Endothelial SCUBE2 Interacts With VEGFR2 and Regulates VEGF-Induced Angiogenesis

Yuh-Charn Lin, Tsu-Yi Chao, Chi-Tai Yeh, Steve R. Roffler, Reiji Kannagi, Ruey-Bing Yang

Objective—Vascular endothelial growth factor (VEGF), a major mediator of angiogenesis, exerts its proangiogenic action by binding to VEGFR2 (VEGF receptor 2), the activity of which is further modulated by VEGFR2 coreceptors such as neuropilins. However, whether VEGFR2 is regulated by additional coreceptors is not clear. To investigate whether SCUBE2 (signal peptide-CUB-EGF domain-containing protein 2), a peripheral membrane protein expressed in vascular endothelial cells (ECs) known to bind other signaling receptors, functions as a VEGFR2 coreceptor and to verify the role of SCUBE2 in the VEGF-induced angiogenesis.

Approach and Results—SCUBE2 lentiviral overexpression in human ECs increased and short hairpin RNA knockdown inhibited VEGF-induced EC growth and capillary-like network formation on Matrigel. Like VEGF, endothelial SCUBE2 was upregulated by hypoxia-inducible factor-1α at both mRNA and protein levels. EC-specific *Scube2* knockout mice were not defective in vascular development but showed impaired VEGF-induced neovascularization in implanted Matrigel plugs and recovery of blood flow after hind-limb ischemia. Coimmunoprecipitation and ligand-binding assays showed that SCUBE2 forms a complex with VEGF and VEGFR2, thus acting as a coreceptor to facilitate VEGF binding and augment VEGFR2 signal activity. SCUBE2 knockdown or genetic knockout suppressed and its overexpression promoted the VEGF-induced activation of downstream proangiogenic and proliferating signals, including VEGFR2 phosphorylation and mitogen-activated protein kinase or AKT activation.

Conclusions—Endothelial SCUBE2 may be a novel coreceptor for VEGFR2 and potentiate VEGF-induced signaling in adult angiogenesis. (***Arterioscler Thromb Vasc Biol*. 2017;37:00-00. DOI: 10.1161/ATVBAHA.116.308546.)**

Key Words: bone morphogenetic protein ■ collagen ■ cysteine ■ dimerization ■ endothelial cell

Angiogenesis, the sprouting of new blood vessels from pre-existing ones, plays a critical role in various physiological processes, including embryonic development, the female reproductive cycle, wound healing, and organ repair in adults.[1] Vascular endothelial growth factor (VEGF) is a major mediator of angiogenesis; it binds to its receptor VEGFR2 (a receptor tyrosine kinase) on the surface of endothelial cells (ECs) and triggers dimerization and transphosphorylation to activate signaling cascades including p44/42 mitogen-activated protein kinases and AKT required for EC migration, proliferation, and tubulogenesis.[2,3] These VEGF responses can be further promoted by a small number of VEGFR2 coreceptors such as neuropilins, heparan sulfate proteoglycans, CD44, and CD146.[4–9] However, additional coreceptors for VEGFR2 that regulate VEGF-induced angiogenesis might exist and remain to be discovered.

SCUBE2 (*S*ignal peptide-*c*omplement protein C1r/C1s, *U*egf, and *B*mp1 [CUB]-*e*pidermal growth factor [EGF] domain-containing protein 2) is the second member of a small, evolutionarily conserved gene family composed of 3 different genes (SCUBE1, 2, and 3) originally identified from human ECs.[10,11] The genes encode ≈1000-amino acid polypeptides organized in a modular fashion with 5 protein domains: an NH_2-terminal signal peptide, 9 tandem repeats of EGF-like motifs, a spacer region, 3 cysteine-rich repeats, and 1 CUB domain at the COOH terminus. These SCUBEs can tether on the cell surface as peripheral membrane proteins by 2 distinct membrane-anchoring mechanisms (ie, electrostatic and lectin–glycan interactions) via their spacer region and the cysteine-rich repeats,[12] and function as coreceptors for many growth factors. For example, SCUBE1 and SCUBE3 behave as a coreceptor in promoting the signaling activity of bone morphogenetic protein, transforming growth factor-β superfamily members, and fibroblast growth factor.[13–16] Although many signaling components of these growth factors have been implicated in angiogenesis during development and disease,[17,18] in vivo vascular phenotypes were not observed in single *Scube*-knockout mouse strains,[19–21] which

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Nonstandard Abbreviations and Acronyms	
AP	alkaline phosphatase
EC	endothelial cell
EC-KO	EC-specific *Scube2* knockout
HIF	hypoxia-inducible factor
HUVEC	human umbilical vein endothelial cell
MLEC	mouse lung endothelial cell
SCUBE2	signal peptide-CUB-EGF domain-containing protein 2
shRNA	short hairpin RNA
VEGF	vascular endothelial growth factor
VEGFR2	vascular endothelial growth factor receptor 2

suggests functional compensation among these SCUBE proteins in the angiogenic endothelium during embryonic development and under normal physiological conditions. Besides being expressed in the normal endothelium,[10,22] SCUBE2 is also highly expressed in hypoxic tumor microvasculature.[22] However, whether SCUBE2 can act as a coreceptor for VEGFR2 and its role in VEGF-induced angiogenesis remain to be determined.

In this study, we investigated whether SCUBE2 is a coreceptor for VEGFR2 and its role in VEGF-induced angiogenesis. SCUBE2 was upregulated by hypoxia-inducible factor-1α (HIF-1α) in response to hypoxic conditions and interacted with VEGFR2 in human ECs, where it acted as a coreceptor with VEGFR2 to facilitate VEGF binding and enhance its downstream signaling, thus promoting VEGF-induced angiogenesis. Endothelial-specific *Scube2* knockout (EC-KO) mice showed no defects in vascular development but did show impaired VEGF-induced neovascularization in implanted Matrigel plugs and impaired recovery of blood flow after induced hind-limb ischemia. SCUBE2 may be a novel coreceptor for VEGFR2 that regulates the VEGF-induced tube formation and proliferation of ECs by fine-tuning VEGFR2-mediated signaling, especially during postnatal angiogenesis induced by ischemia or under hypoxic conditions.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

SCUBE2 Is Expressed in Human ECs and Regulates VEGF-Induced EC Proliferation and Tube Formation

We first confirmed SCUBE2 protein expression in human umbilical vein endothelial cells (HUVECs) by immunostaining (Figure 1A), flow cytometry (Figure 1B), and Western blot analysis (Figure 1C). Confocal immunofluorescence staining and flow cytometry revealed SCUBE2 localized partially on the EC membrane (Figure 1A and 1B). In addition, protein levels were higher in proliferating, subconfluent ECs than growth-arrested, confluent ECs (Figure 1C).

We then investigated a potential role for SCUBE2 in regulating VEGF responses by overexpressing and knocking down SCUBE2 in human ECs. After transduction of recombinant lentivirus encoding the full-length FLAG-tagged SCUBE2 or 2 independent SCUBE2-targeting short hairpin RNAs (shRNA 1 and 2) in HUVECs, the overexpression and knockdown of SCUBE2 were verified by RT-PCR or Western blot analysis (Figure 1D and 1G). SCUBE2 overexpression significantly increased (Figure 1E and 1F) and SCUBE2 knockdown (Figure 1H and 1I) markedly reduced VEGF-induced EC growth and capillary-like network formation on Matrigel. Together, these data support a role for SCUBE2 in modulating VEGF-induced proliferation and tubulogenesis in HUVECs.

Upregulation of SCUBE2 by HIF-1α in HUVECs

Because hypoxia-induced VEGF expression by HIF-1α is critical for postnatal neovascularization,[1] we then investigated whether endothelial SCUBE2 is also upregulated by a similar mechanism. After 12-hour exposure of HUVECs to hypoxia, the expression of SCUBE2 but not SCUBE1 or SCUBE3 was significantly increased at both mRNA and protein levels, which was concomitant with HIF-1α accumulation (Figure 2A and 2B). Furthermore, ChIP assay and promoter mutation analysis confirmed that endogenous HIF-1α could indeed interact with a DNA region that harbors a consensus HIF-binding motif (A/GCTGA) within the SCUBE2 promoter and transactivate SCUBE2 expression in HUVECs with hypoxia treatment (Figure 2C though 2E).

Generation of EC-KO Mice

To further investigate the role of endothelial *Scube2* on VEGF responses in vivo, we knocked out *Scube2* specifically in ECs by crossing mice carrying a conditional Floxed allele of *Scube2* (flanking the exons encoding 9 EGF-like repeats, the spacer region, 3 cysteine-rich motifs, and the CUB domain with *loxP*)[20] with transgenic mice expressing the bacteriophage recombinase Cre under the control of the angiopoietin receptor (Tie2) promoter, which is pan-endothelial expressed (Figure IA in the online-only Data Supplement).[23] Male *Tie2-Cre; Scube2*$^{+/-}$ were bred to female *Scube2*$^{Flox/Flox}$ mice to obtain *Tie2-Cre; Scube2*$^{Flox/+}$ (designated control) and *Tie2-Cre; Scube2*$^{Flox/-}$ (designated EC-KO) mice (Figure IB and IC in the online-only Data Supplement).

To determine the endothelial-specific recombination efficiency of the *Scube2*^{Flox} allele, primary mouse lung endothelial cells (MLECs) were isolated from control and EC-KO mice. Flow cytometry revealed these cell populations highly enriched in ECs because ≈95% of these cells expressed CD31. In addition, both mRNA and protein levels of *Scube2* were efficiently and specifically ablated without obvious compensatory upregulation of *Scube1* and *Scube3* in EC-KO MLECs (Figure ID in the online-only Data Supplement). Consistently, immunostaining of adult mouse lungs verified the complete abrogation of endothelial (but not bronchial epithelial) *Scube2* expression in EC-KO mice (Figure IE in the online-only Data Supplement). Therefore, *Scube2* was efficiently ablated in the endothelial compartment of adult EC-KO mice.

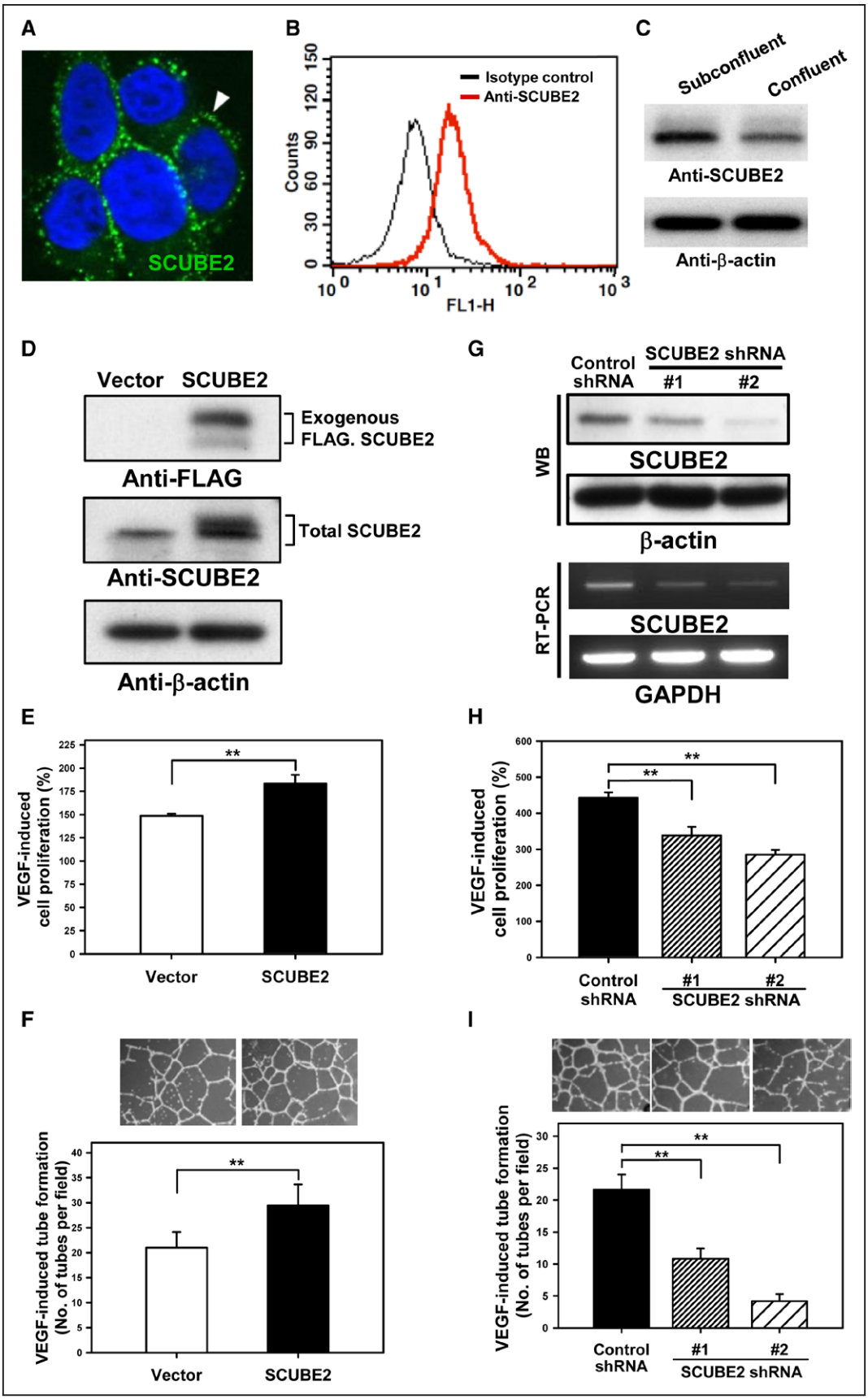

Figure 1. SCUBE2 (signal peptide-CUB-EGF domain-containing protein 2) is expressed at the cell surface of human umbilical vein endothelial cells (HUVECs) and modulates vascular endothelial growth factor (VEGF)–induced HUVEC proliferation and tube formation. **A** and **B**, SCUBE2 immunofluorescence staining (in green) and flow cytometry. Arrowhead indicates SCUBE2 expressed on endothelial (*Continued*)

