ECM2, An Endothelial Specific Filamin A Binding Protein That Mediates Chemotaxis


Objective—We aimed to characterize the expression and function of a novel transcript that bioinformatics analysis predicted to be endothelial specific, called endothelial-specific molecule-2 (ECM2).

Methods and Results—A full-length cDNA was isolated and predicted ECM2 to be a putative 205–amino acid transmembrane protein that bears no homology to any known protein. Quantitative polymerase chain reaction analysis in vitro and in situ hybridization analysis in vivo confirmed ECM2 expression to be exclusively endothelial, and localization to the plasma membrane was shown. Knockdown of ECM2 expression in human umbilical vein endothelial cells using siRNA resulted in both reduced chemotaxis and impaired tube formation on matrigel, a solubilized basement membrane, both processes involved in angiogenesis. A yeast 2 hybrid analysis using the ECM2 intracellular domain identified filamin A as an interacting protein. This interaction was confirmed by precipitation of filamin-A from endothelial cell lysates by a GST-tagged intracellular domain of ECM2.

Conclusion—This study is the first to characterize a novel cell surface protein ECM2 that regulates endothelial chemotaxis and tube formation, and interacts with filamin A. These studies implicate a role for ECM2 in angiogenesis via modulation of the actin cytoskeleton. (Arterioscler Thromb Vasc Biol. 2008;28:1640-1646)

Key Words: endothelial genes • transmembrane proteins • filamin • cell signaling • chemotaxis • angiogenesis
SMART RACE cDNA Amplification kit (BD Biosciences). The ECSM2 transcript was amplified by PCR on total human umbilical vein endothelial cell (HUVEC) cDNA using the upstream primer 5'-tacctggagagcacagcctaca-3' designed to include the XhoI site and the downstream primer 5'-tacctggagagcacatcctg-3' designed to include the SacII site and inserted into the pBluescript vector (Stratagene).

Quantitative PCR
cDNA was prepared using total RNA and the random priming High-Capacity cDNA Archive kit (Applied Biosystems). For the larger cell line screen, standard curve analysis was performed to obtain relative expression levels for ECSM2 and the housekeeping gene β-2-microglobulin to which ECSM2 expression was normalized. To prepare the primary cell type screen, the housekeeper genes flotillin-2, ubiquitin C, and β-actin were chosen using the method described by Vandesompele et al with the software geNorm.14 Data were analyzed using a method described by Pfaffl.15

In Situ Hybridization
In situ hybridization analysis was performed using radioactively labeled probes as described by Poulsom et al.4 In some cases slides were also stained with CD34 antibody using a method described by Jeffery et al.20 The ECSM2 transcript specific probe (nucleotides 15-980) was used to generate the in situ hybridization probe.

Subcellular Localization Studies
ECSM2 was tagged at the C terminus with either myc (pCDNA3.1 Myc-His) or GFP (pEGFP-N1). HUVEC were transfected with these or pEGFP-N1 using Transpass D2 transfection reagent (NEB) for 4 hours. Cells were used for assays at 48 hours after transfection, cells were either viewed live in PBS, or cells were fixed before analysis. Fixed cells were stained with either phalloidin (A12380 Alexa Fluor 568, Molecular probes) to visualize actin, or a yeast 2 hybrid screen using the ECSM2 intracellular domain (residues 120 to 147). ECSM2 was predicted to have no known homologues and contained no functional signal sequence and 27 amino acid transmembrane acids, and extensive database searches revealed that ECSM2 sequence did not match any database sequences other than the ECSM2 gene mapped to human chromosome 5 at position 5q31, and spans 10.3 kb.

The conceptual ECSM2 protein comprised 205 amino acids, and extensive database searches revealed that ECSM2 had no known homologues and contained no functional domains. Analysis of the predicted amino acid sequence revealed that ECSM2 contained a putative transmembrane domain (residues 120 to 147). ECSM2 was predicted to contain multiple O-linked glycosylation sites and a single N-linked glycosylation site at residue 96 by the presence of the universal acceptor sequence Asn-X-(Ser/Thr). Antisera was raised to the intracellular domain of ECSM2, and Western blotting using this revealed that endogenous protein comprised at least 3 bands ranging in size from 40 to 60 kDa (supplemental Figure I). The identification of the bands was confirmed by the observation that they were greatly reduced (supplemental Figure I). The identification of the bands was confirmed by the observation that they were greatly reduced (supplemental Figure I).

