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REVIEW

Lipid rafts: integrated platforms for vascular organization offering therapeutic opportunities

Anna Laurenzana · Gabriella Fibbi · Anastasia Chillà · Giancarlo Margheri · Tommaso Del Rosso · Elisabetta Rovida · Mario Del Rosso · Francesca Margheri

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Abstract Research on the nanoscale membrane structures known as lipid rafts is relevant to the fields of cancer biology, inflammation and ischaemia. Lipid rafts recruit molecules critical to signalling and regulation of the invasion process in malignant cells, the leukocytes that provide immunity in inflammation and the endothelial cells that build blood and lymphatic vessels, as well as the patterning of neural networks. As angiogenesis is a common denominator, regulation of receptors and signalling molecules critical to angiogenesis is central to the design of new approaches aimed at reducing, promoting or normalizing the angiogenic process. The goal of this review is to highlight some of the key issues that indicate the involvement of endothelial cell lipid rafts at each step of so-called 'sprouting angiogenesis', from stimulation of the vascular endothelial growth factor to the choice of tip cells, activation of migratory and invasion pathways, recruitment of molecules that guide axons in vascular patterning and maturation of blood vessels. Finally, the review addresses opportunities for future studies to define how these lipid domains (and their constituents) may be manipulated to stimulate the so-called 'normalization' of vascular networks within tumors, and be identified as the main target, enabling the development of more efficient chemotherapeutics and cancer immunotherapies.

Keywords Endothelial cell · Angiogenesis · Vascular guidance · Vessel maturation · Vessel normalization · Caveolin · Tip cell

Abbreviations

ECs	Endothelial cells
EPCs	Endothelial progenitor cells
ECM	Extracellular matrix
VEGF	Vascular endothelial growth factor
LRs	Lipid rafts
GPI	Glycosylphosphatidylinositol
ssBLMs	Solid-supported bilayer lipid membranes
Cav-1	Caveolin-1
Cav	Caveolin
PTRF	Polymerase I and transcript release factor
VEGFR2	Vascular endothelial growth factor receptor 2
KDR	Kinase insert domain receptor
FLK1	Fetal liver kinase 1
VEGFR1/Flt1	Vascular endothelial growth factor receptor 1/fms-related tyrosine kinase 1
Prxs	Peroxirederoxins
ROS	Reactive oxygen species
MMP	Metalloprotease

A. Laurenzana and G. Fibbi contributed equally to this work.

A. Laurenzana · G. Fibbi · A. Chillà · E. Rovida · M. Del Rosso (✉) · F. Margheri (✉)
Section of Experimental Pathology and Oncology, Department of Experimental and Clinical Biomedical Sciences, University of Florence, Viale GB Morgagni 50, 50134 Florence, Italy
e-mail: delrosso@unifi.it

F. Margheri
e-mail: fmargheri@unifi.it

G. Margheri
Institute of Complex Systems (ISC), Consiglio Nazionale delle Ricerche (CNR), Florence, Italy

T. Del Rosso
Department of Physics, Pontificia Universidade Catolica do Rio de Janeiro, Rio de Janeiro, Brazil

M. Del Rosso
Istituto Toscano Tumori, Florence, Italy

TACE	Tumor necrosis alpha converting enzyme
MMPs	Matrix metalloproteinases
uPAR	Urokinase-type-plasminogen activator receptor
MT1-MMP	Membrane-type-1-MMP
DEP1	Density-enhanced tyrosine phosphatase
ERK1/2	Extracellular signal-regulated kinase 1/2
Robos	Roundabouts
UNC5B	Unc-5 homolog B
Nrps	Neuropilins
Slit1-3	Slit homolog 1-3
RGMa	Repulsive guidance molecule a
FLRT3	Fibronectin and leucine rich transmembrane protein 3
Semas	Semaphorins
vSMC	Vascular smooth muscle cell
PDGFRβ	Platelet-derived growth factor receptor β
S1P	Sphingosine-1-phosphate
TGF β1	Transforming growth factor-β1 (TGFβ1)
ALK1	Activin receptor-like kinase 1
ALK5	Activin receptor-like kinase 5
TIE2	TEK tyrosine kinase
Ang	Angiopoietin
n-3 PUFA	*n*-3 polyunsaturated fatty acids

Introduction

The vascular system, a complex and highly branched tubular network made up of endothelial cells (ECs), ensures the simultaneous and efficient transport of gases, liquids, nutrients, signalling molecules and circulating cells between tissues and organs. Insufficient blood vessel supply causes tissue ischaemia in cardiovascular diseases, whereas new vessel formation allows nutrients, oxygen and signalling molecules to be more available to inflamed tissues and tumors; it also facilitates cancer cell dissemination to distant organs in metastasis [1, 2]. Blood vessels can form anew after tubular organization of endothelial progenitor cells (EPCs; vasculogenesis) or by budding from pre-existing vessels (sprouting angiogenesis), the latter also being integrated by local recruitment of EPCs [3, 4]. Vessel growth is an example of coordinated proliferation, migration, matrix adhesion, guidance and differentiation that results in specialized tissues. During vessel formation, ECs degrade the underlying basement membrane and migrate in the interstitial extracellular matrix (ECM), before proliferating and undergoing cavitation to produce new tubular-like structures. In particular, a subset of cells located at the leading edge of the sprouting vessels, termed 'tip cells', acquires motile, invasive behavior and extends filopodia [2, 5, 6]. The tip and stalk cells of endothelial sprouts express a large array of pro-angiogenesis systems that regulate the temporal and spatial interaction of ECs and ECM. Such systems, regulated by the balance of pro- and anti-angiogenesis factors, include the vascular endothelial growth factor (VEGF) receptor [7], the receptor for the urokinase plasminogen activator [8], the ephrin and Eph-receptor tyrosine kinases [9], the Notch complex [10], integrins [11], cadherins [12, 13] and NADPH oxidase (Nox) [7], to cite only those most studied. The function of many of these systems is boosted by angiogenesis factor-dependent localization of the relevant molecules in specialized microdomains of the cell surface known as caveolar lipid rafts (LRs), signalling platforms that, in many cell types including ECs [14], recruit functionally important molecules involved in cell–matrix interactions and cell signalling. Such microdomains are enriched with cholesterol and glycosphingolipids; they are resistant to cold detergent extraction and show lower buoyant density than most of the plasma membrane [15]. These membrane domains are also enriched with membrane proteins such as caveolins, the Src family of kinases [16, 17] and glycosylphosphatidylinositol (GPI)-anchored proteins [18]. Only a limited number of membrane proteins are known to associate with LRs, but they are of paramount significance because many receptors for extracellular signals, including those involved in EC invasion and guidance, localize in LRs. In this review, we focus on the emerging role of LR microdomains in the regulation of the angiogenesis process.

Lipid rafts and caveolae

The membrane model of Singer and Nicholson [19] postulated a uniform lipid bilayer with randomly floating proteins, but it was soon realized that the membranes were not uniform and that there were clusters of lipids organized in a more ordered state within the generally disordered lipid milieu of the membrane. Such clusters are now referred to as LRs, which are defined as lipid-ordered phases of the cell membrane formed mainly from tightly packed sphingomyelin and cholesterol, rather than the other parts of the membrane that are mainly made up of phospholipids, forming the lipid-disordered phase. These specialized membrane microdomains compartmentalize cellular processes by serving as organizing centers for the assembly of signalling molecules, influencing membrane fluidity and membrane protein trafficking, and regulating neurotransmission and receptor trafficking [20, 21]. Although more ordered and tightly packed than the surrounding surface membrane, LRs float freely in the membrane bilayer [22]. Our concept of LRs has evolved with the realization that the association of raft components is dynamic and that their sizes range from small, short-lived, nanoscale ensembles (<50 nm in diameter) to more stable membrane domains,

with sizes possibly reaching 500 nm in diameter [23]. However, as the average size of LRs is well below the resolution of light microscopy, until 15 years ago, there was a concern about visualizing LRs in cell membranes, because the existing microscopic approaches did not support the 'raft concept' [24]. A key issue was the method used to define an LR component. LRs were defined as the insoluble residue remaining after non-ionic detergent solubilization of cell membranes (hence the definition of LRs as detergent-resistant membranes or DRMs). If a protein became detergent soluble after treatment with methyl-β-cyclodextrin, which extracts cholesterol from the cell membrane, it was considered to be a raft component. If a biological process was inhibited by cyclodextrin treatment, it was considered to be raft dependent [25]. Although such criteria still hold true and have been adopted in most publications, advances in technology have permitted clarification of the 'raft concept'. Visualizing rafts with new microscopic techniques (reviewed in Simons and Gerl [25]) has allowed the identification of LRs as a heterogeneous collection of domains differing both in protein and lipid composition and in temporal stability. This new concept is embodied in the consensus definition of an LR proposed at the 2006 Keystone Symposium: 'Lipid rafts are small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein–protein and protein–lipid interactions [26].