Figure 1 Continued. cell membrane. Nuclei are stained with DAPI in blue. **C**, Western blot analysis of SCUBE2 protein expression in subconfluent (proliferating) and confluent (nonproliferating) HUVECs. **D–F**, SCUBE2 overexpression enhances VEGF-induced HUVEC proliferation and tubulogenesis. Exogenous SCUBE2 expressed in HUVECs transduced with an empty lentivirus or recombinant lentivirus encoding an FLAG-tagged SCUBE2 (**D**). Effect of SCUBE2 overexpression on VEGF-induced HUVEC cell growth (**E**). Data are mean±SD and represent percent increased relative to nonstimulated cells from 3 independent experiments. $^{**}P<0.01$. In vitro Matrigel angiogenesis assay of effect of SCUBE2 overexpression on VEGF-induced tube formation (**F**). Representative images are shown at top and quantified by counting the total number of tubules per field. Data are means±SD calculated from 3 independent experiments. $^{**}P<0.01$. **G–I**, SCUBE2 knockdown decreases VEGF-induced HUVEC proliferation and tube formation. SCUBE2 expression was downregulated by 2 independent SCUBE2-targeting shRNA lentiviruses (SCUBE1-shRNA 1 and 2) in HUVECs (**G**). A luciferase shRNA lentivirus was a negative control (control shRNA). Effect of SCUBE2 knockdown on VEGF-induced (**H**) HUVEC cell growth and (**I**) tube formation. Data are mean±SD and represent percent increased relative to nonstimulated cells from 3 independent experiments. $^{**}P<0.01$. In vitro Matrigel angiogenesis assay of effect of SCUBE2 knockdowns on VEGF-induced tube formation. Representative images are shown at top and quantified by counting the total number of tubules per field (**I**). Data are mean±SD calculated from 3 independent experiments. $^{**}P<0.01$.

Control and EC-KO mice were recovered at the expected Mendelian ratio and showed the normal pattern of weight gain, so vasculogenesis and angiogenesis were sufficient for normal development. We observed no phenotypic differences in general health and behavior between control and EC-KO animals. EC-KO females animals reproduced normally and had normal litter sizes, which suggested sufficient reproductive angiogenesis to allow for colony propagation. Together, these data suggest that physiological angiogenesis was grossly unaffected by endothelial inactivation of *Scube2*.

Responses to Exogenous VEGF and Adult Angiogenesis Are Impaired in *Scube2* EC-KO Mice

To explore the potential function of endothelial *Scube2* in VEGF-stimulated angiogenesis in vivo, we used Matrigel plug assay with Matrigel mixed with VEGF (+) or saline (−) administered subcutaneously to age- and sex-matched EC-KO and control mice with recovery 7 days later (Figure 3). On gross examination, Matrigel plugs containing VEGF from control mice were reddish-brown (Figure 3A). However, plugs containing VEGF from EC-KO mice were paler than control plugs (Figure 3A). Consistently, total hemoglobin content, a measure of intact vessel formation associated with the amount of newly formed capillary network, in VEGF-containing Matrigel plugs was reduced ≈50% for EC-KO mice (Figure 3B). To ensure that this hemoglobin difference was caused by reduced microvasculature density, blood vessel infiltration in implants was quantified by immunostaining with anti-CD31 antibody (a marker of endothelia; Figure 3C and 3D). In contrast to Matrigel-implanted control mice, implanted EC-KO mice showed decreased vascularization, even in saline-containing plugs (Figure 3C). Furthermore, the microvascular density of VEGF-containing plugs was lower by 50% in EC-KO than in control mice (Figure 3D), and no large vascular tubes were seen (Figure 3C), which suggests that the VEGF-induced adult angiogenic response depends on endothelial *Scube2* in vivo.

To further evaluate the proangiogenic effect of endothelial *Scube2*, we used a second adult neovascularization model (ie, hind-limb ischemia): the common femoral artery was ligated in control and EC-KO mice, and blood flow as well as vascular regeneration and angiogenesis were monitored over time by laser Doppler imaging and anti-CD31 immunostaining, respectively. Laser-Doppler analyses revealed a similar degree of reduced blood flow in ligated limbs of control and EC-KO animals after surgery as compared with nonligated contralateral limbs (Figure 3E and 3F), which indicates comparable postsurgery ischemia in both strains. In control mice, blood flow recovered to almost baseline levels by 21 days after surgery. However, blood flow recovery was significantly impaired in EC-KO animals (Figure 3E and 3F). Consistently, histology of the gastrocnemius (calf) muscle showed lower anti–CD31-positive capillary density induced by ligation in EC-KO than that in control mice at 21 days (Figure 3G and 3H). Therefore, functional *Scube2* may be required in the endothelium for revascularization after ischemia induced by femoral artery ligation.

SCUBE2 Colocalizes and Interacts With VEGFR2 and Potentiates VEGF Binding to VEGFR2 in HUVECs

Because SCUBE2 can regulate VEGF responses both in vitro and in vivo (Figures 1 and 3) and SCUBE proteins can function as coreceptors for signaling receptor serine/threonine kinases or receptor tyrosine kinases,[13–16] we examined whether SCUBE2 colocalizes and interacts with VEGFR2 in HUVECs. Confocal microscopy and coimmunoprecipitation experiments indeed revealed that VEGF promotes their colocalization at the plasma membrane and enhances the association of SCUBE2 with VEGFR2, peaking at 10 minutes after VEGF stimulation in HUVECs (Figure 4A and 4B). This result was further elaborated in HEK-293T cells transfected with SCUBE2 and VEGFR2 expression plasmids, showing that the CUB domain of SCUBE2 can interact with the extracellular immunoglobin-like folds of VEGFR2 (Figure II in the online-only Data Supplement). In addition, this SCUBE2–VEGFR2 interaction seemed specific because anti-SCUBE2 immunoprecipitation did not pull down other receptor tyrosine kinases such as VEGFR1 and EGFR or another VEGR2 coreceptor neuropilin-1 (Figure III in the online-only Data Supplement). Most important, besides binding to VEGFR2, SCUBE2 could directly bind to VEGF-A_{165} via its EGF-like repeats (Figure IV in the online-only Data Supplement). We have performed additional coimmunoprecipitation experiments to verify whether SCUBE1 or SCUBE3 can also bind to VEGF or VEGFR2. Unlike SCUBE2 that can specifically bind to VEGF, SCUBE1 and SCUBE3 cannot interact with VEGF, suggesting that SCUBE1 or SCUBE3 may not function as a coreceptor for VEGF (Figure V in the online-only Data Supplement).

To evaluate whether SCUBE2 could indeed increase VEGF binding to VEGFR2, we first produced a functional AP-VEGF

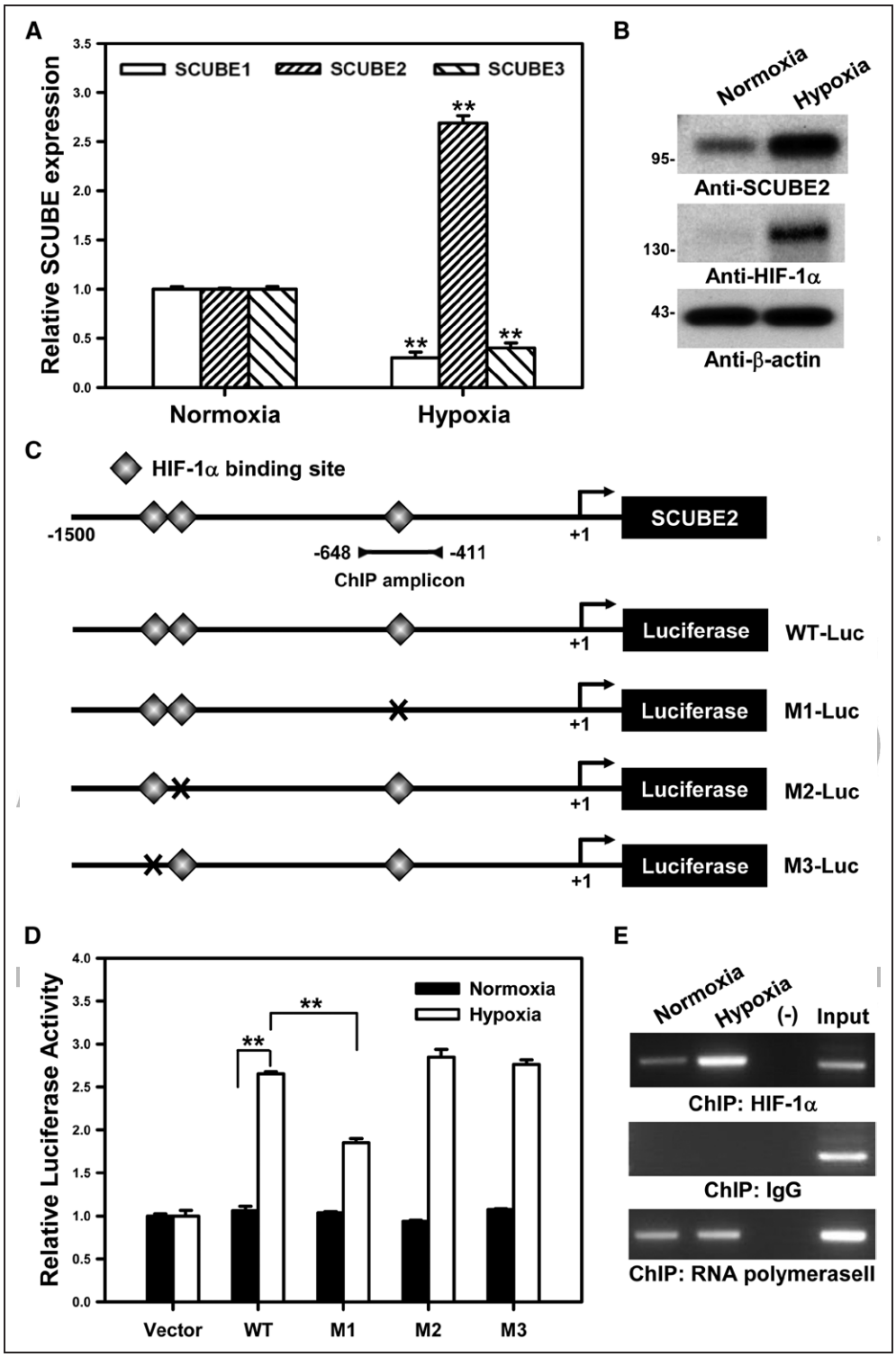

Figure 2. Hypoxia mediated hypoxia-inducible factor-1α (HIF-1α)–induced upregulation of SCUBE2 (signal peptide-CUB-EGF domain-containing protein 2) in human umbilical vein endothelial cells (HUVECs). **A** and **B**, SCUBE2 was upregulated in HUVECs in response to hypoxia. HUVECs were cultured under normoxia or hypoxia (1%) for 12 h. Expression of SCUBE2 at both mRNA and protein levels verified by quantitative RT-PCR (**A**) and Western blot (**B**) analyses. Data are mean±SD from 3 independent experiments. ***P*<0.01 compared with normoxia. **C**, Scheme of the SCUBE2 promoter reporter constructs containing the native (wild type [WT]) or mutated (M1, M2, and M3) HIF-1α–binding sites. The location of promoter (–1500 to +1) of *SCUBE2* gene is shown by a lateral line. The HIF-1α–binding sites are marked by diamonds, and the mutant sites are shown as × marks. The primers and amplicon region used in the ChIP assay are labeled (**upper**). **D**, HIF-1α transactivates SCUBE2 promoter-driven luciferase activity. Quantification of activity of *SCUBE2* WT or mutated promoter constructs transiently transfected into HUVECs. Luciferase activity was normalized to Renilla luciferase activity to control for transfection efficiency. **E**, HIF-1α directly binds to the SCUBE2 promoter with hypoxia. ChIP assay of in vivo binding of HIF-1α protein to the promoter of *SCUBE2* in HUVECs analyzed by PCR with specific primers for HIF-1α amplifying a 238-bp fragment. Lack of cell lysates (–) was a negative control. Data are mean±SD from 3 independent experiments. ***P*<0.01.

