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

Fabrice Soncin[1], Virginie Mattot, Frédéric Lionneton, Nathalie Spruyt, Frédéric Lepretre[2], Agnès Begue and Dominique Stehelin

CNRS UMR 8526 and [2]Service Régional de Cytogénétique Moléculaire, Institut de Biologie de Lille, 1, rue Calmette, 59021 Lille Cedex, France

[1]Corresponding author
e-mail: fabrice.soncin@ibl.fr

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 arteriosclerosis. 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.

Keywords: angiogenesis/endothelium/smooth muscle/migration/vezf1

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 (Leveen *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 (S1P), 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 S1P 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 (Papapetropoulos *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*$^{+/-}$ 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 (Orlidge 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.

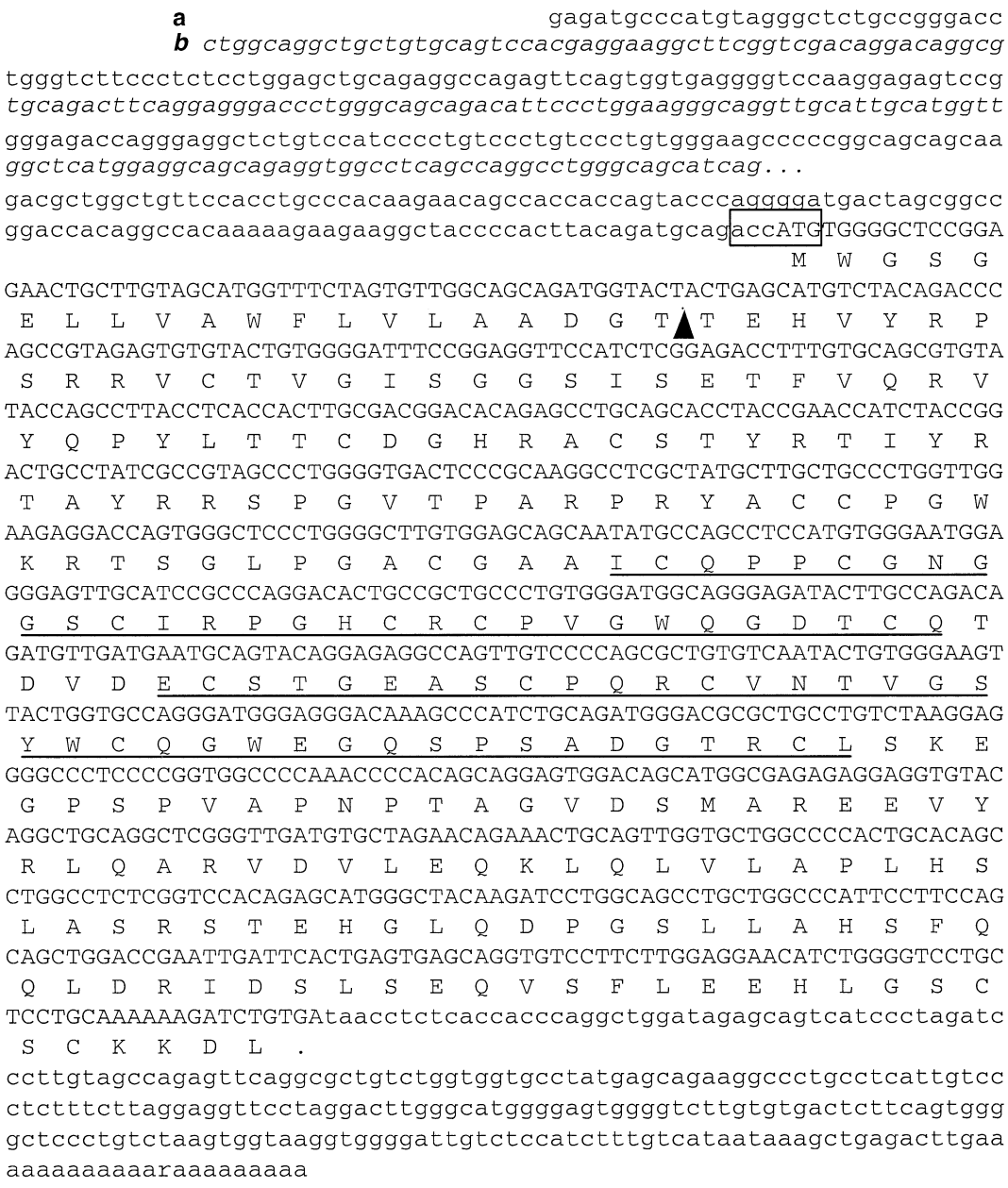


Fig. 1. Sequence of the mouse VE-statin cDNA and protein. The complete 1371 bp sequence of VE-statin-a. VE-statin-b differs from VE-statin-a in the first 169 bp (italics). The coding sequence is shown in upper case, the minimal Kozak sequence is boxed, the arrowhead indicates the signal peptide potential cleavage site, and EGF-like modules are underlined.

