The emerging role of tetraspanin microdomains on endothelial cells

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Abstract
Tetraspanins function as organizers of the cell surface by recruiting specific partner proteins into tetraspanin-enriched microdomains, which regulate processes such as cell adhesion, signalling and intracellular trafficking. Endothelial cells appear to express at least 23 of the 33 human tetraspanins, and a number of recent studies have demonstrated their importance in endothelial cell biology. Tetraspanin CD151 is essential for pathological angiogenesis, which may in part be due to regulation of its main partner proteins, the laminin-binding integrins α3β1, α6β1 and α6β4. CD9 and CD151 are essential for leucocyte recruitment during an inflammatory response, through the formation of pre-assembled nano-platforms containing the adhesion molecules ICAM-1 (intercellular adhesion molecule 1) and VCAM-1 (vascular cell adhesion molecule 1), which ultimately coalesce to form docking structures around captured leucocytes. Tetraspanin CD63 also facilitates leucocyte capture by promoting clustering of the adhesion molecule P-selectin. Finally, Tspan12 is required for blood vessel development in the eye, through regulation of Norrin-induced Frizzled-4 signalling, such that Tspan12 mutations can lead to human disease. Future studies on these and other endothelial tetraspanins are likely to provide further novel insights into angiogenesis and inflammation.

Introduction
Endothelial cells line all blood and lymphatic vessels and are central to the regulation of vascular development and function. They are important in maintaining vascular integrity, in regulating contraction and relaxation of vascular smooth muscle to control blood pressure, in promoting leucocyte transendothelial migration during an immune response, and in angiogenesis, which is the growth of new vessels from existing vasculature. Regulation of endothelial cell function is thus important in several major diseases. These include cancer, by the provision of a blood supply to a developing tumour through angiogenesis, and in inflammatory diseases such as atherosclerosis that can lead to heart attack and stroke [1–3].

The role of endothelial cells in health and disease is regulated by a large repertoire of cell-surface glycoproteins and associated signalling machinery. An emerging concept in the study of membrane proteins is that the fact that many are not randomly positioned on the plasma membrane, but are instead compartmentalized into distinct regions, or microdomains. Examples of such microdomains include neurological and immune synapses [4], lipid rafts [5], caveolae [6] and microdomains formed by tetraspanin proteins [7]. On endothelial cells, understanding precisely how proteins are regulated within membrane microdomains may provide new treatments for diseases ranging from inflammation to cancer. Indeed, recent publications have highlighted a key role for tetraspanins in such processes, and these studies are the focus of the present review.

Tetraspanins as membrane organizers
Tetraspanins are transmembrane proteins found in animals, plants and certain multicellular fungi, and are highly conserved through evolution [7–10]. The general structure of tetraspanins is characterized by four transmembrane domains, intracellular termini and two extracellular loops (Figure 1). Together, these regions form a compact rod-shaped tetraspanin structure [11] of between approximately 25 and 55 kDa, depending on the extent of N-linked glycosylation. The intracellular termini are palmitoylated, which facilitates tetraspanin–tetraspanin interactions and the formation of so-called tetraspanin microdomains or the ‘tetraspanin web’. The large extracellular region contains a CCG (Cys-Cys-Gly) motif and between two and six other cysteine residues, which form disulfide bonds that are important in maintaining a mushroom-like structure composed of five α-helices [12].

The large extracellular domain appears to be important for the interaction of tetraspanins with specific partner proteins, which include some integrins and other adhesion molecules, IgSF (immunoglobulin superfamily) proteins, transmembrane enzymes and various other single and multi-transmembrane proteins [8,9]. For many of these partner proteins, the mechanism by which they are regulated

Key words: CD9, CD63, CD151, endothelium, tetraspanin, Tspan12
Abbreviations used: eNOS, endothelial nitric oxide synthase; FEVR, familial exudative vitreoretinopathy; Fzd4, Frizzled-4; HUVEC, human umbilical vein endothelial cell; ICAM-1, intercellular adhesion molecule 1; IgSF, immunoglobulin superfamily; Lp5, low-density-lipoprotein receptor-related protein 5; SAGE, serial analysis of gene expression; UP, uroplakin; VCAM-1, vascular cell adhesion molecule 1