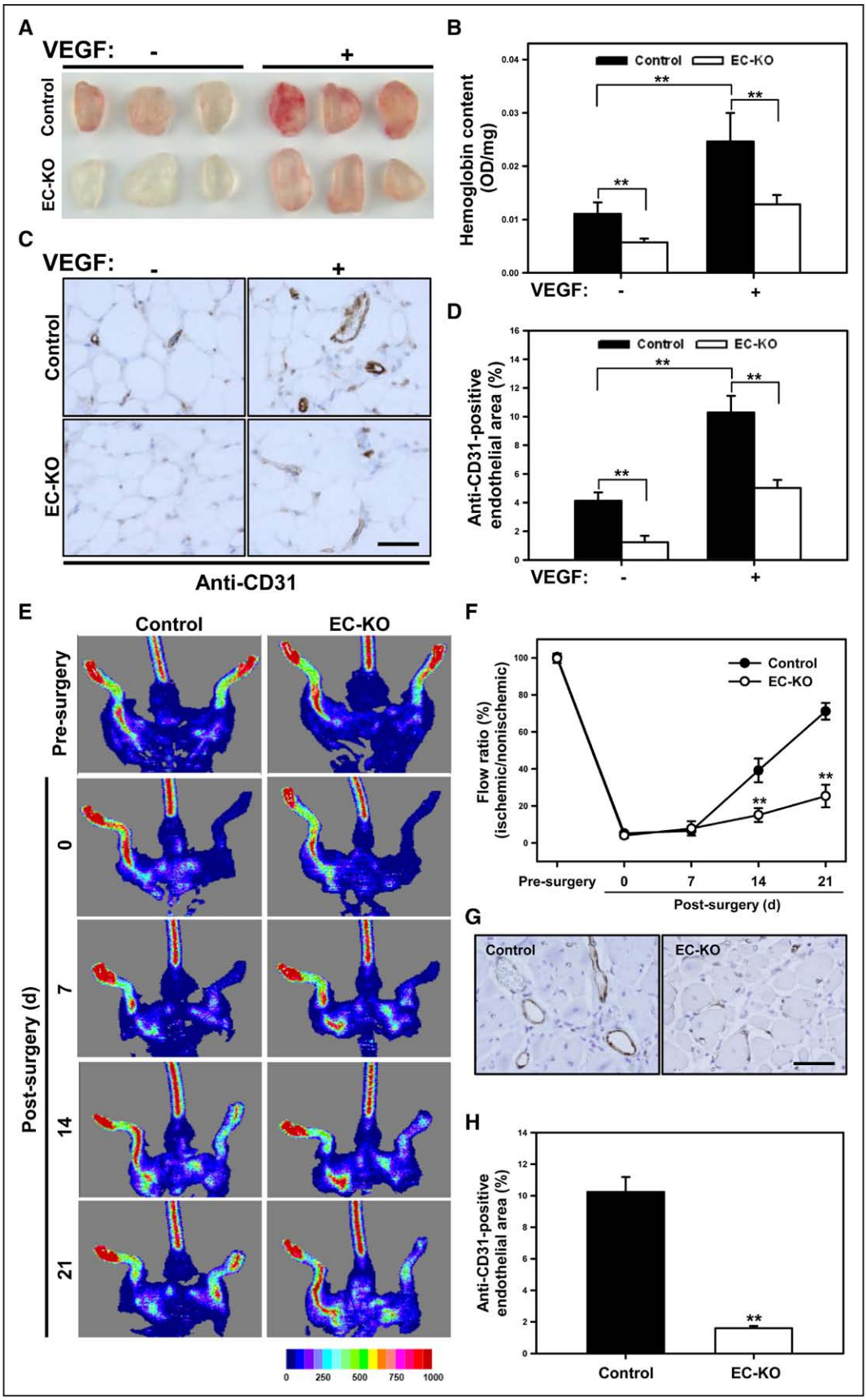

Figure 3. Attenuation of adult angiogenesis in endothelial cell (EC)-specific *Scube2* knockout (EC-KO) mice. **A–D**, Matrigel plug assays. **A**, Representative photographs of Matrigel plugs containing saline (–) or VEGF (+) removed from control and EC-KO mice at 7 d post injection. **B**, Hemoglobin content of saline (–) and VEGF (+)–supplemented Matrigel plugs from control and EC-KO mice. Data are (*Continued*)

Figure 3 Continued. mean±SD (n=5). **P<0.01. **C**, Anti-CD31 staining of sections of Matrigel plugs containing saline (–) or VEGF (+) excised from control and EC-KO mice. Scale bar=40 μm. **D**, Vascularization of Matrigel plugs as determined by anti-CD31 antibody positivity by using ImageJ. Data are mean±SD (n=5). **P<0.01. **E–H**, Hind-limb ischemia–induced neovascularization. Representative laser-Doppler images (**A**) and quantification of hind-limb blood flow (**B**) before and after right femoral artery ligation in mice. Representative images (**C**) and quantification of anti-CD31 immunostaining of calf blood vessels (**D**) at 21 d after femoral artery ligation in control and EC-KO mice. Scale bar=100 μm. Data are mean±SD (n=6). **P<0.01 compared with control.

protein (alkaline phosphatase [AP]–VEGF fusion protein; Figure VIA and VIB in the online-only Data Supplement) as described.[24] As compared with control AP protein, AP-VEGF protein showed binding on HUVECs endogenously expressing VEGFR2 (Figure VIC through VIE in the online-only Data Supplement). Interestingly, SCUBE2 overexpression increased (Figure VIE in the online-only Data Supplement) and SCUBE2 knockdown or genetic knockout decreased AP-VEGF binding as compared with corresponding control HUVECs (Figure VIC and VID in the online-only Data Supplement). Consistently, Scatchard analysis showed that the binding affinity of VEGF to VEGFR2 was greater by a factor of ≈3 with VEGFR2 coexpressed with SCUBE2 than with VEGFR2 alone in HEK-293T cells (K_d=0.21 versus 0.58 nmol/L; Figure 4C). Together, these data suggest that SCUBE2 acts as a coreceptor for VEGFR2 and potentiates VEGF binding to VEGFR2 (Figure 4D).

SCUBE2 Modulates VEGFR2 Phosphorylation and Downstream Signaling in HUVECs

We next investigated whether SCUBE2 facilitates VEGF-activation signals in HUVECs. To this end, we used VEGF as a stimulator and SCUBE2 shRNA knockdown or SCUBE2 overexpression to evaluate the function of SCUBE2 on VEGF-induced signaling. VEGF could induce VEGFR2 Tyr1059 phosphorylation, the p44/42 mitogen-activated protein kinase signaling cascade, and AKT activation. However, SCUBE2 shRNA knockdown reduced (Figure 5A and 5B) and SCUBE2 overexpression (Figure 5C and 5D) enhanced VEGF-induced VEGFR2 phosphorylation, the p44/42 mitogen-activated protein kinase signaling cascade, and AKT activation. Our data strongly indicate that as a VEGFR2 coreceptor, SCUBE2 is involved in regulating VEGF-induced VEGFR2 downstream signals in HUVECs. We have determined the phosphorylation status of SCUBE2 on treatment of VEGF in HUVECs. After VEGF stimulation, anti-SCUBE2 immunoprecipitates were blotted with anti-phosphotyrosine or anti-phosphoserine/threonine (p-Ser/Thr) pan-specific antibody, respectively. SCUBE2 seems unphosphorylated on treatment with VEGF ≤30 minutes. However, further phosphoproteomic analysis will be needed to verify whether or not SCUBE2 is phosphorylated after VEGF stimulation (Figure VII in the online-only Data Supplement).

Scube2 Modulates VEGF Signaling in MLECs

Similar to our knockdown experiments in HUVECs (Figure 1G through 1I), VEGF-induced cell proliferation and tube formation were markedly attenuated in EC-KO MLECs as compared with control MLECs (Figure 6A and 6B). We further assessed the effect of endothelial *Scube2* gene deletion on VEGF-induced signaling. We stimulated control and EC-KO MLECs with 50 ng/mL VEGF for 0, 10, 20, and 30 minutes. In agreement with SCUBE2 shRNA knockdown in HUVECs, VEGF signaling, determined by phosphorylation of VEGFR2, p44/42 mitogen-activated protein kinase, and AKT, was markedly lower in EC-KO than that in control MLECs (Figure 6C and 6D).

Decreased Microvessel Outgrowth From Aortic Explant of *Scube2* EC-KO Mice

We further investigate the role of SCUBE2 in angiogenesis using the ex vivo aortic ring sporting assay, in which we compared the angiogenic potential of aortic fragments derived from control and EC-KO mice. The aortic rings, isolated from control and EC-KO mice, were treated with PBS or VEGF, and the angiogenic response was determined for each individual aortic ring explant by quantifying the number of growing microvessels and by measuring the total length occupied by the newly formed microvessels. Quantification of the number of the tube-like structures (sprouts) and of the length of sprouts in response to VEGF (30 ng/mL) at 5 days showed a significant decrease of both parameters in EC-KO compared with control aortic rings (Figure 6E and 6F). Therefore, these ex vivo results confirm in vitro data and support the role of SCUBE2 in VEGFR2-mediated angiogenesis.

Discussion

Although SCUBE2 was originally identified as an endothelial gene more than a decade ago,[10] its functional relevance in vascular biology during physiological or pathological conditions has not been reported. Even with global inactivation of *Scube2*, we did not observe abnormalities in vasculogenesis and angiogenesis during mouse embryonic development,[20] which suggests possible gene and functional redundancy compensated by *Scube1* and *Scube3* genes. Alternatively, the VEGF signaling levels are sufficient so that any potential enhancement by *Scube2* during embryonic vascular development is redundant. In the present study, we show that hypoxia mediated HIF-1α–upregulated SCUBE2 at the EC surface, which could interact directly with VEGFR2 and function as a coreceptor to facilitate VEGF binding and VEGF-induced signal transduction, thus playing a critical role in adult angiogenesis.

Several lines of evidence suggest the role of SCUBE2 as a coreceptor for VEGFR2. First, SCUBE2 physically associates with VEGFR2 in a complex in overexpressed HEK-293T cells and in human ECs endogenously expressing both molecules. Second, SCUBE2 co-operates with VEGF and potentiates its binding to VEGFR2 to enhance VEGF-induced VEGFR2 phosphorylation and downstream signaling, which results in EC proliferation and capillary network formation. Moreover, VEGF-induced cellular responses and signals in HUVECs could be genetically enhanced by overexpression of SCUBE2 and suppressed by SCUBE2 knockout (EC-KO) and shRNA-mediated knockdown.

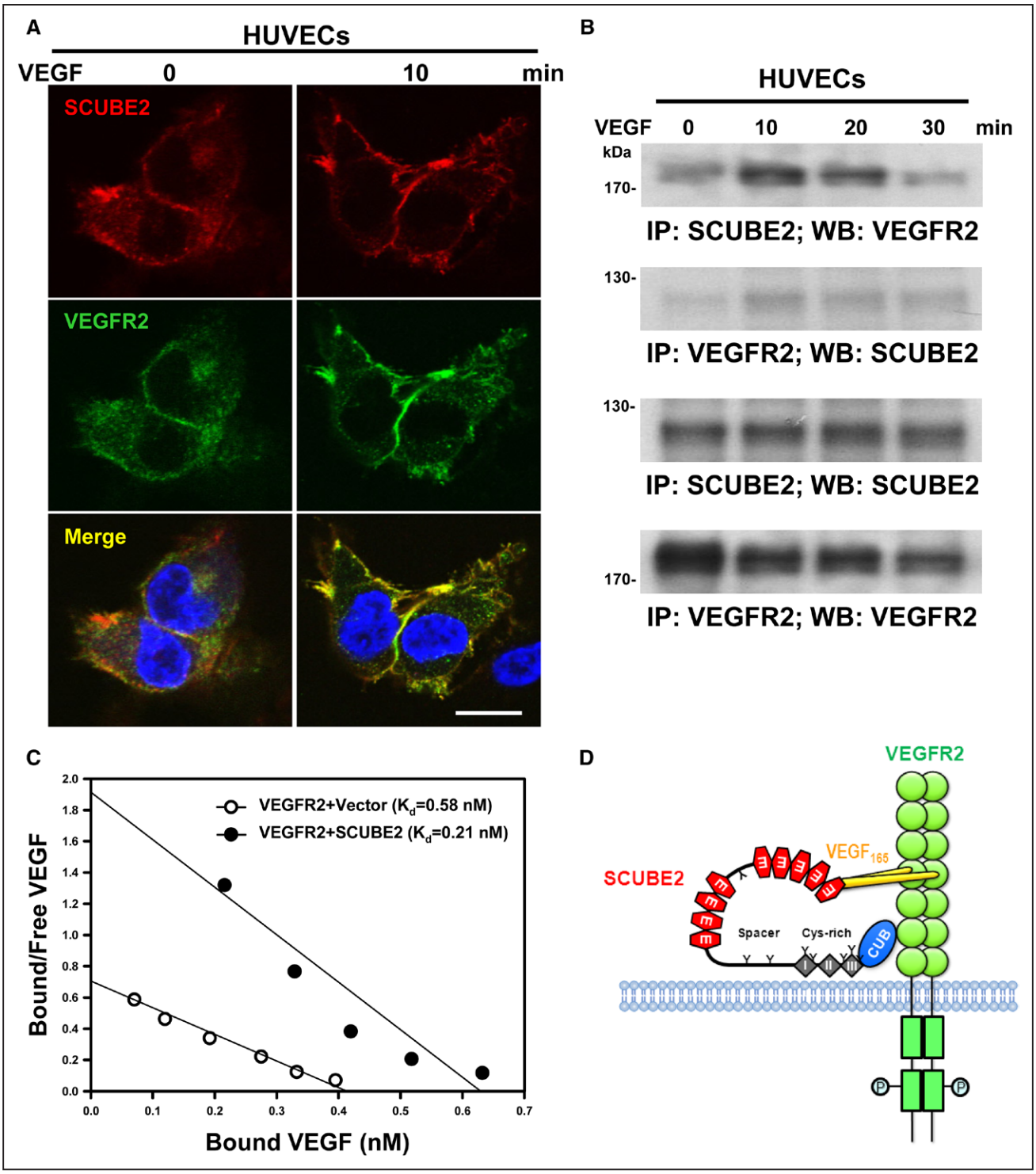

Figure 4. SCUBE2 (signal peptide-CUB-EGF domain-containing protein 2) colocalizes and interacts with vascular endothelial growth factor receptor 2 (VEGFR2) and enhances vascular endothelial growth factor (VEGF) binding to VEGFR2 in human umbilical vein endothelial cells (HUVECs). **A**, VEGF promotes SCUBE2 and VEGFR2 colocalization at plasma membrane. Confocal microscopy of SCUBE2 colocalizing with VEGFR2 on endothelial cell (EC) plasma membrane (**A**). SCUBE2 localization detected by anti-SCUBE2 antibody and Alexa Fluor 594–conjugated IgG (red). VEGFR2 detected by anti-VEGFR2 antibody and Alexa Fluor 488–conjugated IgG (green). Overlay demonstrates colocalization of SCUBE2 with VEGFR2 on EC membranes (yellow). Scale bar=10 μm. **B**, The time course effect of VEGF on SCUBE2 and VEGFR2 interaction. Coimmunoprecipitation experiments of individual anti-SCUBE2 or anti-VEGFR2 antibody pulling down each other's protein. **C**, SCUBE2 increases the binding affinity of VEGF to VEGFR2. Binding of AP-$VEGF_{165}$ to HEK-293T cells expressing the indicated combination of VEGFR2 and SCUBE2 expression plasmids determined colorimetrically; data were plotted by the Scatchard method. The $VEGF_{165}$ binding affinity is indicated. **D**, Endothelial SCUBE2 acts as a coreceptor for VEGFR2 to augment VEGF signaling. SCUBE2 binds VEGF and VEGFR2 through its EGF-like repeats and the CUB domain and forms a complex to promote VEGF-induced VEGFR2 phosphorylation and downstream signaling in ECs.

