Functionally defining the endothelial transcriptome, from Robo4 to ECSCR

Ana Raquel Verissimo, John M.J. Herbert, Victoria L. Heath, John A. Legg, Helen Sheldon, Maud Andre, Rajeeb Kumar Swain and Roy Bicknell

Institute for Biomedical Research, Birmingham University Medical School, Vincent Drive, Birmingham B15 2TT, U.K.

Abstract

We have applied search algorithms to expression databases to identify genes whose expression is restricted to the endothelial cell. Such genes frequently play a critical role in endothelial biology and angiogenesis. Two such genes are the roundabout receptor Robo4 and the ECSCR (endothelial-cell-specific chemotaxis regulator). Endothelial cells express both Robo1 and Robo4, which we have knocked down using siRNA (small interfering RNA) and then studied the effect in a variety of in vitro assays. Both Robo4 and Robo1 knockdown inhibited in vitro tube formation on Matrigel™. Transfection of Robo4 into endothelial cells increased the number of filopodial extensions from the cell, but failed to do so in Robo1-knockdown cells. Separate immunoprecipitation studies showed that Robo1 and Robo4 heterodimerize. We conclude from this and other work that a heteroduplex of Robo1 and Robo4 signals through WASP (Wiskott–Aldrich syndrome protein) and other actin nucleation-promoting factors to increase the number of filopodia and cell migration. Knockdown of the transmembrane ECSCR protein in endothelial cells also reduced chemotaxis and impaired tube formation on Matrigel™. Yeast two-hybrid analysis and immunoprecipitation studies showed that, in contrast with the roundabouts, ECSCR binds to the actin-modulatory filament A. We conclude that all three of these genes are critical for effective endothelial cell migration and, in turn, angiogenesis.

Endothelial markers and genes

Angiogenesis is the development of new blood vessels from existing vasculature [1]. This process is fundamental for normal development and healing, but it also occurs in over 70 different disease states, including diabetic retinopathy, rheumatoid arthritis, psoriasis and cancer [2]. The endothelium is a single layer of simple squamous endothelial cells that line the interior surface of blood vessels of the entire human vascular system [1]. A number of endothelial markers have been known for many years, including vWF (von Willebrand factor) an adhesive glycoprotein, Fli-1 a transcription factor, several receptors including the VEGF (vascular endothelial growth factor) receptors [Flt-1 (Fms-like tyrosine kinase 1), KDR (kinase insert domain receptor)] and the angiopoietin receptors Tie1 and Tie2/TEK (endothelial tyrosine kinase). Since vascular endothelium is not a homogeneous tissue, Kuzu et al. [3] reported the diagnostic usefulness of using a panel of antibodies against vWF, CD31 and CD34 to identify tumours of vascular origin, as they gave reliable immunostaining even of formalin-fixed paraffin-wax-embedded sections.

The advent of modern sequencing and expression analysis combined with advances in bioinformatics data mining has led to the recent discovery of several novel endothelial genes. Our group has used a bioinformatics data mining strategy that combined EST (expressed sequence tag) cluster expression analysis in the human Unigene index and SAGEmap xProfiler to identify novel endothelial-specific genes. Four novel genes were identified: ECSMs (endothelial-cell-specific molecules) 1–3 and magic roundabout (similar to the axon-guidance protein roundabout Robo1) [4]. We have also identified EndoPDI (endothelial protein disulfide-isomerase), highly expressed in endothelial cells [5]. mRNA and protein levels of EndoPDI are induced by hypoxic conditions and it was shown to have a protective role only in cells exposed to hypoxia, in contrast with PDI (protein disulfide-isomerase) which displayed no differences between cell protection under hypoxic or normoxic conditions. Furthermore, we showed using in situ hybridization that EndoPDI expression is very rare in normal tissues, being expressed preferentially in tumour endothelium and other hypoxia-associated lesions. These data indicate that EndoPDI is a potential target for tumour endothelial therapies.

Robo4/Slit: anti- or pro-angiogenic?

The protein similar to Robo1 identified by bioinformatics was shorter than the other members of the roundabout

Key words: angiogenesis, chemotaxis, endothelial-cell-specific chemotaxis regulator (ECSCR), WASP, Robo4/Slit

Abbreviations used: ARRA, apoptosis regulator through modulating IAP (inhibitor of apoptosis) expression; CIP, cellular inhibitor of apoptosis; ECSTM, endothelial-cell-specific molecule; ECSSC, endothelial-cell-specific chemotaxis regulator; EndoPDI, endothelial protein disulfide-isomerase; ERK, extracellular-signal-regulated kinase; G3, glutathione transferase; HEK, human embryonic kidney; HUVEC, human umbilical vein endothelial cell; MAPK, mitogen-activated protein kinase; Robo, roundabout; siRNA, small interfering RNA; VEGF, vascular endothelial growth factor; vWF, von Willebrand factor; WASP, Wiskott–Aldrich syndrome protein.

