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Tetraspanins and vascular functions

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Tetraspanins are multiple membrane-spanning proteins that likely function as the organizers of membrane microdomains. Tetraspanins associate with other membrane-bound molecules such as cell-adhesion proteins, growth factor receptors, and Ig superfamily members and regulate key cellular processes such as adhesion, migration, and fusion. Tetraspanins are widely expressed in vascular and haematopoietic cells and are involved in both physiological and pathological processes related to angiogenesis, vascular injury, thrombosis, and haemostasis. A wide body of evidence suggests that tetraspanins directly regulate the development and functions of the vascular system and the pathogenesis of vascular diseases. This article reviews current understanding of the roles of tetraspanins in vascular functions.

1. Introduction

Tetraspanins comprise a group of integral membrane proteins that contain four conserved hydrophobic transmembrane domains (TM1-TM4), intracellular N- and C-termini, and small (EC1) and large (EC2) extracellular domains.[1–5] A schematic representation of tetraspanin structure is shown in *Figure 1*. The EC2 domain of most tetraspanins features four–six cysteine residues, glycosylation sites, and a highly conserved 'CCG' motif. The 'CCG' motif and conserved strong polar residues in the TM domains are considered to be the signature structural elements for all tetraspanins. The EC2 region, together with transmembrane regions, confers structural and functional specificities to tetraspanins and is also important in mediating tetraspanin interactions with other membrane-bound proteins such as integrins[1–5] and Ig superfamily proteins.[6] Tetraspanin-containing multimolecular complexes are designated as 'tetraspanin web' or 'tetraspanin enriched microdomains' (TEM) as the isolation of a given tetraspanin typically yields a range of other tetraspanins and associated transmembrane proteins.[2,7,8] However, with the exception of CD151-integrin α3 binding, most tetraspanin-transmembrane protein interactions are probably not based on the direct amino-acid-residue–amino-acid-residue interactions at the EC regions because these interactions

are relatively weak and readily disrupted under the so called 'high stringency' detergent conditions used for cell lysate preparation (e.g. 1% Triton X-100).

As the organizers of membrane microdomains, tetraspanins regulate vital cellular events such as adhesion, spreading, migration, and fusion (*Figure 2*). The biochemical characteristics of tetraspanin microdomains and the regulatory roles of tetraspanins in phenomena such as malignancy, immune cell regulation, and viral infections have been thoroughly reviewed in several articles.[9,10–13] Recent studies underline that tetraspanins actively traffic between plasma membrane and intracellular vesicular compartments. The trafficking of tetraspanins is accompanied by the vesicular release and trafficking of other membrane proteins.[14,15] Another emerging theme is that tetraspanins appear to regulate pericellular proteolysis near the plasma membrane leading to the altered cell motility and adhesiveness.[16–19] Although the biochemical and biophysical nature of tetraspanin activities remains unknown, existing evidence has expanded the function of tetraspanins as molecular 'facilitators' or 'organizers' at the plasma membrane to both intracellular vesicles and the extracellular micro-environment.
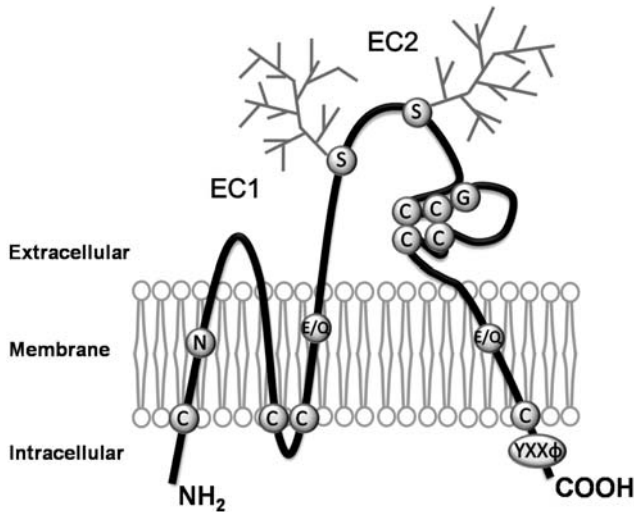
Tetraspanins exhibit diverse tissue distribution patterns. For example, CD9, CD63, and CD151 are widely expressed in a variety of tissue including vascular and haematopoietic cells, whereas others such as TSSC6 are expressed only in the haematopoietic cells. For the 33 identified human tetraspanins, the expression and function of most newly identified tetraspanins in the vascular system remain to be elucidated. Because vascular events such as neointimal

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formation, angiogenesis, and thrombosis are tightly regulated by cell adhesion proteins, e.g., integrins, and tetraspanins are key players in adhesion and migration,

tetraspanins may regulate important pathophysiological phenomena of the vascular system. Indeed, the roles of tetraspanins in thrombosis, vascular morphogenesis, and vascular remodelling are beginning to be increasingly understood. Considering the rapidly growing interest in vascular tetraspanin functions, we discuss the biology of various vascular tetraspanins and their functional role in regulating pathophysiological processes related to the cardiovascular system in this review, which is distinct from earlier reviews focusing on other functional aspects of tetraspanins such as malignancy, immune response, and viral infection.