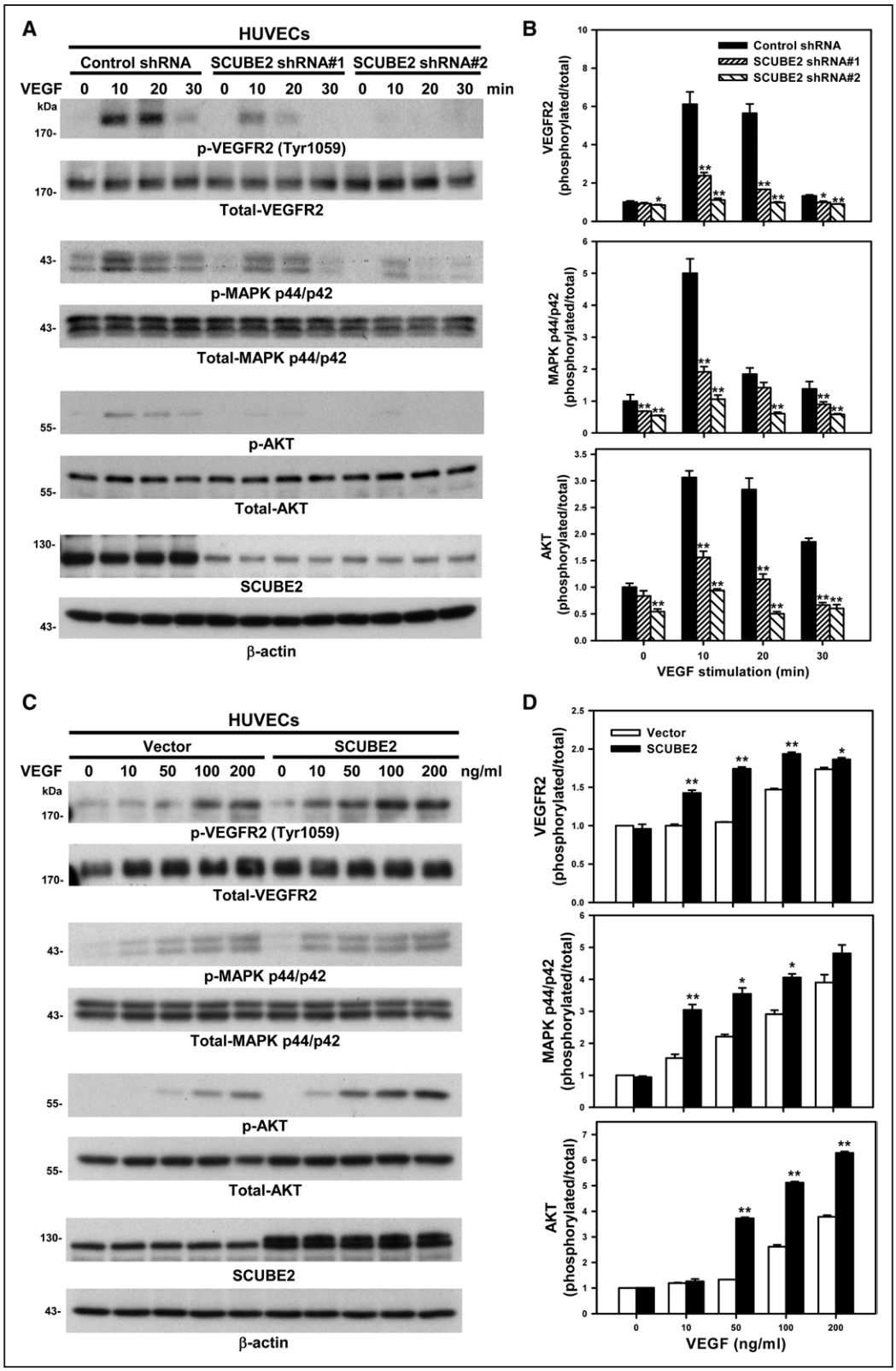

Figure 5. SCUBE2 (signal peptide-CUB-EGF domain-containing protein 2) modulates vascular endothelial growth factor receptor 2 (VEGFR2) phosphorylation and downstream signaling in human umbilical vein endothelial cells (HUVECs). **A** and **B**, SCUBE2 knockdown impairs vascular endothelial growth factor (VEGF) signaling in HUVECs. Western blot analysis of VEGF signaling in HUVECs (**A**) and quantification (**B**) of VEGF-induced phosphorylation of VEGFR2 at Tyr1059, p44/42 mitogen-activated protein kinase (MAPK) at Thr202/Tyr204 and AKT at Ser473 with control, and SCUBE2 shRNA knockdown in HUVECs. Data are mean±SD from 3 independent experiments. **P*<0.05; ***P*<0.01 compared with control. **C** and **D**, Western blot analysis of VEGF signaling in ECs (**C**) and quantification (**D**) of VEGF-induced phosphorylation of VEGFR2 Tyr1059, p44/42 MAPK Thr202/Tyr204, and AKT Ser473 in control and SCUBE2-overexpressing ECs. Data are mean±SD from 3 independent experiments. **P*<0.05; ***P*<0.01 compared with vector.

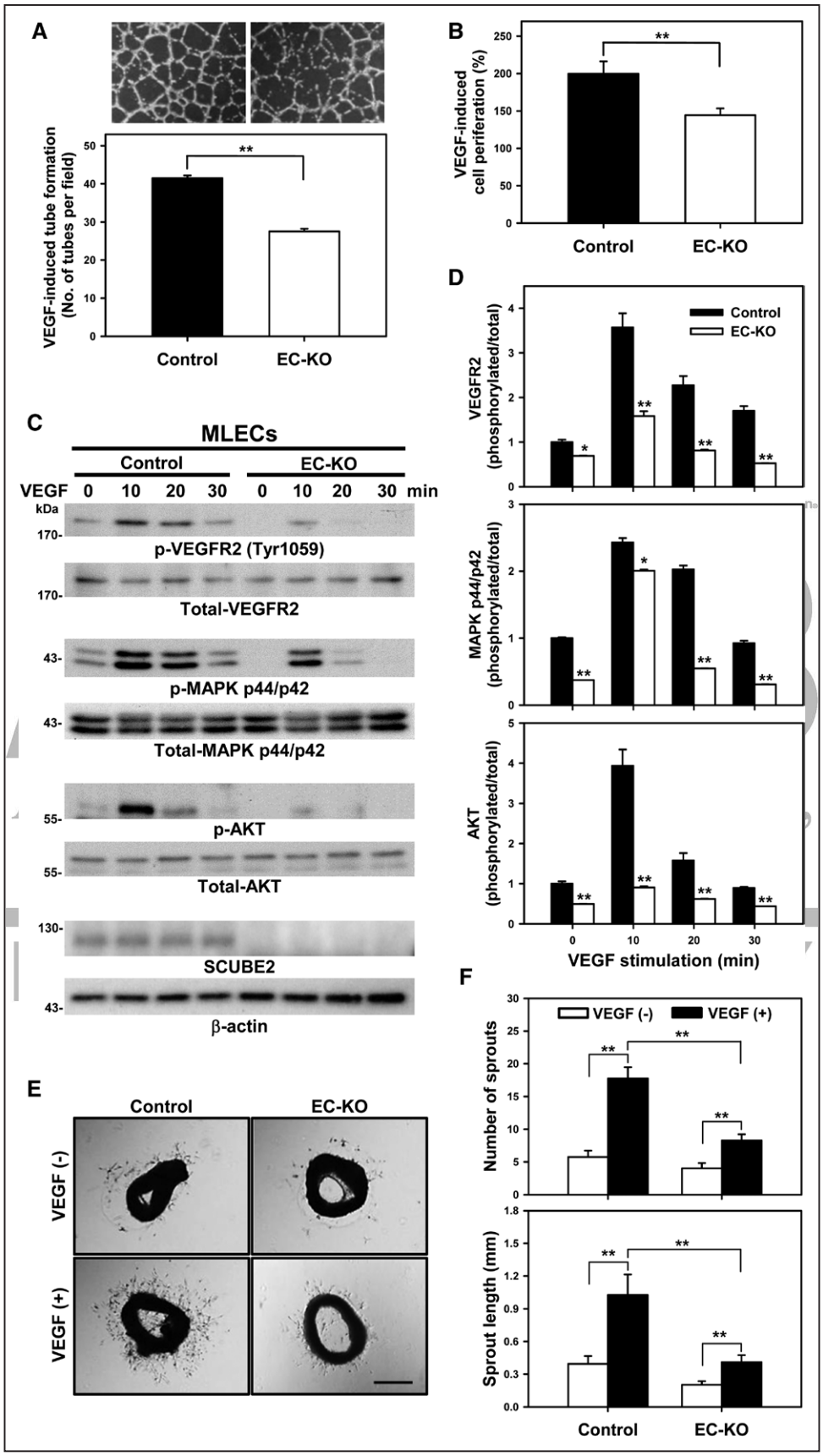

Figure 6. Attenuation of vascular endothelial growth factor (VEGF) responses and signaling in endothelial cell (EC)-specific *Scube2* knockout (EC-KO) mouse lung ECs (MLECs). **A** and **B**, VEGF-stimulated tubulogenesis (**A**) and proliferation (**B**) of EC-KO MLECs. Data are mean±SD from 3 independent experiments. $^{**}P<0.01$ compared with control. **C** and **D**, Western blot analysis of VEGF (*Continued*)

Figure 6 Continued. signaling in MLECs (**C**) and quantification (**D**) of VEGF-induced phosphorylation of VEGFR2 Tyr1059, p44/42 MAPK Thr202/Tyr204, and AKT Ser473 in control and EC-KO MLECs. Data are mean±SD from 3 independent experiments. *P<0.05; **P<0.01 compared with control. **E**, Photomicrographs show the angiogenic response of 5-d culture collagen-embedded thoracic aorta ring explants from control and EC-KO mice. Scale bar=1 mm. **F**, Quantification of the number and length of microvessel sprouts. The angiogenic response was determined for each individual aortic ring explant by quantifying the number of growing microvessels (**left**) and by measuring the total length occupied by the newly formed microvessels (**right**).

Besides *Scube2*-KO mice, many other KO mouse strains also show normal vascular development but impaired postnatal angiogenesis and vascular remodeling. For instance, deficiency of a neuropilin-related transmembrane protein, endothelial and smooth muscle cell-derived neuropilin-like[25] or an immunoglobulin-like cell adhesion molecule, and nectin-like protein-5[26] in mice did not affect embryonic vascular formation but impaired postnatal angiogenesis after ischemia. Although why the absence of these genes is not compensated in ischemia-induced adulthood angiogenesis is unknown, we noted that SCUBE2 but not SCUBE1 or 3 was specifically upregulated by HIF-1α in HUVECs under hypoxia (Figure 2). This unique hypoxic induction of SCUBE2 might explain in part its distinctive role in regulating blood flow recovery after hind-limb ischemia (Figure 3).

Although several other VEGFR2 coreceptors such as neuropilins[6,7] and CD46v6[5] have been implicated in VEGFR2 signaling and angiogenesis, unlike neuropilins, which interact with VEGFR2 and depend on VEGF treatment,[6,7] SCUBE2 and VEGFR2 were preformed in a complex independent of VEGF stimulation in our study. Different VEGFR2 coreceptors may potentiate VEGF signal transduction by distinct mechanisms that may elicit divergent downstream signals, thereby leading to different EC functions depending on the disease status. Therefore, further elucidating the molecular functions of these coreceptors in VEGF-mediated angiogenesis is important.

Another interesting point is that membrane tethering of SCUBE proteins was mediated at least by a polycationic stretch in their spacer region via electrostatic interactions with anionic heparin sulfate proteoglycans expressed on the cell surface.[12,27] Because VEGF has been described as a heparin-binding growth factor[28] and shown to bind to heparin sulfate proteoglycans,[4] a full-scale postnatal angiogenic response may involve co-operation between SCUBE2, heparin sulfate proteoglycans, VEGFR2, and VEGF on the EC surface.

Of note, we and others have recently found that SCUBE2 can positively regulate sonic hedgehog signaling,[20,29–31] which has been implicated in neovessel formation.[32] In addition, endothelial SCUBE2 might collaborate with VE-cadherin (a major adhesive protein of adherens junctions in ECs) essential in supporting vascular stability and promoting angiogenesis[33] because epithelial SCUBE2 could indeed associate with E-cadherin (a major adhesive protein of adherens junctions in epithelium) in maintaining and driving the epithelial molecular phenotype.[34] Therefore, the involvement of SCUBE2 in angiogenesis was not restricted to the action of VEGF, although further studies are needed to clarify whether SCUBE2 participates in postnatal angiogenesis mediated by sonic hedgehog or VE-cadherin signaling.