Nevertheless, there is still controversy over the existence of LRs [27], as well as their real composition. A recent study concluded that cholesterol does not play a direct role in LR organization [28]. In that review, the authors used high-resolution, secondary ion mass spectrometry to directly map the distributions of isotope-labeled cholesterol and sphingolipids in fibroblast plasma membranes. Although cholesterol depletion reduced the abundance of LRs, cholesterol was evenly distributed throughout the plasma membrane and not enriched within LRs. Thus, the authors ruled out favorable cholesterol–sphingolipid interactions dictating plasma membrane organization in fibroblasts. As LRs are disrupted by drugs that depolymerize the cell's actin cytoskeleton, the authors suggested that cholesterol must affect sphingolipid organization via an indirect mechanism involving the cytoskeleton instead. Recent technological advances have allowed the building of artificial LR-like biomimetic membranes, mimicking physiological cell membranes, on solid supports (solid-supported bilayer lipid membranes), which provide excellent templates for studying in vitro LR interactions with several biological molecules. These biomimetic systems are formed by depositing ternary or binary mixtures of phospholipids, sphingolipids and cholesterol, enriched with LR-hosted receptors, on to a hydrophilic surface (typically mica or amorphous silica), via self-assembly or vesicle fusion. Thanks to their mobility on these surfaces, sphingolipids and cholesterol can freely come out of the lipid-disordered phase [29, 30] or assemble in a lipid-ordered macroarea, covering all the surfaces available [31, 32]. The ligand–receptor affinity can be easily demonstrated optically using surface plasmon resonance techniques [30–33].

It has now been firmly established that there are two major types of LRs: those that contain the cholesterol-binding protein caveolin-1 (Cav-1) in the inner leaflet and those that do not. Caveolin is an integral membrane protein that exists in three isoforms: Cav-1, Cav-2 and Cav-3. Although Cav-1 is widely expressed in many tissues, including endothelium, Cav-3 is muscle specific; Cav-2 is co-expressed with Cav-1 which it needs for stabilization and plasma-membrane localization (reviewed in Sotgia et al. [34]). Cav-1, highly expressed in ECs, is organized into specific domains [34]. Both the N- and C-termini of each Cav-1 monomer face the cytoplasm. Some 12–18 Cav-1/Cav-2 monomers form a polymerized filamentous structure [35]. The Golgi body is the subcellular location in which cholesterol, sphingolipids and Cav-1 polymers self-aggregate and generate vesicles that are transported to the plasma membrane to form caveolar LRs, giving the membrane characteristic, spherical, flask-like invaginations (caveolae or 'little caves') that can be detected with electron microscopy, can be widely represented at the external surface of ECs and was first described by GE Palade in 1953 [36]. The caveolar LRs of ECs, which occur in ordered linear arrays over the entire cell body [37], have been involved in many cellular functions such as endocytosis (transcytosis, pinocytosis, potocytosis), signal transduction, mechanotransduction and cholesterol trafficking [38]. However, as caveolin exists in cells that do not show morphological caveolae, efforts have been made to find other proteins producing these unique plasma membrane invaginations via a partnership with caveolin. Recent evidence has shown that caveolar LR formation requires the activity of the protein cavin (also called polymerase I and transcript release factor) [39]. Cavin is a peripheral membrane protein that binds to the phosphatidylserines within the caveolar LR [40]. Cavin and caveolin molecules exist in a stoichiometric ratio in caveolar LRs that are close together [41]. Therefore, cavin appears to be essential for the formation of morphological caveolar LRs. Another family of proteins described in LRs are the flotillin1–2/reggie1–2 complexes, originally described as neuronal proteins in retinal ganglion cells, and expressed during post-injury axon regeneration [42]. Flotillin/reggies oligomerize via their C-terminal domains and are involved in the endocytosis of GPI-anchored proteins

[43]. Although caveolae have a diameter of 70–120 nm [44], planar LRs have one of 1–1,000 nm and are enriched in GPI-anchored proteins and flotillins [43, 44].

Although caveolin, flotillins/reggies and other proteins (reviewed in Lucero and Robbins [45]) can be permanently associated with LRs, other proteins are temporarily associated with them, mainly on the basis of protein post-translational modifications such as the addition of a GPI anchor, palmitoylation and myristoylation [45]. LRs may vary in their cholesterol and caveolin content, which, in turn, is related to LR enrichment in membrane and signalling receptors [18]. Caveolar and planar LRs may both undergo clustering according to alternative models proposing that either (1) non-LR receptors translocate into LRs after ligand binding, resulting in signal transduction, or (2) separate but close LRs may cluster only after ligand binding and receptor activation [46]. Whatever the case, clustering modifies LRs, thereby triggering new functions such as the creation of functional platforms that can be exploited for the signalling networks required in specific biological effects, such as angiogenesis [47, 48].

Involvement of LRs in specific steps of sprouting angiogenesis

The data that are available for the involvement of LRs in every step of sprouting angiogenesis show how LR clusters, which form on the surface of ECs subjected to an angiogenic challenge, provide an integrated platform that directs the formation, guidance, patterning and stabilization of the new vessels. The data that are reviewed here refer only to angiogenesis from pre-existing vessels, because this is only one of the features of tumor angiogenesis [49]. Tumor cells and the tumor microenvironment produce several angiogenic factors, including VEGF, that induce robust endothelial sprouting angiogenesis; however, they are also vasculogenic and actively participate in neovascularization through vascular mimicry [50], differentiation from tumor stem cells to tumor ECs [51] and co-option of vessels [52]. These tumor-specific features reflect the neoplastic transformation-related phenomenon: tumor cells express various embryonic genes, normally silent after birth and in adult life, that maintain the property of directing an ancestral vascular tube formation process [53].

The very beginning: LRs and VEGF stimulation

As a response to hypoxia, tissues overexpress pro-angiogenic growth factors [54]. The most important molecule controlling blood vessel morphogenesis is VEGFA, a member of a larger family of angiogenesis regulators including VEGFB, VEGFC and placental growth factor (PlGF) [55]. Alternative splicing of VEGFA can produce isoforms with anti-angiogenic properties [56]. The final balance of various isoforms controls angiogenesis. Binding of VEGFA to its receptor VEGFR2 (KDR or FLK1) promotes this, according to repetitive cycles that stop when excess anti-angiogenesis factors are present. Of VEGF isoforms and matrix proteins such as the thrombospondins [57], upregulation of VEGFR1/Flt1 is particularly important because it is a sort of VEGF buffer, based on its properties of weak signalling capacity and high VEGF affinity [58].

The main pro-angiogenic receptor VEGFR2 is present in EC caveolar LRs by association with Cav-1, which negatively regulates receptor activity in the basal state [59]. On binding, VEGFA promotes the release of VEGFR2 from caveolar LRs, which occurs together with tyrosine phosphorylation of Cav-1 and VEGFR2, and their co-localization at focal complexes on the edge of EC lamellipodia [60]. These events happen at the distal end of each sprout, which contains a specialized, highly motile and invasive EC termed a ‘tip cell’, provided with dynamic filopodia reminiscent of axonal growth cones [2, 6, 61].

Other functional links relate VEGFA signalling to caveolar LRs. Chronic exposure to reactive oxygen species (ROS) and the resulting oxidative stress, viewed as overproduction of ROS, the failure of antioxidant defence of the organism, or both, play a critical role in human pathology [62], and an excess of ROS production may heavily impair the pro-angiogenic activity of VEGF, despite the well-known redox regulation of VEGFA signalling in angiogenesis [63]. It is now recognized not only that ECs need physiological amounts of ROS to respond properly to a VEGFA challenge [64], but also that VEGF may signal through Nox-derived ROS [7]. This mechanism involves the activation and translocation of the small GTPase Rac1 to the plasma membrane, which stimulates the Nox2-based NADPH oxidase in EC caveolar LRs. ROS derived from this oxidase may reversibly oxidize and inactivate protein tyrosine phosphatases; these negatively regulate VEGFR2 autophosphorylation and the activation of downstream redox signalling events linked to EC proliferation and migration, which in turn contribute to angiogenesis [7, 65]. Therefore, in parallel with classic phosphorylation signalling (Fig. 1), VEGFR2 uses ROS as downstream signal mediators. In fact, several studies have demonstrated the involvement of the superoxide-producing enzyme Nox in VEGF signalling, and EC functions such as adhesion [66] and actin filament assembly [67].