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Figure 1 | Diagrammatic representation of major endothelial tetraspanins and their partner proteins

CD9 and CD151 enhance leucocyte capture by promoting clustering of IgSF adhesion molecules ICAM-1 and VCAM-1. CD63 also enhances leucocyte capture by clustering P-selectin. Tspan12 is essential for development of the retinal vasculature by promoting Norrin-induced signalling by Fzd4 and its co-receptor Lrp5. CD151 is required for pathological angiogenesis, by a mechanism that may involve its regulation of laminin-binding integrins, such as α3β1, α6β1 and α6β4.

by tetraspanins is unknown, and it is unclear whether they associate with one or more specific tetraspanins or with tetraspanin microdomains in general. However, some direct associations have been convincingly demonstrated, namely the laminin-binding integrins α3β1, α6β1 and α6β4 with tetraspanin CD151, the IgSF protein CD19 with tetraspanin CD81, IgSF proteins EWI-2 and EWI-F with related tetraspanins CD9 and CD81, and the urothelial uroplakins UPII and UPIII with tetraspanins UPIa and UPIb respectively. Moreover, in some cases, it has emerged that association with tetraspanins is essential for normal glycosylation (α3β1, CD19, UPII and UPIII), trafficking to the plasma membrane (CD19, UPII and UPIII), adhesive function, signalling and internalization (α3β1 and α6β1) [8,9]. Combining these findings with tetraspanin single-particle tracking data [13,14], a model has been proposed in which tetraspanins and their partners patrol the plasma membrane in relatively small clusters [7,8]. Under certain conditions, such as cross-linking of a partner protein by ligand binding, these clusters may be induced to coalesce into larger clusters, or microdomains, which function as platforms for processes such as adhesion, signalling or internalization. In this model, the tetraspanins can be regarded as having facilitator or fine-tuning function, through the generation of pre-existing complexes of surface proteins and signalling machinery, which enhance the efficiency of cellular functions [7,8].

Consistent with such a fundamental theoretical role for tetraspanins, gene-knockout studies across a range of species have yielded some striking phenotypes [8]. In addition, a human patient and mice deficient in CD81 have impaired B-cell immunity owing to the dramatic reduction in cell-surface expression of CD19, a co-receptor for the B-cell receptor signalling complex [8,15]. Many more interesting knockout phenotypes, in organisms as diverse as humans, mice, fruitflies, nematode worms, plants and fungi, remain in search of a molecular mechanism. Furthermore, knockout phenotypes are often subtle or undetectable, even in cell types in which the deleted tetraspanin is normally expressed at relatively high levels. In these cases, emerging evidence suggests that compensation by related tetraspanins may often be the explanation [7]. Alternatively, researchers may have to design more sophisticated experiments to reveal fine-tuning roles for some tetraspanins.

Tetraspanin expression on endothelial cells

Studies using monoclonal antibodies have definitively identified the following tetraspanins on endothelial cells: CD9, CD63, CD81, CD82, CD151, Tspan4 and Tspan8 [16–19]. However, there are no antibodies against approximately half of the 33 human tetraspanins. We have therefore investigated tetraspanin expression at the mRNA level by analysing large-scale transcriptomic data from publicly available SAGE (serial analysis of gene expression) experiments. HUVECs (human umbilical vein endothelial cells), the widely used primary cell model, were found to express 23 tetraspanins, whereas liver endothelial cells expressed 17 (Figure 2). The reason for the lower number in the latter is likely to be due to the substantially smaller size of the SAGE library. As discussed below, CD63, CD151 and CD9 are relatively well-characterized endothelial tetraspanins, and these were among the most highly expressed in the SAGE libraries (Figure 2). In addition, a number of others were highly expressed, but have not been described before on endothelial cells, including...
Figure 2 | Tetraspanin mRNA expression in endothelial cells
Analyses of publicly available LongSAGE data identified at least 23 endothelial tetraspanins. (A) Freshly isolated HUVECs were from six donors and the total number of SAGE tags in the library was 1289310 (NCBI GEO accession numbers GSM384148 and GSM384149). (B) Liver endothelial cells were isolated from a single donor and the total SAGE tag number in the library was 77759 (NCBI GEO accession number GSM384147).

