

Occlusive vascular disease including atherosclerosis has been postulated as the result of modulated vascular development and adaptation towards vascular injury and mechanical stress. Those adverse responses resulted in neointimal hyperplasia or restenosis which is the growth of new cells and matrix in the inner structure of the vessel wall leading to progressive reduction of blood flow and increased risk of thrombosis. Some theories have been hypothesized, like Response-to-Retention hypothesis. This hypothesis states that the retention of atherogenic lipoproteins associated with the extracellular matrix (ECM) in the arterial intima as the initial event. However it is not clear yet how this ECM and extracellular lipid accumulation precede atherogenesis (1).

Number of proteins with a role in embryogenesis and cancer recurrence have been identified that contain both the EGF (Epidermal Growth Factor) and the CUB (complement subcomponents C1s and C1r) domains, which have been implicated in the regulation of extracellular processes such as developmental patterning, cell signaling, hemostasis, and inflammation (2, 3). Signal peptide-CUB-EGF-like domain containing protein, SCUBE, is a novel, secreted, cell surface glycoprotein expressed in broader spectrum of tissue and cell types besides also reported to be involved during early embryogenesis (4,5,6,7,8). SCUBE1 has been shown to be elevated in both Acute Coronary Syndromes (ACS) and Acute Ischemic Stroke (AIS) (9). SCUBE2 gene was expressed in breast cancer and lung cancer (10,11,12). This gene also implicated in regulating Hedgehog signaling in zebrafish embryo development (8,11). SCUBE3 is highly expressed in osteoblast and has been reported in inducing cardiac hypertrophy in mice (13, 14). However the role of SCUBE2 in vasculature remains unknown. In this present study, we aim to investigate and characterize the SCUBE2 involvement in cell proliferation and cell migration as implicated in developing neointimal hyperplasia formation

Materials and Methods

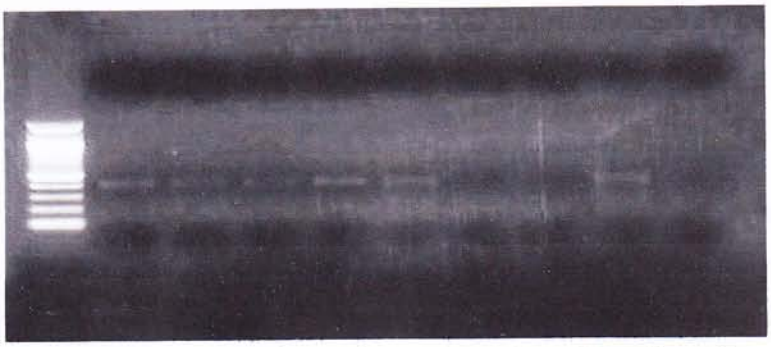
1. Microarray Analysis and Gene Expression
   Ligated carotid artery and sham-operated C57BL/6 mice were assessed for gene expression analysis using microarray analysis.
2. Identification of cells expressing SCUBE2 and full-length cloning SCUBE2
   Human SCUBE2 gene expression is identified using primer pairs 5' AGACCCCAGAAGCTTGGAATA 3' (forward) and 5'TCCCCTCCACATCTTCTG TTT 3'(reverse). Full-length SCUBE2 were obtained by separating 3737 bps SCUBE2 into two fragments which recognized HindIII cleavage site. First fragment with 2455 bps length is identified using primer 5' CCGCAACCGCTGAGCCAT3' (forward) and 5' TCCCCTCCACATCTTCTGTTT3'(reverse). Second pair, read shorter fragment of 1046 bps, is obtained using primer 5' CAAATGGAACCT TCCAAAATGA3'(forward) and 5'AGTGGCAC GTGGGCTGAG3'(reverse).
   Target band was excised and extracted as mentioned in manual (Qiagen Gel Extraction kit, Germany).
3. Transformation and Inoculation.
   Isolated gene was transformed to E.coli DH5α (Takara,Japan) and subject to inoculation with incubation in 370C overnight. Qiagen Miniprep is used to purify the plasmid DNA.
4. Enzyme Restriction - To confirm the correct target, purified plasmid DNA was incubated in 370C with enzyme HindIII. And measure the expected band.