Our study revealed SCUBE2 as a novel VEGFR2 coreceptor that regulates VEGF-induced tube formation and proliferation of ECs by fine-tuning VEGFR2-mediated signaling. However, whether SCUBE2 has clinical importance in the pathogenesis of various angiogenesis-related diseases such as atherosclerosis, diabetic retinopathy, and age-related macular degeneration is unknown. In addition to its expression in normal organ ECs, SCUBE2 is also highly expressed in the ECs of numerous types of human carcinomas and xenografted tumors (Y.-C. Lin et al., unpublished data,). Although further studies are required to validate whether endothelial SCUBE2 is involved in tumor angiogenesis, SCUBE2 may be a promising target molecule for cancer therapy because of its anti-angiogenic effect of SCUBE2 inactivation. Pharmacological blockade of endothelial SCUBE2 may represent a novel therapeutic strategy for angiogenesis-related disorders.

Acknowledgments

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Disclosures

None.

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Highlights

- SCUBE2 (signal peptide-CUB-EGF domain-containing protein 2) expression is upregulated by hypoxia-inducible factor-1α in endothelial cells.
- Responses to vascular endothelial growth factor (VEGF) and adult angiogenesis are impaired in *Scube2* endothelial-specific deletion mice.
- SCUBE2 colocalizes and interacts with VEGFR2 in endothelial cells.
- SCUBE2 potentiates VEGF binding to VEGFR2 and enhances downstream proangiogenic and proliferating signals.
- SCUBE2 is a novel coreceptor for VEGFR2 and promotes VEGF-induced signaling in adult angiogenesis.

Arteriosclerosis, Thrombosis, and Vascular Biology

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SUPPLEMENTAL MATERIAL

Endothelial SCUBE2 interacts with VEGFR2 and regulates VEGF-induced angiogenesis

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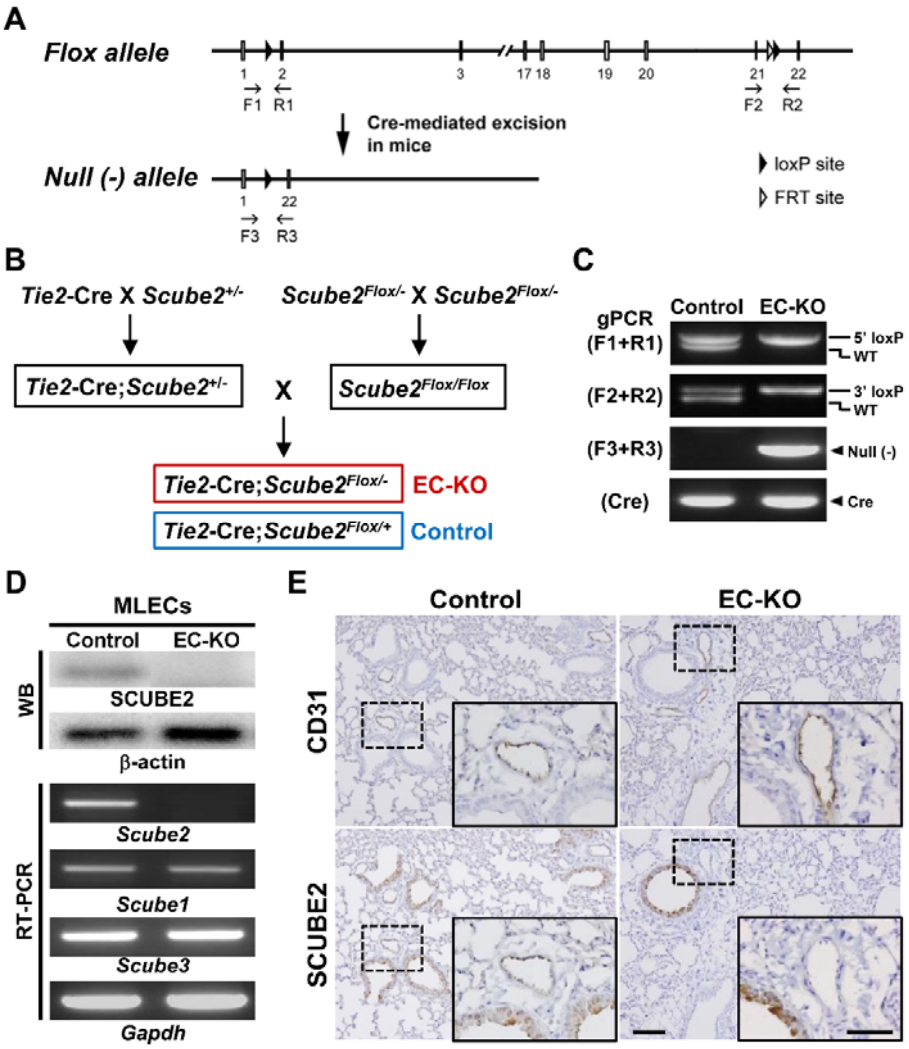
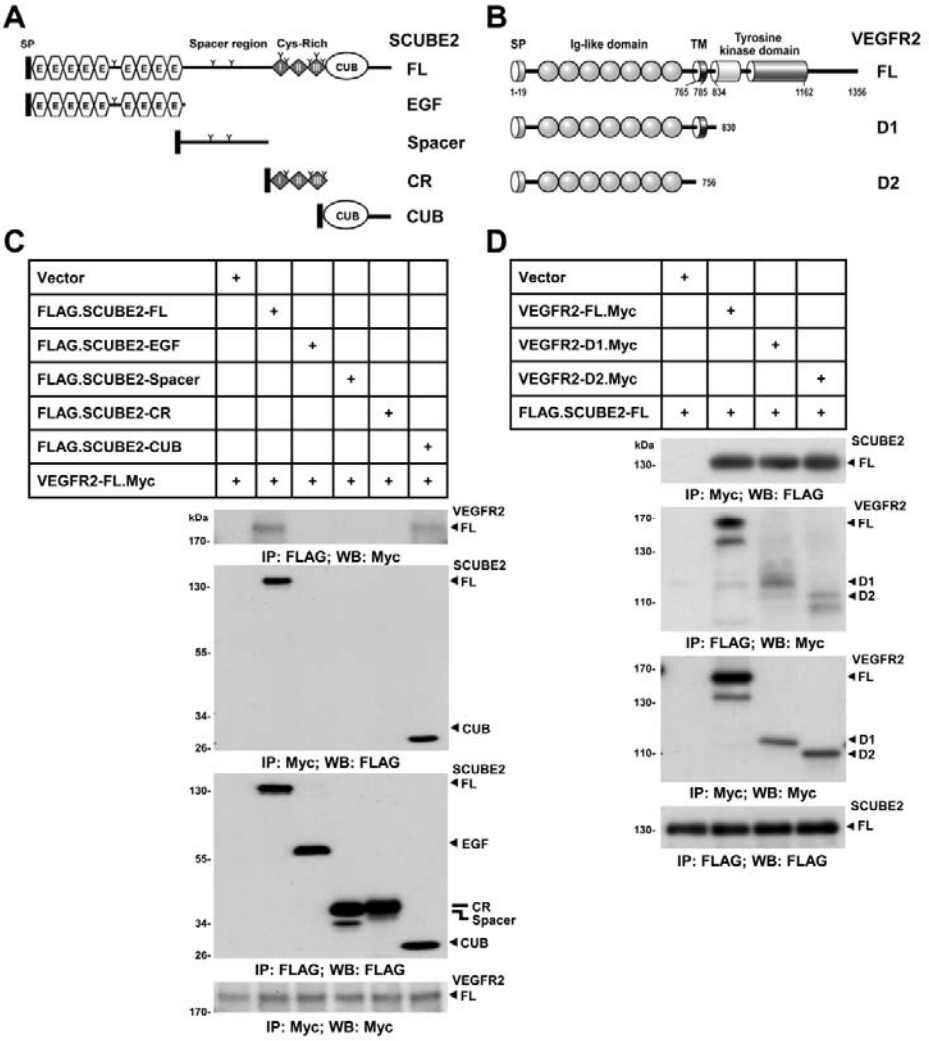
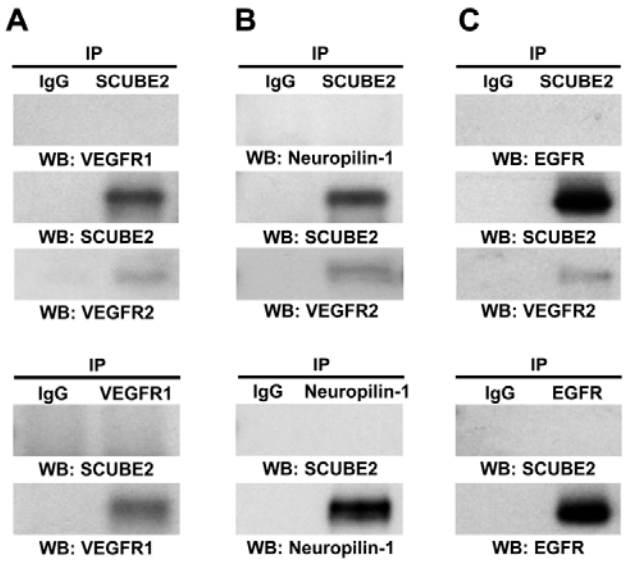


Supplemental Figure I. Generation of endothelial-specific *Scube2* knockout mice. **A**, Schematic diagram of the strategy used to generate endothelial-specific *Scube2*-knockout mice. Conditional *Scube2*-knockout mice were generated with two (5' and 3') targeting vectors in a sequential fashion that introduced two *lox*P sites separated by 65 kb of genomic sequence. The final targeted *Flox* allele contains a *lox*P site in the first intron and a FRT/*lox*P site downstream in the 21st intron of *Scube2*. The locations of primers for genotyping (F1, R1, F2, R2, F3 and R3) are marked in the map. Each exon is numbered under an open box. Filled triangles indicate *lox*P sites and open triangles indicate FRT sites. **B**, Mating scheme to generate endothelial-specific *Scube2* knockout mice (*Tie2*-Cre; *Scube2*$^{Flox/-}$; EC-KO) and control littermates (*Tie2*-Cre; *Scube2*$^{Flox/+}$; Control). EC-KO mice were generated by crossing male *Tie2*-Cre; *Scube2*$^{+/-}$ mice to *Scube2*$^{Flox/Flox}$ female mice. **C**, Genotyping of the *Flox* and *Null* (-) allele by PCR analysis. PCR of genomic DNA from EC-KO or control mice with the primers F1+R1, F2+R2 or F3+R3, respectively. The larger PCR products indicate the presence of the remaining *lox*P site (F1+R1

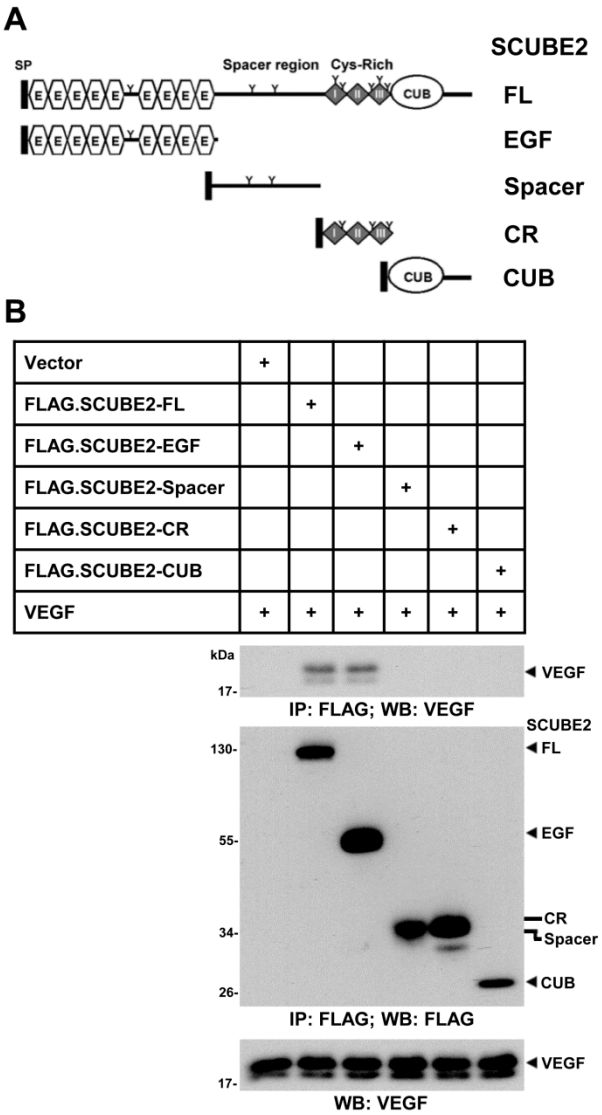
PCR) or the FRT/*lox*P site (F2+R2 PCR). The primers F3 and R3 detected exons 2 to 21 in the null (-) allele. **D** and **E**, Validation of EC-specific deletion of *Scube2* (EC-KO) in mice. RT-PCR and western blot evaluation of *Scube1, 2*, and *3* mRNA and protein levels in control and EC-KO primary mouse lung ECs (MLECs) (D). Immunohistochemistry staining of pan-endothelial marker CD31 and endothelial SCUBE2 expression in control and EC-KO mouse lung tissue (E). Scale bar = 100 or 50 μm (inset).



