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Identification of an angiogenic factor that when mutated causes susceptibility to Klippel–Trenaunay syndrome

Xiao-Li Tian[1,*], **Rajkumar Kadaba**[1,*], **Sun-Ah You**[1,*], **Mugen Liu**[1,2,*], **Ayse Anil Timur**[1], **Lin Yang**[3], **Qiuyun Chen**[4], **Przemyslaw Szafranski**[5], **Shaoqi Rao**[1], **Ling Wu**[1], **David E. Housman**[6], **Paul E. DiCorleto**[3], **David J. Driscoll**[7], **Julian Borrow**[6,†], and **Qing Wang**[1]

1 *Center for Molecular Genetics, Department of Molecular Cardiology, Lerner Research Institute, and Center for Cardiovascular Genetics, Department of Cardiovascular Medicine, The Cleveland Clinic Foundation, and Department of Molecular Medicine, Cleveland Clinic Lerner College of Medicine of Case Western Reserve University, Cleveland, Ohio 44195, USA*

2 *Institute of Genetics, Fudan University, Shanghai 200433, China*

3 *Department of Cell Biology, and*

4 *Cole Eye Institute, The Cleveland Clinic Foundation, Cleveland, Ohio 44195, USA*

5 *Department of Pathology, Baylor College of Medicine, Houston, Texas 77030, USA*

6 *Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA*

7 *Division of Pediatric Cardiology, Mayo Clinic, Rochester, Minnesota 55905, USA*

Abstract

Angiogenic factors are critical to the initiation of angiogenesis and maintenance of the vascular network[1]. Here we use human genetics as an approach to identify an angiogenic factor, *VG5Q*, and further define two genetic defects of *VG5Q* in patients with the vascular disease Klippel–Trenaunay syndrome (KTS)[2,3]. One mutation is chromosomal translocation t(5;11), which increases *VG5Q* transcription. The second is mutation E133K identified in five KTS patients, but not in 200 matched controls. VG5Q protein acts as a potent angiogenic factor in promoting angiogenesis, and suppression of VG5Q expression inhibits vessel formation. E133K is a functional mutation that substantially enhances the angiogenic effect of VG5Q. VG5Q shows strong expression in blood vessels and is secreted as vessel formation is initiated. VG5Q can bind to endothelial cells and promote cell proliferation, suggesting that it may act in an autocrine fashion. We also demonstrate a direct interaction of VG5Q with another secreted angiogenic factor, TWEAK (also known as TNFSF12) [4,5]. These results define VG5Q as an angiogenic factor, establish *VG5Q* as a susceptibility gene for KTS, and show that increased angiogenesis is a molecular pathogenic mechanism of KTS.

Angiogenesis has an essential role in pathological conditions such as cancer and various ischaemic and inflammatory diseases[1,6–9]. KTS (OMIM number 149000) is a congenital vascular disease characterized by malformations of capillary (98% of KTS patients), venous

Correspondence and requests for materials should be addressed to Q.W. (wangq2@ccf.org). The GenBank accession numbers are AY500994 for human VG5Q (hVG5Q) mRNA and amino acid sequences; AY500995 for mouse VG5Q (mVG5Q) mRNA and amino acid sequences; and AY500996 for human VG5Q genomic DNA sequence..

*These authors contributed equally to this work

†Present address: Cancer Research UK, Leukemia Molecular Genetics, Paterson Institute for Cancer Research, Manchester, M20 4BX, UK

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(72%) and lymphatic (11%) vessels, and bony and soft tissue hypertrophy (67%)[2,3]. KTS is commonly sporadic and its aetiology is unknown. To identify a susceptibility gene for KTS, we characterized translocation t(5;11)(q13.3;p15.1), which is associated with KTS[10]. Polymerase chain reaction (PCR) analysis of somatic cell hybrids containing only the derivative chromosome 5 or the derivative chromosome 11 defined the precise locations of two translocation breakpoints (Fig. 1a; see also Supplementary Fig. 1). A novel gene, *VG5Q*, was identified at the 5q13.3 translocation breakpoint. The 5q13.3 translocation breakpoint is located in the promoter of *VG5Q* (Supplementary Fig. 2), and significantly affects the expression of *VG5Q* (see below). The full-length *VG5Q* complementary DNA (4,049 base pairs (bp)) contains a long open reading frame that spans 2,145 bp and encodes a novel protein of 714 amino acids with a forkhead-associated (FHA) domain[11] and a G-patch domain[12] (amino acids 435–508 and 619–663, respectively; Fig. 1b). No genes were identified in the regions 50 kilobases (kb) on either side of the chromosome 11p15.1 breakpoint (Supplementary Fig. 1b).