The role of tetraspanins in angiogenesis
Pro-angiogenic molecules stimulate endothelial cells to proliferate, migrate and form a lumen to create new vessels from the existing vasculature. This requires the co-ordination of multiple adhesion molecules and signalling proteins [1], and there is now considerable evidence that CD151 plays a role in this process. The first definitive data came from analyses of the CD151-knockout mouse [25]. Although CD151 does not seem to be important for development of the vasculature, CD151 was shown to be essential in pathological angiogenesis. When tumour cells were implanted subcutaneously in the knockout mouse, development of smaller tumours with reduced microvessel density was observed. Angiogenesis was also impaired in two other in vivo assays, the Matrigel plug and corneal micropocket assays, and ex vivo in the aortic ring assay. Consistent with a role for CD151 in angiogenesis, endothelial cells from the knockout mouse were functionally defective in a variety of in vitro assays that measured spreading, migration and tube formation [25]. Similarly defective two-dimensional tube formation was observed following CD151 knockdown in human microvascular endothelial cells [26] and in HUVECs (Figure 3).

A pro-angiogenic role for CD151 has been supported by studies that have used adenoviral delivery to overexpress CD151 in vivo. In an early study that used a rat model of myocardial infarction, CD151 delivery increased the number of microvessels in the ischaemic myocardium and appeared to improve heart function [27]. Similar conclusions were drawn from a follow-up study that used a pig model of myocardial infarction [28]. Most recently, CD151 delivery in a rat hindlimb ischaemia model was found to increase capillary density [29]. Moreover, a CD151 mutant that was defective in binding to laminin-binding integrins failed to show increased capillary density [29]. This suggests, but does not prove, that such integrins are important for CD151 pro-angiogenic function.

The precise mechanism by which CD151 regulates pathological angiogenesis is not clear. Since CD151 is essential for normal function of laminin-binding integrins, which are important for cell adhesion, proliferation and migration, one possible mechanism is defective integrin function. Indeed, biochemical studies have shown dramatically impaired integrin complex formation with other proteins in the absence of CD151, presumably because of a failure to localize to tetraspanin microdomains [25]. However, impaired integrin function is unlikely to be the only factor. Indeed, the recent endothelial-specific mouse knockouts for the laminin-binding integrin α3 and α6 chains show that they are actually negative regulators of pathological angiogenesis [30,31]. Comparison of α3 and α6 with CD151-knockout models is complicated, however, by the fact that the α3 knockout lacks α3β1, and the α6 knockout lacks α6β1 and α6β4, whereas the CD151 knockout expresses all three of these endothelial integrins, but in a functionally impaired state.

Angiogenesis-related cell signalling pathways have been studied to address the mechanism by which CD151 regulates...
angiogenesis. The activation of several signalling proteins was impaired when CD151-deficient endothelial cells were plated on Matrigel\textsuperscript{TM}, including the serine/threonine kinase Akt and its downstream target eNOS (endothelial nitric oxide synthase), and the small GTPases Rac and Cdc42 [25]. More recently, the GTPase findings were extended to include the RhoA/Rho-associated kinase/myosin light chain axis, the activation of which was increased in the absence of CD151 [26]. On the basis of these GTPase data, the authors propose a model whereby CD151 promotes stability of the vasculature by enhancing endothelial cell–cell adhesion and reducing contractility [26].

Although such signalling data can help to explain some phenotypes in the absence of CD151, it remains unclear exactly how the absence of CD151 results in aberrant signalling and impaired angiogenesis. It is possible that defects in a variety of CD151-associated proteins are responsible, including laminin-binding integrins and other proteins. For example, CD151 can interact with MT1-MMP (membrane-type 1 matrix metalloproteinase) [32], a matrix metalloprotease which can promote angiogenesis via multiple mechanisms that include degradation of the extracellular matrix. Finally, the recently reported requirement for CD9 in angiogenesis, identified through in vivo CD9 knockdown and the corneal micropocket assay [33], would suggest a general role for tetraspanin microdomains in angiogenesis.

