VE-statin, an endothelial repressor of smooth muscle cell migration

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The recruitment and proliferation of smooth muscle cells and pericytes are two key events for the stabilization of newly formed capillaries during angiogenesis and, when out of control in the adult, are the main causes of atherosclerosis. We have identified a novel gene, named VE-statin for vascular endothelial-statin, which is expressed specifically by endothelial cells of the developing mouse embryo and in the adult, and in early endothelial progenitors. The mouse and human VE-statin genes have been located on chromosome 2 and 9, respectively, they span >10 kbp and are transcribed in two major variants arising from independent initiation sites. The VE-statin transcripts code for a unique protein of 30 kDa that contains a signal peptide and two epidermal growth factor (EGF)-like modules. VE-statin is found in the cellular endoplasmic reticulum and secreted in the cell supernatant. Secreted VE-statin inhibits platelet-derived growth factor (PDGF)-BB-induced smooth muscle cell migration, but has no effects on endothelial cell migration. VE-statin is the first identified inhibitor of mural cell migration specifically produced by endothelial cells.

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Introduction

Angiogenesis is the process by which new blood vessels arise from the established vascular network in response to various angiogenic stimuli. The new capillaries are mainly composed of endothelial cells which initially form a monolayer at the inner border of all blood vessels, in direct contact with the blood circulation. In response to an angiogenic stimulus, these cells are activated to loosen their intercellular junctions, digest the underlying basement membrane, migrate and proliferate in order to form a primary capillary (Carmeliet, 2003). These initial steps and the ensuing stabilization and maturation of new capillaries into fully functional vessels depend on the recruitment and interaction of endothelial cells with mural cells, i.e. smooth muscle cells (SMCs) and pericytes (Carmeliet, 2003). The complex molecular dialogue that takes place during this phase is decisive for the maintenance or the trimming of the newly established capillaries. It involves several soluble factors produced by either or both cell types and their interacting receptors: endothelial cells produce chemotactic, growth and survival factors for mural cells, recruit the mural cells to the newly formed vessel and induce their differentiation (Conway et al., 2001). The platelet-derived growth factor (PDGF)-B–PDGF receptor (PDGFR)-β interaction is a major actor in this process in vivo; inactivation of the pdgf-b gene induces fatal haemorrhages in the embryos (Leeven et al., 1994), due to a reduced coverage by pericytes of microvessels, which appear dilated and tortuous (Lindahl et al., 1997). Inactivation of the pdgfr-β gene induces a similar phenotype (Soriano, 1994; Lindahl et al., 1997), and careful analyses of both phenotypes provided strong evidence that the PDGF-B secreted by endothelial cells induces the differentiation of PDGFR-β-positive cells and that the ligand–receptor interaction is thereafter critical to the establishment of a normal vessel wall (Hellstrom et al., 1999). The phenotypes observed in pdgf-B−/− and pdgfr-β−/− mice are similar to that obtained with the inactivation of the edg-1 gene, which showed a failure of vascular smooth muscle cells and pericytes to migrate around arteries and capillaries (Liu et al., 2000). Edg-1 codes for one of the receptors of sphingosine-1-phosphate (SIP), a multipotent regulator of both SMC and endothelial cell migration and proliferation (for a review see Spiegel and Milstien, 2003). Interestingly, Edg-1 expression is necessary for PDGF-induced cell migration (Hobson et al., 2001), suggesting that PDGF and SIP signalling are somehow linked during mural cell migration and possibly differentiation.