To provide further evidence that *VG5Q* is a susceptibility gene for KTS, we performed a case-control study with 130 unrelated KTS cases and 200 unrelated matched controls. Genetic case-control association studies are a powerful alternative to family-based designs as a method of choice for identifying susceptibility genes for complex diseases as well as sporadic disorders such as KTS[13]. Explicit attempts were made to match the cases with controls for age, gender and ethnicity. Genotyping with population-specific markers[13–15] was used to confirm that our cases matched controls. Allelic frequencies of the markers genotyped, including a single-nucleotide polymorphism P698T in VG5Q, are statistically similar in cases and controls ($P > 0.05$), and they all resemble the frequencies of the Caucasian population (Supplementary Table 1). Mutation analysis of *VG5Q* using the primers listed in Supplementary Table 2 revealed a non-conservative mutation E133K in five independent KTS patients, which resulted in substitution of a negatively charged glutamine residue by a positively charged lysine residue (Fig. 1c, d). All five patients are heterozygous for mutation E133K. Identification of VG5Q mutation E133K in five independent KTS patients suggests that E133 is a mutational hotspot. As E133K occurs within the context of a CpG dimer, it is expected to be a potential hotspot for mutations[16]. Mutation E133Kwas not detected in 200 matched controls. A statistically significant association was thus established between E133K of VG5Q and KTS ($P = 0.009$). This conclusion was also validated using a new genetic-based association study, Genomic Control (estimated λ by mean = max (0.66, 1))[14]. These results suggest that *VG5Q* is a susceptibility gene for KTS and implicate VG5Q in the pathogenesis of the disorder.

VG5Q was clearly detected in cells relevant to KTS, including endothelial cells, vascular smooth muscle cells (VSMCs) and osteoblasts (MG-63) (Fig. 2A–D). Northern blot analysis revealed a single 4.5-kb transcript in human umbilical vein endothelial cells (HUVECs) and human microvascular endothelial cells (HMVECs) (Fig. 2A). Western blot analysis with an anti-VG5Q antibody recognized a predicted 84-kDa protein present in HMVECs and HUVECs (Fig. 2B). VG5Q was also expressed in human heart fibroblast (HHF) and ovarian cancer cells (OV-3), but low expression was detected in kidney cancer cells (RP-45), HeLa cells, and bladder cancer cells (T-24) (Fig. 2D). *VG5Q* was ubiquitously expressed in the human tissues examined (Fig. 2C). Co-immunostaining showed that VG5Q protein co-localized with an endothelial-specific marker, CD31, as well as a VSMC-specific marker, smooth muscle cell α-actin, in blood vessels embedded in various mouse tissues including heart, kidney, tail and limb (Fig. 2E; see also Supplementary Fig. 3).

VG5Q protein was detected mostly in the cytoplasm and around the nuclei of HMVECs (Fig. 2F). In an *in vitro* model of angiogenesis where endothelial cells were plated onto matrigel, VG5Q protein began to redistribute by moving towards the cell periphery and was also detected outside the cell (Fig. 2F, d, f, h). The dynamic re-distribution of VG5Q at this stage resembles

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the secretion pattern of other secreted proteins[17]. These results suggest that VG5Q may be secreted when endothelial cell tube formation is initiated. To confirm that VG5Q is secreted during angiogenesis, a competitive enzyme-linked immunosorbent assay (ELISA) was carried out. Purified VG5Q results in reduced absorbance in the competitive ELISA assay (Fig. 2G). Similarly, the media from matrigel culture also led to significantly reduced absorbance in the competitive ELISA assay compared with the media from plastic culture and blank media (media from matrigel-coated plates without cells) ($P = 0.009$), indicating that the matrigel culture media contained secreted VG5Q. Metabolic labelling with [^{35}S]methionine/[^{35}S] cysteine revealed the presence of VG5Q in the matrigel media of HUVECs transfected with a VG5Q expression construct, but not with vector control (Fig. 2H). These results indicate that angiogenesis accompanies secretion of VG5Q protein. The molecular mechanisms for trafficking and secretion of VG5Q remain to be established; however, it may be released via a non-classical secretory pathway similar to the angiogenic factor FGF-2 (ref. 18).

VG5Q functions as an angiogenic factor. Similar to VEGF, purified wild-type VG5Q protein promoted strong angiogenesis in a chick chorioallantoic membrane (CAM) assay, as shown by the newly formed radiated vessels on the CAM with VG5Q (Fig. 3A). These results suggest that VG5Q is a potent angiogenic factor. Consistent with this finding, using the RNA interference technique, short interfering RNAs (siRNAs) against VG5Q reduced VG5Q expression at both messenger RNA and protein levels, and inhibited tube formation by HMVECs and HUVECs that were plated onto matrigel (Fig. 3B). Similar findings were observed with an antisense oligonucleotide specific to VG5Q (5′-ATCACAAAAATAGTCCCC-3′).