Nevertheless, ROS still have the potential to damage VEGFR2 pathways that need to be controlled to maintain a finely tuned, pro-angiogenic ROS concentration. In particular, the EC caveolar LR-localized VEGFR2 has an

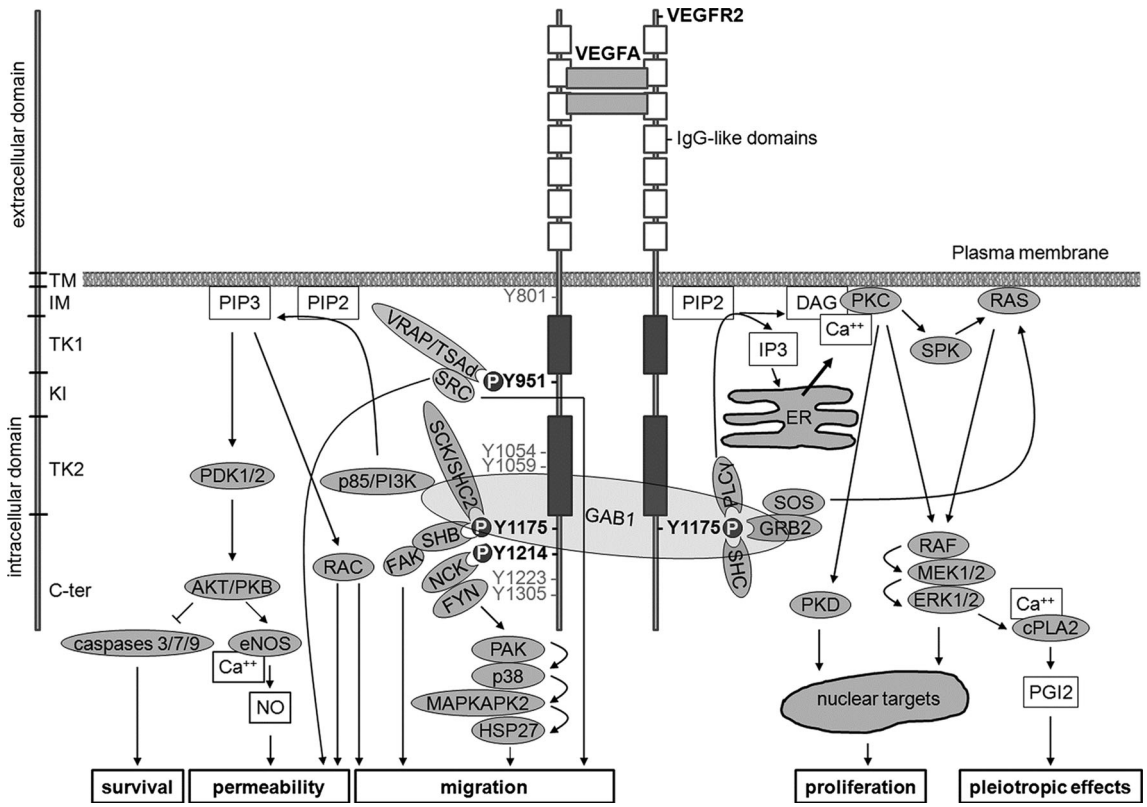


Fig. 1 VEGF signalling elicited by VEGFR2-phosphorylating events. Structure and domains of VEGFR2 are indicated on the *left side*. Dimerized VEGFA (or VEGFC or -D after proteolytic cleavage) binds to second and third IgG-like domains within the extracellular domain of VEGFR2, thus inducing VEGFR2 dimerization and autophosphorylation at several tyrosine residues within the intracellular domain. Signalling elicited by major tyrosine residues phosphorylated in human VEGFR2 951, 1175 and 1214 (corresponding to 949, 1173 and 1212, respectively, in mice) is described. Moreover, phosphorylation at Y1054 and Y1059 is required to achieve maximal kinase activity. Additional tyrosine phosphorylation may occur at Y1223, Y1305, Y1309 and Y1319 residues. However, the role of these events is still unknown. Autophosphorylation at Y801 may precede phosphorylation of Y1054 and Y1059. SH2 domain-containing proteins (grooved oval) interact with tyrosine phosphorylated (P) residues thus activating signalling pathways that elicit several biological effects including increased permeability, survival, migration and proliferation (*lower boxes*). *TM* transmembrane, *IM* intramembrane, *TK* tyrosine kinase, *KI* kinase insert, *C-term* C-terminal, *VEGFA* vascular endothelial growth factor A, *VEGFR2* VEGF receptor 2, *VRAP* VEGFR-associated protein also known as TSAd, T cell-specific adaptor, *SRC* sarcoma, *GAB1* GRB2-(growth factor receptor-bound protein 2) associated binder 1, *GRB2* growth factor receptor-bound protein 2, *PLC-γ* phospholipase C γ, *RAC* Ras-related C3 botulinum toxin substrate, *PIP3* phosphatidylinositol 3,4,5-trisphosphate, *PI3K* phosphatidylinositol 3 kinase, *PDK* phosphoinositide-dependent kinase, *PKB* protein kinase B also known as AKT, RAC-alpha serine/threonine-protein kinase, *BAD* BCL2 associated death promoter, *BCL2* B-cell CLL/lymphoma 2, *eNOS* endothelial nitric oxide synthase, *ER* endoplasmic reticulum, *PLC-γ* phospholipase C γ, *NO* nitric oxide, *FAK* focal adhesion kinase, *SHC* src homology/collagen, *SCK* SHC-like protein also known as SHC2, (Src homology 2 domain containing) transforming protein), *PIP2* phosphatidylinositol (4,5)-bisphosphate, *IP3* inositol (1,4,5)-trisphosphate, *DAG sn*-1,2-diacylglycerol, *PKC* protein kinase C, *ERK1/2* extracellular regulated kinases 1 and 2, *MEK* MAPK/Erk kinase, *HSP27* heat-shock protein 27, *MAPKAP 2/3* MAPK-activating protein kinases 2 and 3, *PAK* p21-activated protein kinase, *p38* p38 mitogen-activated protein kinase, Ca^{++} calcium, *cPLA2* cytosolic phospholipase A2, *SHB* SH2 domain-containing adapter protein B. *SPK* sphingosine kinase, *CDC42* cell division cycle 42

oxidation-sensitive cysteine residue with a functionally active reduced state that is preserved specifically by peroxiredoxin-2 [68]. Peroxiredoxins (Prxs) are a family of proteins with peroxidase activities that degrade H_2O_2 to H_2O, thereby abolishing H_2O_2 toxicity. Prx-2-mediated VEGFR2 protection is achieved by association of the two proteins in the caveolar LRs of ECs [68]. In a recent study, based on proteomic identification of VEGFA-dependent protein enrichment of membrane caveolar LR microdomains in EPCs, we showed that not only Prx-2, but also Prx-6, is located on caveolar LRs, possibly pointing to a convergent role for the two enzymes in safeguarding the redox-sensitive VEGFR2 [69]. In that work, we have shown that the gene ontology (GO) term referred to as 'H_2O_2 metabolic process' (represented by Prx-2, Prx-6 and protein DJ-1) undergoes the highest enrichment (>70-fold) at the EPC caveolar LR level on VEGFA stimulation. Along with this, the GO terms related to the control of apoptosis/programmed cell death

(including some heat shock proteins) are significantly overexpressed in caveolar LRs of VEGFA-challenged EPCs. The caveolar LR localization of such molecules further reinforces the need for EPCs to strictly control ROS production/activity, as well as eliminating and/or inactivating damaged molecules. Such molecules may result from the Nox generation of ROS that are spatially and temporally dependent on VEGFA signalling at caveolar LRs, leading edge/focal adhesion complexes and cell–cell junctions in ECs [7].

What happens at the caveolar LRs of ECs/EPCs upon VEGFA stimulation offers further insights into how changes in the organization of protein caveolar LR microdomains may affect antioxidant defence/pro-survival pathways, supporting the concept of caveolar LRs as 'floating islands of death and survival' [70].