Results
Identification, Cloning, and Sequence Analysis of ECSM2
To obtain the full length ECSM2 transcript sequence, the sequence of an ECSM2 EST (B1823114) was used to design primers for 5’ and 3’ rapid amplification of cDNA ends (RACE). RACE was performed using HUVEC RNA. Sequencing of RACE experimental products revealed the ECSM2 transcript to be 1030 bp in length. Subsequent Northern blot analysis on human breast tissue RNA confirmed the RACE data with expression of a major transcript at approximately 1 kb (data not shown). Primers were subsequently designed to the 5’ and 3’ ends of the full length ECSM2 sequence, which enabled PCR cloning of the transcript from HUVEC cDNA. Translation of the entire cDNA sequence revealed ECSM2 to contain an open reading frame (ORF) of 618 bp with a 67 bp 5’ untranslated region (UTR) and 345 bp 3’ UTR. The complete ECSM2 nucleotide sequence did not match any database sequences other than ESTs (GenBank/EBI). Database searches and fluorescence in situ hybridization analysis (data not shown) revealed that the ECSM2 gene mapped to human chromosome 5 at position 5q31, and spans 10.3 kb.

The conceptual ECSM2 protein comprised 205 amino acids, and extensive database searches revealed that ECSM2 had no known homologues and contained no functional domains. Analysis of the predicted amino acid sequence revealed that ECSM2 contained a putative 24-amino acid signal sequence and 27 amino acid sequence transmembrane domain (residues 120 to 147). ECSM2 was predicted to contain multiple O-linked glycosylation sites and a single N-linked glycosylation site at residue 96 by the presence of the universal acceptor sequence Asn-X-(Ser/Thr). Antisera was raised to the intracellular domain of ECSM2, and Western blotting using this revealed that endogenous protein comprised at least 3 bands ranging in size from 40 to 60 kDa (supplemental Figure I). The identification of the bands was confirmed by the observation that they were greatly reduced as a result of ECSM2 specific siRNA mediated knockdown. The heterogeneous size of ECSM2, which is at least 20 to 40 kDa bigger than the size predicted from the amino acid sequence, strongly suggests that ECSM2 is highly glycosylated. ESTs from several other species showed homology to ECSM2 (Figure 1). Sequence alignment of the hypothetical proteins (Figure 1) showed extensive conservation of the
predicted intracellular domain of the ECSM2 protein, but much less in the extracellular domain. The figure shows that while the transmembrane and intracellular domains showed 75% similarity between zebrafish and man, conservation of the extracellular domain was poor, even within mammals.

Quantitative Analysis of ECSM2 Expression
Quantitative PCR analysis was performed on a selection of human cells to examine the pattern of ECSM2 expression. ECSM2 was expressed in all 4 endothelial cell types investigated but absent in nonendothelial cell lines or primary isolates (Figure 2). After endothelial cells, the highest expression was seen in aortic smooth muscle cells but was only 4% of that in HUVECs. There are 2 vascular smooth muscle cDNA libraries in the public databases, the HCASM2 and Sugana coronary artery smooth muscle cell libraries which between them encode 16,254 ESTs. A blast search of the ECSM2 nucleotide sequence (NM_001077693) against the 2 libraries identified no hits, and it is possible that the 4% signal detected in the real-time PCR analysis of the vascular smooth muscle cells could be attributable to trace contamination of the isolate with endothelium.

In Situ Hybridization Analysis of ECSM2 Expression
In situ hybridization studies of ECSM2 expression were performed to determine expression of ECSM2 in human tissues in vivo. Endothelial restricted expression of ECSM2 was observed in human breast carcinoma (Figure 3A through 3B), human ganglioglioma (Figure 3C through 3D), the skin in a psoriasis patient biopsy (Figure 3E through 3F), placenta (Figure 3G through 3H), and fetal tissue (Figure 3I through 3J). To confirm that expression was endothelial, human breast carcinoma sections were also immunostained for the endothelial specific marker CD34. Colocalization of the signals for ECSM2 and CD34 confirmed endothelial specific expression of ECSM2 in vivo (Figure 3K through 3L).

Subcellular Localization of ECSM2
To examine the cellular localization of ECSM2, the full-length protein was expressed with either myc or GFP fused at its C terminus and transfected into HUVECs. HUVECs were transfected with ECSM2-myc and immunofluorescence performed using antimyc antibodies. This showed that ECSM2 was localized to the membrane, and counterstaining with the nuclear marker 4',6-diamidino-2-phenylindole (DAPI) confirmed that the expression seen was at the plasma membrane.