Another important pathway of regulation of blood vessel establishment and maturation is the angiopoietins (Ang)–Tie-2 interaction. Endothelial and mural cells are the main producer of Ang-1 (Davis et al., 1996) and Ang-2 (Maisonpierre et al., 1997; Witzenbichler et al., 1998), respectively, which both interact with the endothelial-specific receptor Tie-2 and play quite complex roles in angiogenesis: Ang-1 has no effect on cell proliferation (Witzenbichler et al., 1998) but is directly involved in endothelial cell survival (Hayes et al., 1999) as it promotes the activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway (Kim et al., 2000b) and the upregulation of survivin (Papapetroupolous et al., 2000). Ang-1 induces endothelial sprouting and protease release (Kim et al., 2000a), cell migration (Witzenbichler et al., 1998) and tubule formation (Hayes et al., 1999), which are all critical steps of endothelial cell emigration from the established vessels and of angiogenesis. Gene inactivation of either ang-1 or tie-2 led to similar defects in embryos, mainly affecting mural cell recruitment, vessel remodelling and maturation (Dumont et al., 1994; Sato et al., 1995; Suri...
et al., 1996). Although originally described as a natural antagonist of Ang-1 (Maisonpierre et al., 1997), Ang-2 may also lead to cell survival (Kim et al., 2000c), tubule formation and activation of Tie-2 (Teichert-Kuliszewska et al., 2001).

Mural cells produce growth and chemotactic factors toward endothelium, such as vascular endothelial growth factor (VEGF). VEGF is essential to the angiogenic process; it promotes all the initial steps of activation of the endothelial cells by inducing intercellular permeation and matrix degradation, capillary progression by stimulating endothelial cell migration and proliferation, and vessel maintenance by promoting endothelial cell survival (for a review see Carmeliet, 2003). Embryonic blood vessel development is strictly dependent on the dose of VEGF as vegf gene inactivation is lethal to vegf<sup>−/−</sup> embryos (Carmeliet et al., 1996; Ferrara et al., 1996). Inactivation of the VEGF receptor genes flk-1 (Shalaby et al., 1995), flt-1 (Fong et al., 1995, 1999) and of neuropilin-1 and -2 together (Takashima et al., 2002) induce early defects in endothelial differentiation or organization of the primitive vascular network, and lead to embryonic lethality. Mural cells also produce transforming growth factor-β (TGF-β) which, upon activation by cell contact, inhibits endothelial cell proliferation and migration (Orridge and D’Amore, 1987; Sato and Rifkin, 1989; Sato et al., 1990), and induces SMC differentiation and extracellular matrix secretion, thus stabilizing the blood vessels (Dickson et al., 1995; Li et al., 1999). Of particular interest in this pathway is the TGF-β type III receptor endoglin, whose gene inactivation induced defective vascular remodelling and SMC differentiation (Li et al., 1999). Finally, direct contacts between endothelial and mural cells as well as with the extracellular matrix also take part in the dialogue, and the formation of a locally stable and functional vascular tree will ultimately depend on all these complex interactions. Major pathological incidences are the result of a perturbation of these exchanges. In arteriosclerosis, SMC migration and proliferation and a damaged endothelium are key factors for the progression of the initial lesion (Behrendt and Ganz, 2002). In solid tumours, the formation of an irregular and poorly structured blood vessel network is the result of, in part, a lack of coordinated interactions between these cells (Carmeliet, 2003).

Although several growth and chemotactic factors are known to induce the initial recruitment and proliferation of SMCs around capillaries, the factors and the mechanisms by which SMC recruitment is downregulated and ultimately repressed when blood vessels reach maturity are poorly understood. Here, we have identified VE-statin as such a repressor; it may be one of the missing molecular links that are involved in the regulation of SMC recruitment by endothelial cells during angiogenesis.

Results

Characterization of the mouse and human VE-statin cDNAs

The VE-statin cDNA was identified initially as a 3’ end fragment of vezf1, the mouse equivalent of the human DB1 transcription factor (Koyano-Nakagawa et al., 1994; Xiong et al., 1999), and was found thereafter to be an authentic, different transcript (see below and Supplementary data available at The EMBO Journal Online).