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 *db1* 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 *db1* 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)

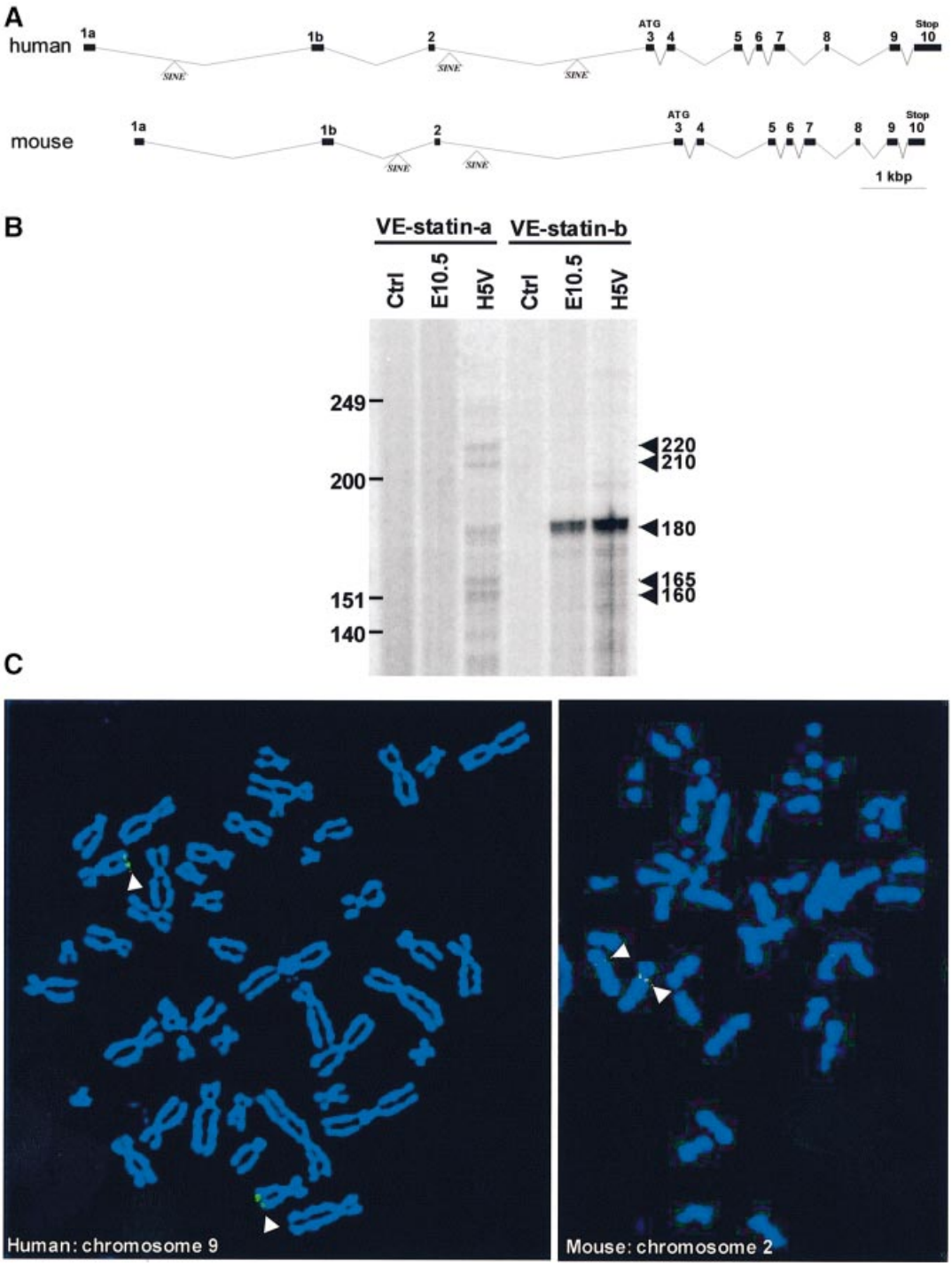


Fig. 2. Structure of the mouse and human *VE-statin* genes. (**A**) Schematic representation of the human and mouse *VE-statin* genes. Numbers and horizontal lines indicate the exons. Exons 1a and 1b code for VE-statin-a and VE-statin-b, respectively. The ATG start codon is located in exon 3 and the stop codon in exon 10. Polyadenylation signals were found in the mouse (ATAATAAAGC) and human (ACAATAAAAA) genomic sequences. SINE = short interspersed nuclear elements. (**B**) RNase protection assay of VE-statin-a and VE-statin-b transcripts was performed on tRNA (Ctrl) or on mouse embryo E10.5 and H5V endothelial cell RNA. VE-statin-a has several (at least six) start sites which span ~60 bp (arrowheads), whereas VE-statin-b has a unique start site. Numbers on the left correspond to size markers. (**C**) FISH analysis of the mouse and human chromosomes using VE-statin-specific fluorescent probes. In both species, a unique signal was found on each corresponding pair of chromosomes (arrowheads).