A critical task: LRs and the choice of tip cells

If all ECs were to react indiscriminately with VEGFA, the relevant section of the vessel might disintegrate, thus compromising tissue perfusion [2]. In fact, only a fraction of ECs become tip cells and initiate the sprouting process, whereas others stay behind ('stalk cells') and maintain the vessel's integrity (for model illustrations of the process, see the literature [2, 3, 71]). The caveolar LR-located Notch system, which has key roles in many differentiation processes, regulates this tip–stalk decision [72, 73]. The Notch receptor is normally triggered by cell-to-cell contact, in which the transmembrane proteins of those cells in direct contact provide the ligands that bind the Notch receptor of adjacent cells. This kind of interaction induces the so-called 'Notch cascade', which consists of Notch ligands and intracellular proteins transmitting the Notch signal to the genome. Involvement of the Notch system in angiogenesis occurs according to this general model. VEGF upregulates expression of the Notch ligand Delta-like 4 (DLL4), which reaches very high levels in tip cells [74–76]. The Notch receptor protein spans the cell membrane of stalk ECs, and DLL4 binding to the extracellular domain induces the proteolytic cleavage and release of the intracellular domain, which enters the stalk EC's nucleus to regulate gene expression [77]. Once Notch's extracellular domain has interacted with the ligand, an ADAM family metalloprotease, known as TACE (tumor necrosis α-converting enzyme), cleaves the Notch receptor just outside the membrane [78], releasing the extracellular portion of Notch, which continues to interact with the ligand. After this first cleavage, an enzyme called γ-secretase cleaves the remaining moiety of the Notch protein just inside the inner leaflet of the cell membrane of the Notch-expressing cell. In addition, the γ-secretase complex, made up of several proteins including presenilin-1, nicastrin, anterior pharynx defective-1 and presenilin enhancer-2, has been shown to be localized to LRs in retinal ECs [79–81]. The activity of γ-secretase releases the intracellular domain of the Notch protein, which moves to the nucleus where it can regulate gene expression by activating the transcription factor CSL [82]. When the intracellular Notch domain binds to CSL, it represses the transcription of VEGFR2/3 in stalk cells, thereby blunting angiogenic sprouting [82]. In addition to DLL4, another transmembrane Notch ligand, Jagged 1, is involved in tip EC selection. Unlike DLL4, Jagged 1 is a positive regulator of angiogenesis and acts by inducing a sugar modification of the Notch receptor [76]. Therefore, caveolar LR-associated Notch signalling may be used to control the sprouting pattern of blood vessels during angiogenesis by selecting the tip ECs that will drive vascular patterning, and the stalk ECs that will be prevented from migrating into the developing sprout. The migratory behavior of connector stalk ECs must be limited to retain a patent connection with the original blood vessel [83, 84].

A further task of VEGF: LRs and activation of migratory and invasion pathways of ECs

In adults, quiescent blood vessels are surrounded on the abluminal surface by a basement membrane, which consists of laminins, collagen type IV, nidogens, and the heparan sulfate proteoglycan perlecan [85–87]. Under the influence of VEGF, ECs selected to become tip cells activate proteolysis-dependent invasion mechanisms within a few minutes to degrade the basement membrane and progress within a provisional ECM. It is, therefore, understandable that there is extensive literature on this issue. However, a number of studies, including gene deletions in mice, have pointed to the essential role of matrix metalloproteases (MMPs) and the urokinase-type plasminogen activator receptor (uPAR)-associated plasminogen activator/plasmin system in the onset of angiogenesis [88–93]. MMP2, MMP9 (also referred to as gelatinases) and MT1-MMP (membrane type-1 MMP) stimulate angiogenesis primarily by ECM degradation, but the activity of these proteases is complex and may include other effects such as the activation of growth factors and cytokines, recruitment of EPCs and the degradation of inhibitors [88, 89]. Comparison of the ability to enhance capillary-like tube formation in a collagen-rich matrix indicated that MT1-MMP enables ECs to form invading tubular structures, whereas MMP2 and MMP9, and their cognate cell-surface receptors β3-integrin and CD44, do not, although they are required to perform efficient angiogenesis [90, 94]. MT1-MMP belongs to a subfamily of MMPs that is anchored to the membrane rather than being

secreted, and this fact makes such proteases prime candidates for coordinating extracellular cues with cellular responses. MT1-MMP proteolytic activity in ECs is closely linked to its regulated presence in specific domains of the plasma membrane, particularly in lamellipodia and filopodia, where MT1-MMP is associated with Cav-1 and integrin αvβ3 in caveolar LRs and is catalytically active [95, 96]. Caveolar LRs provide the main route for MT1-MMP internalization in ECs, but they may contribute to its mobilization to invadopodia, as reported for cancer cells [95, 97]. Once located in caveolar LRs of EC invadopodia, MT1-MMP efficiently degrades the basement membrane of the vessel, which becomes leaky and hyperpermeable to blood plasma proteins [98]. Such vascular hyperpermeability causes leakage from the blood of the ECM proteins fibrinogen, vitronectin and fibronectin [98]. Fibrinogen is subsequently converted into fibrin through the activation of coagulation and, together with other extravasated proteins and pre-existing collagen, forms a new provisional ECM that provides an optimal molecular bed for sprouting vessels. MT1-MMP exerts its influence on both vascular morphogenesis and endothelial tube formation within three-dimensional collagen matrices, where it creates 'vascular guidance tunnels' [90, 95, 99, 100] by proteolysis. Within such physical conduits, ECs are freely able to migrate in an MMP-independent manner, to organize each other and polarize against a fluid–ECM interface at the tunnel wall. Within such interconnected tubular structures, ECs can be induced to collapse by microtubule-depolymerizing agents [101], and the preformed conduits used to regrow tubes or remodel existing tube structures within the spaces [102]. Lumen formation within EC cords in both collagen and fibrin matrices is integrin- and Rho GTPase-dependent, and involves the formation and coalescence of pinocytic intracellular vacuoles together with MT1-dependent ECM proteolysis [90, 99, 100, 102–106]. Intracellular vacuoles coalesce to form intracellular lumina [91, 108].

Molecular mechanisms that coordinate proteolysis with the formation of the cytoskeleton and lumina have been extensively reviewed elsewhere [90]. The presence of fibrin and vitronectin in the angiogenesis provisional matrix is the reason why the caveolar LR-associated, urokinase plasminogen activator receptor (uPAR)-dependent, plasminogen activation system must have efficient angiogenesis. In fact, although several molecular interactions between the MMP and the plasminogen/plasmin (fibrinolytic) system may affect cellular fibrinolysis [107], the main cell-associated fibrinolytic system hinges on uPAR. Also known as CD87, uPAR is a glycoprotein organized into three domains and tethered to the cell membrane with a GPI anchor [108, 109], the presence of which determines the partitioning of uPAR in LRs in many cell types [110–112], including the caveolar LRs of ECs [113–116]. We have shown that LR partitioning of uPAR is also strictly related to its high affinity for the GM1 and GM3 gangliosides, identifying at least three uPAR compartments in human EPCs: the first associated with caveolar LRs, the second with GM1-rich LRs and the third with GM3-rich LRs [116]. Although the traditional role of uPAR is its cell-surface activation of uPA, leading to plasminogen activation that generates plasmin, and cascade activation of the plasmin-related MMPs, uPAR has been shown to contribute to many proteolysis-independent processes, as previously reviewed [117–121]. It can bind directly to vitronectin, which is abundant in the provisional matrix of sprouting vessels, with a domain distinct from its uPA-binding site [122]; even in the absence of a transmembrane and intracellular domain, uPAR serves as a 'signalosome' organizer that is triggered on uPA or vitronectin ligation and by simultaneous interactions with signalling-competent surface integrins and receptors, such as the epidermal growth factor receptor [123]. From a general point of view, the inactive precursor pro-uPA interacts with the receptor uPAR via its growth factor domain, allowing the conversion of uPAR-bound pro-uPA to active uPA [124]. Once activated, uPA cleaves the proenzyme plasminogen to yield active plasmin [125], which activates pro-MMPs to active MMPs, as well as pro-uPA to active uPA [117–121]. In the case of ECs, VEGF interaction with its receptor VEGFR2 rapidly induces prourokinase activation that is dependent on a phosphoinositide 3 (PI3)-kinase-mediated change in integrin affinity, MT1-MMP-mediated activation of MMP2 and subsequent uPAR-bound pro-uPA activation. This VEGF-induced, MMP2-mediated, pro-uPA activation of ECs is responsible for VEGF-dependent local fibrinolytic activity and might be one of the initial steps in the angiogenic process [126]. As a consequence, stimulation of ECs by VEGFA/VEGFR2 engagement leads to a redistribution of caveolar LR-associated MT1-MMP and uPAR to the leading edge of tip cells, thus focusing the proteolytic activity of the growing vessel on the invasive front of migrating ECs [97]. A possible reason for the concentration of tip-cell proteolytic activity in selected spots of the cell membrane could be the need for spatial and temporal control of proteolytic activities for efficient angiogenesis. In fact, excessive proteolysis can cause unwanted damage to the provisional ECM, by degrading and dissolving the three-dimensional structure required for anchoring the migrating cell, as we originally proposed in the 'grip-and-go' model of cell migration [117] on the basis of previous experiments performed in plasminogen activator inhibitor type 1-deficient mice with severely impaired angiogenesis [127, 128].