Result

Microarray Analysis

1452968_at	collagen triple helix repeat containing 1	7.09	97.4	P	13251.8	P
1453125_at	SRY-box containing gene 11	7.21	0.9	A	128.4	P
1453214_at	leucine rich repeat containing 15	8.30	5.4	A	1713.0	P
1453418_at	collagen, type XXIV, alpha 1	9.71	0.9	A	793.4	P
1453486_a_at	signal peptide, CUB domain, EGF-like 2	9.31	1.4	A	905.0	P
1456515_s_at	transcription factor-like 5 (basic helix-loop-helix)	8.15	3.6	A	1017.2	P

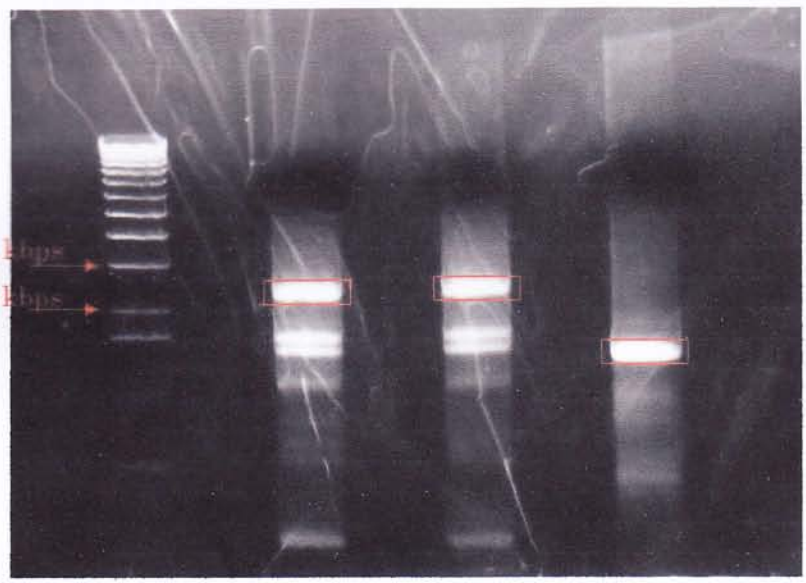
2. SCUBE2 expression in broad spectrum of human cell lines.



100bps 1 2 3 4 5 6 7 8 9

1 : HAEC 3 : HMVEC 5 : HCASMC 7 : Meg-01 9 : U937
2 : HCAEC 4 : HUVEC 6 : THP-1 8 : Jurkat

3. Full-length cDNA of SCUBE2



Discussion

Novel secreted protein, SCUBE2, is shown to be highly expressed in microarray analysis of carotid artery ligation samples. This result leads to an implication of their role during development of neointimal hyperplasia as an early process of atherosclerosis.

Its expression in various human cells lead us into a question of their involvement in cell proliferation, migration and signaling pathway especially in endothelial cells and vascular smooth muscle cells, as main component in vasculature and so far hypothesis points to their contribution for the development of cardiovascular diseases.