Purified VG5Q was able to bind to cultured endothelial cells (Fig. 3C) in calcein AM-based cell adhesion assays. Significantly higher calcein fluorescence (cells bound) was detected with VG5Q protein than control bovine serum albumin (BSA) (Fig. 3C). As VG5Q binds to the surface of endothelial cells and triggers endothelial cell proliferation (see below), it may act on endothelial cells in an autocrine fashion. However, a paracrine action of VG5Q cannot be ruled out as it is also expressed in VSMCs. It remains to be determined whether VG5Q binds to endothelial cells through a receptor. If such a receptor exists it may be protein in nature, as trypsinized endothelial cells, in contrast with endothelial cells collected by cell dissociation buffer, no longer adhered to VG5Q.

Functional characterization of two KTS-associated genetic mutations—one translocation t (5:11) found in one patient and one missense mutation E133K in 5 of 130 patients—validated VG5Q as a susceptibility gene for KTS. First, *VG5Q* promoter with the translocation junction fragment showed threefold higher expression than the wild-type *VG5Q* promoter (Fig. 3D, construct (3) versus construct (2)). These data suggest that the t(5;11) KTS translocation is a functional genetic defect that can lead to over-expression of *VG5Q*, which may result in increased angiogenesis. Second, significant differences in angiogenesis were observed between wild-type and mutant VG5Q with the E133K substitution ($P < 0.05$) (Fig. 3A, j). Mutant VG5Q protein acted as a more potent angiogenic factor than the wild-type protein (wild type versus mutant: Fig. 3A, c versus d at a concentration of 37.5 ngμl^{-1}; e versus f at 75 ngμl^{-1}; g versus h at 150 ngμl^{-1}; summarized in panel j, $P < 0.05$). These results demonstrate that mutation E133K of VG5Q is a functional mutation that acts by a gain-of-function mechanism (increased angiogenesis). Mutation E133K is located within a putative phosphorylation site for glycogen synthase kinase 3 (I132-**E133**-T134-S135-I136-L137-N138-S139), and is only one amino-acid residue away from a putative phosphorylation site for protein kinase casein kinase 1 (S135-I136-L137-N138-S139-K140-D141-H142). The change of the phosphorylation state of the endothelial-cell-specific receptor tyrosine kinase TIE2 can cause vascular venous malformations (one of the clinical features manifested in KTS). So far only two mutations have been identified in TIE2, and both show ligand-independent

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hyperphosphorylation and result in constitutively active TIE2 (ref. 19). Thus, we speculate that mutation E133K may affect the phosphorylation state of VG5Q, resulting in increased angiogenesis. Histological analysis of subcutaneous veins in 33 KTS patients revealed an increase in the number of small venules (capillary veins) in affected KTS tissues[20]. Our 'increased angiogenesis' theory may explain the histological features of KTS. Increased angiogenesis will lead to an increased number of blood vessels, as observed in affected tissues from KTS patients.

To elucidate the molecular mechanism by which VG5Q promotes angiogenesis, we isolated protein factors that associate with VG5Q using the yeast two-hybrid system with VG5Q as the 'bait'. Sequence analysis showed that one isolated cDNA encoded the carboxy-terminal domain of TWEAK (amino acid residues 136–249), a member of the tumour-necrosis factor (TNF) superfamily that induces angiogenesis *in vivo*[21]. The direct physical interaction between VG5Q and TWEAK was demonstrated using glutathione *S*-transferase (GST) pull-down assays with GST–TWEAK protein and *in-vitro*-translated ^{35}S-labelled VG5Q (Fig. 4a). In co-immunoprecipitation assays, the anti-VG5Q antibody specifically precipitated a protein recognized by an anti-TWEAK antibody, validating the interaction between VG5Q and TWEAK *in vivo* (Fig. 4b). Co-immunostaining showed co-localization of the two proteins around the nuclei in HUVECs cultured on plastic dishes (Fig. 4c). In HUVECs initiating endothelial tube formation on matrigel, VG5Q and TWEAK moved to the cell surface (Fig. 4c). Together, these results suggest that VG5Q may promote angiogenesis by interacting with TWEAK.