VEGF-challenged sprouting angiogenesis originates in mature vessels, where ECs are in a confluent state. The response of ECs to VEGF stimulation is reduced by cell density through the increased activity of the density-enhanced tyrosine phosphatase DEP1. High levels of DEP1 impair extracellular signal-regulated kinase (ERK) 1/2 activation, a downstream signalling event of the VEGF/VEGFR system, leading to a downregulation of uPAR synthesis that then blocks angiogenesis [129]. Sparsely growing cells overexpress uPAR as a consequence of DEP1 downregulation, so it is likely that overexpression of uPAR in invading tip ECs may be related to the absence of associated ECs at the leading edge of the sprouting vessel, with inhibition of DEP1 in the tip cell [130]. Other growth factors, such as fibroblast growth factor 2 (FGF2), epidermal growth factor (EGF) and hepatocyte growth factor (HGF), have been shown to induce overexpression of proteolytic activity and angiogenesis in ECs via the PI3-kinase pathway-dependent activation of uPAR-bound pro-uPA [131]. Through domains II and III, uPAR also interacts with α5β1-integrin in ECs, leading to integrin activation and the redistribution required for it to become available to ECM substrates, and providing further support to the 'grip' of invading ECs [132]. In addition, uPAR has been implicated in zymogen coagulation factor XII-dependent angiogenesis [133]. Due to VEGF-dependent vascular hyperpermeability, coagulation factor XII is present within the provisional ECM of the sprouting vessel. It binds to domain II of uPAR on the EC membrane. Factor XII engagement induces uPAR's communication with the cell through a β1-integrin. Cell stimulation via uPAR and the integrin also includes the EGF receptor. These pathways lead to ERK1/2 and Akt phosphorylation, which stimulates EC growth, proliferation and angiogenesis [133].

All of the data published to date about the role of the caveolar LR-associated proteolytic systems in ECs support the hypothesis that uPAR is the organizer and orchestrator of a spatially restricted cell interactome, which drives ECs through the initiation and termination of angiogenesis.

LR association of axon guidance molecule receptors in vascular patterning

The vasculature develops in a way that is similar to the nervous system, forming a complex, highly branched, tree-

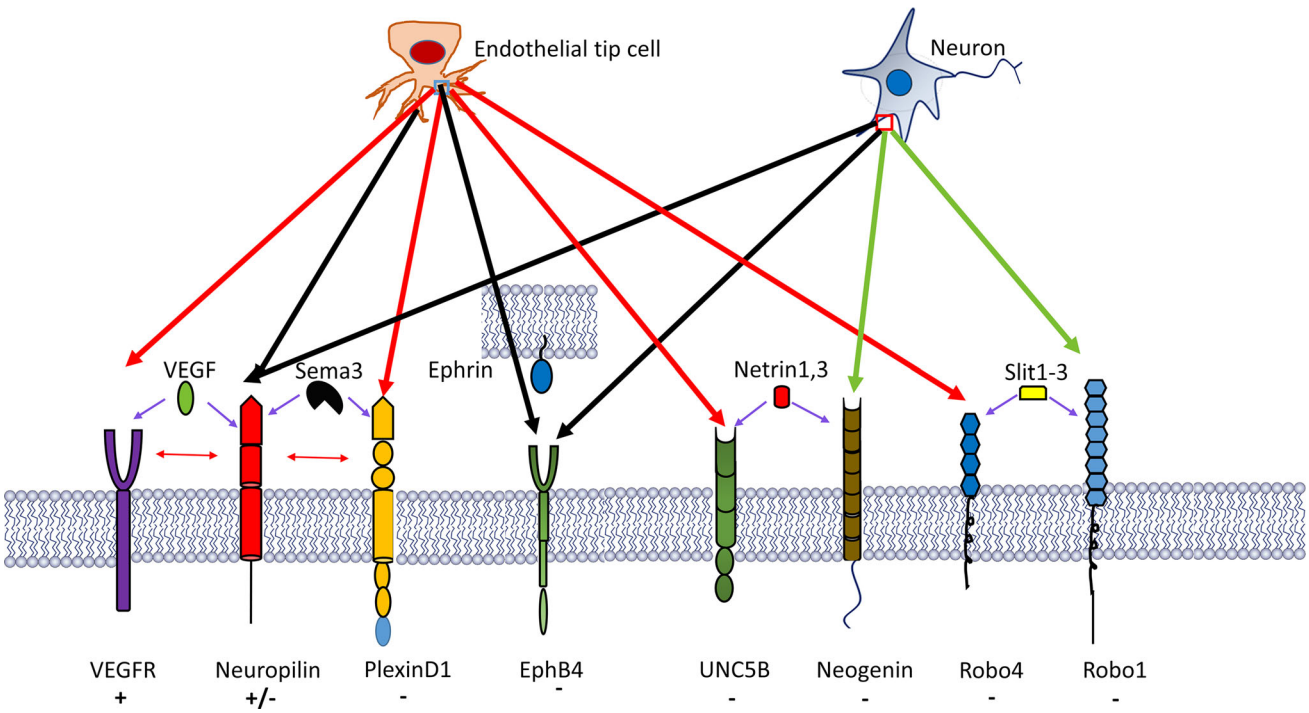


Fig. 2 A number of neural guidance molecular pathways have been recognized to participate in blood vessel branching morphogenesis. These pathways include semaphorin-3A (Sema3A)–plexinD1, ephrinB2–EphB4 and SLIT2–Robo4, which all elicit EC chemorepulsion, whereas SLIT2–Robo1 and VEGF–VEGFR2 (and possibly VEGF–NRP1) have been implicated in endothelial tip cell chemoattraction and elongation. Predominantly EC-expressed receptors are indicated by *red arrows*, receptors with shared expression in the nervous and the vascular system by *black arrows*, and molecules with uncertain expression in the vascular system by *green arrows*. Symbols *plus* and *minus* indicate chemoattraction and chemorepulsion, respectively

like network [2, 134–136]. Surprisingly, the specialized tip ECs, which lead and drive endothelial sprouts, share several features with the guidance structure of the nervous system, the axonal growth cone [2, 135]. Tip cells are highly similar to the growth cones of developing axons, because they extend many filopodia that explore the environment, thereby sensing repulsive or attracting cues. Both structures use common signalling cues to regulate their guidance. Angiogenic ECs express receptors for axon guidance molecules (Fig. 2), including the roundabouts (Robos), UNC5B, neuropilins (Nrps), PlexinD1 and the Eph family receptor tyrosine kinases [136]. In particular, Robo4, UNC5B and PlexinD1 are mainly expressed in the vasculature, whereas Nrps and Eph-receptor expression are shared equally in the vasculature and nervous system.