The longest isolated VE-statin cDNA clone spanned 1.37 kbp (VE-statin-a, Figure 1). Nested 5'-RACE analysis was performed using a mouse E11 embryonic cDNA library in order to confirm that we had isolated the full-length VE-statin cDNA. This allowed the identification of a second VE-statin cDNA (VE-statin-b, 1.4 kbp) which differed from VE-statin-a in the first 169 bp (Figure 1). Regarding the human product, 5’-RACE based on a partial cDNA sequence (accession No. NM_016215) allowed the identification of an additional stretch of 236 bp located 5’ of the deposited sequence. Based on sequence analogy and gene structure, this human product corresponds to mouse VE-statin-b (73% identity with mouse VE-statin-b versus 59% with mouse VE-statin-a). RT–PCR analysis confirmed the existence of VE-statin-a and showed the presence of VE-statin-b in human endothelial cells (not shown).

VE-statin gene structure

The mouse VE-statin gene was isolated and sequenced; it spans >10 kbp and is structured in 11 exons and introns (Figure 2; and table 1 of the Supplementary data), including the alternative exon-1a and -1b, which correspond to the VE-statin-a and VE-statin-b transcripts, respectively. Exon 9 may be alternatively spliced as some clones showed variations in this exon.

The structure of the human VE-statin gene has been compiled from 5’-RACE, PCR amplification and sequence analyses (not shown), and from the available human genome data (accession No. AL354671). It shows very close similarities to the mouse gene organization (Figure 2A).

In order to define precisely the transcription start points of the VE-statin transcripts in addition to the 5’-RACE analysis, primer extension (not shown) and RNase protection assays were performed (Figure 2B). VE-statin-a shows several transcription start points; the longest transcripts extend ~75 bases upstream of the first identified base, and initiation spans >60 bp. This variant was found to be expressed at much lower levels in cells and embryos than the VE-statin-b form, for which a strong and unique transcription start was identified, most probably due to the presence of the CATAAAAAGC box located 42 bases upstream of the first transcript base.

The chromosomal localization by fluorescence in situ hybridization (FISH) using VE-statin probes revealed the presence of the gene exclusively on chromosome 9 (9q34.3–qter) and chromosome 2 (2B), in human and mouse, respectively (Figure 2C). RH mapping (not shown) confirmed the localization of the human gene on chromosome 9 next to the WI-17482 marker at 130.6 and 131.2 Mb (positions in the UDB http://bioinformatics. weizmann.ac.il/udb/ and Santa Cruz http://genome.ucsc. edu/ databases, respectively), on the distal region of the long arm of chromosome 9. It is noteworthy that the human and mouse db1 genes have been mapped to chromosome 17 and 11, respectively, further confirming that db1 and VE-statin are distinct genes.
VE-statin is expressed by endothelial cells in vitro

Both VE-statin variants are expressed in cultured endothelial EOMA, H5V and 1G11 cells (Figure 3A), but not in 3T3 and L929 fibroblasts. For comparison, mDB1 was found to be expressed at similar levels in all tested cell lines. In order to assess the size of the genuine VE-statin transcripts, analysis of expression of the VE-statin isoforms was performed by northern blotting. One major transcript of ~1.6 kb was recognized by each probe (Figure 3B); this size is in accordance with the size of the respective VE-statin cDNAs. Expression of VE-statin-a and -b is high in heart, lung and kidney and not detectable in other tissues in these conditions.