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

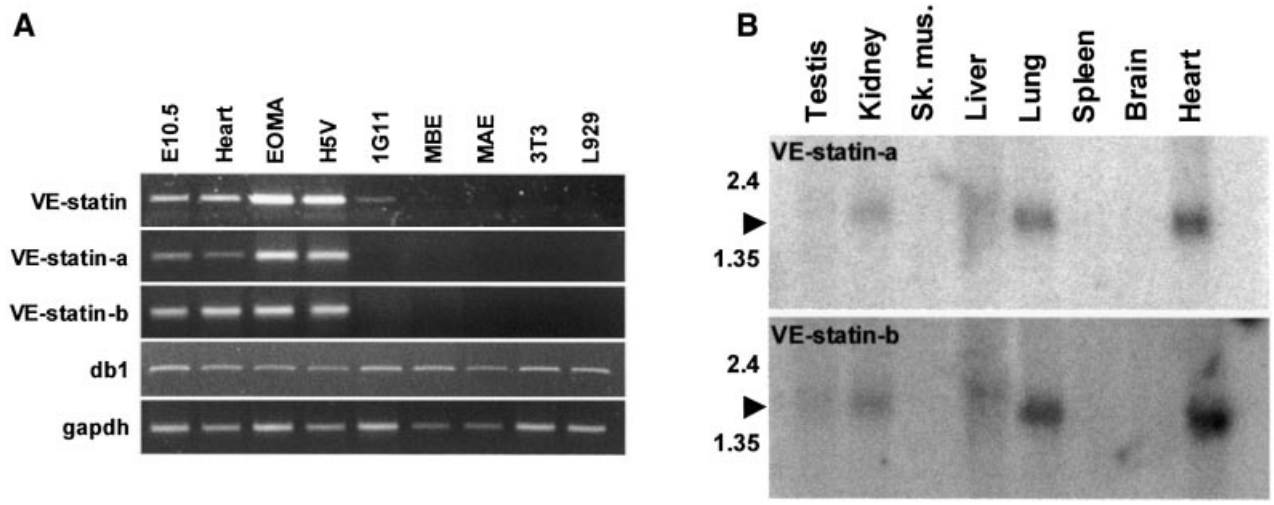


Fig. 3. Analysis of VE-statin transcript expression. (**A**) RT–PCR analysis of expression of the transcripts of VE-statin (total), VE-statin-a, VE-statin-b, db1 and gapdh in total RNA of E10.5 mouse embryo and heart, and in various cell lines. (**B**) Northern blot analysis of expression of VE-statin-a (top) and VE-statin-b (bottom) in various mouse tissues (arrowheads). Numbers indicate the size of markers (kb).

(Figure 4B). 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.

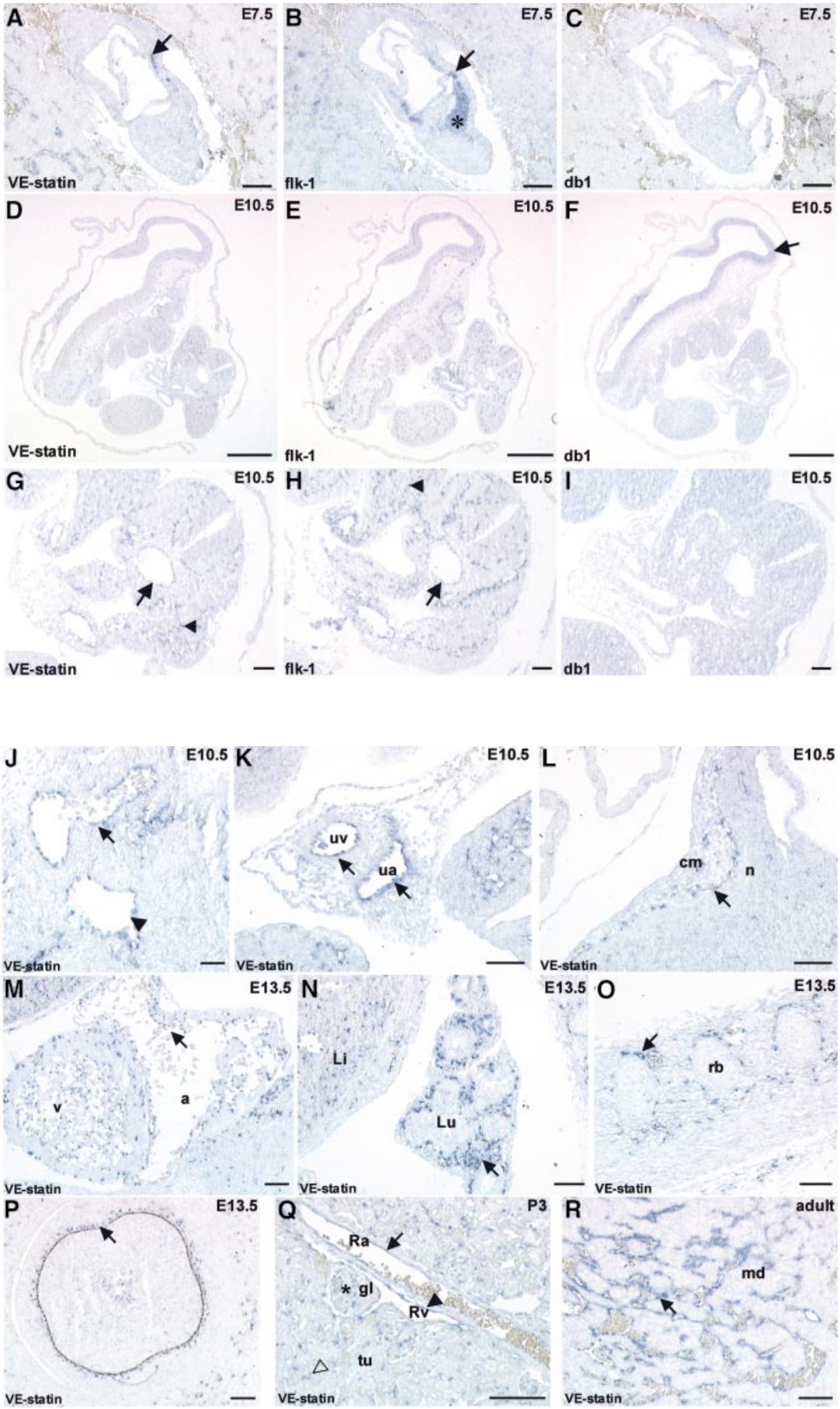
VE-statin protein structure

The translation start of the VE-statin protein was predicted to be located at codon $AUG_{281–283}$ and $AUG_{309–311}$ 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