TWEAK binds to its receptor, fibroblast-growth-factor-inducible 14 (Fn14), as a homotrimer, and it promotes angiogenesis *in vivo*[21] as potently as VEGF and FGF-2, two well-known angiogenic factors[1,18]. TWEAK treatment has been shown to promote cell proliferation and migration of HUVECs[5]. We demonstrated that VG5Q also induces proliferation of HUVECs (thymidine uptake: 680 ± 29 for wild-type VG5Q compared with 524 ± 14 for control (no VG5Q); $P = 0.007$, $n = 8$). The proliferation of HUVECs was also observed for mutant VG5Q with mutation E133K (thymidine uptake: 711 ± 37 compared with control (524 ± 14); $P = 0.001$, $n = 8$), but was not significantly different from wild-type VG5Q ($P > 0.05$, $n = 8$). As angiogenesis is a complex process involving endothelial cell protease secretion, proliferation, migration, adhesion and survival, a major effect of mutation E133K may be more prominent in processes other than proliferation.

KTS is genetically heterogeneous, and other KTS genes remain to be identified. We reported one *de novo* translocation, t(8;14)(q22.3;q13), associated with KTS[22], and our mutation screening failed to identify a VG5Q mutation in the patient with this translocation. Molecular characterization of the t(8:14) translocation may lead to the identification of a new susceptibility gene for KTS. KTS is also expected to be phenotypically heterogeneous. Some KTS patients exhibit an absence of venous valves, hypoplasia of the veins, and complete absence of the deep venous system. Mutations in other KTS genes may account for such phenotypic variations.

Previously, a hypothesis of paradominant inheritance (autosomal lethal genes surviving only in a mosaic state) was proposed to explain the genetic basis of KTS and other syndromes that are characterized by sporadic occurrence and a mosaic distribution of lesions[23]. On the basis of this model, patients with the VG5Q E133K mutation may carry a second mutational hit in *VG5Q* or another gene within the affected tissues. Alternatively, the sporadic nature of KTS may be explained by a model of autosomal-dominant inheritance with incomplete penetrance. Further studies are needed to distinguish between these hypotheses in the future when affected KTS tissues are available for molecular genetic analysis. Future studies will also determine

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whether VG5Q is involved in other pathological conditions associated with abnormal angiogenesis (tumour, ischaemia, inflammation).

As purified VG5Q promotes angiogenesis and suppression of VG5Q expression inhibits vessel formation, this work might have potentially important clinical therapeutic implications. VG5Q may be a target for angiogenic therapies (stimulating new blood vessel growth) or anti-angiogenic therapies (halting new blood vessel growth) in treatment of angiogenesis-dependent disorders such as ischaemic heart disease and cancer.

Methods

Construction of somatic cell hybrids

We constructed and analysed somatic cell hybrids derived from the blood sample of the t(5;11) (q13.3;p15.1) translocation patient as previously described[24].

Mutation analysis

Informed consent was obtained from the participants in accordance with the standards established by local institutional review boards. Genomic DNA was prepared from whole blood with the DNA Isolation Kit for Mammalian Blood (Roche Diagnostic). Single-strand conformation polymorphisms and DNA sequence analyses were carried out as described previously[25–27].

Western blot analysis

A polyclonal antibody against human/mouse VG5Q was developed using a synthetic polypeptide (LAQLRRKVEKLERELRSC) as the immunogen (Biosource International). The immunogen sequence corresponds to a unique portion of the amino terminus of VG5Q and did not match any other sequences in the databases, suggesting specificity of the VG5Q antibody. Western blot analysis was performed as described[28,29].

Expression and purification of human VG5Q protein

The full-length wild-type VG5Q cDNA was cloned into a bacterial expression vector pET-28b (Novagen), resulting in expression construct pET-28VG5Q-wt for 6 × His-tagged VG5Q. The VG5Q mutation E133K was introduced into pET-28VG5Q-wt using PCR-based site-directed mutagenesis, resulting in pET-28VG5Q-mt. The expression constructs were transformed into *Escherichia coli* BL21(DE3) Star, and 6 × His–VG5Q protein was purified using a Ni-NTA agarose column according to the manufacturer’s instructions (Qiagen). The eluted protein was dialysed, and quality of purification was examined by SDS–polyacrylamide gel electrophoresis (PAGE) and western blot analysis.

Chick CAM assay

Fertilized chicken eggs were incubated at 37 °C for 4 days and then opened. The embryos were transferred into Petri dishes (100 mm diameter) and cultured at 37 °C for an additional 4 days to allow the development of CAM. Then, round-glass cellulose discs (3 mm diameter) soaked with either human recombinant VEGF (100 $ng\mu l^{-1}$; Sigma, catalogue number V 7259) or with different concentrations of purified wild-type or mutant VG5Q (37.5 $ng\mu l^{-1}$, 75 $ng\mu l^{-1}$ and 150 $ng\mu l^{-1}$) were placed on the CAMs. The control discs were soaked with the buffer that was used for dialysis and dissolving of VG5Q protein (50 mM Tris-HCl, 150 mM NaCl and 2 mM $MgCl_2$, pH 7.4) or BSA (100 $ng\mu l^{-1}$). On the fifth day after the attachment of discs, the newly formed vessels were examined and visualized with a photomicroscope (Leica MZFLIII) and Spot Advanced software (Diagnostic Instruments). The number of newly formed vessels in the CAM was quantified with ImagePro Plus software (Media Cybernetics).