VE-statin is an endothelial cell-specific gene in vivo

The expression pattern of VE-statin was analysed by in situ hybridization and compared with the expression patterns of dbf1 and of the endothelial-specific flk-1 used here as an early mesodermal-, then endothelial-specific control. Expression of VE-statin strictly coincides with the endothelium throughout development, whereas dbf1 expression does not. At E7.5, VE-statin expression is detected exclusively in the primitive blood islands where the first endothelial cells differentiate. Expression of flk-1 is also found in the primitive blood islands and in the intra-embryonic mesoderm (Yamaguchi et al., 1993; Breier et al., 1996). At E10.5, VE-statin expression (Figure 4A)
overlaps that of flk-1 (not shown) in the endothelial cells of the umbilical vein and artery, of the third and fourth branchial arch arteries, of the dorsal aorta and of the cephalic mesenchyme. db1 expression is ubiquitous in the mouse embryo at this stage, with a higher expression in the neuroepithelium (Figure 4A). At E13.5, the VE-statin expression pattern is still restricted to endothelial cells, as observed in the facial mesenchyme, the brain, the liver, the heart endocardium, the lungs, the vascular networks around the ribs or the pigmented layer of the retina.
We investigated the expression pattern of **VE-statin** in the kidney after birth, as blood vessels still develop and remodel in this organ. In 3-day-old pups, the **VE-statin** expression pattern is strictly restricted to the endothelium. **VE-statin** is expressed both in renal arteries and in veins (Figure 4B), suggesting that its expression is not restricted to the arterial or the venous vascular network at this stage. **VE-statin** expression is also detected in the peritubular and fenestrated glomerular capillaries and in the arteries (arcuate or interlobular), indicating that in this organ, expression is not associated with a particular type of vessel. In pregnant mice, **VE-statin** expression was also detected in blood vessels of the mesometrial deciduum of the uterus.

**VE-statin protein structure**

The translation start of the **VE-statin** protein was predicted to be located at codon AUG<sub>281</sub>-<sub>283</sub> and AUG<sub>909</sub>-<sub>911</sub> of the mouse and human **VE-statin-a** transcripts, respectively, based on the presence of a minimal Kozak translation initiation sequence (Figure 1) (Kozak, 1989) in both species, the presence of stop codons in the other possible reading frames, and a high level of similarities between the human and mouse sequences (79% identities) starting at this position. These reading frames translate into proteins of 29.8 and 29.6 kDa in mouse and human, respectively (Figure 5A). In order to test whether these frames were functional, the full-length **VE-statin-a** and **VE-statin-b** cDNAs were used in *in vitro* translation experiments. One major protein of ~30 kDa was expressed from both cDNAs (Figure 5B); no other specific product could be detected. The open reading frames code for proteins of 275 and 273 amino acids (calculated mol. wts 29.8 and 29.6 kDa) in mouse and human, respectively. A comparison of the protein sequences in the two species shows 78% identity and 87% overall homology (Figure 5A). Database searches for protein domains in **VE-statin** predicted the presence of a conserved, cleavable, signal peptide in the N-terminal part of the human and mouse protein. Two epidermal growth factor (EGF)-like domains are present in both species; they are encoded by separate exons (6 and 7 in mouse). No close resemblance of **VE-statin** to other known factors was found otherwise.

**VE-statin is a secreted soluble protein**

Such protein features are present in several membrane-bound and secreted factors and suggested that the **VE-statin** protein might be exposed on the outer side of the cells or released as a soluble factor. In order to test these hypotheses, the **VE-statin** coding sequence was cloned in-frame with either green fluorescent protein (GFP) or the *Haemophilus influenza* haemagglutinin (HA) epitope in