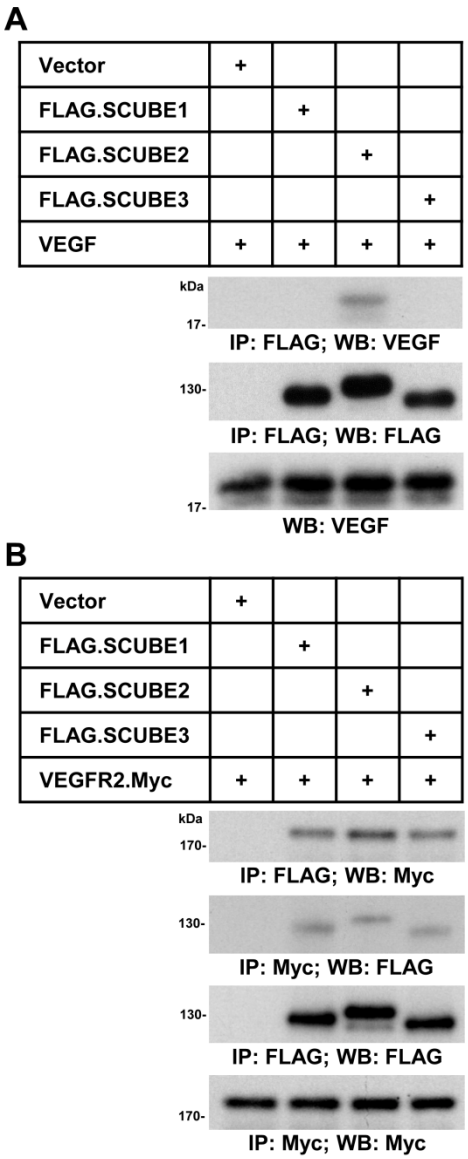
Supplemental Figure II. Molecular mapping of the interacting domains between SCUBE2 and VEGFR2. **A** and **B**, Domain organization of the SCUBE2 and VEGFR2 expression constructs used to map the interacting domains. FLAG epitope was added immediately after the signal peptide sequence at the NH_2-terminus of SCUBE2 constructs (A), and Myc epitope was tagged to the COOH-terminus of VEGFR2 full-length (FL) and its deletion mutants D1 and D2 (B). SP, signal peptide; CR, cysteine-rich. **C** and **D**, The CUB domain of SCUBE2 can interact with the extracellular Ig-like domain of VEGFR2. The expression plasmids encoding Myc-tagged VEGFR2-FL were transfected alone or together with a series of FLAG-tagged SCUBE2 constructs (C) or the plasmids encoding the FLAG-tagged SCUBE2-FL alone or with Myc-tagged VEGFR2-FL, D1 or D2 constructs in HEK-293T cells (D) for 2 days, then cell lysates underwent immunoprecipitation, followed by western blot analysis with indicated antibodies to determine the protein–protein interactions. IP, immunoprecipitation; WB, western blotting.



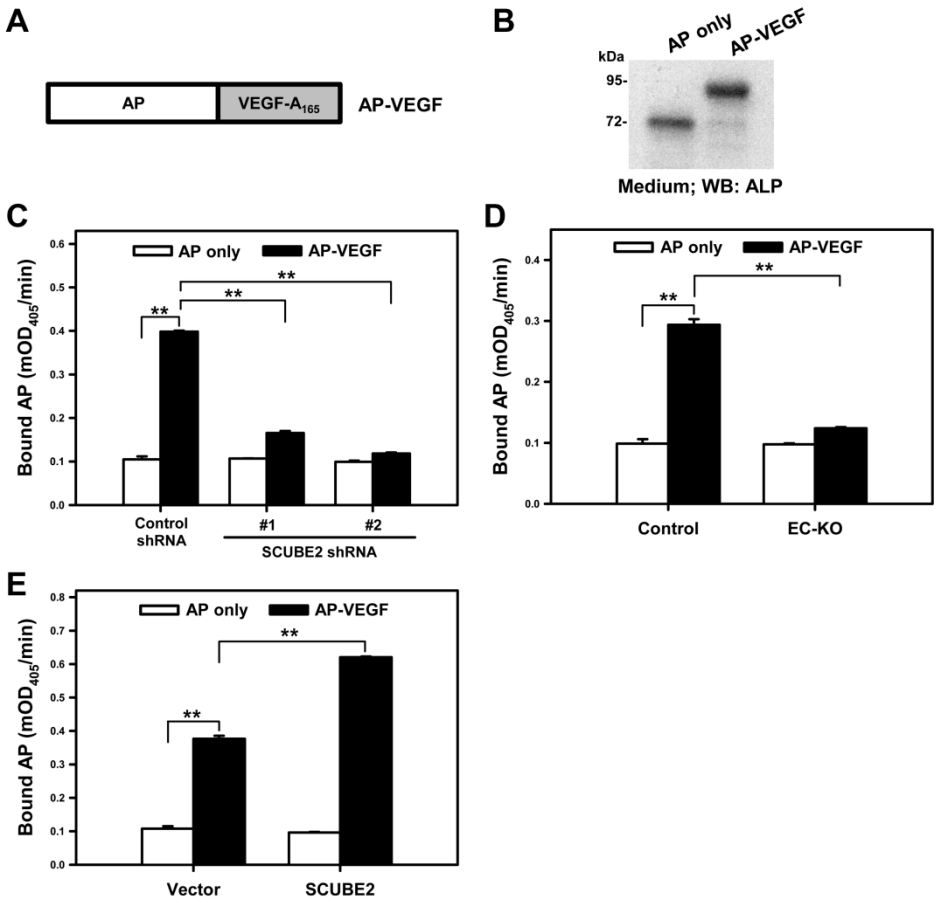
Supplemental Figure III. SCUBE2 specifically interacts with VEGFR2 but not VEGFR1, Neuropilin-1 and EGFR in HUVECs. Anti-SCUBE2 immunoprecipitates were subjected to western blotting with anti-VEGFR1 (A), anti-Neurpilin-1 (B), anti-EGFR (C) or anti-VEGFR2 antibody that service as a positive control to confirm their protein-protein interactions (top panel). A reciprocal pull-down assay was always performed to verify these immunoprecipitation results (bottom panel).



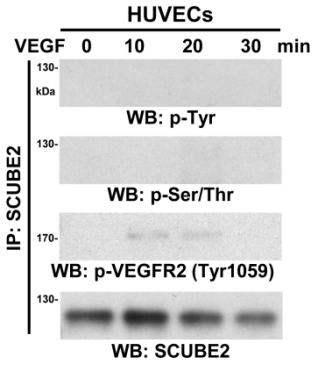
Supplemental Figure IV. VEGF-A_{165} (designated VEGF) directly interacts with the NH_2-terminal EGF repeats of SCUBE2. **A**, Domain organization of the SCUBE2 expression constructs used to map the interacting domains. FLAG epitope was added immediately after the signal peptide sequence at the NH_2-terminus of SCUBE2 constructs. SP, signal peptide; CR, cysteine-rich. **B**, Recombinant VEGF protein was mixed with recombinant FLAG-tagged SCUBE2-FL and its domain-specific protein fragments bound to anti-FLAG M2 antibody-agarose beads or control beads for incubation for 4 h at 4°C, then beads were washed extensively and interaction was visualized by western blot analysis with anti-VEGF antibody. IP, immunoprecipitation; WB, western blotting.



Supplemental Figure V. The interaction between SCUBE protein family and VEGF or VEGFR2. **A**, SCUBE2 can specifically interact with VEGF. Recombinant VEGF (VEGF-A_{165}) protein was mixed with recombinant FLAG-tagged SCUBE1-3 and its proteins bound to anti-FLAG M2 antibody-agarose beads for incubation for 4 h at 4°C, then beads were washed extensively and interaction was visualized by western blot analysis with anti-VEGF antibody. **B**, SCUBE1-3 can interact with VEGFR2. The expression plasmids encoding Myc-tagged VEGFR2 were transfected alone or together with FLAG-tagged SCUBE1-3 constructs in HEK-293T cells for 2 days, then cell lysates underwent immunoprecipitation, followed by western blot analysis with indicated antibodies to determine the protein–protein interactions. IP, immunoprecipitation; WB, western blotting.



Supplemental Figure VI. SCUBE2 modulates the binding of VEGF on ECs that endogenously express VEGFR2. **A** and **B**, Production of an alkaline phosphatase fusion of VEGF-A_{165} (AP-VEGF). Schematic diagram of the AP-VEGF construct (A). Western blot analysis of AP-VEGF expression in conditioned media from HEK-293T cells (B). **C-E**, SCUBE2 potentiates VEGF binding to VEGFR2 in ECs. Binding of AP-VEGF and control AP protein on ECs endogenously expressing VEGFR2. Effect of SCUBE2 knockdown (C), SCUBE2 knockout (D), and SCUBE2 overexpression (E) on AP-VEGF binding compared with corresponding control ECs. Data are means ± SD from 3 independent experiments. **, $P < 0.01$.



Supplemental Figure VII. Effect of VEGF treatment on the phosphorylation status of SCUBE2 in HUVECs. HUVECs treated with VEGF (100 ng/ml) for 10, 20 or 30 min were lysed and immunoprecipitated with anti-SCUBE2 antibody. Anti-SCUBE2 immunoprecipitates were blotted with anti-phosphotyrosine (p-Tyr) or anti-phosphoserine/threonine (p-Ser/Thr) pan-specific antibody, respectively. Note that Tyr1059-phosphorylated VEGFR2 was pulled down and served as a positive control. IP, immunoprecipitation; WB, western blotting.

Online Table I. Oligonucleotide primers used in this study

Gene*	Forward	Reverse	Assay
SCUBE1	TGCGGCGGCGAGCTTGGTGAC	TTTGGAGCGCAGCAGTTTGATGAA	RT-PCR
SCUBE2	TCTTGCCCAGGAAATACTACGACT	TGGGCCAGGACATCAAACAGAG	RT-PCR
SCUBE3	TGGCCCAATGCAAGAATCGTCAGT	TGGGCTAGCACCTCAAAGAAG	RT-PCR
GAPDH	GCCAAAAGGGTCATCATCTC	ACCACCTGGTGCTCAGTGTA	RT-PCR
Scube1	CGGCGGCGAACTTGGTGACTACA	TTGATAAAGGACCGGGGGAACAT	RT-PCR
Scube2	TGACTACCTGGTGATGCGGAAAAC	CAGTGGCGTGTGGGAAGAGTCA	RT-PCR
Scube3	TGCTCCCCGGGCCACTACTAT	AGCGCTGTTGGCCTCACTGGTCTT	RT-PCR
SCUBE2	GCACACGCACGCGCGCACACA	GAAGGGTGCAGAGGGTGTGCT	ChIP
Gapdh	ATCATCCCTGCATCCACTGGTGCTG	TGATGGCATTCAAGAGAGTAGGGAG	RT-PCR
SCUBE1	AACATCCCGGGGAACTACAG	GCAGCCACCATTATTGTCCT	Q-PCR
SCUBE2	CAGGCAGAGTCCTGTGGAGT	TAAAATGCAGCGTTCTCGTG	Q-PCR
SCUBE3	CCTGCTTGTCCTGCTGGT	TCGATGTGGCAGTTGTCAGT	Q-PCR
GAPDH	TGAAGGTCGGAGTCAACGG	AGAGTTAAAAGCAGCCCTGGTG	Q-PCR
Scube2-5'Flox (F1, R1)	ATAGTCGACTGTTGTCCAGTATCTGTTGC	ATAGCGGCCGCTACGACACCCTGGGATAAAG	Genotyping
Scube2-3'Flox (F2, R2)	GGCCATGTCCCTGAAGAAAACTA	TTATGGGGCCAAGACACTCAAA	Genotyping
Scube2-Null (F3, R3)	CTGGGGCCTCTGGGACACTATT	GTTATGGGGCCAAGACACTCAAA	Genotyping
Cre	TTACCGGTCGATGCAACGAGTGATG	GTGAAACAGCATTGCTGTCACTT	Genotyping

**SCUBE1-3* and *GAPDH* are human genes; *Scube1-3* and *Gapdh* are mouse genes.

Detailed Methods

Antibodies and reagents

Anti-SCUBE2 (catalog GX85173), anti-HIF-1α (catalog GTX127309), and anti-phospho-tyrosine (catalog GTX14167) polyclonal antibodies were from GeneTex (Irvine, CA). Anti-phospho-VEGFR2 (Thr1059) (catalog 3817), anti-phospho-MAPK p44/p42 (Thr202/Tyr204) (catalog 9106), anti-MAPK p44/p42 (catalog 9102), anti-phospho-AKT (Ser473) (catalog 9271), anti-AKT (catalog 9272), and anti-EGFR (catalog 2232) antibodies were from Cell Signaling Technology (Danvers, MA). Anti-VEGFR2 and anti-VEGF antibodies were from Thermo Scientific (Rockford, IL, catalog MA5-15157) and Santa Cruz Biotechnology (Santa Cruz, CA, catalog sc-152), respectively. Anti-CD31 (catalog ab28364), anti-phospho-serine/threonine (catalog ab17464), anti-VEGFR1 (catalog ab32152), and anti-neuropilin-1 (catalog ab81321) antibodies were from Abcam (Cambridge, MA). Anti-Ki67 and anti-β-actin antibodies were from DAKO Cytomatation (Glostrup, Denmark, catalog M7249) and NOVUS Biologicals (Littleton, CO, catalog NB600-501), respectively. Recombinant $VEGF_{165}$ protein was from R&D Systems (Minneapolis, MN, catalog 293-VE).

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Tissue sections (5 μm thick) were dewaxed with xylene, rehydrated in graded concentrations of alcohol, treated with 3% H_2O_2 for 20 min, washed with PBS, blocked with blocking solution (PBST supplemented with 2% BSA and 2% normal goat serum) for 1 h, and incubated at room temperature overnight with primary antibody. Antibody binding was detected by using horseradish peroxidase-conjugated antibody and stable 3,3'-diaminobenzidine (DAB) peroxidase substrate. Hematoxylin was used for counterstaining.[5]

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Statistical analysis

Data are presented as mean ± SD and were analyzed by two-tailed paired *t* test. $P < 0.05$ was considered statistically significant.