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Competitive ELISA analysis

HUVECs were plated on Lab-Tek II chamber slides coated with or without matrigel (*in vitro* angiogenesis) for 4 h. The media were collected, incubated for 30 min with an optimum concentration (200 ng ml^{-1}) of anti-VG5Q antibody (determined experimentally with antigen), and transferred to wells coated with peptide immunogen (1μg ml^{-1}, 6 replicates). The bound antibody was detected by secondary horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG, chromogenic reaction, and absorbance reading of the wells. The negative controls include media only and PBS buffer. Positive control is the purified VG5Q protein. Blank media (media from matrigel-coated plates without cells) did not significantly reduce the HRP absorbance ($P = 0.24$) (Fig. 2G), indicating that decreases in absorbance with media from cells from matrigel culture are specific to VG5Q and are not due to the release of inhibitors from matrigel. The purified VG5Q did not give better inhibition in the ELISA assay because anti-VG5Q antibody was saturated.

Metabolic labelling

HUVECs were transiently transfected using HUVEC Nucleofector Kit (Amaxa Biosystems) with pcDNA3.1-VG5Q or vector pcDNA3.1, and metabolic labelling was carried out using Tran^{35}S-label (ICN Biochemicals) as described[30], but with modifications (see Supplementary Methods). Media from ^{35}S-labelled HUVEC transfectants were precipitated with an anti-VG5Q antibody, and precipitates were analysed by SDS–PAGE.

RNA interference

siRNA was selected 75 bases downstream from the start codon. The selected sequences were searched against the NCBI database to ensure that they were unique to *VG5Q*. The sequences for the two selected siRNAs are: 5′ -AAUUGUCAUUAGAUCACCUGU-3′ (siRNA1) and 5′ -AAGAACAAAAAAAACUGGGAC-3′ (siRNA2). The scrambled siRNA sequence is 5′ -GCGCGCUUUGUAGGAUUCG-3′. The siRNA was synthesized by Dharmacon Research.

siRNA (1.6 nmol) was introduced into endothelial cells by transfection with oligofectamine (Invitrogen). Forty-eight hours after transfection, the adherent cells were collected by trypsinization. The cells were plated at a density of 0.2 million cells cm^{-2} on Lab-Tek II chamber slides (Nalge Nunc International) coated with matrigel. Endothelial cell tube formation was examined 24 h later.

Cell adhesion assays

HUVECs were labelled with calcein AM (Molecular Probes), and incubated with 96-well plates coated with 2μg ml^{-1} of purified VG5Q or BSA (control). The unbound cells were removed by aspiration. Wells were washed with PBS, and read in a CytoFluor II Fluorescence Reader to measure fluorescence of adhering cells (see Supplementary Methods).

Protein–protein interactions

The cDNA segment encoding the extracellular domain of TWEAK (amino acids 93–249) was subcloned into pGEX-4T-1 to create the GST–TWEAK fusion protein. *In vitro* protein–protein interactions were carried out with pull-down assays using the GST–TWEAK fusion protein and *in-vitro*-translated, ^{35}S-labelled VG5Q produced in rabbit reticulocyte lysates using the TNT-coupled reticulocyte system (Promega). Co-immunoprecipitation was performed as described[28].

Cell proliferation assays

Endothelial cell proliferation assays were carried out with purified VG5Q using the [^{3}H] thymidine-uptake method (see Supplementary Methods).