Fig. 4. *In situ* expression pattern of *VE-statin, flk-1* and *db1*. (Top panel) *In situ* hybridization analysis of expression of *VE-statin* (**A**, **D** and **G**), *flk-1* (**B**, **E** and **H**) and *db1* (**C**, **F** and **I**) in E7.5 (A–C) and E10.5 (D–I) mouse embryos. At E7.5, *VE-statin* (A) and *flk-1* (B) are expressed in the primitive blood islands of the yolk sac (arrows). *flk-1* is also expressed in the intra-embryonic mesoderm (B, asterisk). *db1* expression is not detected at this stage (C). At E10.5, *VE-statin* (D) and *vegfr-2* (E) are similarly expressed in the forming blood vessels. A higher magnification of the caudal part of the embryo shows the parallel expression patterns of *VE-statin* (G) and *flk-1* (H) in the endothelium (dorsal aorta, arrow; hind limb vessels, arrowhead). At this stage, *db1* is ubiquitously expressed in the embryo, with a higher expression in the neuroepithelium (F, arrow). *db1* expression is never detected in the endothelium (I). Bars represent 100 μm in (A–C) and (G–I), and 1 mm in (D–F). (Bottom panel) *VE-statin* expression analysis in E10.5 (**J**–**L**) and E13.5 (**M**–**P**) embryos, in 3-day-old pups (**Q**) and in adult tissues (**R**). At E10.5, *VE-statin* expression is detected in the third and fourth branchial arch arteries (J, arrow and arrowhead, respectively), in the umbilical vein and artery (K) and in the endothelial cell precursors of the cephalic mesenchyme (L). At E13.5, *VE-statin* is expressed in endothelial cells of the endocardium (M), in the primitive pulmonary vascular network (N), in the blood vessels surrounding the ribs (O) and in the vascular network associated with the pigmented layer of the retina (P). After birth, *VE-statin* expression is detected in the renal artery (Q, arrow) and vein (arrowhead), in the glomerular capillary network (Q, asterisk) and in the peritubular capillaries (Q, open arrow). In the pregnant female, *VE-statin* is strongly expressed in the endothelial cells of the blood vessels of the mesometrial deciduum (R, arrow). a = atrium; cm = cephalic mesenchyme; gl = glomerulus; Li = liver; Lu = lung; md = mesometrial deciduum; n = neuroepithelium; Ra = renal artery; rb = rib; Rv = renal vein; tu = renal tubules; ua = umbilical artery; uv = umbilical vein; v = ventricule. Bars represent 100 μm.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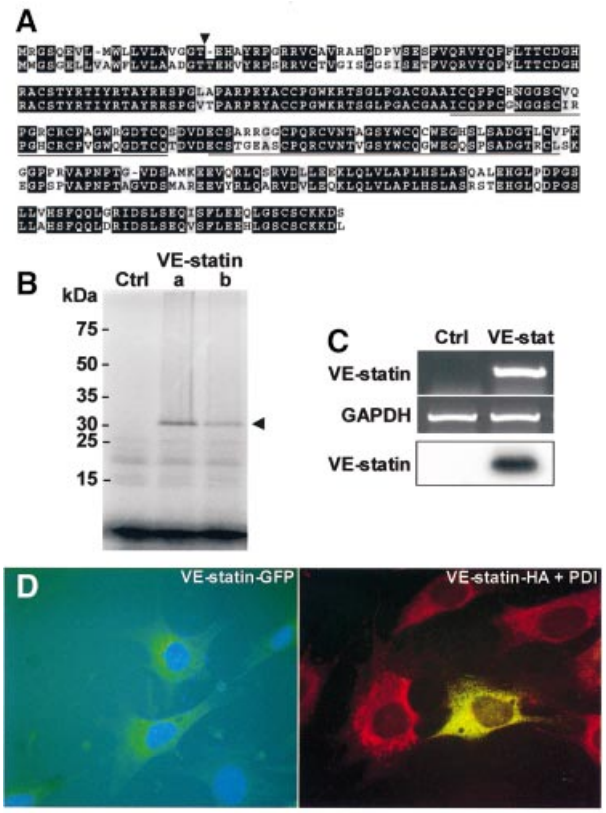

Fig. 5. Expression of the VE-statin protein. (**A**) Homology alignment of the human (top) and mouse (bottom) VE-statin protein sequences. Identical amino acids at matching positions are boxed in black; conservative substitutions are boxed in grey. Legends are as in Figure 1. (**B**) The complete VE-statin-a (a) and VE-statin-b (b) cDNA or control empty vector (Ctrl) were used in *in vitro* translation experiments. A single VE-statin protein migrating at the predicted size of 30 kDa is produced in both cases. (**C**) Top two lanes: RT–PCR analysis of expression of VE-statin 24 h after transfection of 3T3 cells with the pcDNA3 control (Ctrl) or pVE-statin-HA (VE-stat) vectors. Bottom lane: immunoblot analysis of expression of the VE-statin protein in extracts of pcDNA3- (Ctrl) and pVE-statin-HA- (VE-stat) transfected cells. (**D**) Cells were transfected with the pVE-statin–GFP (left) or pVE-statin-HA (right) expression vector, stained the next day with Hoechst reagent and visualized for direct fluorescence (GFP, left) or incubated with anti-HA- (green) and anti-protein disulfide isomerase- (red) specific antibodies. Incubation with secondary antibodies yielded no fluorescent signal (not shown).