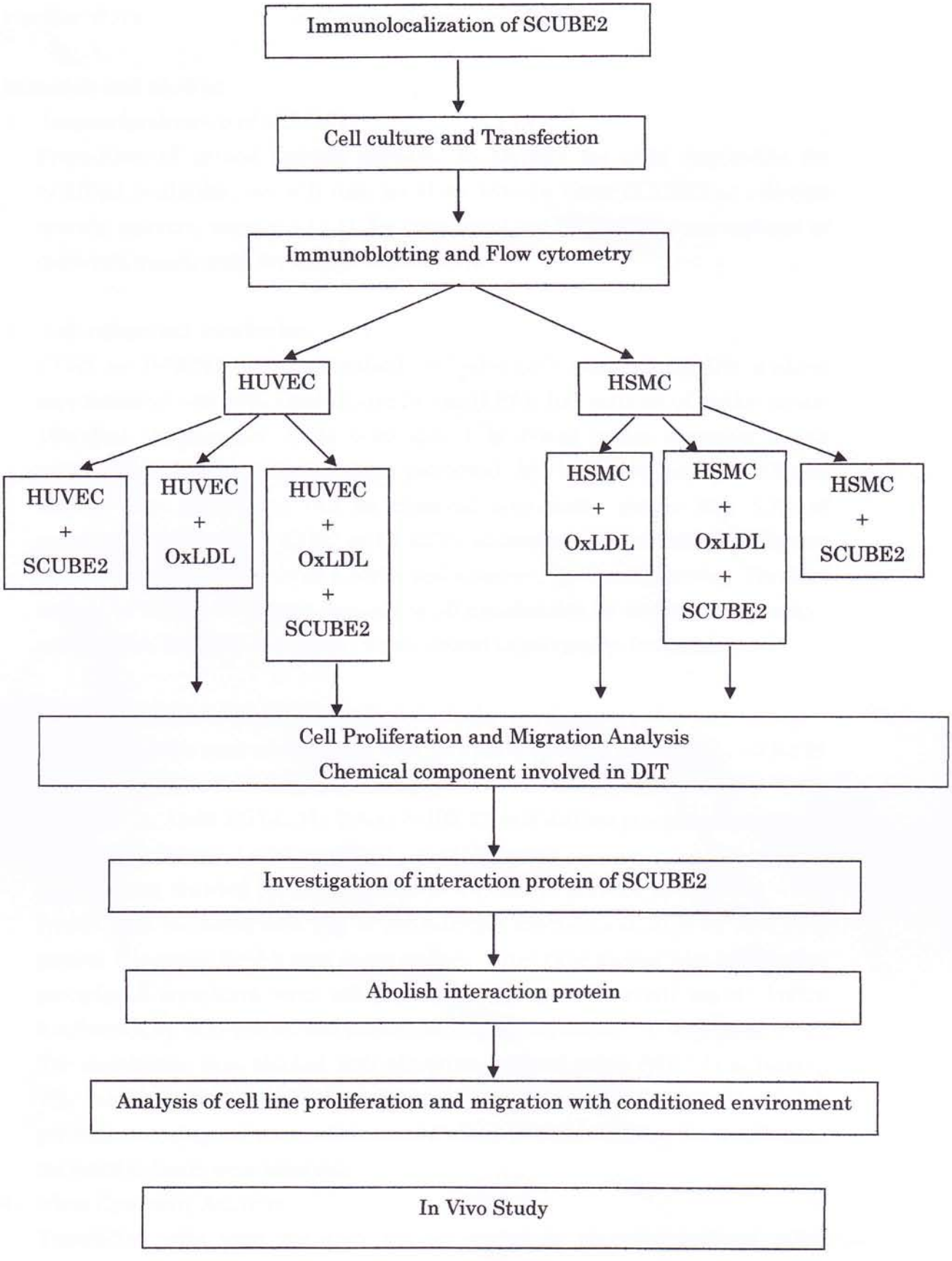
Possible Difficulties

SCUBE2 gene is a GC rich contain gene. To obtain a definite gene target with PCR and ligation was taking time since large difference of melting temperature between forward primer and reverse primer. Ligation of GC rich contain gene with longer fragment is sometime troublesome which up till know there are no such explanations available to describe the phenomenon.

Further Work

We rigorously investigate SCUBE2 localization in human vascular disease sample and emerge for their role in cell proliferation and migration.

Core Plan for Further Work on SCUBE2



Further Work

Materials and Method

1. Immunolocalization of SCUBE2

   Preparation of carotid ligation samples. To identify the cells responsible for SCUBE2 production, we will stain serial sections for either SCUBE2 or cell-type specific markers, namely, CD 31 for endothelial cells, CD 68 for macrophage or α-smooth muscle actin for smooth muscle cells.

2. Cell culture and transfection

   COS7 or HEK293 were maintained in Dulbecco's modified Eagle's medium supplemented with 10% Fetal Bovine Serum (FBS), 100 units/ml of penicillin and 100μg/ml streptomycin. Cells were seeded in 6-well plates overnight before transfection. The transfection was performed by calcium-phosphate-mediated method. The transfection will be observed consistently greater than 90% of transfection efficiency in COS7 or HEK293, accessed by the transfection of green fluorescence protein vector as reporter and examined by flow cytometry. The total amount of DNA will be kept constant in all transfections by supplementing empty vector DNA. HUVEC and HSMC were cultured as previously described.