Figure 1. A, Alignment of ECSM2 orthologs, the transmembrane and intracellular domains show greater conservation than the extracellular domain, conserved regions are highlighted in gray. The signal peptide region was predicted by Signal P software and 27 amino acid transmembrane domain was predicted by Dense Alignment Surface Analysis. More detailed legends for Figures 1 through 5 may be found in the supplemental materials.

Figure 2. Quantitative PCR analysis of ECSM2 expression by cell lines in vitro. A, ECSM2 expression in 4 endothelial (HUVEC, HDMEC, MEC, NEC) and 8 nonendothelial cell lines. B, The level of ECSM2 expression in endothelial human primary cell isolates (HUVEC, HDMEC) and 5 nonendothelial isolates.
and not the nuclear membrane. Examination of live cells expressing ECSM2-GFP showed localization at the cell surface and particularly on cellular protrusions such as filopodia (Figure 4B). ECSM2-GFP–expressing HUVECs were also fixed and stained with phalloidin to visualize F-actin, which is known to associate around the plasma membrane. Membrane expression of ECSM2 mostly colocalized with F-actin expression, particularly where many filopodia were present (Figure 4C).

Functional Studies With siRNA

ECSM2 expression was reduced to 20% of normal levels in HUVECs using 2 independent siRNA duplexes D1 and D2, compared to mock transfected or negative control duplex transfected cells (Figure 5A). Reduction of ECSM2 protein was confirmed by Western blotting (supplemental Figure I). Cells were then assayed in a scratch wound, chemotaxis and matrigel assays. Cells expressing reduced ECSM2 showed no change in their ability to move in a scratch wound assay (data not shown) but showed a severe defect in their ability to undergo chemotaxis in response to a gradient of fetal calf serum and endothelial growth supplements (Figure 5B), and an impairment in their ability to form tubes when plated on matrigel (Figure 5C and 5D). In the tube forming assay it was found that the cells with reduced ECSM2 formed a less stable and connected network. The matrigel assays were quantitated by counting the numbers of nodes containing 1, 2, 3, or 4 or more branch points (1 branch point indicating an unconnected end). In cells with knockdown of ECSM2, there was an increase in the nodes containing 1 or 2 branch points and a reduction in the nodes containing three or more branch points. This reduced complexity was more evident at 12 hours compared with 6 hours, suggesting that these cells are less able to form a stable network resulting in retraction of some of the connections and an increase in unconnected branch points. Introduction of siRNA into cells can give artifactual results attributable to induction of interferon (INF) secretion from the transfected cell (the so-called INF response). Real-time PCR quantitation of the INF sensitive genes (ISG20 and OAS1) showed no difference between transfectants and controls (data not shown) and confirmed that these duplexes do not induce the INF response at 50 nmol/L 48 hours after transfection. Finally, the duplexes are too small to bind to Toll3 and produce off target effects.22

Identification of Filamin A as an ECSM2 Binding Protein

To determine the mechanism by which ECSM2 was acting, proteins interacting with the intracellular domain of ECSM2 were identified by yeast 2 hybrid. The intracellular domain of ECSM2 (residues 147 to 205) was used as bait in a yeast 2 hybrid analysis of a human placental library. Three clones isolated encoded the C-terminal region of filamin A. The 3 clones encoded amino acids 1658 to 1846 and 2092 to 2310, which are in the 15 to 16 and 19 to 21 beta repeat sheets regions respectively. To confirm this interaction, the intracellular domain of ECSM2 fused to GST was used to pull down endogenous filamin A from a HUVEC lysate revealing a filamin A band at 280 kDa which was absent in the GST-fusion control (Figure 6).

Discussion

Endothelial cells are one of the most transcriptionally active cell types, as they are involved in many physiological processes and play a role in the development of diseases such as atherosclerosis and cancer. As a result, many laboratories seek to identify novel endothelial specific genes. There is a particular interest in the identification of membrane spanning proteins that respond to environmental signals at the cell surface and transduce that information to the cytoskeleton. In this study, we describe the identification and characterization of the novel transmembrane protein ECSM2, a previously...
uncharacterized filamin A binding protein involved in endothelial chemotaxis and tube formation in vitro.