2. Specific roles of tetraspanins in vascular functions

2.1 CD9

Human CD9 (Tspan29) was first detected by its reactivity with the monoclonal antibody (mAb) BA-1 raised against a leukaemia cell line NALM-6.[20] Using specific DNA probes, the gene encoding CD9 was localized to the short arm of chromosome 12.[21] The primary structure of CD9 was elucidated in 1991 when CD9 cDNA was isolated from a megakaryocytic library. The CD9 gene consists of eight coding regions (exons) that span about a 20 kb region of genome. Transcription of the CD9 gene generates a 1.4 kb mRNA that encodes a protein of 228 amino acids with an apparent molecular weight of a 24 kDa on SDS–PAGE.[22] This report also confirmed that the mature protein contained four hydrophobic sequences representative of four transmembrane spanning domains. CD9 expression has been demonstrated on several cells such as macrophages, eosinophils, basophils, fibroblasts, epithelial cells, neuronal cells, oocytes, and
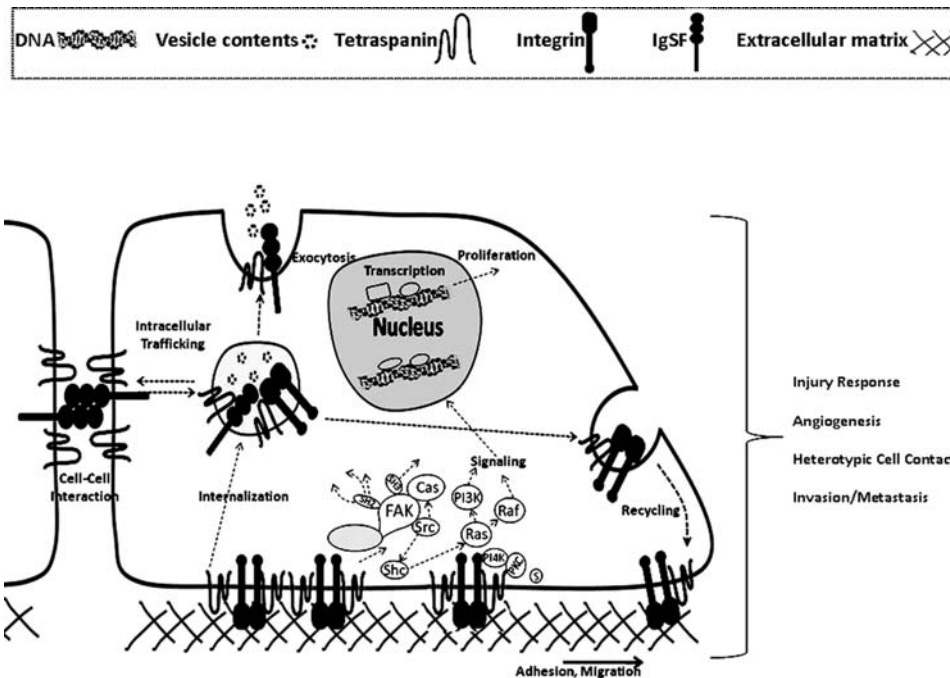

Figure 1 Schematic drawing of the structure of tetraspanins. Tetraspanins are composed of four transmembrane, an intracellular N- and C-termini, and two extracellular (one shorter, EC1, and one longer, EC2) domains. The conserved motifs or residues featured by tetraspanins are denoted. Most tetraspanins contain four or six cysteine residues in EC2, and two of those are in a highly conserved 'CCG' motif. Most of tetraspanins have glycosylation sites in EC2 as indicated as the squares, while CD9 is glycosylated in EC1. In each tetraspanin, there are several cysteine residues proximal to the interface of the inner leaflet and cytosol, which are the sites for palmitoylation and characteristic strong polar residues in the transmembrane domains. Tetraspanins also contains 'YXXΦ' sorting motif in the C-terminal cytoplasmic domain. C, Cysteine; E, Glutamic acid; G, Glycine; Q, Glutamine; N, Aspargine; S, Serine; Y, Tyrosine; X, any aminoacid; Φ, Hydrophobic aminoacid.