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**Fig. 3.** Analysis of **VE-statin** transcript expression. (A) RT–PCR analysis of expression of the transcripts of **VE-statin** (total), **VE-statin-a**, **VE-statin-b**, db1 and gapdh in total RNA of E10.5 mouse embryo and heart, and in various cell lines. (B) Northern blot analysis of expression of **VE-statin-a** (top) and **VE-statin-b** (bottom) in various mouse tissues (arrowheads). Numbers indicate the size of markers (kb).
medium. Despite several attempts, no significant amounts of VE-statin were found in the membrane fractions of surface-biotinylated cells (not shown), suggesting that VE-statin is not exposed on the outer membrane of these cells. On the other hand, VE-statin was detected in the conditioned medium of pVE-statin-HA-transfected cells (Figure 6A), whereas no protein was detected in the conditioned medium of non-transfected or mock-transfected cells. The apparent size of secreted VE-statin is slightly higher than the predicted molecular mass, suggesting that the protein is post-translationally modified. Pulse–chase and immunoprecipitation experiments confirmed that VE-statin is a secreted protein; metabolically labelled [35S]VE-statin is detected beginning 2 h after the end of the pulse period in the cell supernatant, and accumulates thereafter. Concomitantly, [35S]VE-statin is chased from the cells, with a calculated intracellular half-life of ~1.5 h (Figure 6B, cells). Secretion of VE-statin in the supernatant is prevented when the cells are cultured in the presence of the secretion inhibitor brefeldin A (Figure 6B) or, to a lesser extent, with monensin (not shown).

**VE-statin reduces SMC migration but not proliferation**

Since VE-statin is expressed by endothelial cells and released in the culture medium as a secreted molecule, we next investigated its effects on the closest neighbour to endothelial cells in vivo, i.e. mural cells. So far, all our attempts to produce a recombinant active VE-statin protein using various expression systems have failed. In order to obtain significant amounts of post-translationally modified and secreted VE-statin (see above and Discussion), VE-statin-containing medium was produced by transfecting 3T3 cells with the pVE-statin-HA expression vector (Figure 5C) and setting up conditions for standardized production of serum-free conditioned medium.

VE-statin did not affect the growth rate of aortic SMCs (AoSMCs) (Figure 7A), as shown by the strictly similar
growth rates obtained either in the absence or presence of minimal amounts of donor calf serum used to sustain cell survival and growth.

Secondly, the effect of VE-statin on cell migration was checked using the wound assay performed on confluent monolayers of AoSMCs in serum-free conditions. In basal
medium, cell migration after 2 days was very limited and increased only slightly in the presence of PDGF-BB (not shown). The migration rate of AoSMCs grown in control medium was twice as high as that obtained in unconditioned medium (not shown, and Figure 7B and C) and was increased 1.5-fold further when 80 ng/ml PDGF-BB was added (Figure 7B and C). On the other hand, when cultured in VE-statin-containing medium, the basal AoSMC migration rate was reduced to that observed with basal medium, and PDGF-BB had almost no stimulatory effects on cell migration (Figure 7B and C).

The inhibitory effect of VE-statin on cell migration was evaluated further using the Boyden chamber assay; AoSMC migration in control medium was stimulated 2.6-fold in the presence of PDGF-BB, while this effect was significantly repressed in the presence of VE-statin (Figure 7D). As these effects could reflect an indirect change of the conditioned medium by a 3T3 autocrine loop during production rather than a direct effect of VE-statin, the same experiments were conducted using VE-statin-immunodepleted conditioned medium. In these conditions, the VE-statin-depleted medium no longer affected PDGF-induced cell migration; the migration rates became similar to that of the controls (Figure 7E), thus showing that VE-statin is a direct inhibitor of PDGF-induced AoSMC migration.

In order to evaluate the specificity of the observed effects, VE-statin was also assessed for its potential effects on primary human umbilical vein endothelial cell (HUVEC) migration, using VEGF as chemoattractant. VEGF stimulated HUVEC migration 2-fold both in control and in the presence of VE-statin (Figure 7F), showing that VE-statin has no effects on endothelial cell migration.