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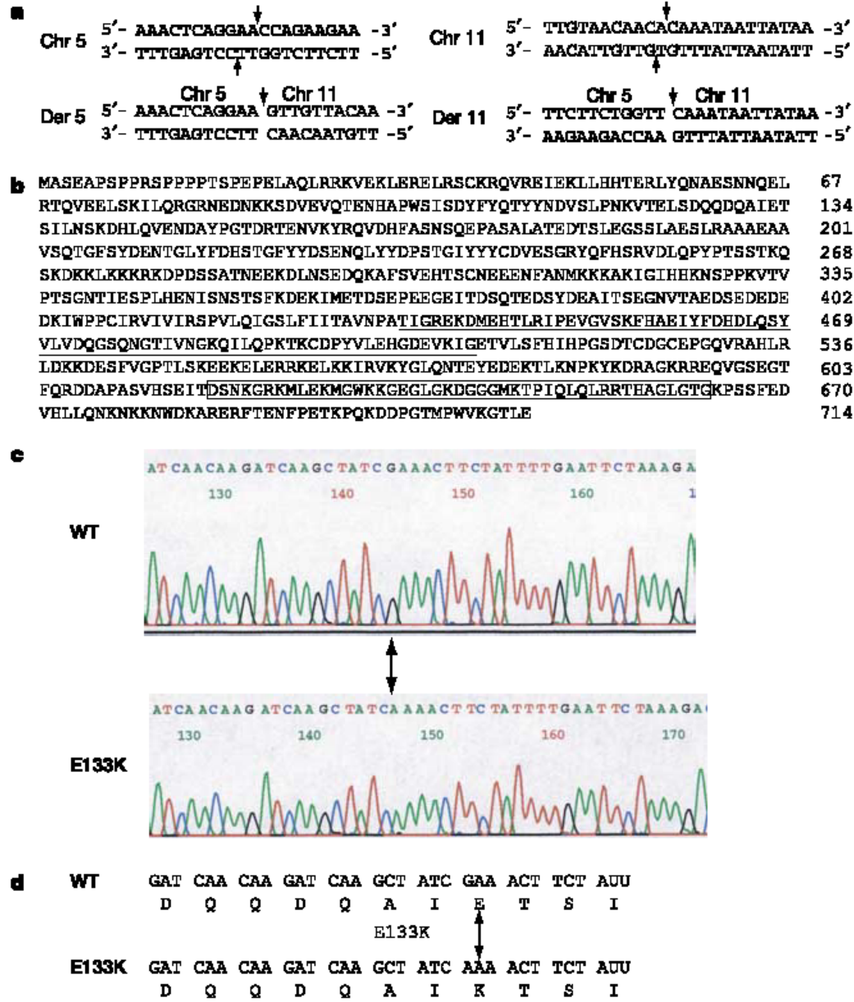

Figure 1.

Positional cloning of the *VG5Q* gene. **a**, Definition of chromosome breakpoints involved in translocation t(5;11)(q13.3;q15.1) associated with KTS. Chr 5/Chr 11, normal chromosomes; Der 5/Der 11, derivative chromosomes. **b**, The amino acid sequences of human *VG5Q* protein. The FHA domain (435–508) and a G-patch domain (619–663) are indicated. The estimated pI of VG5Q is 5.2. **c**, **d**, Identification of VG5Q mutation E133K in five independent KTS patients. **c**, DNA sequence analysis (G to A substitution at codon 133). **d**, Schematic representation of mutation E113K (substitution of a glutamic acid residue by a lysine residue).

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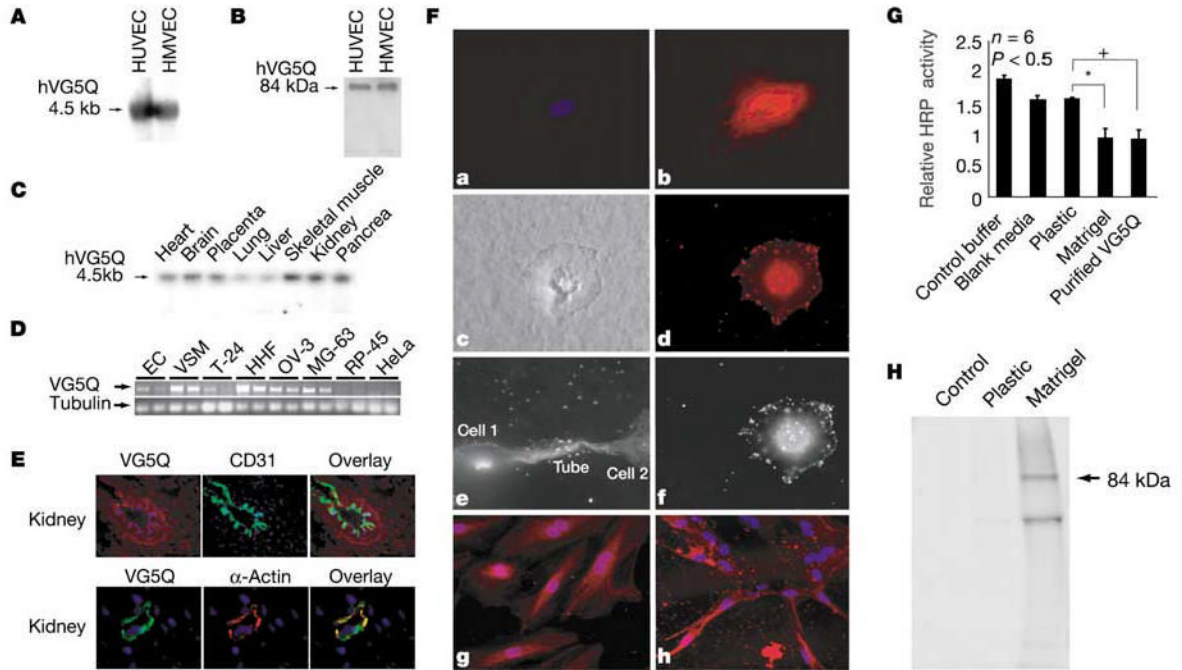

Figure 2.