We originally identified ECSM2 (Genbank DQ462572) by bioinformatics,9 and it was named Endothelial Cell Specific Gene-2 (ECSM2) because of its putative endothelial restricted expression but has otherwise remained a hypothetical protein. This work has confirmed the ECSM2 mRNA as a 1030-bp transcript and validated its highly restricted endothelial expression experimentally. Quantitative PCR analysis of primary cell isolates and cell lines cultured in vitro showed that ECSM2 was expressed only in endothelial cells (Figure 2). In situ hybridization analysis of ECSM2 expression in a range of human tissues showed that the ECSM2 transcript displayed highly restricted endothelial expression, with little or no expression observed in other cell types (Figure 3). Endothelial expression of ECSM2 was confirmed by immunostaining of adjacent sections for the endothelial marker CD34. Our findings are supported by the work of Ho et al, who reported that an EST now known to belong to ECSM2 was the most endothelial specific of 64 endothelial genes identified in a combined bioinformatics and microarray screen.3 Thus, ECSM2 shows potential as an endothelial marker, and the ECSM2 transcript has been used as a measure of endothelial cell contamination24 and differentiation.12

The ECSM2 gene is expressed from a 10.3-kb locus on human chromosome 5q31; it encodes a 205 amino acid
protein, which sequence analysis predicted to contain a signal sequence and transmembrane region. Extensive database searches revealed the ECSM2 to be unique in that it bore no homology to any known protein and contained no functional domains. Putative ECSM2 orthologs were identified in other vertebrate species (Figure 1). Sequence alignment of the orthologs revealed that they all contained a highly conserved intracellular domain and a highly variable extracellular domain. The domain specific conservation of ECSM2 may prove to be significant in understanding the function of the protein. The conservation may indicate that there is a strong selective pressure to maintain the integrity of the intracellular domain sequence, or alternatively, interaction with another factor (eg, a virus) may have driven rapid evolution of the extracellular domain. The size of the ECSM2 protein is more than twice that predicted from the amino acid structure, and it is heterogeneous in nature. This experimental evidence, combined with a large number of serine and threonine residues predicted to be sites of O-linked glycosylation in the extracellular domain, which are conserved across species, strongly suggests that ECSM2 is heavily O-glycosylated. A single N-linked glycosylation site is not conserved beyond primates and unlikely to be functionally critical.

Cell surface expression of ECSM2 was confirmed by using an ECSM2-GFP fusion protein in HUVECs (Figure 4). ECSM2 expression was uniform across the entire membrane in 293 embryonic kidney cells (data not shown). By contrast, in HUVECs expression was concentrated at certain points such as the filopodia, which may be a feature of ECSM2 expression in the presence of its interacting proteins.

A mechanism for controlling the localization of ECSM2 within the plasma membrane is through the intracellular interactions of ECSM2 with other proteins. To investigate ECSM2 protein interactions, yeast 2 hybrid analysis was performed using the intracellular domain of ECSM2 as bait to screen a human placental cDNA library. The screen identified a putative interaction between filamin A and ECSM2, and this was subsequently confirmed by using an ECSM2 fusion protein to precipitate endogenous filamin A from HUVEC lysate (Figure 6). Filamin A belongs to the filamin family of actin binding proteins that link actin filaments at the cell membrane and help maintain cell structure. Filamins are considered to be key players in mammalian cell locomotion.25

Mammalian filamins exhibit marked promiscuity in their protein interactions and have been shown to bind to more than 30 different proteins.26 Despite the diversity of the interacting proteins the regions of the filamin that they bind to are principally specified by their function. Thus, a few smaller proteins that participate in signaling processes recognize repeats 1 to 15. In contrast, receptor proteins that make up the largest group of interactors all recognize repeats 16 to 24, and it is this interaction that mediates cross-talk between the extracellular environment and the actin matrix. The interaction of the intracellular domain of ECSM2 with repeats 19 to 21 (and to a lesser extent 15 and 16) are consistent with ECSM2 bearing such a role in endothelial cells that are known to express filamin A.27

An effect of ECSM2 knockdown on endothelial movement in a chemotaxis (Boyden chamber) but not a chemokinetic (scratch wound) assay is in accord with the cells sensing a ligand concentration gradient via ECSM2. The media used as chemoattractant in the Boyden chamber was complex (serum, endothelial cell growth supplements), and at this point it is not clear what an ECSM2 ligand could be. A second possibility is that ECSM2 may act to modulate another, for example, vascular endothelial growth factor, chemokinetic signal. It is also not known what function the extensive O-glycosylation of ECSM2 performs, but in other proteins it has been shown to confer resistance to proteases and modulate the intracellular trafficking of proteins among others. Further work, including in vivo studies, is needed to define the exact role of ECSM2 in angiogenesis and vascular biology.

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Disclosures
None.