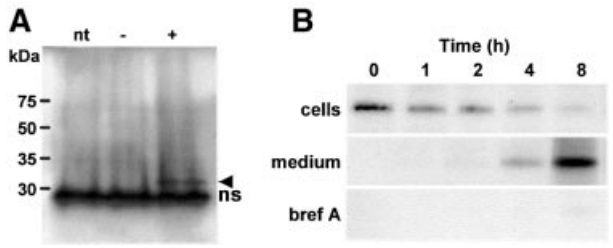

Fig. 6. VE-statin is a secreted protein. (**A**) 3T3 fibroblasts were transfected or not (nt) with the pcDNA3 vector (–) or pVE-statin-HA expression vector (+). The next day, the culture medium was removed, fresh medium was added and the cells were cultured further for 6 h. Secreted VE-statin (arrowhead) was immunoprecipitated from the filtered medium and detected by western blotting. ns = non-specific signal. (**B**) Pulse–chase analysis of VE-statin secretion. 3T3 cells were transfected in conditions similar to those in (A). The following day, they were metabolically labelled for 1 h and chased for up to 8 h in the absence (medium) or presence of the secretion inhibitor brefeldin A (bref A). Supernatants and cell extracts (cells) were collected at the indicated times. VE-statin was immunoprecipitated, and analysed by SDS–PAGE and autoradiography. The results are representative of three independent experiments performed in similar conditions.

expression vectors and used for overexpression in 3T3 cells, which do not normally express VE-statin (Figure 5C).

3T3 cells transfected with the control GFP vector showed a typical pattern of diffuse cytoplasmic and strong nuclear fluorescent staining (not shown), and fusion with VE-statin prevented the GFP from entering the nucleus (Figure 5D). Co-immunofluorescence experiments showed that VE-statin is not associated with the cytoskeleton/focal contacts or with the mitochondria (not shown). Rather, VE-statin is found in the endoplasmic reticulum (ER), as the observed staining is compatible with a co-localization with protein disulfide isomerase (Figure 5D), a specific marker of this cellular compartment (Freedman, 1989).

This latter finding prompted us to analyse the presence of the protein on the cell membrane and in the culture 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-translationnally modified. Pulse–chase and immunoprecipitation experiments confirmed that VE-statin is a secreted protein; metabolically labelled [^{35}S]VE-statin is detected beginning 2 h after the end of the pulse period in the cell supernatant, and accumulates thereafter. Concomitantly, [^{35}S]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