Figure 2 Schematic drawing showing cellular functions of tetraspanins. Tetraspanins associate with integrins, Ig superfamily proteins, and other transmembrane proteins. Additionally, tetraspanins are in molecular complex with cytoplasmic proteins, such as syntenin-1 (s) and signalling molecules. Tetraspanin containing complexes are designated as 'tetraspanin web' or 'tetraspanin enriched microdmians' (TEM). In addition to serving as organizers at the plasma membrane, tetraspanins are also enriched in the intracellular vesicles. Trafficking of tetraspanins between various cellular compartments tightly regulates exocytosis and trafficking of their associated partners. Tetraspanins in conjunction with other membrane proteins regulate cell–extracellular matrix adhesion, cell–cell interactions, cell migration, and modulate intracellular signaling events. Tetraspanins regulate phenomena such as vascular injury response, angiogenesis, heterotypic cell contacts, and tumour invasion and metastasis.

various tumours and tumour cell lines.[23] CD9 regulates phenomena such as cell morphology, migration, proliferation, cell fusion, and tumour cell metastasis.[2,24] CD9 is the first tetraspanin for which knock out (KO) mice were generated, and oocytes derived from CD9 KO mice have drastically reduced fertility rates.[25] In various cell systems, CD9 exists in molecular complexes with β1 and β3 integrins,[26,27] Ig superfamily proteins,[6] membrane-anchored heparin-binding epidermal growth factor (HB-EGF)-like growth factor,[28] and other tetraspanins.[2]

Smooth muscle cells (SMC) constitute a major component of muscular arteries. Endothelial damage in the vessel wall elicits phenotypic changes in the SMC leading to the formation of neointimal layer. Neointimal hyperplasia directly contributes to the pathogenesis of restenosis after angioplasty and predisposes vessels to occlusion by platelet-rich thrombi.[29] Therefore, membrane proteins regulating SMC phenotypes can profoundly influence the progression of atherothrombosis culminating in vessel occlusion. CD9 is expressed in cultured SMC as well as in the SMC of the vessel wall following vascular injury.[30,31] Specific effects of tetraspanin CD9 in the regulation of SMC phenotypes and in neointima formation following vascular injury have been studied using various model systems.[30–33] Earlier reports clearly established an association between CD9 and β1 integrins in SMC and suggested that CD9 levels are upregulated in SMC undergoing switch from the synthetic to proliferative phenotype. In addition, a peri-vascular electrical injury to the femoral artery suggested that the extent of neointima formation was not significantly altered in CD9 KO mice when compared with that of the wild-type controls.[32] More recently, antibody perturbation and adenoviral CD9 gene delivery studies in a carotid ligation injury model conclusively established a connection between CD9 and neointimal hyperplasia.[30] In vitro experimental results using human coronary artery SMC indicated that CD9 is important in regulating both proliferative and migratory phenotypes of SMC.[30] CD9 in SMC associates with various β1 integrins including the fibronectin receptor α5β1.[30,31] Since vascular injury is associated with tissue remodelling involving extracellular matrix (ECM) reorganization, we predict that CD9 and its associated integrins play a critical role in the overall healing response of the injured vessel.

CD9 in other model systems associates with certain growth factors belonging to epidermal growth factor (EGF) family. In this regard, CD9 specifically associates with the membrane anchored HB-EGF-like growth factor receptors and regulates its juxtacrine growth factor activity.[28] Such findings warrant future investigations to study the relative importance of other CD9 associated proteins in the vascular injury response. When exogenously expressed, CD9 significantly upregulates PI-3K/Akt signalling pathway in SMC as well as in other model systems.[23,30] As PI-3K/Akt plays an important pivotal role in the modulation of SMC cell migration and proliferation,[34,35] it can be speculated that augmentation of PI3-K/Akt activity by CD9 may play a critical role in CD9-induced SMC phenotypic changes. Future studies are required to delineate upstream molecular signalling mechanisms that lead PI-3K/Akt enhanced activation by CD9 and to define the downstream events that affect SMC phenotypes upon PI-3K activation.