Finally, an emerging theme in tetraspanin research concerns the potential for tetraspanins to regulate the composition and target cell selection of exosomes [34]. These are membrane-bound vesicles, of 30–100 nm in diameter, that are formed by exocytosis of multivesicular bodies from a wide range of cell types. Exosomes appear to exert their functions via the transport of proteins, mRNA and microRNA between cells, and have been implicated in angiogenesis. Importantly, exosomes contain relatively high levels of tetraspanins [34]. Zöller and colleagues have demonstrated that tumour cells expressing Tspan8, which is known to be associated with poor prognosis, can promote systemic angiogenesis [16]. Moreover, their Tspan8-positive exosomes are targeted to endothelial cells via $\alpha_4\beta_1$ (very late antigen 4) integrin interaction with VCAM-1 (vascular cell adhesion molecule-1) [35]. Such targeting induces proliferation, migration and sprouting of endothelial cells, and maturation of endothelial progenitor cells. The authors speculate that tumour-induced angiogenesis by Tspan8-positive exosomes has therapeutic potential for cancer treatment [35].

**Tetraspanins promote endothelial-leucocyte adhesion**

Extravasation of leucocytes from the bloodstream to sites of infection involves a complex series of events including leucocyte tethering to the inflamed endothelium, rolling, firm adhesion, crawling and transendothelial migration [3]. These events are dependent on multiple interactions between the leucocyte and the apical membrane of the endothelial cells. Of particular importance is an actin-based adhesion complex on endothelial cells that includes the IgSF proteins ICAM-1 (intercellular adhesion molecule 1) and VCAM-1, which bind to leucocyte integrins $\alpha_L\beta_2$ (LFA-1 [lymphocyte function-associated antigen 1]) and $\alpha_4\beta_1$ respectively. Formation of this complex reinforces adhesion of the leucocyte to the endothelium, to prevent bloodflow forcing detachment, and facilitates the process of extravasation [3].

Barriero et al. [36] have demonstrated that ICAM-1 and VCAM-1 co-localize with CD9 and CD151 at the edges of circular docking structures, which form on the apical...
endothelial cell surface upon attachment of leucocytes under inflammatory conditions. Knockdown of either tetraspanin resulted in partial reductions of ICAM-1 and VCAM-1 surface expression, accompanied by reduced leucocyte adherence and transmigration. In further experiments using a non-endothelial cell line model system, CD9 was shown to promote adhesion of ICAM-1 and VCAM-1 to their respective integrin ligands [36]. A follow-up study further characterized these tetraspanin-enriched docking structures using scanning electron microscopy, FRAP (fluorescence recovery after photobleaching) and FRET (fluorescence resonance energy transfer)—FLIM (fluorescence lifetime imaging microscopy) [13]. Barreiro et al. [13] showed that integrin binding of either ICAM-1 or VCAM-1 led to recruitment of the other into the docking structure, and this appeared to be independent of linkage to the actin cytoskeleton and was not mediated by ICAM-1 or VCAM-1 interactions. In fact, preferential CD9–ICAM-1 and CD151–VCAM-1 interactions resulted in pre-assembled complexes that were approximately 300 nm in diameter on endothelial cells [13], similar in size to tetraspanin-enriched complexes observed previously on the HeLa epithelial cell line [37]. The authors proposed that these pre-formed ‘sticky’ nano-platforms would facilitate leucocyte capture by accelerating the process of docking structure formation around the leucocyte [13].

In addition to promoting leucocyte binding, an endothelial tetraspanin was reported recently to promote tumour cell binding to lung endothelial cells, since this was reduced in a CD151-deficient mouse model for metastasis [38]. Taken together with the pro-angiogenic role for CD151 described in the previous section, this suggests that CD151 holds considerable promise as an anti-tumour drug target.