**Discussion**

VE-statin is a novel endothelial-specific secreted ligand which shows no close homology with other known factors produced by these cells. Expression of the *VE-statin* gene is detected in endothelial precursors at a very early stage of the endothelial differentiation program (E7.5). When compared with other major endothelial markers, *VE-statin* is not expressed in the uncommitted embryonic mesoderm at E7.5; its expression starts slightly after that of *flk-1*, the earliest known marker of endothelial commitment (Shalaby et al., 1995). Extra-embryonic (blood island) expression of *VE-statin* is concomitant with that of *tie-2* (Sato et al., 1993; Schnürch and Risau, 1993) and of *VE-cadherin* (Breier et al., 1996), and starts at least 0.5 days earlier than *tie-1* (Dumont et al., 1995). Expression of all these early endothelial markers proved to be essential for the establishment of the vasculature: expression of *flk-1* is critical for early endothelial differentiation and blood island formation (Shalaby et al., 1995), *VE-cadherin* and *tie-2* play essential roles in endothelial survival, vasculogenesis and angiogenesis (Dumont et al., 1994; Sato et al., 1995; Carmeliet et al., 1999; Gory-Fauré et al., 1999), whereas *tie-1* is involved in the maintenance of endothelium integrity (Sato et al., 1995). Thereafter and throughout embryonic development, *VE-statin* is expressed wherever endothelial cells are present, with a pattern of expression similar to that of the other endothelial markers *tie-1, tie-2* (Dumont et al., 1995) and *VE-cadherin* (Breier et al., 1996). It is somehow different from *flk-1*, as *VE-statin* is still expressed in established vessels. VE-statin constitutively marks endothelial cells; no obvious variations of *VE-statin* expression have been observed in the embryonic or adult tissues that have been analysed so far. In particular, VE-statin is expressed by endothelial cells regardless of their origin (vein or artery) or vessel size.

The sequence of VE-statin provided little indication of the possible roles of the protein, apart from the presence of a bona fide signal peptide and of two EGF-like modules. EGF-like modules are present in a large number of membrane-bound and secreted proteins, ranging from one EGF-like module to several tens per molecule. However, their presence is not a clear indication of function; they are critical for protein–protein recognition, the enzymatic activities of several blood coagulation proteins such as factor IX, factor VII, factor X and protein C, and the binding of urokinase to its receptor (for a review see Stenflo et al., 2000). EGF-like modules are also involved in cell surface protein–protein recognition such as between the *Drosophila* Notch and Delta receptors (Fehon et al., 1990). This implies that VE-statin may interact with other proteins after secretion; one such obvious target is its putative receptor on the surface of SMCs, which remains to be identified.

The finding that VE-statin has an effect on SMC migration and not on proliferation was quite surprising as, so far, the limited number of known natural inhibitors of SMC migration also repress cell proliferation. These include TGF-β (Bjorkerud, 1991; Ma, 2000), PTEN (Huang and Kontos, 2002) and SIP, depending on the EDG receptors present on the responsive cells (Liu et al., 2000; Kluk and Hla, 2001; Ryu et al., 2002). Since VE-statin has no effect on endothelial cell migration while these cells express significant amounts of it, endothelial cells do not seem to have an autocrine loop of down-regulation of their migration in response to VE-statin, possibly because they do not express a functional receptor. As already mentioned, a major issue will be the identification of the VE-statin receptor(s) on the surface of SMCs and the understanding of the triggered intracellular signalling. It would also be of particular interest to assess the possible interplay between VE-statin, PDGF-B and SIP in PDFGR-β and EDG-1 signalling during SMC migration.

Our work adds a new molecular actor in the dialogue between endothelial and mural cells, i.e. an endothelial inhibitor of mural cell recruitment. Several questions arise from this initial description: is VE-statin the only such inhibitor produced by endothelial cells, in which case it is expected to play a central role during embryonic development or, as is more probably the case, are there other such specific regulators of this mural cell key function? What is the physiological role of VE-statin *in vivo*? VE-statin is expressed very early during endothelial differentiation and later in all embryonic blood vessels. Its function as an inhibitor of SMC migration suggests that it acts in a preventative role in the developing embryo by repressing SMC recruitment before the first mural cells associate with the capillaries, thus delaying premature blood vessel maturation in the fast growing and demanding tissues. It may also prevent an over-recruitment
of SMCs around the newly established capillaries. Interestingly, VE-statin would, however, not affect the formation of vascular walls of already SMC-colonized vessels because it has no effects on SMC proliferation. Later, and in the adult when the vessels are stabilized by mural cells, VE-statin may be expressed in order to prevent the migration of SMCs away from the vessel, thus participating in their stabilization. It could also participate in other functions in the vessels, such as preventing SMC apoptosis. Such a role on SMC metabolism is certainly expected to be important in arteriosclerosis and in the stabilization of tumour blood vessels. VE-statin gene inactivation is currently under way and should provide details of the role of this gene in blood vessel formation during embryonic development.