3. Immunoprecipitation and Western Blot Analysis

   Transfected cells were washed once with phosphate-buffered saline and lysed for 15 minutes on ice in 0.5 ml of lysis buffer (20mM Tris-HCl, pH 7.5, 150 nM NaCl, 1mM EDTA, 1mM 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 4^0C for 15 minutes at 10,000xg. Cells lysates were incubated with 1μg of the indicated antibody and 20 μl of 50% (v/v) protein A-Agarose for 2 h 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 phosphate-buffered saline (pH 7.5) containing 1% gelatin and 0.05% Tween 20 and were blotted with the horseradish peroxidase-conjugated goat anti-mouse IgG for 1 h. After washing the membranes, the reactive bands were observed.

4. Flow Cytometry Analysis

   Transfected cells were collected and suspended in phosphate-buffered saline

containing 2% FBS in a volume of 0.25 ml. A total of 1 μg of purified anti-FLAG M2 antibody and fluorescence isothiocyanate-conjugated goat anti-mouse secondary antibody (1:100 dilution) were added sequentially; each were incubated for 45 min on ice.

5. Isolation of lipoproteins and oxidation.

Differential centrifugation was used to isolate LDL from plasma as previously described (16). The LDL was oxidized by adding 20-100 μM CuSO4 per 2 mg/ml LDL, followed by incubation at 37^0C. The extent of oxidation was characterized by measurement of relative electrophoretic mobility (REM) and lipid peroxidates.

6. Cell proliferation and migration

HUVEC and HSMC were cultured with oxLDL and SCUBE2. Cell proliferation and cell migration will be observed on 24 h, 48 h, and 72 h.

Cell Proliferation and Migration Assay : Cell proliferation is using BrdU according to company instruction.

Cell migration assay is using Boyden chamber (Millipore, Bedford, MA) migration assays were performed as described previously. Briefly, serum-starved cells were detached, resuspended in migration medium (DMEM containing 0.5% BSA), and counted. Both sides of MilliCell chambers were precoated with rat tail collagen (5 μg/ml in PBS) overnight at 4 °C, washed with PBS, and air-dried. Chambers containing serum-starved cells (1×10^5 cells/0.3 ml) were placed in 24-well dishes containing DMEM with 0.5% BSA with or without OxLDL and SCUBE2 at the indicated concentrations. Transiently transfected migratory cells on the membrane underside were identified by GFP fluorescence, and the migration of stable cell lines was visualized by Crystal Violet staining (0.1% Crystal Violet, 0.1 M borate, pH 9.0, 2% EtOH) and cell counting (cells/field using a 40× objective).

7. Generation of GST fusion proteins and GST pull-down assay

COS7 cells were lysed in RIPA lysis buffer with protease and protease inhibitor (150 mM NaCl, 50 mM Tris-HCl (pH7.5), 1% Noninet P-40 (NP40), 1mM Sodium Vanadate, 5 mM Sodium Fluoride, 1 mM phenylmethylsulfonylfluoride (PMSF), 1 mg/ml aprotinin, 1 mg/ml pepstatin and 1 mg/ml leupeptin) at 4^0C for 30 min. Cellular proteins were pre-cleared with glutathione agarose beads for 2 h. After centrifugation, the cellular extract was incubated with GST fusion protein immobilized with glutathione agarose beads and incubated overnight at 4^0C with

tumbling. The beads were washed six times and the bound proteins were eluted and subjected to SDS-PAGE followed by visualization by coomasie blue of the protein of interest.

Expected Result

Detectable SCUBE2 in endothelial cells and vascular smooth muscle cells in carotid ligation samples, leads to DNA transfection of this gene into COS7 cell line which is expected in resulting the secreted protein of SCUBE2. Identification and confirmation of this protein will use Western Blot with expected size is 250 kDa.

Identified and confirmed-SCUBE2 secreted protein, will be observed for their involvement in cell proliferation and migration as implicitly reported for their involvement in embryogenesis and cancer development. To investigatigate their action, we will use BrdU assay and Boyden Chamber migration assay. At this investigation, we will mimick the proses which assume to develop diffuse intimal thickening by using OxLDL in cell culture medium. In their process, SCUBE2 might interact with the other protein or bind to other molecules which is thought to activate their function.

To identify cellular proteins that interact specially with the SCUBE2 protein fused to glutathione S-transferase will synthesize in E.coli recovered in glutathione agarose and incubated with whole cell extracts prepared from COS7 cells. This approach is well-established technique for detection of novel protein interactors. The identify of purified proteins will be verified by Western Blot analysis using SCUBE2 antibody.

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