A further recent report has demonstrated that clustering of partner proteins to promote their adhesive function is not restricted to ICAM-1 and VCAM-1, but can now be extended to CD63 and P-selectin [39]. Unlike other endothelial tetraspanins, which are concentrated at cell–cell junctions, CD63 largely localizes to late endocytic organelles such as Weibel–Palade bodies and lysosomes [19]. P-selectin has a similar distribution, and is rapidly trafficked to the surface of activated endothelial cells, where it promotes leucocyte recruitment through binding to its leucocyte counter-receptor, PSGL-1 (P-selectin glycoprotein ligand 1). On such activated endothelial cells, CD63 and P-selectin were shown to associate using two techniques, a proximity ligation assay and immunogold labelling followed by scanning electron microscopy [39]. Moreover, the absence of CD63 resulted in reduced surface expression and clustering of P-selectin. As a consequence, P-selectin function appeared to be impaired, since leucocyte rolling and tethering to cremaster venules was dramatically reduced in CD63-knockout mice. Moreover, neutrophil extravasation in CD63-knockout mice was delayed in a peritonitis model, thus mimicking the phenotype of P-selectin-knockout mice [39]. Taken together with the regulation of ICAM-1 and VCAM-1 by CD9 and CD151, endothelial tetraspanins are proving to be key regulators of leucocyte recruitment during the inflammatory cascade.

**Tetraspanin regulation of vascular development**

During development of the mammalian retina, formation of the two intra-retinal capillary beds is regulated by the canonical Wnt signalling pathway, and alterations to this pathway disrupt retinal vascular development [40]. For example, mutations in the Wnt receptor Fzd4 (Frizzled-4), or its co-receptor Lrp5 (low-density-lipoprotein receptor-related protein 5), cause FEVR (familial exudative vitreoretinopathy) which is characterized by incomplete retinal vascular development, potentially leading to impaired vision and blindness. In addition to small, secreted Wnt ligands, Fzd4 can also be activated via the secreted cysteine knot protein Norrin, which is structurally unrelated to Wnts, and Norrin mutations lead to FEVR [40].

Junge et al. [20] have shown recently that the tetraspanin Tspan12 has an important role in retinal vascular development via regulation of Norrin-induced Fzd4 signalling. Tspan12-deficient mice showed retinal vasculature defects that were similar to those reported previously in mice deficient for Norrin, Fzd4 or Lrp5. A genetic interaction between Tspan12 and Norrin or Lrp5 was demonstrated by impaired retinal vasculature sprouting in compound Tspan12/Norrin and Tspan12/Lrp5 heterozygotes. In addition, Tspan12 promoted Norrin-induced Fzd4 signalling in a cell line model system, but had no effect on Wnt-induced signalling. Importantly, an interaction between Tspan12 and Fzd4/Lrp5 was demonstrated by co-immunoprecipitation in an overexpression system. Moreover, the capacity of Tspan12 to rescue signalling by mutants of Norrin and Fzd4, which were known to exhibit defective oligomerization, led the authors to propose that Tspan12 regulates the signalling pathway by promoting Fzd4 clustering [20].

Following this discovery in gene-targeted mice, four studies have identified autosomal dominant mutations in Tspan12 that are responsible for human FEVR [21–24]. Tspan12 thus becomes the fourth gene to be responsible for human FEVR, after Norrin, Fzd4 and Lrp5, and may account for approximately 3–6% of cases [22,24]. Since all types of mutation were identified, and disease severity was similar for different mutations, it was proposed that haploinsufficiency of Tspan12 causes FEVR [21]. However, it remains to be determined whether any of the missense mutations could be functioning as dominant-negatives, for example by disrupting complexes of wild-type Tspan12 with Fzd4. Moreover, further studies are required to determine the precise mechanism by which Tspan12 regulates Fzd4/Lrp5 signalling, and whether this pathway could be drug-targeted to treat the neovascularization that occurs in conditions such as diabetic retinopathy, age-related macular degeneration and retinopathy of prematurity.

**Future directions**

Researchers have so far focused their studies on the relatively highly expressed endothelial tetraspanins CD9, CD63 and CD151, for which monoclonal antibodies and knockout
mouse models are available. Such studies have revealed essential roles for CD151 in pathological angiogenesis and for CD9, CD63 and CD151 in leucocyte capture during an inflammatory response. Other endothelial tetraspanins remain largely unstudied, in part due to a lack of antibody reagents. Nevertheless, the recently reported role for Tspan12 in blood vessel development in the eye and subsequent identification of Tspan12 mutations causing human disease underlines the need to study the full complement of endothelial tetraspanins. Only then can we fully understand their organizing role on the cell surface and unlock their potential as therapeutic targets.

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