Expression profile of VG5Q and dynamic redistribution and secretion of VG5Q protein during angiogenesis. **A**, **B**, VG5Q expression in HMVECs and HUVECs determined by northern (**A**) and western blot analyses (**B**). **C**, Tissue expression pattern of *VG5Q* determined by northern blot analysis. **D**, Expression of VG5Q in different cell lines determined by RT–PCR. Tubulin, internal control. **E**, Immunostaining of mouse kidney for VG5Q protein expression. The nucleus was stained with DAPI (blue signal). CD31, endothelial cell marker; α-actin, VSMC marker. **F**, HMVECs were cultured on plastic (**a**, **b**, **g**) and matrigel (**c**–**f**, **h**). VG5Q protein, red; nucleus, blue (DAPI). **c**, **d**, **f**, One hour on matrigel; **e**, 4 h on matrigel; **h**, 24 h on matrigel. **G**, Competitive ELISA analysis to show that VG5Q is secreted. Relative HRP activity, absorbance reading of the wells. An asterisk indicates statistical significance ($P < 0.05$). **H**, Detection of VG5Q in the media by metabolic labelling and immunoprecipitation, indicating that cells secrete VG5Q into the media. Control, HUVECs transfected with pcDNA3.1; plastic and matrigel, HUVECs transfected with pcDNA3.1-VG5Q and plated on either plastic or matrigel plates, respectively. The 84-kDa band indicated by an arrow represents VG5Q. The nature of the small 45-kDa band is unknown, but may be a cleaved VG5Q product. Faint signal was also observed in the media from cells cultured on plastic dishes. This may suggest weak secretion of VG5Q under this condition.

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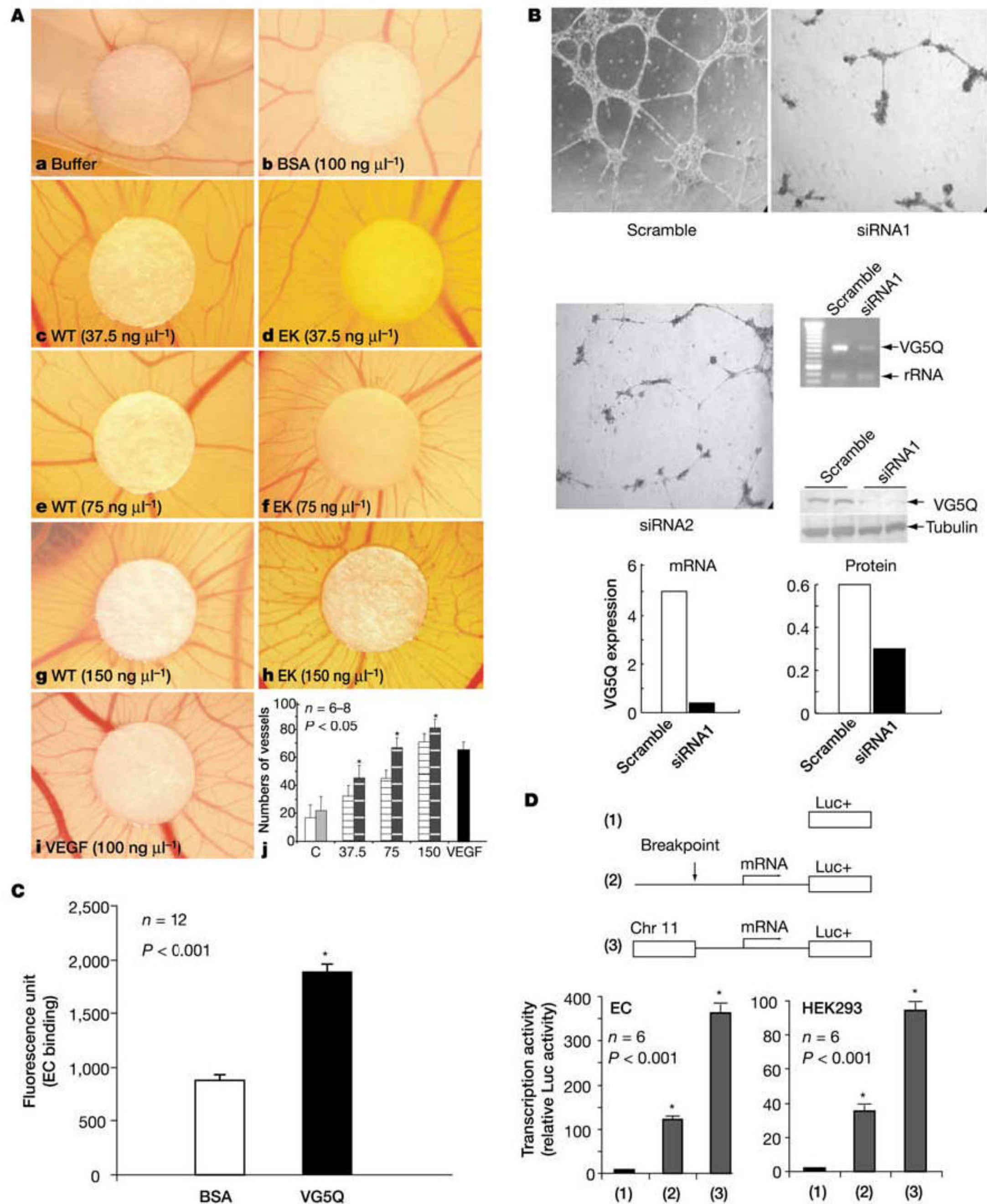