Slits and roundabouts

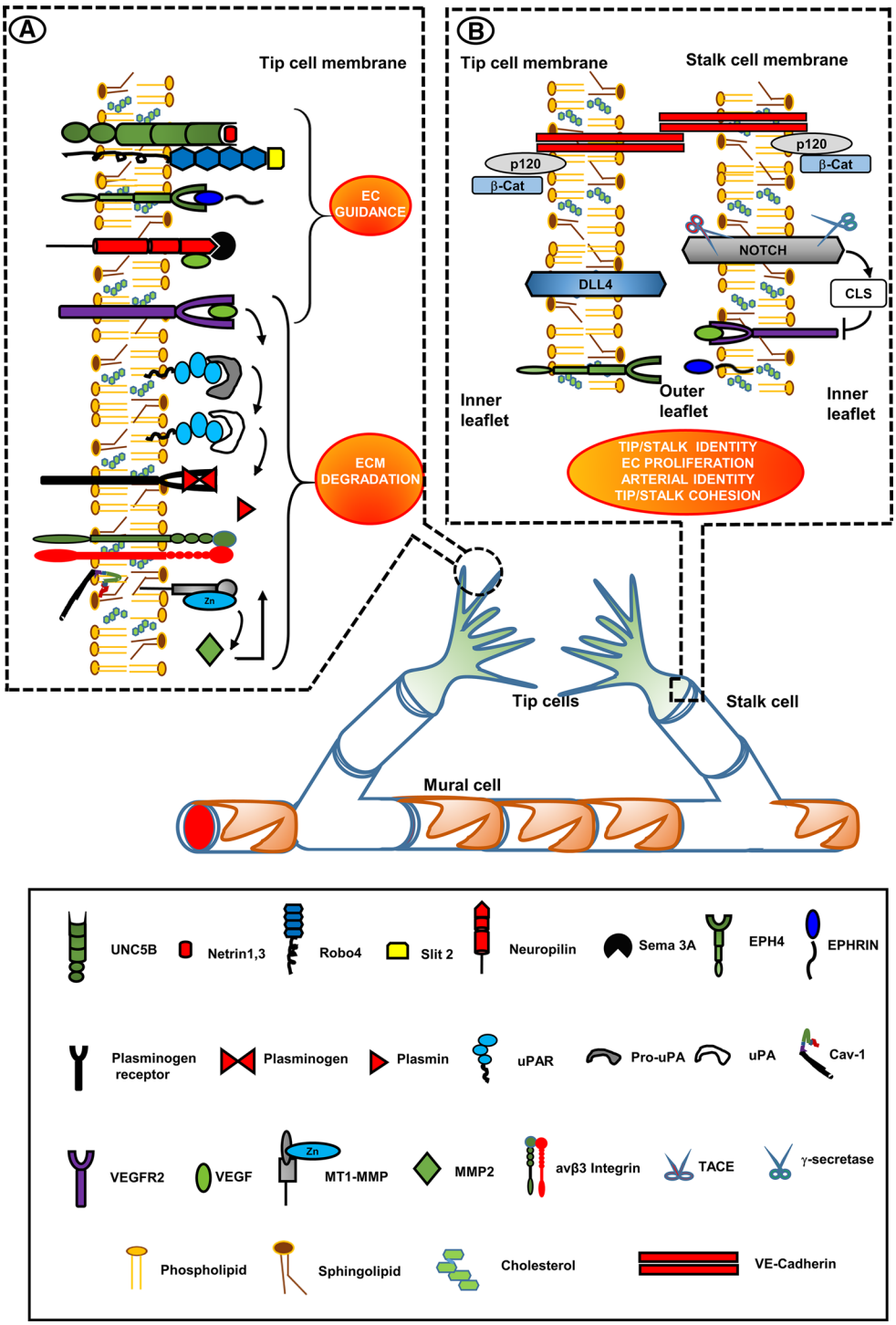
Robo4 (also referred to as 'magic roundabout') is a receptor for Slit1–3 glycoproteins, a family of ligands produced by many tissues, including tumors [137, 138]. In axonal growth, Slit proteins have an evolutionarily conserved role in the guidance of repulsion [139]. However, in angiogenesis, conflicting reports implicate them as both pro- and anti-angiogenic molecules. There are three vertebrate Slit proteins (Slit1–3), which are secreted ligands for the four members of the Robo family of receptors (Robo1–4). To date, it seems that any Slit protein can interact with any Robo receptor. Researchers interested in angiogenesis have focused on Slit2, because it is expressed in angiogenic tissues. Of the Robo receptors, Robo4 has received the most attention because it is expressed specifically in the vasculature and upregulated at sites of angiogenesis. Robo4/Slit2 interaction negatively regulates angiogenesis by inhibiting VEGF/VEGFR2 signalling [140]. However, other studies have identified Slit2 as a positive regulator of angiogenesis through interactions with either Robo4 or Robo1 [141, 142]. Conclusive demonstration of Robo1 function in ECs is lacking, because vascular phenotypes in Robo1 knockout mice have not been reported [135], even though small interfering RNA (siRNA) knocks out Robo1 but not Robo4 in ECs, so impairing migration to VEGF in vitro [143]. Whatever the case, the evidence relating Robo4 to LRs is mainly based on its interaction with other partner receptors. In ECs, Robo4 interacts with UNC5B, another EC guidance receptor that, because of its palmitoylation [144], selectively partitions in LRs; such co-localization is functionally important for vessel integrity and angiogenesis inhibition [145]. Furthermore, Robo4 is co-immunoprecipitated with soluble FLT1 (VEGFR1), which is an LR-associated molecule in podocytes [146].

Netrins and UNC5B

Netrins are a class of proteins involved in axon guidance; their structure resembles the ECM protein laminin. The netrin family is composed mostly of secreted proteins, which serve as bifunctional signals: attracting some neurons (chemotropic) but repelling others (chemorepellent) during brain development. Like Robo4, the netrin receptor UNC5B is vasculature specific, and expressed in tip and arterial ECs, and sprouting capillaries [135, 136]. UNC5B activation by Netrin-1/3 prevents filopodia extension in ECs and negatively regulates capillary branching in vessel patterning [147, 148]. Palmitoylation induces UNC5B partitioning in LRs of ECs [144], allowing inclusion of netrin–UNC5B repulsive activity among LR-dependent effects. Netrin-4, another netrin-negative regulator of angiogenesis, does not bind UNC5B directly, but it has been shown to bind to neogenin, an additional netrin receptor molecule that recruits UNC5B to mediate the anti-angiogenic activity of Netrin-4 [149]. The evidence clearly shows that UNC5B–neogenin interaction, which mediates the anti-angiogenic activity of Netrin-4, is an LR-driven process. Blocking neogenin-LR association influences axonal path finding and lowers axonal membrane cholesterol, a process that disrupts LRs and restores neuron locomotor function after spinal cord injury [150]. Another UNC5B ligand, namely FLRT3 (fibronectin and leucine-rich transmembrane protein 3), has been identified [151]. Overall, these data suggest that UNC5B has many ligands and interactors that trigger UNC5B activation and vascular patterning by LR recruitment. Netrins may also have bifunctional activities in the vasculature, because pro-angiogenic netrin activities have been reported [135].

Semaphorins, plexins and neuropilins

Semaphorins (Semas) are a large family (eight classes of molecules have been described so far) of secreted and membrane-bound proteins, characterized by the presence of a common Sema domain, originally described as axon guidance cues and later shown to be regulators of vascular patterning. Semas signal via two receptor families, plexins and neuropilins (Nrps). Membrane-bound Semas bind and signal directly through plexins, whereas most class 3 secreted Semas (Sema3A–3G) are known to bind to a holoreceptor complex that consists of Nrps as the ligand and plexins as the signal-transducing subunit [152, 153]. The exception to this rule is Sema3E, which binds to the vasculature-restricted plexinD1 receptor directly and independently of the Nrps [154]. Blood vessels deflect from chick embryo somites that overexpress Sema3E, indicating that this protein mediates EC repulsion,



restricting blood vessel growth in mice [154]. In axonal growth, LRs mediate the inhibitory effects of Sema3A on growth cones in *Xenopus* spinal neurons [155]. Disruption of LRs by depletion of membrane cholesterol effectively blocks Sema3A-induced repulsion and the extension of growth cones in Xenopus spinal neuron cultures [156]. Furthermore, brief exposure to Sema3A increases the association of Nrp-1 with LRs, implying asymmetrical receptor–LR association and localized signalling in the growth cones during guidance responses. Activation of mitogen-activated protein kinases (MAPKs) on Sema3A treatment appears to depend on the integrity of LRs and is required for Sema3A-induced growth cone repulsion [157]. These data support a role for LRs in mediating growth cone

◀**Fig. 3** Vessel development: lipid-raft-localized molecules involved in vessel branching morphogenesis. Therapeutic targets. *Insets* show selected LRs of the tip-cell filopodia (*inset* **a**) and of the tip-cell/stalk-cell interface (*inset* **b**). *Inset 1* VEGFR2 is located in filopodia [59, 60]. EC guidance receptors are in LRs and their cognate ligands generally induce EC repulsion [140, 144, 145, 147, 148]. VEGF family members also bind to a different extracellular sequence of Nrps, resulting in attraction of vascular structures [155] in a way similar to VEGFA-VEGFR2 interaction. Upon VEGFA/VEGFR2 interaction, MT1-MMP is associated with cav-1 and integrin αvβ3 in caveolar-LRs of filopodia, where it activates pro-MMP2 to MMP2 that is then bound to β3 integrins in LRs [95, 96, 126]. MMP2 activated uPAR-bound pro-uPA to uPA [124], which cleaves the proenzyme plasminogen to yield active plasmin [125] that in turn activates pro-MMPs to active MMPs, as well as pro-uPA to active uPA [117–121]. *Inset 2* the Notch system is located in LRs of the stalk cell. VEGFR2 stimulation upregulates overexpression of the Notch ligand protein Dll4 in the rear moiety of the tip-cell, which leads to CSL-mediated silencing of VEGFR2 expression in stalk-cell [71]. The stalk cell becomes insensitive to VEGF stimulation. Tip-cell/stalk-cell connection is warranted by VE-cadherins (not associated to LRs), and by the LR-associated Eph-Ephrin system [2]. The *red–orange* ellipses identify the LR-directed processes, important in vessel development, that may be potentiated by forcing induction of LR formation to promote functional recovery and normalization of angiogenesis in tumors and other angiogenesis diseases