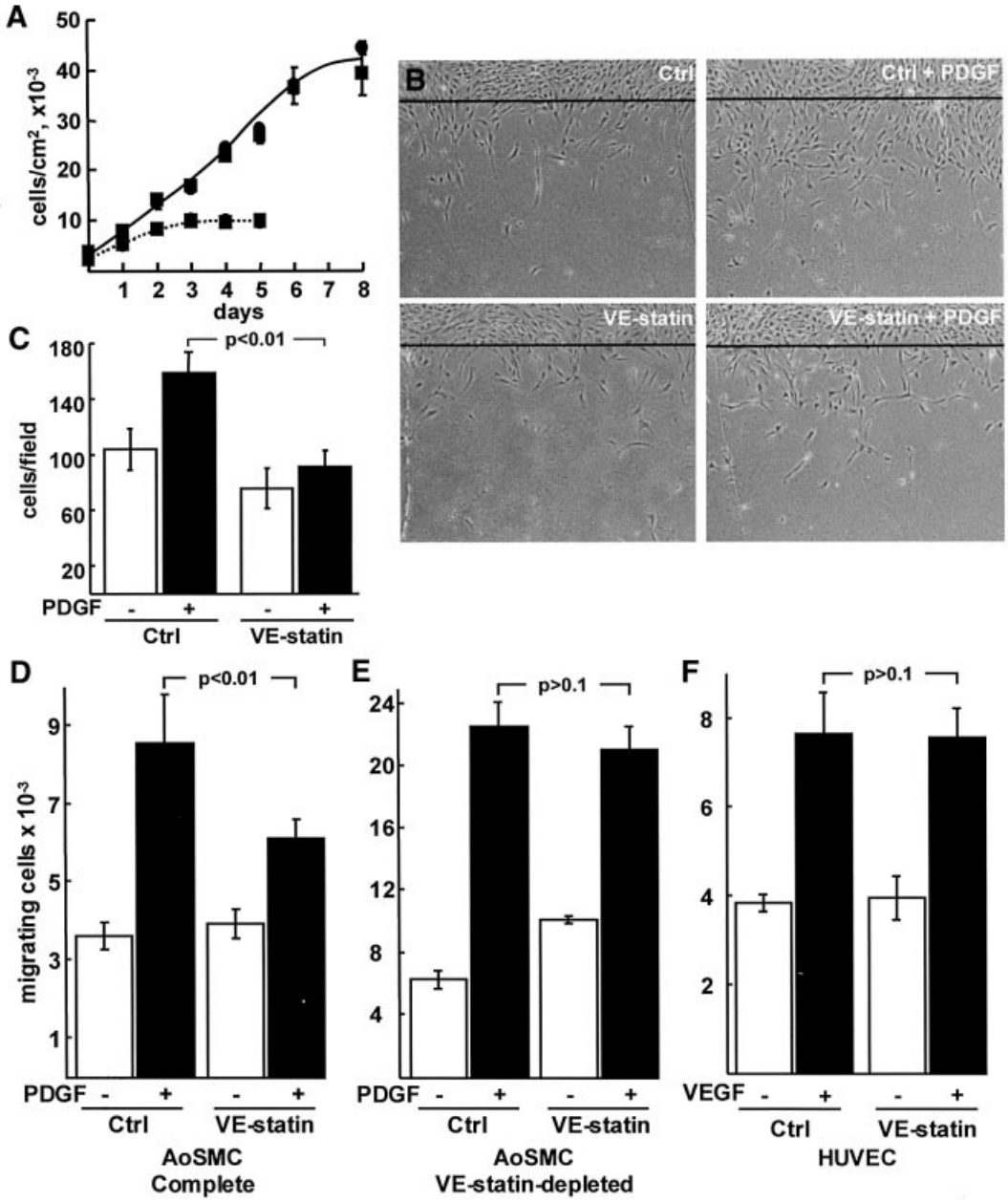

Fig. 7. VE-statin inhibits PDGF-induced AoSMC migration, not proliferation. (**A**) AoSMCs were plated at low density and cultured in control (filled circles) or VE-statin-containing (filled squares) medium, in the presence of 50 ng/ml PDGF-BB and in the absence (dotted line) or presence of 2% donor calf serum (solid line). Data are presented as mean ± 95% confidence intervals. (**B**) AoSMC were cultured until a confluent monolayer was formed. The monolayers were then wounded with a razor blade and the cells cultured in serum-free control (Ctrl) or VE-statin-containing medium in the absence (–, open bars) or presence of 80 ng/ml PDGF-BB (+, filled bars). The cells were cultured for 2 days and the monolayers observed by phase contrast microscopy. The bar indicates the location of the wound. (**C**) The migration rates in (B) were measured by counting the number of cells that had migrated beyond the wound mark after 48 h. A range of 3–7 identical fields per sample were recorded using a digital camera and analysed. The data are presented as mean ± 95% confidence intervals; they are representative of two experiments performed using two independent batches of conditioned medium. (**D**) AoSMCs (58 000 cells/cm^2) were plated in the upper chamber of culture inserts in control (Ctrl) or VE-statin-containing, serum-free medium and in the absence (–, open bars) or presence of 80 ng/ml PDGF-BB (+, filled bars). Two days later, the cells that had crossed the porous membrane were counted. The data are presented as mean ± 95% confidence intervals; they are representative of three experiments performed using three independent batches of conditioned medium. (**E**) AoSMCs were plated in the upper chamber of culture inserts in conditions similar to those in (D), but incubated in VE-statin-depleted conditioned medium. The removal of VE-statin restores the stimulatory effects of PDGF on AoSMC migration. The data are presented as mean ± 95% confidence intervals; they are representative of two experiments performed using two independent batches of conditioned medium. (**F**) Primary HUVECs were assayed for migration using the Boyden chamber assay as above in control (Ctrl) or VE-statin-containing medium and in the absence (–, open bars) or presence of 10 ng/ml VEGF (+, filled bars) used as chemoattractant. The data are presented as mean ± 95% confidence intervals; they are representative of two independent experiments.

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 S1P, 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 S1P 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 (H5V) and EOMA endothelioma, 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 ACAAAAAGAAGAAGGCTACC and CAGCGGCAGTGTCCTGGGCG 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 (1.7×10^6 and 1.6×10^6 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.influenza* HA epitope-coding sequence in-frame with the VE-statin CDS into pcDNA3.

5′-RACE and gene fragments isolation

Nested 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 CGGAGCCCCACATGGTCTGCATC and TCTTTTTGTGGCCTGTGGTCCGG, and CCACATCAGCAGCACCTCCTGAGAG and GAGCCCCTCATGGCCTGTGCCTCCA, respectively. *VE-statin* gene isolation was performed both by probing λ libraries (1–1.8 × 10^6 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 ^{32}P-labelled VE-statin-a and -b probes using the TCCCACAGGGACAGGGACAGGGGATGGACAGAGCC and ATGCTGCCCAGGCCTGGCTGAGGCCACCTCTGCTG 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, H5V endothelial cells or control yeast tRNA, 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 ^{32}P-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 GCTGTCTGGTGGTGCCTATG and TTATTATGACAAAGATGGAG 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 db1 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-^{35}S 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). Gels were fixed, soaked in Amplify reagent (Amersham-Biosciences) and autoradiographed.

Transfection and production of conditioned medium

3T3 cells (15 000 cells/cm^2) were plated in 78.5 cm^2 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 EBM-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/cm^2 in 4 cm^2 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/cm^2) were plated in 28 cm^2 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/cm^2) 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 $VEGF_{165}$ (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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References

Behrendt,D. and Ganz,P. (2002) Endothelial function. From vascular biology to clinical applications. *Am. J. Cardiol.*, **90**, 40L–48L.

Bjorkerud,S. (1991) Effects of transforming growth factor-β1 on human arterial smooth muscle cells *in vitro*. *Arterioscler. Thromb.*, **11**, 892–902.

Breier,G., Breviario,F., Caveda,L., Berthier,R., Schnürch,H., Gotsch,U., Vestweber,D., Risau,W. and Dejana,E. (1996) Molecular cloning and expression of murine vascular endothelial-cadherin in early stage development of cardiovascular system. *Blood*, **87**, 630–641.

Carmeliet,P. (2003) Angiogenesis in health and disease. *Nature Med.*, **9**, 653–660.

Carmeliet,P. *et al*. (1996) Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature*, **380**, 435–439.

Carmeliet,P. *et al*. (1999) Targeted deficiency or cytosolic truncation of the VE-cadherin gene in mice impairs VEGF-mediated endothelial survival and angiogenesis. *Cell*, **98**, 147–157.

Conway,E.M., Collen,D. and Carmeliet,P. (2001) Molecular mechanisms of blood vessel growth. *Cardiovasc. Res.*, **49**, 507–521.

Davis,S. *et al*. (1996) Isolation of angiopoietin-1, a ligand for the Tie2 receptor by secretion-trap expression cloning. *Cell*, **87**, 1161–1169.