Within the vascular tissue CD9 expression is not limited to the SMC of the medial layer. As studies have shown endothelial cells (ECs) derived from various tissue sources express CD9. For example, EC derived from bovine retina,[36] human umbilical vein,[37,38] saphenous vein grafts,[39] and lymphatic vessels[40] all express significant amounts of CD9. CD9 in EC is attributed to be important in several cellular events. Using anti-CD9 mAbs, Klein-Soyer et al.[39] showed a regulatory role for CD9 in EC migration. In cultured human umbilicial vein cells (HUVEC), CD9 is predominately localized to EC junctions.[41] Furthermore, anti-CD9 mAbs inhibited transwell haptotactic migration of EC to fibronectin as well as EC migration into wound areas in an in vitro scratch assay. However, in contrast with SMC, the effects of anti-CD9 mAbs appeared to be limited to the regulation of EC migration as EC proliferation was unaltered upon anti-CD9 mAb treatment.[39] However, anti-CD9 mAb treated HUVEC were reported to have a diminished platelet-induced proliferative response.[42]

In addition to EC migration, CD9 is implicated to modulate leucocyte transendothelial migration.[38] Both neutralizing anti-CD9 antibody and recombinant CD9 protein corresponding to the EC2 domain inhibited leucocyte migration.[38] During the process of leucocyte transendothelial cell migration, CD9 redistributed and clustered near the regions of EC contacts with leukocytes. Thus, by differentially clustering with the adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), CD9 enriched microdomains in the plasma membrane of EC may take part in regulating the endothelial barrier function.

In addition, the regulatory role of CD9 in mediating cell–cell interactions is not limited to homotypic aggregation events as evidence suggests that CD9 indeed can regulate heterotypic cell contacts. For example, very early observations suggested that CD9 mAbs can enhance neutrophil adhesion to the endothelium.[43] Furthermore, anti-CD9 mAbs inhibited transendothelial migration of melanoma cells suggesting that CD9 modulates tumor cell–EC interactions.[44] More recent evidence indicates that in activated EC, CD9 containing tetraspanin microdomains take part in the formation of specialized structures designated as endothelial adhesion platforms (EAP) that are biochemically distinct from lipid rafts.[45] Ligation of CD9 with antibodies resulted in tetraspanin clustering and recruitment of ICAM-1 and VCAM-1 to the docking structures providing the first direct evidence for the role of tetraspanins in heterotypic cellular interactions.[45]

As CD9 is abundantly expressed in platelets as well as in the endothelium, it remains to be determined whether similar heterotypic cell contact regulatory mechanisms are operational in platelet interactions with the vascular endothelium. A putative role for CD9 in the regulation of EC matrix metalloproteinase (MMP) activity has been investigated.[19] In mouse lung endothelial cells (MLEC), a specific knockdown of CD9 does not appear to interfere with the MMP-2 activity, whereas CD151 knockdown in these cells significantly enhances MMP2 activity and its collagenolytic activity. Thus, it appears that tetraspanins differentially regulate EC functions and this feature may be due to the ability of tetraspanins to reorganize membrane protein complexes. Other than SMC and EC, the involvement of CD9 in cardiac myocyte growth and heart muscle hypertrophy has also been reported.[46] Adenoviral-mediated exogenous CD9 expression in mouse cardiac myocytes inhibited cardiac

muscle hypertrophy and reduced mortality following myo-cardial infarction.[46]

2.2 CD63

CD63 or Tspan30 is primarily an intracellular tetraspanin and is mainly localized to late endosomal and lysosomal compartments. In the vascular system, CD63 is relatively well studied in platelets and EC. In EC, CD63 was identified as a component of Weibel-Palade bodies,[47,48] which store and secrete von Willebrand factor (vWF). Together with P-selectin, CD63 is one of the few proteins known to reside in the membrane of Weibel-Palade bodies.[48–50] Although it is not well understood how CD63 is targeted to Weibel-Palade bodies, vWF clearly plays a role in CD63 trafficking into these structures.[51,52] CD63 and P-selectin are initially recruited to the vWF storage granules that further develop into Weibel-Palade bodies, suggesting that the luminal vWF contains a sorting signal that targets CD63 and P-selectin.[47] During the exocytosis of vWF, the surface expressions of CD63 and P-selectin in EC are transiently enhanced due to the fusion between the Weibel-Palade body membrane and the plasma membrane,[47] which likely contributes to the leuco-cyte attachment to the endothelium.