Materials and methods

Cells
Mouse heart (HSV) and EOMA endothelium, brain capillary (MBE) and aortic (MAE) normal endothelial cells, lung endothelial line 1G11, 3T3 and L929 (ATCC) fibroblasts, human primary AoSMCs and HUVECs (Clonetics) were cultured using standard conditions.

Cloning
The initial VE-statin probe was produced from reverse-transcribed mouse E10.5 embryonic total RNA using 50 ng of each oligonucleotide ACAAAAAGAAAGAAGCTTAC and CCCGGGAGATGCTGGG-GG in High Fidelity PCR master mix (Roche). PCR amplification [94°C for 2 min; 35 cycles of 94°C for 30 s, 54°C for 30 s, 72°C for 1 min; then 72°C for 7 min] was performed and the 416 bp product was cloned, sequenced and used to probe mouse embryonic and ovarian λ libraries (1.7 × 106 and 1.6 × 106 phages, respectively). The full-length VE-statin-a and VE-statin-b CDNA were cloned into the pcDNA3 vector (Invitrogen) and used to generate the various VE-statin probes.

The pVE-statin-GFP vector was constructed by cloning the VE-statin CDS in the pEGFP-N1 vector (BD-Clontech). The pVE-statin-HA expression vector was constructed by cloning the H.influenzae HA epitope-coding sequence in-frame with the VE-statin CDS into pcDNA3.

5'-RACE and gene fragments isolation
Nestled 5'-RACE analysis was performed on human normal ovary and mouse E7.5 embryonic CDNA (Marathon-ready CDNA, Clontech) as recommended, but using the High Fidelity PCR master mix (Roche) and the mouse and human oligonucleotides CGCGAGCCCACATGTTCGTAGCTC and TCTTTTGGGTCTTGAGCCG, and CCACATGCACGACATCTGGAG and GAGGGCTCATGGCCCTGTGCTTCA, respectively. VE-statin gene isolation was performed both by probing λ libraries (1–1.8 × 106 clones each) using CDNA and genomic VE-statin probes, and by PCR fragment amplification using the mouse GenomeWalker (Clontech) and various sets of specific oligonucleotides.

RNase protection assay
Two specific genomic fragments of 850–900 bp which encompassed −150–175 bp of either exon 1a or exon 1b were amplified from a 17 kbp VE-statin genomic fragment using High Fidelity PCR master mix (Roche). The amplified fragments were cloned and used to generate 32P-labelled VE-statin-a and -b probes using the TCCCAGGGGACAAGGACGGATGCCAGAC and ATGCTGGGTGGCTGCTGGATCGGGCCACATCTGCTGG primers, respectively, and the Riboprobe Gemini system II (Promega). The RNase protection assays were performed using 5 µg of total RNA isolated from mouse E10.5 embryonic, HSV endothelial cells or control yeast rRNA, using RPA III (Ambion).

Northern blotting
Human and mouse multiple tissue northern blot membranes (Clontech) were incubated with human or mouse VE-statin-a or VE-statin-b antisense 32P-labelled probes, respectively, in ExpressHyb hybridization solution (Clontech) at 68°C for 1 h. The membranes were washed three times in 2× SSC, 0.05% SDS at 50°C, dried and autoradiographed.