References


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Methods

Bioinformatics - ECSM2 was identified as a gene preferentially expressed in endothelial cells by analysis of expression data in EST and SAGE databases as described previously (9). The ECSM2 genomic location and putative ECSM2 orthologs were found by BLAST and BLAT analysis of genomic and EST databases (GenBank™/EBI).

Cell culture - Human umbilical vein endothelial cells (HUVEC) and human dermal microvascular endothelial cells (HDMEC) were obtained from TCS cell works (UK), MEC (Myometrial microvascular endothelial cells) and NEC (Normal endometrial microvascular endothelial cells) were a gift from Dr Leonid Nikitenko (University of Oxford). Endothelial cells were cultured in MCDB 131 medium (Invitrogen) containing 20% fetal calf serum (Sigma-Aldrich), 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 5 IU/ml heparin and 50 μg/ml endothelial cell growth supplement (Sigma, Dorset, UK). Cells were routinely split 1 in 3 and were used up to 8th passage. Human embryonic kidney cells (293), Human embryonic lung fibroblast (HEL), Human T lymphoblast, acute T-cell lymphatic leukemia (JURKAT), Human lung fibroblast (MRC-5), Human peripheral blood leukocyte, acute promyelocytic leukemia (HL60), Human breast adenocarcinoma (MCF-7) Human glioblastoma-astrocytoma (U87-mg), Human neuroblastoma (SK-N-SH) and Human melanoma (SK23) were obtained from Cancer Research UK Central Services and grown in Dulbecos Modified Eagles Medium containing 10% fetal calf serum. Human epidermal keratinocytes, Human hepatocytes and Human peripheral blood lymphocytes were purchased from TCS cell works and used immediately after thawing for RNA extraction.

Identification of full length cDNA sequence and cloning of ECSM2 cDNA - 5’ and 3’ rapid amplification of cDNA ends (RACE) was used to determine the full sequence of the ECSM2 transcript. RACE was performed using the SMART RACE cDNA Amplification kit (BD Biosciences, Clontech, UK), according to the manufacturer’s instructions. Briefly, total RNA extracted from HUVEC in TRI reagent (Sigma) was reverse transcribed to 5’ and 3’ RACE ready cDNA and 5’ and 3’ RACE reactions performed using the ECSM2 gene specific primers 5’- cccacagtgaacctcaggctgacc-3’ and 5’- agcaactttgggtccccatgagtcc-3’ for the 5’ and 3’ reactions respectively. RACE reactions were subjected to agarose gel electrophoresis to confirm a single major PCR product and the products TA cloned using the TOPO TA cloning kit (Invitrogen) prior to sequencing. To clone the ECSM2 transcript, total RNA extracted from HUVEC was reverse transcribed using Superscript II reverse transcriptase (Invitrogen). The ECSM2 transcript was amplified by PCR on total HUVEC cDNA using the upstream primer 5’- tactcgagatggacagagcctccactga-3’ designed to include the XhoI site and the downstream primer 5’- taccgcggcacctcatcactttccttgc -3’ designed to include the Sac II site and inserted into the pbluescript vector (Stratagene, UK). The open reading frame of ECSM2 (nucleotides 68-685) was amplified by PCR using the upstream primer 5’- ctcggctcgaggctgacatgggcaccgcaggagcc -3’ designed to include the XhoI site and the downstream primer 5’-ccaggaatctttaaagccctacctgagag -3’ designed to include the EcoR1 site and inserted into the pbluescript vector.

Quantitative PCR - Total RNA was extracted from cells in culture using TRI reagent (Sigma) and cDNA prepared using the random priming High-Capacity cDNA Archive kit (Applied Biosystems, UK), according to the manufacturers instructions. Assays on Demand Taqman™ probes to ECSM2 (Hs_00416227) and β-2-microglobulin were obtained from Applied Biosystems, UK, and quantitative PCR reactions to amplify each of these genes performed separately. Reactions were prepared in triplicate using 12.5 μl Taqman™ Universal PCR Master Mix (Applied Biosystems, UK), 1.25 μl Taqman™ Primers and Probe, and 5 ng template cDNA (in 5μl volume) in a total reaction volume of 25 μl, and performed on a Rotor-Gene RG3000 thermal cycler (Corbett Research, UK) using the following cycling conditions; 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. To relate levels of ECSM2 and β-2-microglobulin in each sample to a known standard, quantitative PCR was also performed for each gene on 20, 10, 5,
2.5, 1.25, 0.625 and 0.3125 ng of cDNA. Standard curves were generated by linear regression using log (Ct) versus log (cDNA (ng)), and the relative expression levels for each gene calculated in each sample from Ct values using the standard curve. Relative ECSM2 expression was then determined using the standard curve method. To perform the primary cell type screen, Exiqon™ probe technology was used. The housekeeper genes flotillin-2, ubiquitin C and b-actin were chosen using the method described by Vandesompele et al. (2002) with the software geNorm (16). Primer and probe sets were designed for ECSM2 and the housekeeper genes using the ProbeFinder software (Roche). The probe primer set used for ECSM2 was 5’ – cacagagccagtttcttcca – 3’ and gettgctctcctctct – 3’ with exiqon™ probe 03. The reactions were prepared using 12.5 ml of absolute qPCR mastermix (Abgene, UK), 400 nM of forward and reverse primers, 0.25 ml of the appropriate Exiqon™ probe and 5 ng of template (in 5 ml volume), in a total volume of 25 ml. The same cycling conditions were used as for the TaqmanTM system. Data was analysed using a method described by Pfaffl (17) ensuring the same threshold value was used in all samples for a given gene when determining raw Ct values.