Several lines of evidence suggest that CD63 is functionally involved in vascular cell adhesion. First, CD63 antibodies inhibit monocyte and neutrophil adhesion to serum-coated substratum and EC layer, respectively.[53,54] Second, CD63 physically associates with several integrins such as $\alpha3\beta1$, $\alpha6\beta1$, and $\alpha L\beta2$,[55,56] which are found in the vascular system. Third, a CD63 mAb induces integrin $\alpha L\beta2$-dependent neutrophil adhesion to HUVEC, and integrin $\alpha L\beta2$ is upregulated in CD63-activated neutrophils so that these neutrophils become competent to bind HUVEC.[56] Moreover, CD63 likely regulates cell migration based on the observations of altered cell motility upon CD63 antibody treatment and CD63 overexpression.[57,58]

Mechanistically, CD63 appears to regulate exocytosis at the cellular level. For example, the Weibel-Palade bodies in EC undergo exocytosis upon inflammatory stimulation while an antibody to CD63 inhibits IgE-mediated release of histamine in basophils.[59] Although the activities of protein phosphatases (such as receptor-linked tyrosine phosphatase α), protein kinases (such as Src),[56,60–62] and lipid kinases (such as type II PI-4kinase) were detected in CD63 immuno-precipitates, little knowledge has been gained in understanding the CD63-mediated signalling pathways. Recently, CD63 was found to bind syntenin-1, a PDZ domain-containing adapter protein.[63] CD63 C-terminal cytoplasmic domains and syntenin-1 PDZ domains are needed for the interaction while the C-terminus of syntenin-1 stabilizes the inter-action.[63] Syntenin-1 retards CD63 internalization and the deletion of the N-terminal 100 residues of syntenin-1 completely blocks CD63 internalization.[63] In addition, CD63 associates with membrane-type 1 MMP (MT1-MMP) to facilitate its lysosomal degradation,[64] functions as a cellular receptor of tissue inhibitor of metalloproteinase-1 (TIMP-1),[65] and interacts with H, K-ATPase β-subunit to enhance its internalization.[66]

2.3 CD151

CD151 or Tspan24, originally identified as platelet-EC tetra-span antigen 3 (PETA-3), is widely expressed in ECs, SMCs, and epithelial cells, and in haematopoietic cells such as platelets and megakaryocytes.[67–69] CD151 shows stable and stoichiometric association with laminin-binding integrins $\alpha3\beta1$, $\alpha6\beta1$, $\alpha6\beta4$, and $\alpha7\beta1$ in a number of cell systems and functions together with the integrins in a variety of cel-lular processes.[70–72] Much of the knowledge on biological properties of CD151 and its associated integrins is gained in the areas of cell motility,[73–75] cell–cell adhesion,[76–78] cell–ECM adhesion[75,79] and tumour metastasis.[74,80] In the vessel wall, CD151 is expressed in the SMC-enriched medial layer, fibroblast-enriched adventitial layer, and endothelium. Although the expression of CD151 in athero-sclerotic arteries appears to be increased when compared with the normal arteries,[81] precise regulatory role of CD151 in SMC functions has so far not been investigated.

In cultured HUVEC, intracellular CD151 accounts for up to 66% of the total CD151 and is mostly localized to the endo-somal/lysosomal vesicles. The cell surface CD151 in ECs is localized at cell–cell junctions,[41] a pattern also seen with tetraspanins CD9 and CD81, which are the primary tetraspa-nins associating with CD151.[41,82] Remarkably, the tetraspa-nin web formed by CD9, CD81, and CD151 on EC are critical for proper adhesive function of ICAM-1 and VCAM-1, and appropriate distribution of CD151 in the webs on EC is required for lymphocyte transendothelial migration and can strengthen the firm adhesion of lymphocytes during extravasation.[38] In addition, CD151 has been implicated to regulate several essential functions of EC including migration, invasion, and spreading.[41,82,83]

Accumulating evidence reveals that CD151 is an important regulator of vasculogenesis and angiogenesis. First, CD151 regulates EC cable formation in in vitro Matrigel assays.[82–84] Second, CD151 expression via viral vectors substantially increases microvessel density in the rat ventricular myocar-dium undergoing ischaemic infarction.[85] Last, CD151 KO results in mice defective in pathological angiogenesis as evi-denced by in vivo Matrigel plug assays, corneal micropocket and tumour implantation assays, and ex vivo aortic ring assays.[83] Despite these striking vascular abnormalities, the physiological vascular morphogenic process appears to be unaffected in CD151 KO mice.[83]