1 To whom correspondence should be addressed (email: r.bicknell@bham.ac.uk).
**Figure 1** | Proposed models describing the current knowledge of Robo4 signalling in endothelial cells

The three models are based mainly on the work of Jones et al. [14], Sheldon et al. [19] and Kaur et al. [21], as indicated. Arp2/3, actin-related protein 2/3 complex; IRSp53, insulin receptor substrate of 53 kDa; NWASP, neuronal WASP; WIP, WASP-interacting protein.

family and was named Robo4. *In vitro*, it was expressed only in endothelial cells. The endothelial specificity of Robo4 *in vivo* was confirmed by *in situ* hybridization, immunohistochemistry and by constructing a mouse expressing LacZ from the Robo4 promoter [6,7]. Interestingly, Robo4’s primary location in adult tissues corresponds to sites of active angiogenesis, such as tumour vessels [6]. Seth et al. [8] also observed Robo4 overexpression in tumour endothelial adult cells in a range of solid tumours compared with that on endothelium in normal tissues. Wang et al. [9] found that, in HUVECs (human umbilical vein endothelial cells), binding of Slit2 to Robo1 induces cell migration. Robo1 and Robo 4 are both up-regulated in colorectal cancer tumours, whereas in mouse development, it is not necessary [14]. Although the deletion of Robo4 in mouse embryos is not lethal, Robo4<sup>AP/AS</sup>-knockout mice display defects in vascular integrity, especially in pathological conditions associated with vascular leakage induced by VEGF. Robo4/Slit2 inhibited cell migration, tube formation and vascular leak *in vivo* by blocking Src family kinase activation (Figure 1). Interestingly, it was found that Robo4 is expressed in stalk, but not tip, cells, as would be expected by analogy to neural roundabouts [14].

In early studies Park et al. [12] suggested that, despite structural and location differences between Robo4 and the other family members, Slit2 would also be a ligand for this protein. Cells treated with Slit2 showed impaired migration. Accordingly, Seth et al. [8] found that either the addition of Slit2 or Robo4 overexpression are able to block VEGF-mediated cell migration. They suggest that this is achieved via the Ras/Raf/MEK [MAPK (mitogen-activated protein kinase)/ERK (extracellular-signal-regulated kinase)/ERK pathway, since Robo4 down-regulates ERK and FAK (focal adhesion kinase). So far, as pointed out by Bicknell [15], no direct molecular interaction between Slit2 and Robo4 has been demonstrated, and X-ray studies suggest an absence of or at best weak binding. The study by Park et al. [12] was performed in HEK (human embryonic kidney)-293 cells with overexpressed proteins, but it is unknown whether this system represents *in vivo* interactions in the endothelial cells. In addition, there is no spatial or temporal overlapping of Slits and Robo4 expression in zebrafish embryos [13].

In contrast with Park et al. [12], we were unable to show an interaction between Robo4 and any Slit either by immunoprecipitation or BiaCore analysis. Under the same conditions, we observed interaction between Robo1 and Slits 1, 2 and 3 [16]. The administration of soluble extracellular domain of Robo4 resulted in angiogenesis inhibition *in vivo* and impaired endothelial cell proliferation, tube formation and endothelial cell migration stimulated by VEGF or bFGF (basic fibroblast growth factor) [16]. These results suggest some caution in interpreting the findings by Jones et al. [14], as discussed in [15]. Also, the lack of evidence for a molecular interaction suggests a Slit2-independent mode of action for Robo4. Accordingly, Robo4 knockdown in endothelial cells impaired the chemotactic response to serum, which was not observed on Robo1 knockdown. Slit2 binds to endothelial cells expressing both Robos 1 and 4, but not to Robo4-knockdown cells. Interestingly, Slit2 seems to act in a Robo4-independent manner as it is able to stimulate migration in the absence of Robo4 [17].

Our data are supported by a recent study in retinal cells which demonstrates that siRNA (small interfering RNA) knockdown of Robo4 inhibits attachment, spreading, migration, proliferation and tube formation. In addition, in hypoxia, but not in normoxia, Robo4 seems to increase endothelial cell survival [18].

In order to shed some light on the molecular mechanisms by which roundabout proteins regulate endothelial processes, we knocked down Robo1 and Robo4 in HUVECs [19]. Both siRNA knockdowns resulted in impaired migration and tube formation. WASP (Wiskott–Aldrich syndrome protein) and WASP-interacting protein actin-nucleating complex were
both identified as binding proteins for Robo4 intracellular domain by yeast two-hybrid and GST (glutathione transferase) pull-down. We also showed that Robo1 and Robo4 heterodimerize and Robo1 is essential for Robo4-induced filopodia formation. Taking these results together, we hypothesize that Slit2 exerts its effects by binding to Robo1 in the heterodimeric complex with Robo4, rather than to Robo4 itself (Figure 1). This is a valid assumption, taking into account that both Robos are expressed in the vertebrate endothelium [9].

In our GST pull-down, we also identified Mena, which signals downstream from Cdc42 and IRSp53 (insulin receptor substrate of 53 kDa) to initiate filopodia formation [20]. Kaur et al. [21] found that Robo4 activates Cdc42 and Rac1 Rho GTPases in zebrafish endothelial cells and lower amounts of these latter proteins are present in Robo4 knocked-down embryos (Figure 1). Furthermore, angioblasts isolated from these embryos search actively for direction, which suggests a role for Robo4–Rho GTPases in attractive vascular guidance. Rho GTPases, upon activation, target many effector proteins to induce actin cytoskeleton changes. A group of such proteins are the WASP family [22].