FISH and RH mapping
The bacterial artificial chromosome (BAC) clone 611D20 (Incyte Genomics) was used as human probe; a plasmid containing −17 kbp of specific mouse genomic DNA was used as murine probe. The human primer set GCTGTCGTTGCTGCTATG and TTATTTGAC-AAGAGTGGG was used to screen the Stanford radiation hybrid panel G3.

In situ hybridization
In situ hybridization was performed using a 1.3 kb mouse VE-statin cDNA probe, a 1 kb vegfr-2 mouse CDNA or a 800 bp dbl mouse CDNA. Sense and antisense probes were synthesized from linearized plasmids using 350 µM digoxigenin-UTP (Roche), as described in Wilkinson and Nieto (1993). In situ hybridization was performed on 5 µm paraffin sections as in Mattot et al. (1995). Digoxigenin was detected by immunohistochemistry essentially as in Wilkinson and Nieto (1993).

Pulse-chase
The day following transfection, the medium was replaced by Met/Cys-free Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) and the cells incubated for 30 min prior to the addition of 250 µCi of Pro-Mix L-35S labelling mix (Amersham-Biosciences). Cells were labelled for 45 min, brefeldin A (GolgiPlug, Promega) was then added or not, and the cells incubated further for 15 min, at which time the medium was replaced with DMEM containing 0.2% bovine serum albumin (BSA) (chase) and brefeldin A where indicated. Medium and cell extracts were collected at the indicated times and immunoprecipitated (see Supplementary data). Cells were fixed, soaked in Amplify reagent (Amersham-Biosciences) and autoradiographed.

Transfection and production of conditioned medium
3T3 cells (15 000 cells/cm2) were plated in 78.5 cm2 culture dishes and transfected the next day with or without 5 µg of pcDNA3 or pVE-statin-HA vectors using Exgen 500. Cells were incubated for 6 h and the medium was changed for culture medium.

When needed, the next day, conditioned media were produced by replacing the medium with serum-free and growth factor-free SmBM or EBV-2 basal media (Clonetics) containing 0.2% BSA (Sigma) and further incubation for 24 h. Conditioned media were collected and filtrated (0.22 µm), and cell extracts were analysed for transfection efficiency.

Conditioned medium was depleted of VE-statin by incubating in the presence of 10 µg/ml purified anti-HA polyclonal antibody (Convance) overnight at 4°C followed by immunoadsorption on protein A/protein G (50:50)–Sepharose beads for 1 h at 4°C and filtration (0.22 µm).

Analysis of proliferation
AoSMCs were plated at 3500 cells/cm2 in 4 cm2 culture dishes and cultured for 24 h, at which time the medium was replaced with control or VE-statin-containing medium, 50 ng/ml PDGF (R&D) and 2% donor calf serum (Hyclone) where indicated; culture medium was changed every other day. Cells were counted using a haemocytometer (Coulter, Coultronics).

Analysis of cell migration
For wound assays, AoSMCs (50 000 cells/cm2) were plated in 28 cm2 culture dishes and cultured until confluence. The monolayers were wounded using a razor blade and rinsed twice with basal medium. VE-statin-containing or control medium was added with or without 80 ng/ml PDGF and the cells were cultured further for 2 days.

For Boyden chambers assays, AoSMCs or HUVECs (58 000 cells/cm2) were plated in the upper chamber of cell culture inserts (8 µm pore size; Becton-Dickinson) in control or VE-statin-containing medium and in the presence or absence of 80 ng/ml PDGF or 10 ng/ml VEGF165 (Peprotech), respectively. After 2 days, the cells that had migrated to the lower compartment were collected by trypsin/EDTA treatment and counted (Z2 Coulter, Becton Dickinson).

GenBank accession numbers
The mouse VE-statin-a and -b sequences have been deposited in the GenBank database under accession Nos AY239289 and AY239290, respectively.

Supplementary data
Supplementary data are available at The EMBO Journal Online.

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