Mechanistically, studies in epithelial cells indicate that CD151 associates with $\alpha3\beta1$ integrin and regulates cell–cell contact through organizing junctional complexes containing cadherin, β-catenin, PKC, and PTPμ.[76,77] Also, CD151, like its partner integrin $\alpha6\beta4$, is a constitutive component of hemi-desmosomes,[78] a major cell–matrix adhesion machinery in epidermis. At the molecular level, CD151 promotes cell adhesion strengthening mediated by integrin $\alpha6\beta1$ and potentiates the ligand-binding activity of integrin $\alpha3\beta1$ by stabilizing the activated conformation of this integrin.[79,86] Intracellularly, CD151 expression leads to the PKC- and Cdc42-dependent actin cytoskeletal reorganization, a process critical for both adhesion and migration.[76,77] In EC, both overexpression and knockout studies indicate that CD151 can upregulate eNOS, Akt, and Rac activities, which are apparently needed for endothelial cell–cell adhesion and angiogenesis.[83,85] Endothelial CD151 also promotes the col-lagenolytic activity and TEM association of MT1-MMP, an ECM remodelling enzyme involved in angiogenesis.[41] However, the precise mechanisms of CD151 controlling EC adhesion, migration, and angiogenesis remain largely unknown.

Within haematopoietic cells, CD151 expression is mostly restricted to platelets, megakaryocytes, erythrocytes, and

activated T lymphocytes.[69,87,88] A C-terminal deletion mutation of human CD151 presents with severe defects of erythropoiesis,[89] suggesting the connection between CD151 and the proper functioning of haematopoietic cells. However, CD151-null mice are normal in the development of haematopoietic cells and proliferation of T lymphocytes,[90–92] CD151 appears to modulate cell adhesion in haematopoietic cells such as the homotypic cell–cell adhesion of erythroleukaemia and megakaryoblastic cells.[87,91] In human T lymphocytes, CD151 expression is transactivated by Tax in response to human T cell leukaemia virus type 1 (HTLV-1) infection where CD151 promotes α5β1 integrin-dependent adhesion to fibronectin in HTLV-1-positive T cells.[93,94] In addition, CD151 may also play a role in human immunodeficiency virus type 1 (HIV-1) entry into macrophages. Recombinant soluble forms of the EC2 domains of CD151 as well as tetraspanins CD9, CD63, and CD81 are capable of inhibiting HIV-1 virion uptake perhaps by altering the organization of CD4-HIV complexes within tetraspanin web that are required for membrane fusion events.[95]

3. Function of tetraspanins in haemostasis and thrombosis

3.1 CD9

Platelets express at least five different tetraspanin members, CD9, CD151, CD63, TSSC6,[11] and Tspan9,[96] of which CD9 has the highest surface expression. At 50 000–80 000 copies per platelet, CD9 is approximately equimolar with that of fibrinogen receptor integrin GPIIb/IIIa.[97] CD9 in platelets is in a molecular complex with the GPIIb/IIIa and other platelet membrane glycoproteins CD36,[98] GPIb/V/IX complex, the integrin-associated protein (IAP),[26] and with CD63.[99] CD9 interactions with GPIIb/IIIa are disrupted by strong non-ionic detergents such as Triton-X100, and with the detergents such as digitonin that disrupt tetraspanin–tetraspanin interactions.

All described anti-human CD9 monoclonal antibodies (mAbs) bind in the EC2 region of CD9 and induce platelet aggregation via FcγRII-mediated crosslinking mechanism. Antibody ligation to CD9 results in activation of G proteins leading to phosphoinositide hydrolysis and inhibition of adenylate cyclase. These events mediated by phospholipase C, required neither secreted ADP nor thromboxane generation.[100] Due to the propensity of CD9 mAbs to actuate FcγRII-dependent mechanisms, employing intact anti-CD9 IgG in functional assays has made understanding the specific contribution of CD9 in platelets a challenge.[101,102] Soluble F(ab')2 fragments to CD9 derived from intact mAbs do not elicit such pro-aggreagtory response.[103] However, immobilized F(ab')2 are capable of inducing dense granule release and platelet aggregation, suggesting that immobilization or direct clustering of CD9 contributes to initiation of a platelet signal.[104]

Recent studies performed using monovalent Fab fragments of CD9 mAbs reveal that engagement of CD9 with Fab promotes aggregate stability and enhanced fibrinogen binding to GPIIb/IIIa under threshold concentrations of agonists.[105] Similarly, recombinant protein corresponding to the CD9 EC2 domain inhibits low-dose agonist induced platelet aggregation (J. Kotha *et al.*, unpublished observations). Scanning electron microscopy with immunogold labelling

with CD9 mAb suggest that CD9 is enriched at the regions of platelet–platelet contacts and is localized with the GPIIb/IIIa in the alpha granules and pseudopodial process of activated platelets.[106] Confocal laser scanning microscopy studies on platelets at various stages of spreading reveal that in addition to high-density localization of CD9 to platelet–platelet contact sites, filopodial extensions show markedly enhanced CD9 staining.[105]