In situ hybridization - In situ hybridization analysis was performed using radioactively labelled probes as described by Poulson et al 1998 (18). In some cases slides were also stained with CD34 antibody using a method described by Jeffery et al 2003 (19). The ECSM2 transcript specific probe (nucleotides 15-980) was used to generate the in situ hybridization probe.

Subcellular localization studies - To generate Myc tagged ECSM2, the ECSM2 open reading frame was amplified by PCR using 5’- tctggaagcttatgg gcaccgcaggagcc -3’ the upstream primer designed to include the Hind III site and the down stream primer 5’- cggaggatacgagaacctctct tcgag -3’ designed to include the BamH1 site and inserted into the pCDNA3.1 Myc-His vector (Invitrogen). To generate a green fluorescent protein (GFP) tagged ECSM2, the ECSM2 open reading frame was amplified by PCR using the upstream primer 5’- tctggaagcttatgg gcaggagcc -3’ designed to include the Hind III site and the down stream primer 5’- cggaggatacgagaacctctct tcgag-3’ designed to include the BamH1 site and inserted into the pEGFP-N1 vector (BD Biosciences, UK). To examine ECSM2-GFP expression, HUVEC were seeded on sterile glass coverslips in 6 well plates, the following day cells were transfected with ECSM2-GFP plasmid using Fugene 6 (Roche, UK) for 293 cells or Transpass D2 transfection reagent (NEB) for HUVEC according to the manufacturers instructions. Two days after transfection, cells were either viewed live in PBS, or cells were fixed prior to analysis with either anti-myc or phallolidin staining. Fixation was performed for 10 minutes in 4% formaldehyde, neutralised in 50mM NH4Cl for 10 minutes and permeabilised in 0.1% Triton-X-100 for 4 minutes. For anti-myc staining, the coverslips were incubated in 9E10 anti-myc (CRUK) and Alexa Fluor 488 anti mouse secondary (Invitrogen) for 30 minutes each. To visualize F-actin, fixed cells were stained with phallolidin (A12380 Alexa Fluor 568 (Molecular probes, UK)) according to manufacturers instructions. After fixation and staining, coverslips were mounted onto slides using Vectorsheild with 4’-diamidino-2-phenylindole (DAPI) (Vector Laboratories, USA) to visualize nuclei. 293 cells were viewed on a Zeiss Axioplan microscope (Zeiss, UK) using Smartcapture 2 image acquisition software and images merged in Adobe Photoshop. HUVEC cells were viewed using a Zeiss LSM 510 confocal microscope using Zeiss LSM Image Browser image analysis software.