Dickson,M.C., Martin,J.S., Cousins,F.M., Kulkarni,A.B., Karlsson,S. and Akhurst,R.J. (1995) Defective haematopoiesis and vasculogenesis in transforming growth factor-β1 knock out mice. *Development*, **121**, 1845–1854.

Dumont,D.J., Gradwohl,G., Fong,G.H., Puri,M.C., Gertsenstein,M., Auerbach,A. and Breitman,M.L. (1994) Dominant-negative and targeted null mutations in the endothelial receptor tyrosine kinase, *tek*, reveal a critical role in vasculogenesis of the embryo. *Genes Dev.*, **8**, 1897–1909.

Dumont,D.J., Fong,G.H., Puri,M.C., Gradwohl,G., Alitalo,K. and Breitman,M.L. (1995) Vascularization of the mouse embryo: a study of flk-1, tek, tie and vascular endothelial growth factor expression during development. *Dev. Dyn.*, **203**, 80–92.

Fehon,R.G., Kooh,P.J., Rebay,I., Regan,C.L., Xu,T., Muskavitch,M.A. and Artavanis-Tsakonas,S. (1990) Molecular interactions between the protein products of the neurogenic loci Notch and Delta, two EGF-homologous genes in *Drosophila*. *Cell*, **61**, 523–534.

Ferrara,N., Carver-Moore,K., Chen,H., Dowd,M., Lu,L., O'Shea,K.S., Powell-Braxton,L., Hillan,K.J. and Moore,M.W. (1996) Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature*, **380**, 439–442.

Fong,G.-H., Rossant,J., Gertsenstein,M. and Breitman,M.L. (1995) Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature*, **376**, 66–70.

Fong,G.H., Zhang,L., Bryce,D.M. and Peng,J. (1999) Increased hemangioblast commitment, not vascular disorganization, is the primary defect in flt-1 knock-out mice. *Development*, **126**, 3015–3025.

Freedman,R.B. (1989) Protein disulfide isomerase: multiple roles in the modification of nascent secretory proteins. *Cell*, **57**, 1069–1072.

Gory-Fauré,S., Prandini,M.H., Pointu,H., Roullot,V., Pignot-Paintrand,I., Vernet,M. and Huber,P. (1999) Role of vascular endothelial-cadherin in vascular morphogenesis. *Development*, **126**, 2093–2102.

Hayes,A.J., Huang,W.Q., Mallah,J., Yang,D., Lippman,M.E. and Li,L.Y. (1999) Angiopoietin-1 and its receptor Tie-2 participate in the regulation of capillary-like tubule formation and survival of endothelial cells. *Microvasc. Res.*, **58**, 224–237.

Hellstrom,M., Kalen,M., Lindahl,P., Abramsson,A. and Betsholtz,C. (1999) Role of PDGF-B and PDGFR-β in recruitment of vascular smooth muscle cells and pericytes during embryonic blood vessel formation in the mouse. *Development*, **126**, 3047–3055.

Hobson,J.P., Rosenfeldt,H.M., Barak,L.S., Olivera,A., Poulton,S., Caron,M.G., Milstien,S. and Spiegel,S. (2001) Role of the sphingosine-1-phosphate receptor EDG-1 in PDGF-induced cell motility. *Science*, **291**, 1800–1803.

Huang,J. and Kontos,C.D. (2002) Inhibition of vascular smooth muscle cell proliferation, migration and survival by the tumor suppressor protein PTEN. *Arterioscl. Thromb. Vasc. Biol.*, **22**, 745–751.

Kim,I., Kim,H.G., Moon,S.O., Chae,S.W., So,J.N., Koh,K.N., Ahn,B.C. and Koh,G.Y. (2000a) Angiopoietin-1 induces endothelial cell sprouting through the activation of focal adhesion kinase and plasmin secretion. *Circ. Res.*, **86**, 952–959.

Kim,I., Kim,H.G., So,J.N., Kim,J.H., Kwak,H.J. and Koh,G.Y. (2000b) Angiopoietin-1 regulates endothelial cell survival through the phosphatidylinositol 3′-kinase/Akt signal transduction pathway. *Circ. Res.*, **86**, 24–29.

Kim,I., Kim,J.H., Moon,S.O., Kwak,H.J., Kim,N.G. and Koh,G.Y. (2000c) Angiopoietin-2 at high concentration can enhance endothelial cell survival through the phosphatidylinositol 3′-kinase/Akt signal transduction pathway. *Oncogene*, **19**, 4549–4552.

Kluk,M.J. and Hla,T. (2001) Role of the sphingosine 1-phosphate receptor EDG-1 in vascular smooth muscle cell proliferation and migration. *Circ. Res.*, **89**, 496–502.

Koyano-Nakagawa,N., Nishida,J., Baldwin,D., Arai,K. and Yokota,T. (1994) Molecular cloning of a novel human cDNA encoding a zinc finger protein that binds to the interleukin-3 promoter. *Mol. Cell. Biol.*, **14**, 5099–5107.

Kozak,M. (1989) The scanning model for translation: an update. *J. Cell Biol.*, **108**, 229–241.

Leveen,P., Pekny,M., Gebre-Medhin,S., Swolin,B., Larsson,E. and Betsholtz,C. (1994) Mice deficient for PDGF B show renal, cardiovascular and hematological abnormalities. *Genes Dev.*, **8**, 1875–1887.

Li,D.Y., Sorensen,L.K., Brooke,B.S., Urness,L.D., Davis,E.C., Taylor,D.G., Boak,B.B. and Wendel,D.P. (1999) Defective angiogenesis in mice lacking endoglin. *Science*, **284**, 1534–1537.