Significance of CD9 in platelet function and haemostasis has also been studied using CD9 KO mice model. Despite being the most abundant tetraspanin on platelets, CD9 deficiency does not appear to alter agonist-induced platelet aggregation or expression of the platelet activation marker, P-selectin (F. Lanza, personal communication). Tail bleeding assays suggest that CD9 KO mice have a decreased bleeding tendency that is in total contrast with that of other tetraspanin KO models (CD151 and TSSC6). However, CD9 KO platelets display an increased αIIbβ3 activation without changes in total levels of platelet αIIbβ3. These results point to CD9 as a repressor of integrin αIIbβ3 activation in platelets (F. Lanza, personal communication). The exact role of CD9 in platelet function and haemostasis remains elusive, and it is yet to be determined whether CD9 Fabs or other agents that functionally perturb CD9 carry a potential therapeutic value in regulating thrombosis and haemostasis.

3.2 CD63

CD63 is a tetraspanin expressed abundantly in the platelets. In contrast with CD9, CD63 is mostly localized to the dense granules and lysosomes of resting platelets and redistributes to platelet surface only upon platelet activation. Thus, CD63, along with P-selectin, is routinely used as a surface marker for platelet activation. CD63 is also expressed in several megakaryocytic cell lines.[107] As a constitutively expressed protein in the platelet granules, CD63 was found to be associated with the platelet storage pool deficiency disorders such as the Hermansky–Pudlak Syndrome.[108] Biochemical studies show that the surface translocated CD63 preferentially complexes with the CD9- and GPIIb/IIIa-containing multimeric complexes.[109] A direct role for CD63 in the platelet granule release and in altering GPIIb/IIIa-fibrinogen interactions remain to be determined. Immunoelectron microscopy studies demonstrate that CD63 and type II PI-4 kinase colocalize at both internal membranes of resting platelets and filopodia of thrombin-activated platelets.[110]

Antibody perturbation of platelets with anti-CD63 mAbs exhibits incomplete spreading on fibrinogen and impairs tyrosine phosphorylation of focal adhesion kinase (FAK).[99] CD63 also modulates platelet spreading on the fibrinogen-immobilized substratum in an integrin GPIIb/IIIa-dependent manner.[99] Despite these characteristics, perturbation of CD63 function in platelets via mAbs does not alter platelet adhesion and activation *per se*. Recent evidence from CD63-null mice indicated CD63 is not required for platelet development, activation, and adhesion to collagen under shear flow and thrombus formation both *in vitro* and *in vivo*. However, when compared with the wild-type, CD63-deficient platelets consistently displayed slightly but statistically insignificantly stronger responses in standard

aggregation assay and reduced reversibility of aggregation at the low and intermediate concentrations of agonist.[111]

3.3 CD151

CD151 is expressed in human as well as murine platelets. Like CD9, CD151 also complexes with GPIIb/IIIa.[91] However, unlike CD9-GPIIb/IIIa complexes, CD151-GPIIb/IIIa interactions are retained under stringent detergent conditions such as 1% Triton X-100.[91] Similar to CD9, certain mAbs against human CD151 induce platelet aggregation via FcRγII-mediated cross-linking mechanism.[112] Despite having no changes in GPIIb/IIIa expression, platelets from CD151 KO mice have impaired GPIIb/IIIa outside-in signalling and exhibit diminished spreading on fibrinogen and reduced formation of filopodia.[91] Other haemostatic anomalies in CD151 KO mice include delayed clot retraction and reduced agonist-induced platelet aggregation response to collagen, ADP, and PAR-4 agonist peptide.[91] However, platelet adhesion to immobilized fibrinogen and the alpha and dense granules secretion by platelets appear to be unaffected by CD151 deficiency.[91]

3.4 TSSC6 and Tspan9

TSSC6 or Tspan32 was shown to be expressed in murine platelets. Platelets derived from the TSSC6 KO mice exhibit unstable haemostasis (including increased tail bleeding time, blood lost, rebleeding, and emboli), delayed clot retraction, reduced spreading on fibrinogen, and reduced aggregation to low dosages of PAR-4 agonist peptide and collagen.[113] Whether or not TSSC6 is expressed in human platelets and has a functional role in human thrombosis and haemostasis is yet to be determined. Tspan9 is a recently identified platelet tetraspanin that selectively complexes with the platelet collagen receptor, GPVI, and α6β1 but not with adhesion receptors GPIb and GPIIb-IIIa.[100] The role of Tspan9 has not been determined.