Yeast two hybrid screen - The yeast two hybrid screen was performed using the BD Matchmaker Library Construction and Screening kit (Clontech, BD Biosciences, UK) according to the manufacturers instructions. A bait construct comprising of the ECSM2 intracellular domain (amino acids 148-205) was amplified by PCR using the upstream primer 5’- tgcatta ggttcaagt tgcgg -3’ designed to include the Ned 1 site and the down stream primer 5’- gtcgaccaagacccatccttcgag -3’ designed to include the Sal 1 site and inserted into the pGBK7 vector. To prepare a cDNA library using the BD library construction system a library cDNA fragments was generated by random priming from poly A’ placental RNA. The reverse transcription reaction incorporated adaptor ends onto the cDNA fragments that
enabled the incorporation into the linearized pGADT7 library vector upon transformation into yeast. The two hybrid screen was performed by co-transformation of the ECSM2-pGBKT7 (GAL4 binding domain) bait vector, the Smal linearized pGADT7 (GAL-4 activation domain) library vector and the placental library cDNA into the yeast strain AH109. AH109 yeast harbour the reporter genes HIS3 and ADE2 under the control of GAL4 binding sites, they are also unable to grow in tryptophan deficient or leucine deficient media, enabling the selection of the TRP1 and LEU1 nutritional markers on the pGBKT7 and pGADT7 vectors respectively. The AH109 co-transformation reaction was initially plated onto triple drop out medium (SD/-His/-Trp/-Leu) and yeast colonies with a positive phenotype were then subjected to a more stringent screen by testing for growth on quadruple drop out medium (SD/-Ade/-His/-Trp/-Leu). To identify potential interacting clones, colony PCR was performed on the positive colonies to amplify library fragments. The fragments were subjected to agarose gel electrophoresis and bands extracted and sequenced. Clones were sequenced and identified by BLAST search analysis against the GenBank™/EBI databases. Clones of interest were analyzed further by isolating the pGADT7 vectors from the original yeast colonies and transforming them into bacteria to obtain plasmid preparations. The pGADT7 library plasmids were then co-transformed into AH109 yeast with the ECSM2-pGBKT7 bait vector and screened on quadruple drop out medium to reconfirm the interaction.

Precipitation assay using GST fusion protein – The ECSM2 intracellular domain (ECSM2-ICD) (amino acids 148-205) was amplified by PCR using the upstream primer 5’ – tagtaggatccagtgtcggaagag – 3’ designed to include the BamHI site and the downstream primer 5’ tagtaggaattcttaaagtctctgctg – 3’ designed to include the EcoRI site and inserted into the GST expression vector pGEX2TK. pGEX2TK and pGEX2TK-ECSM2-ICD were transformed into BL21(DE3) pLysS E. coli. Cultures of E. coli were grown to an optical density at 600 nm of 0.4 and induced with 1 mM isopropyl b-D-1-thiogalactopyranoside for 3 hours at 30 °C. Bacteria were lysed by one freeze thaw cycle and resuspension in E. coli lysis buffer (50 mM Tris pH 7.5, 100 mM NaCl, 2 mM EGTA, 2 mM EDTA, protease inhibitor cocktail (Sigma), followed by sonication and addition of Triton X-100 to a final concentration of 1%. The lysates were incubated for 30 minutes on ice and then centrifuged at 20 000 g for 15 minutes at 4 °C. To perform the precipitation, bacterial lysate containing 5 mg of GST-ECSM2-ICD was incubated with 25 ml of glutathione agarose (GE Healthcare) for 2 hours at 4 °C on a rotating wheel. The glutathione agarose was washed twice with E. coli lysis buffer and twice with NP40 lysis buffer (1% NP40, 10 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, protease inhibitor cocktail (Sigma), 10 mM NaF, 2 mM sodium orthovanadate). 1 ml of HUVEC lysate from 10^8 cells was used per precipitation and incubated with the glutathione agarose/GST fusion for 2 hours at 4 °C on a rotating wheel. HUVEC were lysed with NP40 lysis buffer, followed by incubation on ice for 30 min and centrifugation at 20 000 g for 10 min at 4 °C to remove cell debris. The precipitation samples were washed 3 times in NP40 lysis buffer containing 700 mM NaCl followed by addition of SDS-PAGE sample buffer. Samples were separated using SDS-PAGE and either Coomassie stained for visualisation of the GST fusion proteins, or immunoblotted using anti-filamin A monoclonal antibody (Chemicon) using standard protocols.

Preparation of rabbit ECSM2 specific antisera
The GST-ECSM2 intracellular domain fusion protein was used as the immunogen to make anti-ECSM2 antisera. Rabbits were immunised initially with 400 μg in complete Freund’s adjuvant and boosted a further 5 times with 100 μg in incomplete Freund’s adjuvant. Sera from the terminal bleed was used at a dilution of 1/500 for western blotting.
**Full Figure legends**

**Figure 1.** A, Alignment of ECSM2 orthologs, the transmembrane and intracellular domains show greater conservation than the extracellular domain, conserved regions are highlighted in grey. The signal peptide region was predicted by Signal P software28 and 27 amino acid transmembrane domain was predicted by Dense Alignment Surface Analysis29. Accession numbers for the derived sequences are as follows: Mouse: NP_001028313; Opossum: XP_001376521; Human: NP_001071161; Monkey: C0580079; Sheep: DY510184; Dog: XP_549577; Zebrfish: XP_001345388.