Figure 3.

VG5Q is an angiogenic factor and both VG5Q E133K and KTS translocation t(5;11) are functional mutations. **A**, Angiogenesis promoted by VG5Q protein was determined by CAM assays. EK, VG5Q with mutation E133K. **B**, Effect of siRNA against VG5Q on endothelial tube formation. Scramble, control siRNA. Suppression of VG5Q expression by siRNAs was determined by RT–PCR (ribosomal RNA as control) and western blot analyses (tubulin as control). **C**, Binding of VG5Q to endothelial cells by cell adhesion assays. **D**, The t(5;11) (q13.3;p15.1) translocation associated with KTS increases expression of VG5Q. Results represent mean of triplicate cultures ± standard deviation from three independent experiments. EC, endothelial cell.

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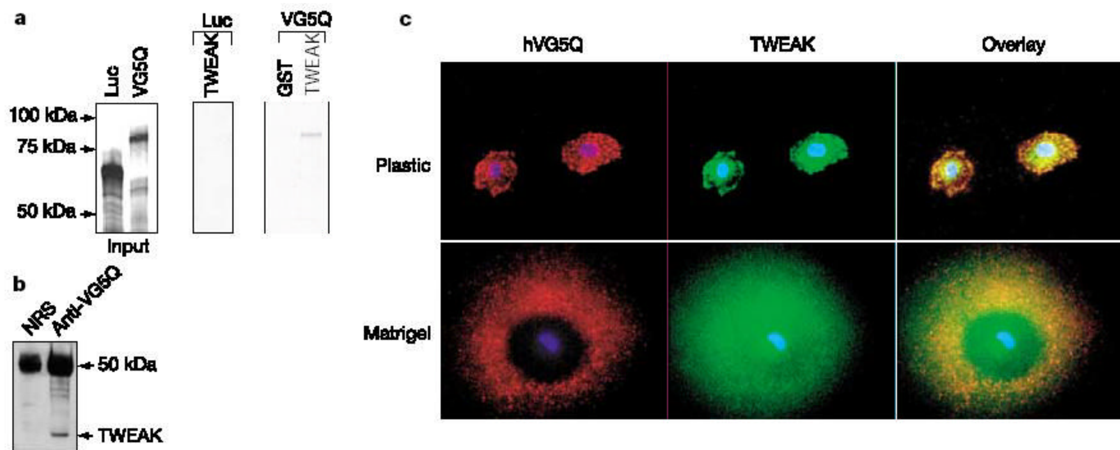

Figure 4.

VG5Q interacts with TWEAK. **a**, Pull-down assays using GST–TWEAK. Input, ^{35}S-labelled luciferase (Luc, about 61 kDa) and VG5Q (about 84 kDa). Right panel, binding of VG5Q with GST–TWEAK, but not with GST; middle panel, no interaction between GST–TWEAK with luciferase. **b**, Co-immunoprecipitation of TWEAK with VG5Q from HVSMC protein extract by a rabbit anti-VG5Q antibody. Bound materials were analysed by western blot using a goat anti-TWEAK antibody. NRS, normal rabbit serum as a negative control. 50-kDa band, IgG cross-reaction. **c**, Co-localization of VG5Q and TWEAK in HUVECs. VG5Q, red signal; TWEAK, green; nuclei, blue (DAPI).