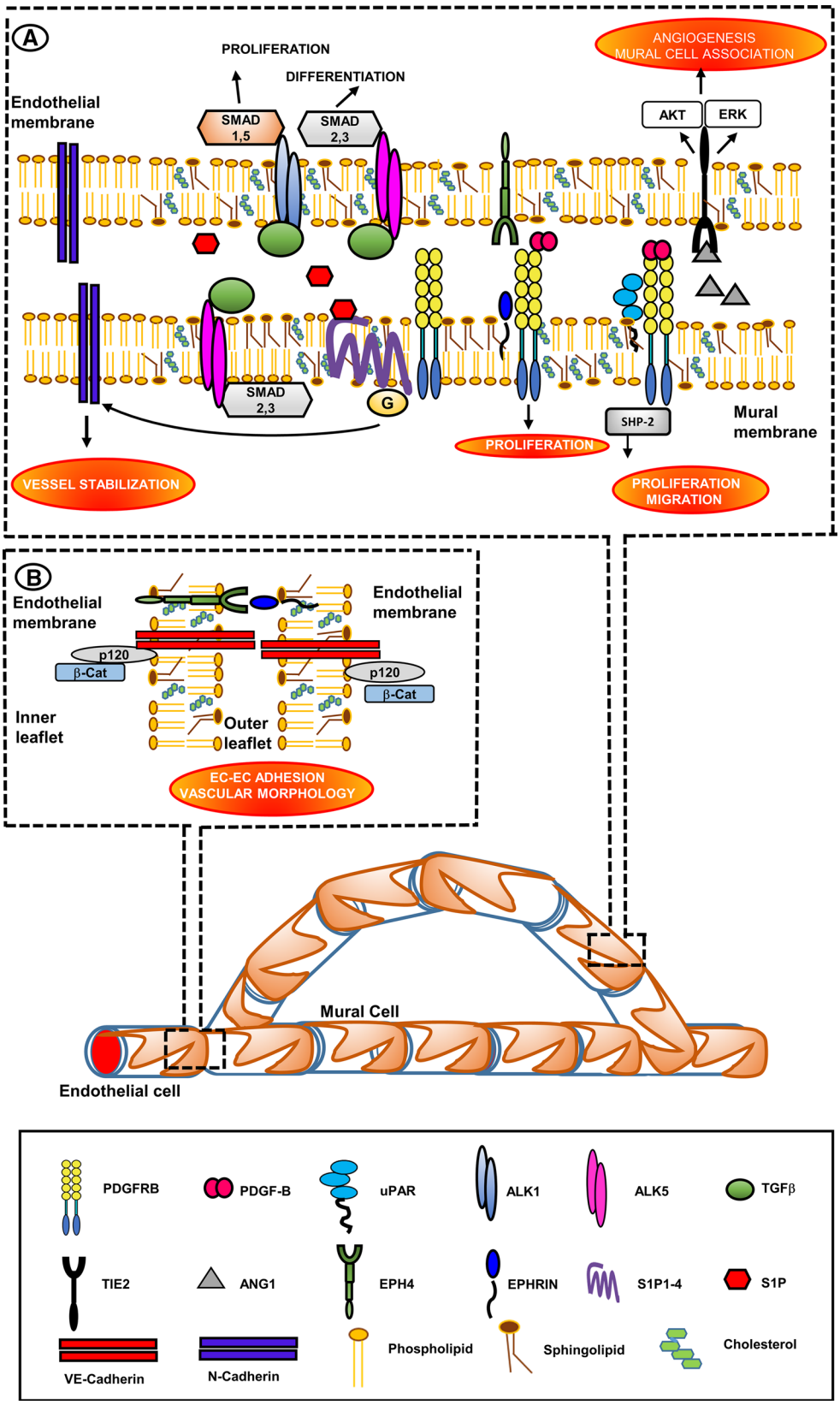
guidance, by providing a molecular platform for the localized assembly of ligand–receptor complexes and their downstream effectors for cytoskeletal rearrangement and local protein synthesis, including Nrp-1, plexins, Src family kinases, Rho GPTases and MAPKs [156].

Besides Semas, VEGF family members also bind to the extracellular domain of Nrps by recognition of a different sequence [155]. In this case, the final result is attraction of vascular structures. In both vessels and axonal growth cones, the members of this system are located in LRs, as also shown by the LR-dependent endocytosis of Nrp1 induced by Sema3C in ECs [158] and by flotillin-mediated endocytic events that dictate cell type-specific responses to Sema3A in cortical neurons [159]. Although the guidance of Nrps in response to Semas in the nervous system is mainly repulsive and mediates growth cone collapse [160], Nrps are attractive in vessels and mediate tip EC extension and directional vessel sprouting in response to the VEGF family. Figure 3 shows a scheme summarizing the integrated activities of LR-associated molecules in tip-cell filopodia and at the tip-cell/stalk-cell interface.

Ephrins and Eph receptors, a multi-purpose system involved in vascular guidance, EC–EC interaction and mural cell recruitment

The Eph-receptor tyrosine kinases and their membrane-bound ligands, the ephrins, mediate cell contact-dependent signalling that controls several aspects of nervous system development, as well as vascular differentiation, guidance, assembly and angiogenesis [135]. Ephrins are cell-surface proteins that are either attached via a GPI anchor (ephrin-A subclass) or a transmembrane sequence (ephrin-B subclass). Ephs have also been classified as EphA and EphB, based on their binding preference to ephrin-A or -B [161]. Besides activating the cognate Eph receptors (forward signalling), ephrins have receptor-like properties, being capable of transduction by themselves (reverse signalling) [161]. Several Ephs and ephrins are present in the vasculature. The ephrin-A1 ligand and its receptor EphA2 are expressed in developing vessels during tumor neovascularization [162], but also in tumor cells, and have been related to EC migration and VEGF expression [163]. Binding of ephrin-B2 to EphB4 modulates EC–EC interactions and is essential in angiogenesis, whereas ephrin-B2 expression in mural cells, such as pericytes and vascular smooth muscle cells (vSMCs), controls their motility, adhesion and recruitment in vessel wall assembly [164]. Among the class B ligands and receptors, ephrin-B2 and EphB4 selectively mark the endothelia of arteries and veins, respectively, and their expression in mice is essential for the angiogenic growth of embryonic vasculature [165]. In the adult, ephrin-B2 expression is upregulated in physiological and pathological angiogenesis [166, 167]. In line with the repulsive role of ephrin/Eph in the nervous system, EphB4 and reverse signalling by ephrin-B2 act as negative regulators of branching angiogenesis by promoting circumferential growth of blood vessels and suppressing EC sprouting [168]. Overall, these data indicate that the precise role of ephrin-B2 and EphB4 in the vasculature remains unclear [135].

Accumulating evidence suggests that both ephrins and Eph receptors are loosely preclustered in LRs of the plasma membrane, forming low-affinity ephrin–ephrin and Eph–Eph dimers, and that ephrin docking may cause an Eph-receptor rearrangement that triggers stable aggregation into larger Eph–ephrin clusters, which may fuse together into larger signalling platforms on Eph receptor–ephrin binding [161, 169–173]. Moreover, it was found that receptor tyrosine kinases, including Eph family receptors, are concentrated and highly organized in caveolar LRs in the neuronal plasma membrane [174]. All the available evidence supports an important role for LR clustering in the initiation, propagation and maintenance of Eph signal transduction events. An interesting paper has highlighted the principle that Eph signalling may be different from outside and inside LR microdomains, which impart the stability of certain dimers of the Eph transmembrane domain, providing proper oligomerization of the receptor, and thus initiating the formation of bigger LRs with large ephrin–Eph signalling clusters within the plasma membrane. This kind of activation has been referred to as a


A
PROLIFERATION
DIFFERENTIATION
ANGIOGENESIS
MURAL CELL ASSOCIATION
Endothelial membrane
SMAD 1,5
SMAD 2,3
AKT
ERK
SMAD 2,3
G
SHP-2
Mural membrane
PROLIFERATION
VESSEL STABILIZATION
PROLIFERATION MIGRATION
B
Endothelial membrane
Endothelial membrane
p120
β-Cat
p120
β-Cat
Inner leaflet
Outer leaflet
EC-EC ADHESION VASCULAR MORPHOLOGY
Mural Cell
Endothelial cell
PDGFRB
PDGF-B
uPAR
ALK1
ALK5
TGFβ
TIE2
ANG1
EPH4
EPHRIN
S1P1-4
S1P
VE-Cadherin
N-Cadherin
Phospholipid
Sphingolipid
Cholesterol