**Figure 2.** Quantitative PCR analysis of ECSM2 expression by cell lines in vitro. A, The level of ECSM2 expression in four endothelial and 8 non-endothelial cell lines; HUVEC (human umbilical vein endothelial cells), HDMEC (human dermal microvascular endothelial cells), MEC (myometrial microvascular endothelial cells), NEC (normal endometrial microvascular endothelial cells), JURKAT (lymphoblast, acute T cell leukaemia), HELF (human embryonic lung fibroblast), MRC-5 (lung fibroblast), HL60 (peripheral blood leukaocyte, acute promyelocytic leukaemia), U87-mg (glioblastoma-astrocytoma), SK-N-SH (neuroblastoma), SK23 (melanoma). Expression levels are shown relative to the level in HUVEC which was arbitrarily set at 1, +/− SD. B, the level of ECSM2 expression in human primary cell isolates including two endothelial (HUVEC and HDMEC) and five non-endothelial isolates: HBEC (human bronchial epithelial cells), hepatocytes, MRC-5 fibroblasts and peripheral blood lymphocytes. Data was analyzed using the method described by Pfaffl17 and is shown +/− SE.

**Figure 3** In situ hybridization analysis of ECSM2 expression in human tissues. Lightfield (A, C, E, G, I and K) and darkfield (B, D, F, H, J and L). ECSM2 expression was restricted to the endothelium in all tissues analyzed. A and B, expression of ECSM2 in large and small vessels of breast carcinoma (some small vessels are indicated by arrows in A). C and D, ECSM2 expression restricted to the endothelium of ganglioglioma tissue (some small vessels are indicated by arrows in C). E and F, ECSM2 expression in vessels of the skin from a psoriasis biopsy (arrows indicate vessels in E). G and H, ECSM2 expression in the endothelium of placental tissue, (the line of corresponding arrows in G and H indicates ECSM2 expression by individual endothelial cells, the single arrow in G shows a transversely cut vessel). I and J, endothelial expression of ECSM2 in fetal tissue (arrows in I indicate small vessels and vessel walls). K and L, ECSM2 expression in breast carcinoma with vessels also stained for the endothelial marker CD34 (indicated by the brown staining in K), confirming the endothelial restricted expression of ECSM2. Scale bars represent 100 μm.

**Figure 4.** Subcellular localization of ECSM2. A, Localisation of myc-tagged ECSM2 in HUVEC. HUVEC were transfected with ECSM2-myc, fixed and immunofluorescence performed using anti-myc antibodies; cells were counterstained with 4′-diamidino-2-phenylindole (DAPI) (blue) to visualize nuclei. B, HUVEC expression of ECSM2-GFP. HUVEC expressing ECSM2-GFP were viewed live by confocal microscopy. ECSM2-GFP expression localized to the cell membrane, with strongest expression seen on cellular protrusions (as is seen in the higher magnification image on the right). C, HUVEC expression of ECSM2-GFP and F-actin. HUVEC expressing ECSM2-GFP were fixed and stained with phallolidin (red) and DAPI (blue) to visualize F-actin and the nuclei respectively, the cells were viewed by confocal microscopy. ECSM2-GFP was expressed at the membrane and cellular protrusions, with some co-localization to F-actin. Scale bars represent 20 μm.

**Figure 5.** siRNA knockdown of ECSM2 results in reduced chemotaxis and tube formation and matrigel. HUVEC were mock transfected or transfected with a negative control duplex (Neg) or two ECSM2 specific duplexes (D1 and D2). After 48 hours cells were harvested and used in chemotaxis and matrigel assays. A, Levels of ECSM2 message relative to actin
message were measured using reverse transcription and quantitative PCR, levels were normalised to the mock transfected cells. B Cells were assayed for their ability to migrate in a chemotaxis assay using fetal calf serum and endothelial growth supplements as chemoattractant, data is pooled from 9 wells from 3 independent experiments per condition. C, Cells were seeded on to matrigel and pictures taken after 12 hours. D, Quantitation of the matrigel assays: nodes containing 1, 2, 3 or ≥4 branch points were quantitated from 6 fields of view per condition. The matrigel assay was repeated 5 times with similar results.

Supplemental Figure I. Western blot of ECSM2 protein. HUVEC were either mock transfected or transfected with a negative control duplex, or two ECSM2 specific duplexes (D1 and D2). After 48 hours cells were lysed and blotted for ECSM2 or tubulin.