The current literature on Robo4 has been somewhat controversial, especially regarding whether Slit2 is a real ligand for Robo4 and their anti- or pro-angiogenic effects. A better understanding of the molecular mechanisms underlying the role of Robo4 in angiogenesis is needed. Nevertheless, the weight of evidence suggests an involvement in cytoskeletal rearrangements that modulate filopodia formation.

A novel endothelial-cell-specific chemotaxis regulator
The endothelial specificity of ECSCR (endothelial-cell-specific chemotaxis regulator) (formerly ECSM2) has been confirmed [23]. ECSCR is a glycosylated type I transmembrane protein concentrated in filopodia. Knockdown by siRNA resulted in reduced chemotaxis and impaired tube formation on Matrigel™. A yeast two-hybrid screen identified filamin A as an interacting protein for the intracellular domain. This was confirmed by GST pull-down. These data implicate a role for ECSCR in angiogenesis via modulation of the actin cytoskeleton (Figure 2). Mouse and zebrafish orthologues were identified. The alignment of ECSCR orthologues indicates that the transmembrane and intracellular domains show greater conservation than the extracellular domain [24].

These results were confirmed independently by Ma et al. [25]. They observed that transfection of ECSCR–GFP (green fluorescent protein) into HEK-293 cells gave rise to the formation of filopodia and destruction of actin stress fibres formed by filament bundling. These cells contained diminished overall tyrosine phosphorylation signalling compared with the controls. Additionally, Ma et al. [25] described that ECSCR can cross-talk with the EGFR (epidermal growth factor receptor) to attenuate the EGF (epidermal growth factor)-induced cell migration, possibly by inhibiting the Shc/Ras/ERK (MAPK) pathway (Figure 2).

A recent paper described a protein called ARIA [apoptosis regulator through modulating IAP (inhibitor of apoptosis) expression] as a novel gene [26]. ARIA is in fact identical with ECSCR, although the authors failed to acknowledge the earlier papers first describing ECSM2/ECSCR in 2000 [4] and 2008 [24]. ARIA/ECSCR is expressed in mouse embryonic vasculature and also in endothelial cells in vitro and in vivo. The molecular size of the protein (∼60 kDa) is in accordance with our data. ECSCR siRNA knockdown in HUVECs resulted in significant reduction of apoptosis. The authors did not observe any effects of siRNA knockdown on cell migration or proliferation and, unfortunately, they gave no description on how the Boyden chamber assays were performed other than that the attractant was VEGF [26]. In contrast, Armstrong et al. [24] showed that ECSCR knockdown strongly inhibits migration in a Boyden chamber when fetal bovine serum is the chemoattractant. However, knockdown had no effect on a scratch wound healing assay (V.L. Heath, unpublished work). We conclude that ECSCR probably mediates endothelial chemotaxis, but not chemokinesis. The chemoattractant present in serum has not been identified, but from the data of Ikeda et al. [26], it is presumably not VEGF.

The expression of cIAP (cellular IAP) -1 and -2 was found to be increased in knocked down endothelial cells and 20S PMSA-7 (proteasome subunit α7) was identified as a binding partner for ECSCR intracellular domain by a yeast two-hybrid screening and confirmed by co-precipitation [26]. No mention of interaction of ECSCR and filamin A was reported in this study. ECSCR appears to regulate endothelial cell survival by modulating cIAP-1 and cIAP-2 pro-apoptotic protein degradation by the proteasome (Figure 2). Like Ikeda et al. [26], we also observed tube

Figure 2 | Proposed models describing the current knowledge of ECSCR signalling in endothelial cells
The three models are based mainly in the work of Armstrong et al. [24], Ma et al. [25] and Ikeda et al. [26], as indicated. EGFR, epidermal growth factor receptor; PMSA-7, proteasome subunit α7.
formation disruption after 12 h in siRNA-treated HUVECs. However, they report tube stabilization on these cells after 4 days. The structure stability of the Matrigel™ we use does not allow us to perform an assay longer than 24 h. Also, they observed that non-transfected cells have greater apoptosis, therefore there are fewer living cells available to form tubes. That may be an explanation for why those cells display decreased tube formation compared with siRNA-treated ones. Matrigel™ plugs, mouse ischaemic retina model and tumour xenografts suggest that siRNA knockdown enhances angiogenesis in vivo by decreasing apoptosis.

During 2008, at least three different groups submitted their work on ECSCR for publication. So far, even though there is already some discrepancy in some results, ECSCR seems to be an interesting and promising target for angiogenesis therapies, due to its endothelial specificity and role in cell motility and apoptosis.

Funding

This work was funded by Cancer Research UK [programme grant number C4719/A6766 (to R.B.)].

References


Received 6 July 2009
doi:10.1042/BSI0371214

©The Authors Journal compilation ©2009 Biochemical Society