Supplemental References

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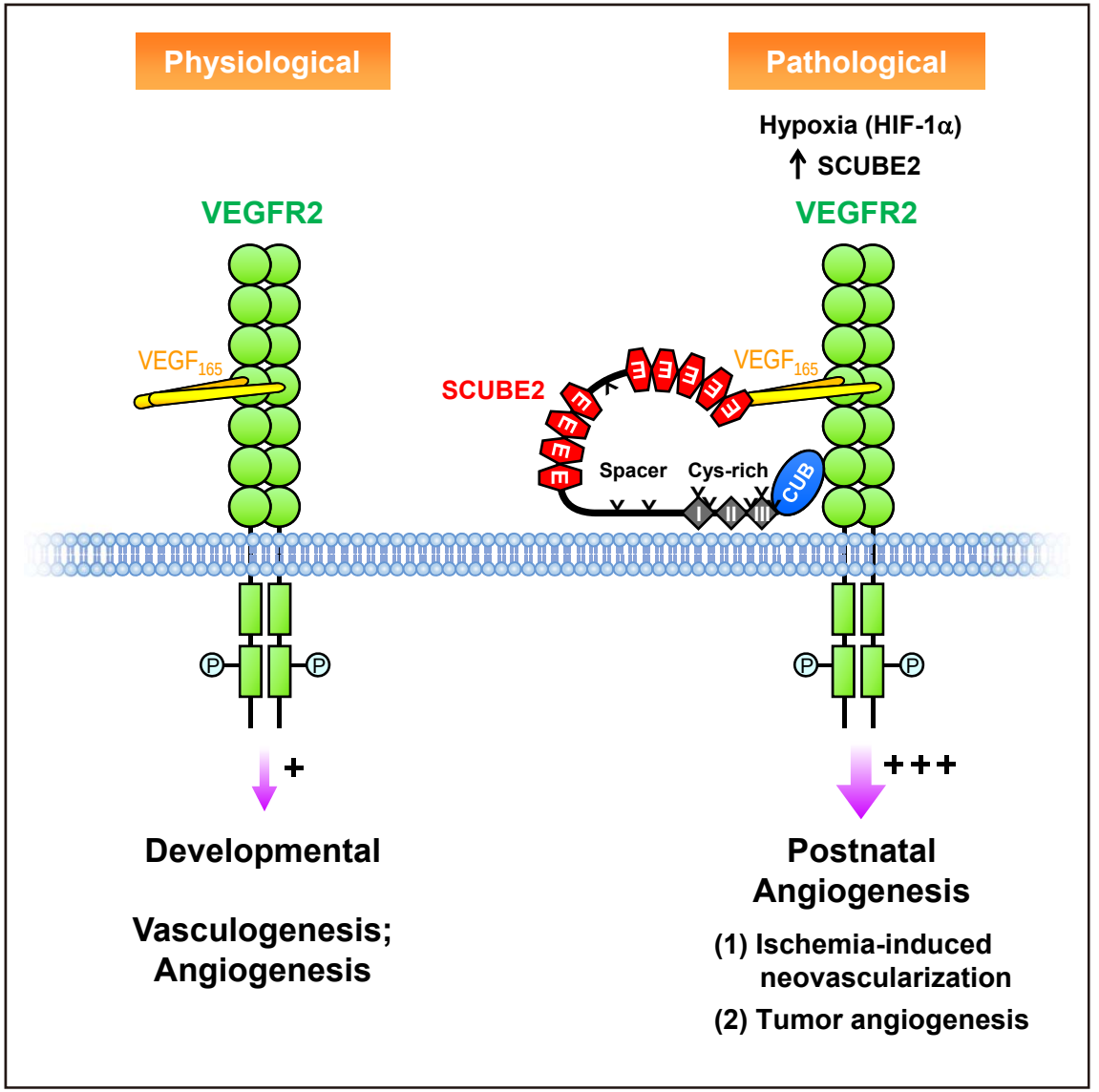
Physiological
Pathological
Hypoxia (HIF-1α)
↑ SCUBE2
VEGFR2
VEGFR2
VEGF165
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Spacer
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CUB
I
II
III
P
P
P
P
+
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SUPPLEMENTAL MATERIAL

Endothelial SCUBE2 interacts with VEGFR2 and regulates VEGF-induced angiogenesis

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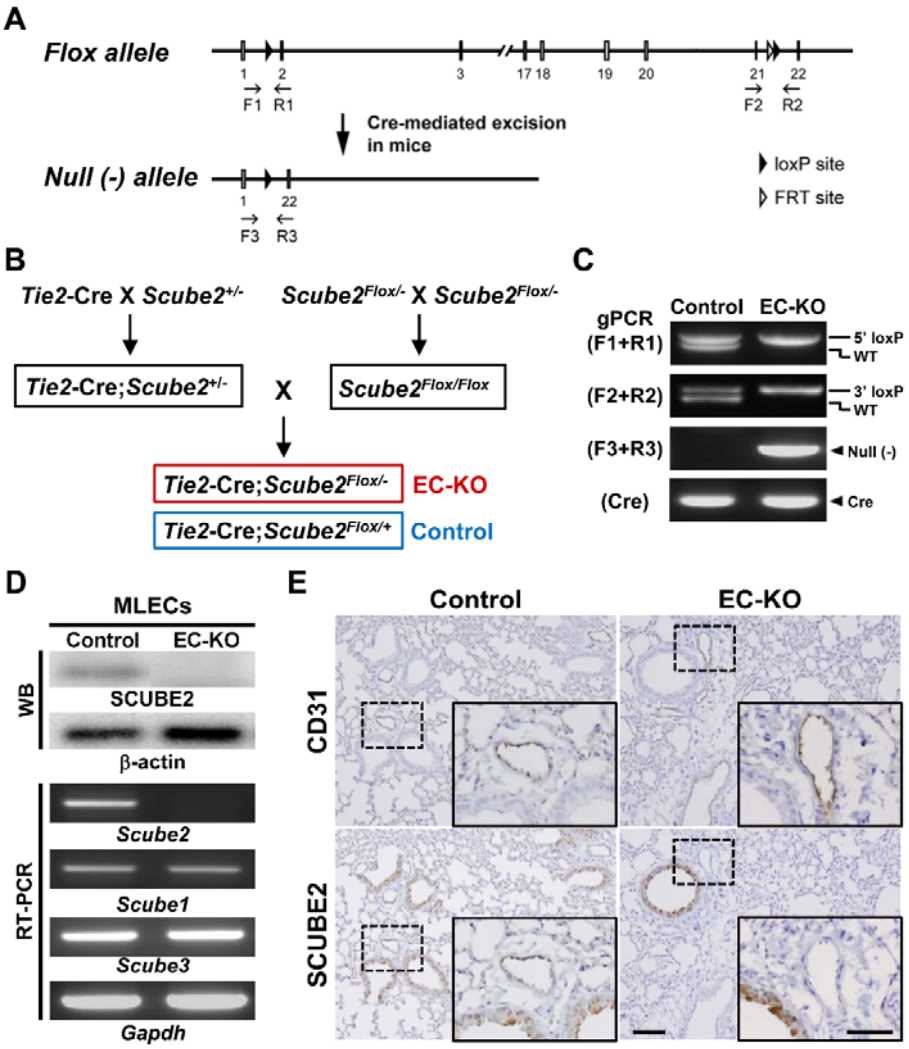
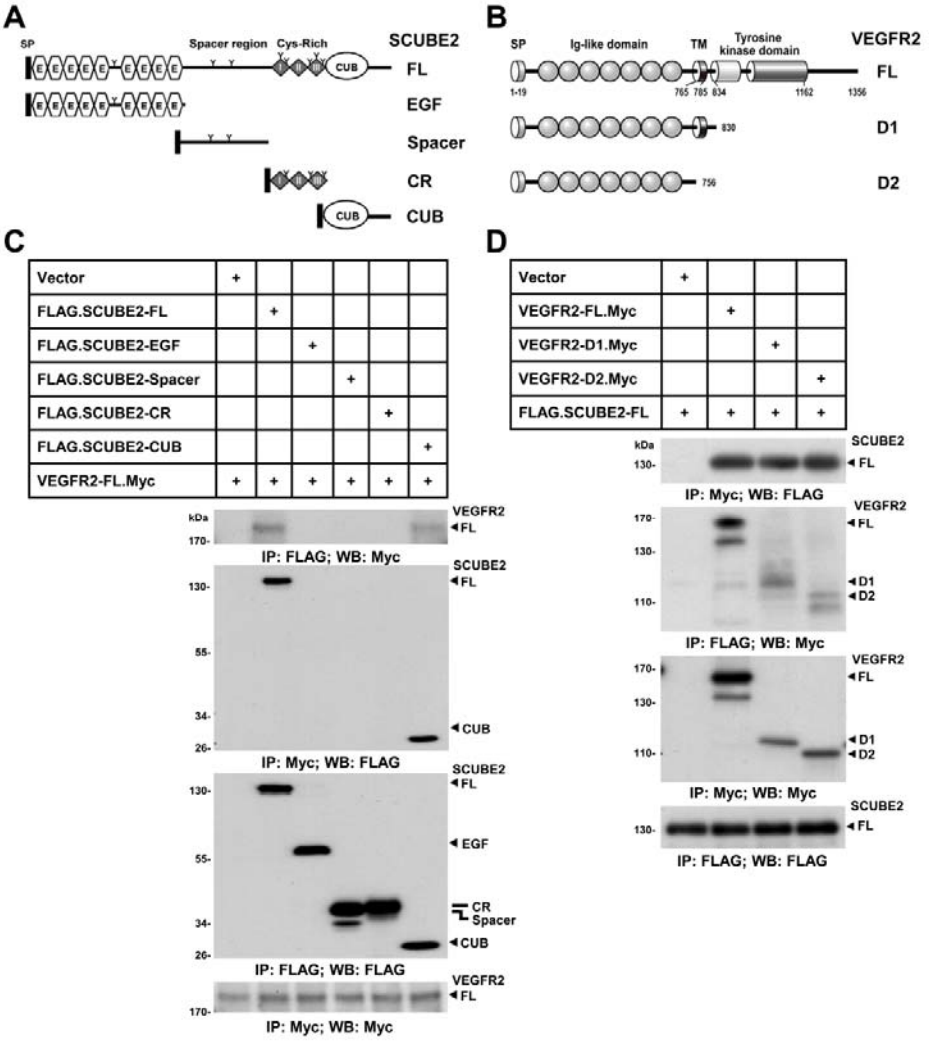
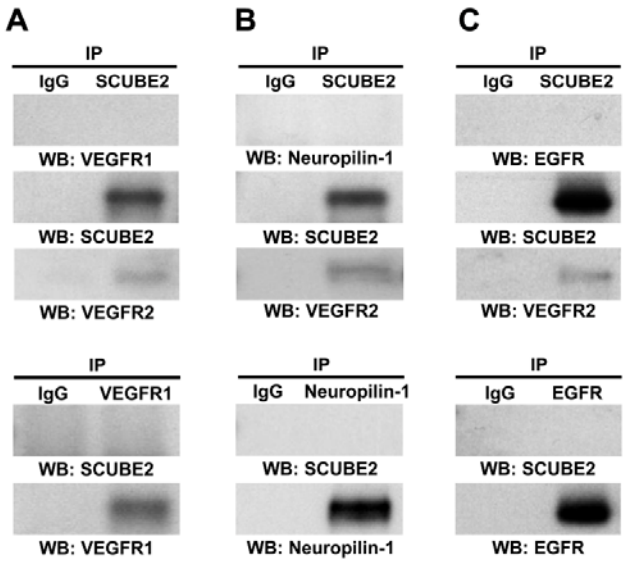


Supplemental Figure I. Generation of endothelial-specific *Scube2* knockout mice. **A**, Schematic diagram of the strategy used to generate endothelial-specific *Scube2*-knockout mice. Conditional *Scube2*-knockout mice were generated with two (5' and 3') targeting vectors in a sequential fashion that introduced two *lox*P sites separated by 65 kb of genomic sequence. The final targeted *Flox* allele contains a *lox*P site in the first intron and a FRT/*lox*P site downstream in the 21st intron of *Scube2*. The locations of primers for genotyping (F1, R1, F2, R2, F3 and R3) are marked in the map. Each exon is numbered under an open box. Filled triangles indicate *lox*P sites and open triangles indicate FRT sites. **B**, Mating scheme to generate endothelial-specific *Scube2* knockout mice (*Tie2*-Cre; *Scube2*$^{Flox/-}$; EC-KO) and control littermates (*Tie2*-Cre; *Scube2*$^{Flox/+}$; Control). EC-KO mice were generated by crossing male *Tie2*-Cre; *Scube2*$^{+/-}$ mice to *Scube2*$^{Flox/Flox}$ female mice. **C**, Genotyping of the *Flox* and *Null* (-) allele by PCR analysis. PCR of genomic DNA from EC-KO or control mice with the primers F1+R1, F2+R2 or F3+R3, respectively. The larger PCR products indicate the presence of the remaining *lox*P site (F1+R1

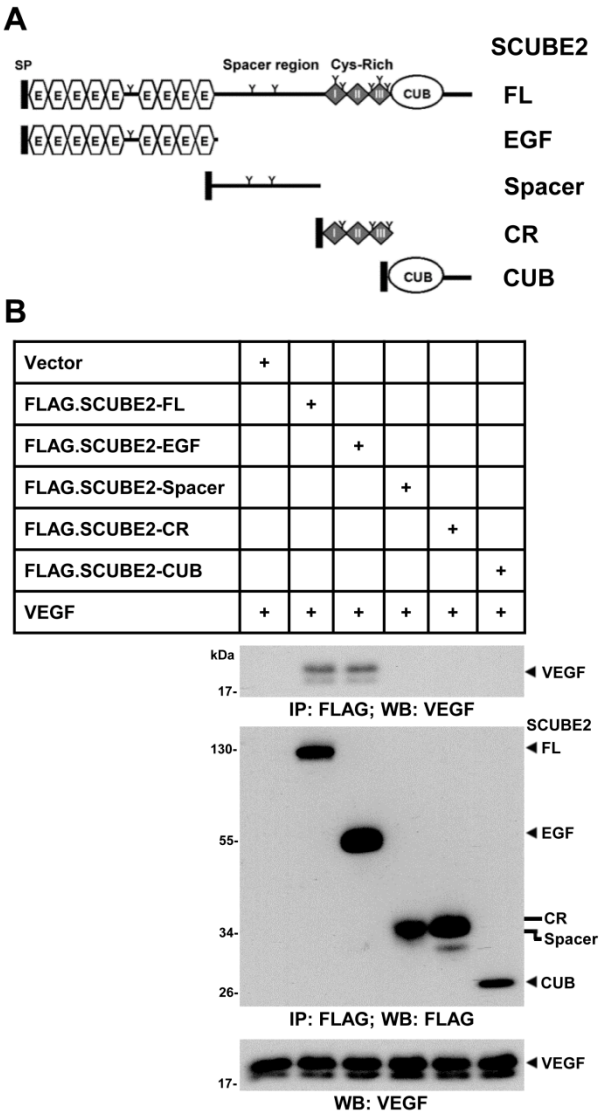
PCR) or the FRT/*lox*P site (F2+R2 PCR). The primers F3 and R3 detected exons 2 to 21 in the null (-) allele. **D** and **E**, Validation of EC-specific deletion of *Scube2* (EC-KO) in mice. RT-PCR and western blot evaluation of *Scube1, 2*, and *3* mRNA and protein levels in control and EC-KO primary mouse lung ECs (MLECs) (D). Immunohistochemistry staining of pan-endothelial marker CD31 and endothelial SCUBE2 expression in control and EC-KO mouse lung tissue (E). Scale bar = 100 or 50 μm (inset).