4. Concluding remarks

Evolutionarily, tetraspanin expression is primarily restricted to multicellular organisms,[114,115] suggesting that their primordial function is in modulating intercellular interactions. Functional association of tetraspanins with cell motility is evident even in organisms such as fungi where a tetraspanin homolog directs the formation of cellular protrusive structure that is important for fungal cell invasive activity.[116] In vascular tissue and platelets, tetraspanins clearly regulate cell migration and intercellular interactions (*Table 1*). Thus, a common theme for tetraspanin function in vascular tissue appears to be the modulation of the adhesive and migratory properties of cells. Another characteristic feature for vascular tetraspanins is their localization in cellular vesicles, suggesting that they regulate vesicle trafficking and release events. For example, all four tetraspanins discussed are either largely or partially localized in intracellular granules or vesicles of platelets. Their translocation to the cell surface upon activation correlates with the platelet aggregation. In addition, the vesicular trafficking of CD151 regulates cell migration while EC movement is likely involved in CD151-mediated neovascularization.[15,83]

Earlier studies have highlighted the importance of tetraspanins in the regulation of various vascular events. Several critical questions emerge from the earlier observations. For example, how do tetraspanins coordinate cell–matrix and cell–cell adhesions in vascular events? How do tetraspanins coordinate motogenic and mitogenic activities during vascular morphogenesis, remodelling, and vascular injury response? What are the precise roles that vascular tetraspanins play in vesicle trafficking and release? How are the tetraspanins in vesicular compartments connected to cell adhesion and migration? How important are the intricate interactions within tetraspanin webs for fine-tuning specific phenotypes of platelets, SMC, and EC? Could tetraspanins serve as prognostic indicators for vascular disease or as targets for drug therapy?

Table 1 The distribution, partners, and functions of vascular tetraspanins

Tetraspanin	Cell type	Protein interactions	Specific functions
CD9	Smooth muscle cells	α5β1	Migration, proliferation, signalling
		α2β1	
		α3β1	
	Endothelial cells	ICAM-1	Transendothelial migration of leucocytes
		VCAM-1	
		α3β1	
	Platelets	GPIIb/IIIa	Aggregation, signalling
		GPIb/V/IX	
CD151	Smooth muscle cells	Not done	Not done
	Endothelial cells	Tetraspanins such as CD9 and CD81	Cell–ECM adhesion
		Integrins such as α3β1, α6β1, and α5β1	Cell–cell adhesion
		IgSF proteins such as ICAM-1 and VCAM-1	Cell migration
		MT1-MMP	Angiogenesis
	Platelets	GPIIb/IIIa	Aggregation, spreading, adhesion to fibrinogen
		Tetraspanins such as CD9	
CD63	Smooth muscle cells	Not done	Not done
	Endothelial cells	vWF	Vesicle trafficking
	Platelets	GPIIb/IIIa	Spreading, signalling
		Type II PI-4K	

To answer these outstanding questions, the challenges lie in unraveling the functional associations of the tetraspanin web, elucidating the specific signalling pathways that regulate cell phenotypes, and understanding the trafficking mechanism of tetraspanins in vascular cells. Another key issue to be addressed is the interpretation of the functional data obtained from tetraspanin KO models as other tetraspanins can provide functional compensation.[117] Whether or not such mutual functional compensatory phenomena contribute significantly to the overall cell phenotypes observed in these models must be determined before a specific function can be assigned to a tetraspanin.

Based on the fundamental nature of these outstanding questions and key issues, we believe that the future progress largely depends on the integrated approaches that combine biophysical and high resolution imaging, signalling and genetic, or *in vitro* and *in vivo* studies. Also, because of the functional versatility of tetraspanins, their specific effector functions may be unique to the vascular system. Future directions will entail the extensive use of vascular models to determine the exact mechanism of vascular tetraspanin function. Furthermore, given the importance of VSMC in the pathogenesis of multiple vascular diseases, functional analysis of tetraspanins in this understudied cell system is predicted to be fruitful. Moreover, besides the tetraspanin members discussed in this review, the expression characteristics and functional roles of many novel and less studied human tetraspanins in vascular system are largely unclear and will become a major exploring area in the coming years. Finally, translational research linking tetraspanins to vascular pathology will provide useful insights into the discovery of preventive measures, diagnostic markers, and therapeutic agents for vascular diseases.

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