Lindahl,P., Johansson,B.R., Levéen,P. and Betsholtz,C. (1997) Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. *Science*, **277**, 242–245.

Liu,Y. *et al*. (2000) Edg-1, the G protein-coupled receptor for sphingosine-1-phosphate, is essential for vascular maturation. *J. Clin. Invest.*, **106**, 951–961.

Maisonpierre,P.C. *et al*. (1997) Angiopoietin-2, a natural antagonist for Tie2 that disrupts *in vivo* angiogenesis. *Science*, **277**, 55–60.

Mattot,V., Raes,M., Henriet,P., Eeckhout,Y., Stéhelin,D., Vandenbunder,B. and Desbiens,X. (1995) Expression of interstitial collagenase is restricted to skeletal tissue during mouse embryogenesis. *J. Cell Sci.*, **108**, 529–535.

Orlidge,A. and D'Amore,P.A. (1987) Inhibition of capillary endothelial cell growth by pericytes and smooth muscle cells. *J. Cell Biol.*, **105**, 1455–1462.

Papapetropoulos,A., Fulton,D., Mahboubi,K., Kalb,R.G., O'Connor,D.S., Li,F., Altieri,D.C. and Sessa,W.C. (2000) Angiopoietin-1 inhibits endothelial cell apoptosis via the Akt/survivin pathway. *J. Biol. Chem.*, **275**, 9102–9105.

Ryu,Y., Takuwa,N., Sugimoto,N., Sakurada,S., Usui,S., Okamoto,H., Matsui,O. and Takuwa,Y. (2002) Sphingosine-1-phosphate, a platelet-derived lysophospholipid mediator, negatively regulates cellular Rac activity and cell migration in vascular smooth muscle cells. *Circ. Res.*, **90**, 325–332.

Sato,T.N., Qin,Y., Kozak,C.A. and Audus,K.L. (1993) tie-1 and tie-2 define another class of putative receptor tyrosine kinase genes expressed in early embryonic vascular system. *Proc. Natl Acad. Sci. USA*, **90**, 9355–9358.

Sato,T.N. *et al*. (1995) Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. *Nature*, **376**, 70–74.

Sato,Y. and Rifkin,D.B. (1989) Inhibition of endothelial cell movement by pericytes and smooth muscle cells: activation of a latent transforming growth factor-β1-like molecule by plasmin during co-culture. *J. Cell Biol.*, **109**, 309–315.

Sato,Y., Tsuboi,R., Lyons,R., Moses,H. and Rifkin,D.B. (1990) Characterization of the activation of latent TGF-β by co-cultures of endothelial cells and pericytes or smooth muscle cells: a self-regulating system. *J. Cell Biol.*, **111**, 757–763.

Schnürch,H. and Risau,W. (1993) Expression of tie-2, a member of a novel family of receptor tyrosine kinases, in the endothelial cell lineage. *Development*, **119**, 957–968.

Shalaby,F., Rossant,J., Yamaguchi,T.P., Gertsenstein,M., Wu,X.-F.,

Breitman,M.L. and Schuh,A.C. (1995) Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature*, **376**, 62–66.

Soriano,P. (1994) Abnormal kidney development and hematological disorders in PDGF β-receptor mutant mice. *Genes Dev.*, **8**, 1888–1896.

Spiegel,S. and Milstien,S. (2003) Sphingosine-1-phosphate: an enigmatic signalling lipid. *Nature Rev. Mol. Cell. Biol.*, **4**, 397–407.

Stenflo,J., Stenberg,Y. and Muranyi,A. (2000) Calcium-binding EGF-like modules in coagulation proteinases: function of the calcium ion in module interactions. *Biochim. Biophys Acta*, **1477**, 51–63.

Suri,C., Jones,P.F., Patan,S., Bartunkova,S., Maisonpierre,P.C., Davis,S., Sato,T.N. and Yancopoulos,G.D. (1996) Requisite role of angiopoietin-1, a ligand for the Tie2 receptor, during embryonic angiogenesis. *Cell*, **87**, 1171–1180.

Takashima,S. *et al*. (2002) Targeting of both mouse neuropilin-1 and neuropilin-2 genes severely impairs developmental yolk sac and embryonic angiogenesis. *Proc. Natl Acad. Sci. USA*, **99**, 3657–3662.

Teichert-Kuliszewska,K. *et al*. (2001) Biological action of angiopoietin-2 in a fibrin matrix model of angiogenesis is associated with activation of Tie2. *Cardiovasc. Res.*, **49**, 659–670.

Wilkinson,D.G. and Nieto,M.A. (1993) Detection of messenger RNA by *in situ* hybridization to tissue sections and whole mounts. *Methods Enzymol.*, **225**, 361–373.

Witzenbichler,B., Maisonpierre,P.C., Jones,P., Yancopoulos,G.D. and Isner,J.M. (1998) Chemotactic properties of angiopoietin-1 and -2, ligands for the endothelial-specific receptor tyrosine kinase Tie2. *J. Biol. Chem.*, **273**, 18514–18521.

Xiong,J.W., Leahy,A., Lee,H.H. and Stuhlmann,H. (1999) Vezf1: a Zn finger transcription factor restricted to endothelial cells and their precursors. *Dev. Biol.*, **206**, 123–141.

Yamaguchi,T.P., Dumont,D.J., Conlon,R.A., Breitman,M.L. and Rossant,J. (1993) Flk-1, an flt-related receptor tyrosine kinase is an early marker for endothelial cell precursors. *Development*, **118**, 489–498.

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