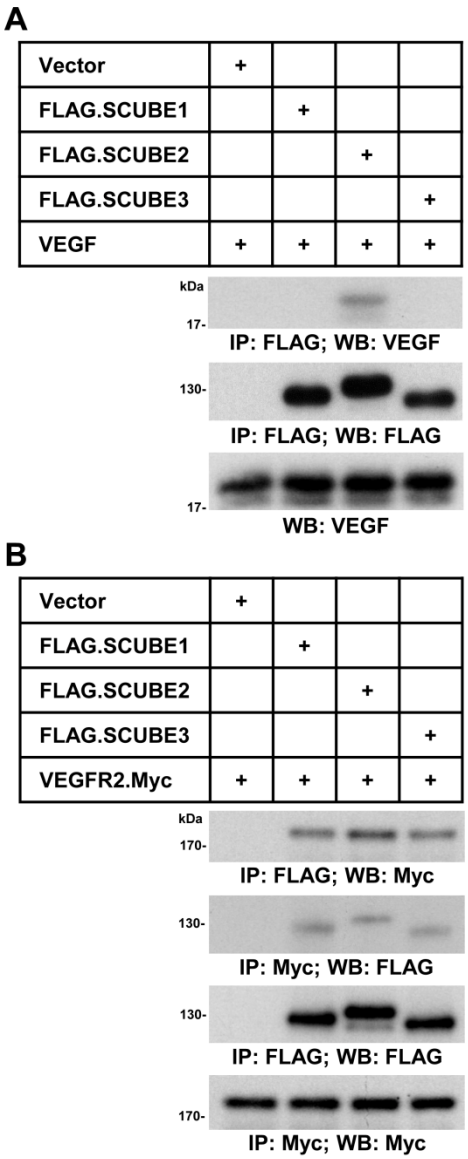
Supplemental Figure II. Molecular mapping of the interacting domains between SCUBE2 and VEGFR2. **A** and **B**, Domain organization of the SCUBE2 and VEGFR2 expression constructs used to map the interacting domains. FLAG epitope was added immediately after the signal peptide sequence at the NH_2-terminus of SCUBE2 constructs (A), and Myc epitope was tagged to the COOH-terminus of VEGFR2 full-length (FL) and its deletion mutants D1 and D2 (B). SP, signal peptide; CR, cysteine-rich. **C** and **D**, The CUB domain of SCUBE2 can interact with the extracellular Ig-like domain of VEGFR2. The expression plasmids encoding Myc-tagged VEGFR2-FL were transfected alone or together with a series of FLAG-tagged SCUBE2 constructs (C) or the plasmids encoding the FLAG-tagged SCUBE2-FL alone or with Myc-tagged VEGFR2-FL, D1 or D2 constructs in HEK-293T cells (D) for 2 days, then cell lysates underwent immunoprecipitation, followed by western blot analysis with indicated antibodies to determine the protein–protein interactions. IP, immunoprecipitation; WB, western blotting.



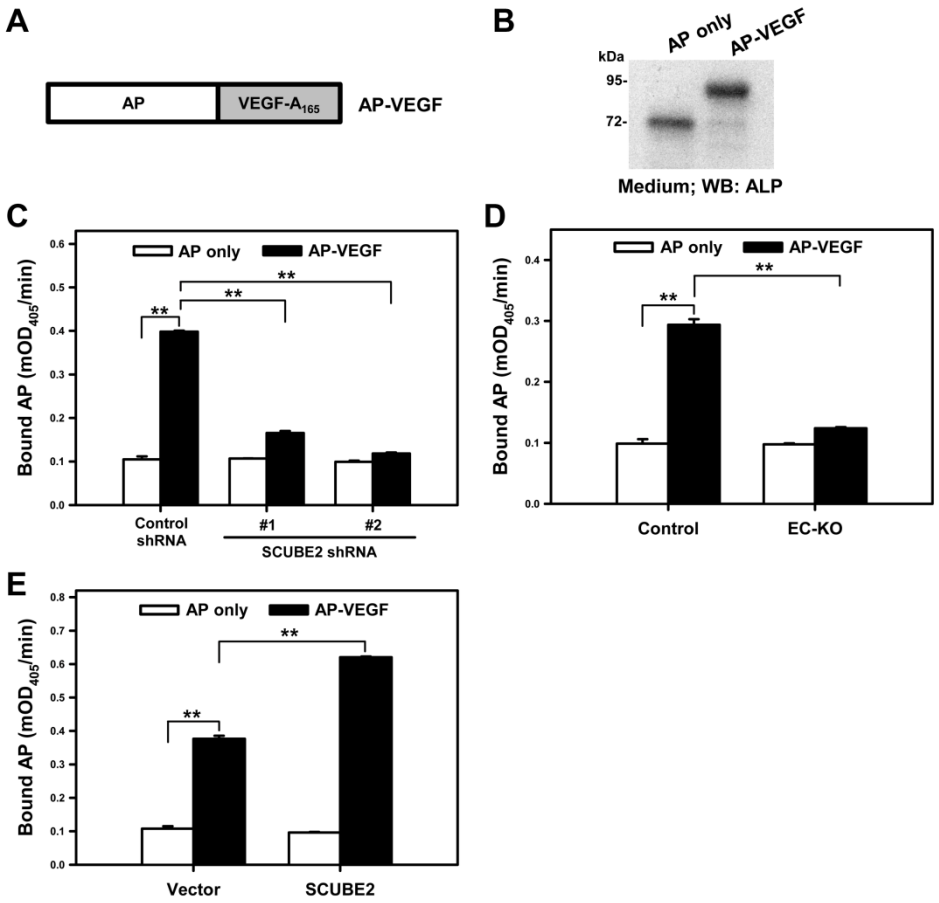
Supplemental Figure III. SCUBE2 specifically interacts with VEGFR2 but not VEGFR1, Neuropilin-1 and EGFR in HUVECs. Anti-SCUBE2 immunoprecipitates were subjected to western blotting with anti-VEGFR1 (A), anti-Neurpilin-1 (B), anti-EGFR (C) or anti-VEGFR2 antibody that service as a positive control to confirm their protein-protein interactions (top panel). A reciprocal pull-down assay was always performed to verify these immunoprecipitation results (bottom panel).



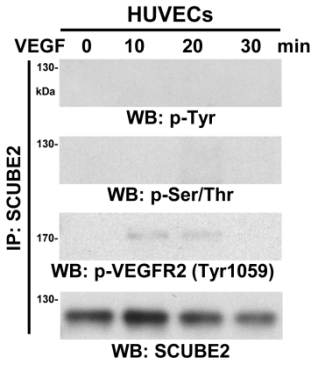
Supplemental Figure IV. VEGF-A_{165} (designated VEGF) directly interacts with the NH_2-terminal EGF repeats of SCUBE2. **A**, Domain organization of the SCUBE2 expression constructs used to map the interacting domains. FLAG epitope was added immediately after the signal peptide sequence at the NH_2-terminus of SCUBE2 constructs. SP, signal peptide; CR, cysteine-rich. **B**, Recombinant VEGF protein was mixed with recombinant FLAG-tagged SCUBE2-FL and its domain-specific protein fragments bound to anti-FLAG M2 antibody-agarose beads or control beads for incubation for 4 h at 4°C, then beads were washed extensively and interaction was visualized by western blot analysis with anti-VEGF antibody. IP, immunoprecipitation; WB, western blotting.



Supplemental Figure V. The interaction between SCUBE protein family and VEGF or VEGFR2. **A**, SCUBE2 can specifically interact with VEGF. Recombinant VEGF (VEGF-A_{165}) protein was mixed with recombinant FLAG-tagged SCUBE1-3 and its proteins bound to anti-FLAG M2 antibody-agarose beads for incubation for 4 h at 4°C, then beads were washed extensively and interaction was visualized by western blot analysis with anti-VEGF antibody. **B**, SCUBE1-3 can interact with VEGFR2. The expression plasmids encoding Myc-tagged VEGFR2 were transfected alone or together with FLAG-tagged SCUBE1-3 constructs in HEK-293T cells for 2 days, then cell lysates underwent immunoprecipitation, followed by western blot analysis with indicated antibodies to determine the protein–protein interactions. IP, immunoprecipitation; WB, western blotting.



Supplemental Figure VI. SCUBE2 modulates the binding of VEGF on ECs that endogenously express VEGFR2. **A** and **B**, Production of an alkaline phosphatase fusion of VEGF-A_{165} (AP-VEGF). Schematic diagram of the AP-VEGF construct (A). Western blot analysis of AP-VEGF expression in conditioned media from HEK-293T cells (B). **C-E**, SCUBE2 potentiates VEGF binding to VEGFR2 in ECs. Binding of AP-VEGF and control AP protein on ECs endogenously expressing VEGFR2. Effect of SCUBE2 knockdown (C), SCUBE2 knockout (D), and SCUBE2 overexpression (E) on AP-VEGF binding compared with corresponding control ECs. Data are means ± SD from 3 independent experiments. **, $P < 0.01$.



Supplemental Figure VII. Effect of VEGF treatment on the phosphorylation status of SCUBE2 in HUVECs. HUVECs treated with VEGF (100 ng/ml) for 10, 20 or 30 min were lysed and immunoprecipitated with anti-SCUBE2 antibody. Anti-SCUBE2 immunoprecipitates were blotted with anti-phosphotyrosine (p-Tyr) or anti-phosphoserine/threonine (p-Ser/Thr) pan-specific antibody, respectively. Note that Tyr1059-phosphorylated VEGFR2 was pulled down and served as a positive control. IP, immunoprecipitation; WB, western blotting.

Online Table I. Oligonucleotide primers used in this study

Gene*	Forward	Reverse	Assay
SCUBE1	TGCGGCGGCGAGCTTGGTGAC	TTTGGAGCGCAGCAGTTTGATGAA	RT-PCR
SCUBE2	TCTTGCCCAGGAAATACTACGACT	TGGGCCAGGACATCAAACAGAG	RT-PCR
SCUBE3	TGGCCCAATGCAAGAATCGTCAGT	TGGGCTAGCACCTCAAAGAAG	RT-PCR
GAPDH	GCCAAAAGGGTCATCATCTC	ACCACCTGGTGCTCAGTGTA	RT-PCR
Scube1	CGGCGGCGAACTTGGTGACTACA	TTGATAAAGGACCGGGGGAACAT	RT-PCR
Scube2	TGACTACCTGGTGATGCGGAAAAC	CAGTGGCGTGTGGGAAGAGTCA	RT-PCR
Scube3	TGCTCCCCGGGCCACTACTAT	AGCGCTGTTGGCCTCACTGGTCTT	RT-PCR
SCUBE2	GCACACGCACGCGCGCACACA	GAAGGGTGCAGAGGGTGTGCT	ChIP
Gapdh	ATCATCCCTGCATCCACTGGTGCTG	TGATGGCATTCAAGAGAGTAGGGAG	RT-PCR
SCUBE1	AACATCCCGGGGAACTACAG	GCAGCCACCATTATTGTCCT	Q-PCR
SCUBE2	CAGGCAGAGTCCTGTGGAGT	TAAAATGCAGCGTTCTCGTG	Q-PCR
SCUBE3	CCTGCTTGTCCTGCTGGT	TCGATGTGGCAGTTGTCAGT	Q-PCR
GAPDH	TGAAGGTCGGAGTCAACGG	AGAGTTAAAAGCAGCCCTGGTG	Q-PCR
Scube2-5'Flox (F1, R1)	ATAGTCGACTGTTGTCCAGTATCTGTTGC	ATAGCGGCCGCTACGACACCCTGGGATAAAG	Genotyping
Scube2-3'Flox (F2, R2)	GGCCATGTCCCTGAAGAAAACTA	TTATGGGGCCAAGACACTCAAA	Genotyping
Scube2-Null (F3, R3)	CTGGGGCCTCTGGGACACTATT	GTTATGGGGCCAAGACACTCAAA	Genotyping
Cre	TTACCGGTCGATGCAACGAGTGATG	GTGAAACAGCATTGCTGTCACTT	Genotyping

**SCUBE1-3* and *GAPDH* are human genes; *Scube1-3* and *Gapdh* are mouse genes.