◀**Fig. 4** Vessel maturation: lipid-rafts as platforms of vessel stabilization by promotion of EC/mural cells and EC/EC interactions. Therapeutic targets. *Insets* show the LRs-located molecules that promote vessel stabilization and maturation by allowing mural cells (pericytes in capillaries and vSMS in larger vessels)/ECs (*inset* **a**) and EC/EC interactions (*inset* **b**). *Inset* **a** PDGFRβ is located in LRs of mural cells [181]. PDGFRβ requires the presence of partners for a proper signaling. uPAR associates with PDGFRβ in vSMCs. Assembly of this complex is necessary for signaling and initiation of functional changes in vSMCs mediated by the tyrosine phosphatase SHP-2. The complex is assembled on LRs [182, 183]. In vSMCs Ephrin-B2 interacts with PDGFRβ and controls its distribution and signaling, thereby promoting vSMCs proliferation [184]. PDGFRβ function also involves cooperation with LR-associated S1P receptors [185], thereby stimulating EC recruitment of pericytes and vSMCs [186]. S1P derives from EC sphingolipids that are marker constituents of LRs [187]. The main role of S1P1 receptor involves the trafficking of the cell adhesion molecule N-cadherin, which is not a LR-associated molecule, to the EC-mural-cell contact zone [188]. Tie2 associates with LRs following stimulation with Ang1 [193]. Caveolar-LRs facilitate the degradation of TGFβ receptors and therefore the turn-off of TGFβ signaling [190], thus controlling excess TGFβ activity. *Inset* **b** VE-cadherin is specific to EC adherent junctions and is necessary for vascular morphogenesis. VE-cadherin is associated to LRs. VE-cadherin is in turn associated with p120-catenin, and this interaction is necessary for VE-cadherin recruitment in LRs [13]. Ephrins and Eph receptors form clusters in LRs, providing low-affinity ephrin–ephrin and Eph–Eph dimers. Ephrin docking triggers stable aggregation into larger Eph-ephrin clusters which may fuse together into larger signaling platforms following Eph receptor-ephrin binding [161, 169–173]. The *red–orange* ellipses identify the LR-directed processes that have the chance to favor EC–EC interaction (to prevent endothelial fenestrations and vascular leakage), and vessel wall integrity upon EC-mural cells interaction. Even if N-cadherins are not associated to LRs, their overexpression is a LR-dependent event directed by S1P1 receptor. Potentiation of LR formation may therefore have the chance to promote functional recovery and normalization of angiogenesis in tumors and other angiogenesis diseases where endothelial fenestrations, vascular leakage and loss of mural cells compromise vessel wall integrity

'rotation-coupled activation mechanism' which may take place during Eph-receptor signalling [175].

Lipid rafts and the maturation of blood vessels

The stepwise transition from a growing vasculature to a quiescent and functional vascular network has been referred to as 'maturation' [2]. This implies the suppression of EC proliferation, blocking of proteolytic degradation of the provisional matrix and of sprouting, stabilization of preformed vascular structures, differentiation of specific vascular structures such as valves, fenestrations and tight junctions, and juxtaposition of mural cells, such as pericytes and vSMCs. Mural cell recruitment is a particularly important event, which is fulfilled by pericytes around capillaries and vSMCs around larger diameter vessels. Their ontogeny from progenitor stem cells is still a matter of intensive research [176–178].

PDGF-B receptor and its partners

Expression of the platelet-derived growth factor receptor β (PDGFRβ) tyrosine kinase in pericytes and vSMCs is required for mural cell proliferation, chemotactic migration and incorporation into the vessel wall [179, 180]. PDGF-B depends on LRs for cell signalling, which implies the presence of PDGFRβ in LRs [181]. PDGFRβ requires the presence of partners for proper signalling in mural cells. The uPAR associates with PDGFRβ, which serves as a transmembrane adaptor for uPAR in vSMCs. Assembly of this complex is necessary for transduction of intracellular signalling and the initiation of functional changes in vSMCs. The tyrosine phosphatase SHP-2 mediates these processes, after co-localization with PDGFRβ and uPAR: such a multi-molecular complex is assembled on LRs, the disruption of which precludes SHP-2 phosphorylation, its association with PDGFRβ and vSMC functional responses [182, 183]. Another critical association of PDGFRβ is that with ephrin-B2: in vSMCs ephrin-B2 controls PDGFRβ distribution in the plasma membrane, endocytosis and signalling. In fact, an absence of ephrin-B2 leads to redistribution of PDGFRβ from caveolar LRs to clathrin-associated membrane fractions, impairing vSMC proliferation [184]. Accordingly, mutant mice lacking ephrin-B2 expression in vSMCs develop vessel wall defects and aortic aneurysms [184]. PDGFRβ function also involves cooperation with a further LR-associated family of G-protein-coupled receptors which bind sphingosine-1-phosphate (S1P), a sphingolipid secreted by ECs [185]. The four receptors for S1P, referred to as S1P1–4, are expressed in mural cells, and the number of pericytes and vSMCs recruited in S1P1-deficient mice is compromised [186]. S1P derives from sphingolipids that are marker constituents of LRs, so, in this case LRs not only harbor the relevant receptors, but are also likely to be the source of the cognate ligand [187]. The main role of S1P1 in ECs involves trafficking of the cell adhesion molecule N-cadherin to the EC-mural cell contact zone [188]. ECs express two different classic cadherins—vascular endothelial (VE) cadherin and neural (N) cadherin—with distinct functions in the vasculature. VE-cadherin is specific to EC-adherent junctions and necessary for vascular morphogenesis. It is associated with LRs. LR-associated VE-cadherin is, in turn, associated with p120-catenin, and this interaction is necessary for VE-cadherin recruitment in LRs [13]. N-cadherins show diffuse localization on the EC surface and interact with mural cells for vessel stabilization [13].

TGFβ1 and vascular maturation: a role for LRs?

The differentiation of progenitor cells into mural cells is controlled by transforming growth factor-β1 (TGFβ1)

[189]. TGFβ1 is also involved in vascular formation through activin receptor-like kinase (ALK)-1 and ALK5. ALK5, which is expressed ubiquitously, phosphorylates Smad2 and Smad3, whereas EC-specific ALK1 activates Smad1 and Smad5 [190]. There is evidence that Smad2/3 signalling in ECs is indispensable for the maintenance of vascular integrity via the fine-tuning of N-cadherin, VE-cadherin and S1P1 expression in the vasculature [191]. It is well documented that TGFβ receptors are endocytosed via clathrin-coated vesicles. They may also enter cells via caveolar LRs [192]. Although receptor endocytosis is not essential for TGFβ signalling, clathrin-mediated endocytosis has been shown to promote TGFβ-induced Smad activation and transcriptional responses. Caveolar LRs are regarded as signalling centers for a wide variety of receptors, but are particularly involved in the facilitation of TGFβ-receptor degradation and therefore the turning off of TGFβ signalling [192]. Hence caveolar LRs have the role of controlling excess TGFβ activity.

TIE receptors and angiopoietins

Endothelial cells express TIE2, a tyrosine kinase receptor for angiopoietin (Ang) ligands [193]. Signalling by Ang1, expressed by mural cells, and by TIE2 of ECs promotes angiogenesis and mural-cell association with the endothelium, in addition to reducing vascular leakage [193, 194]. The major intracellular signalling systems activated by TIE2 in response to Ang1 include the Akt and ERK1/2 pathways. TIE2 is not detectable in the LR fraction of human umbilical vein endothelial cells (HUVECs) unless they are first stimulated with Ang1. After stimulation, a fraction of TIE2 associates tightly with the LRs. Treatment of HUVECs with the LR-disrupting agent methyl-β-cyclodextrin selectively inhibits Ang1-induced Akt phosphorylation [195]. Therefore, LRs serve as signalling platforms for TIE2 in vascular ECs, especially for the Akt pathway. Figure 4 shows a scheme summarizing the integrated activities of LR-associated molecules in EC-mural cells and EC–EC interactions for the vessel stabilization.

Conclusions

LRs and vessel ‘normalization’, a therapeutic opportunity for tumors and other non-malignant angiogenic diseases

Given the importance and overall activity of LR platforms as organizers of ‘pro-angiogenic’ molecular assemblies in sprouting ECs, how should we exploit these LR properties for a possible therapeutic approach to control angiogenesis? This would include angiogenesis in pathological cancers and its lack in diseases of ischaemic origin [196]. Anti-angiogenic therapy has shown promise as a treatment for several cancers, opening a new avenue to anti-cancer therapy [50]. The anti-VEGF antibody bevacizumab has shown the best activity among the more than 20 anti-angiogenic drugs that have been investigated to date in clinical trials for several types of cancer. In combination with various chemotherapeutic agents, bevacizumab significantly prolongs progression-free survival for a period of time (3–6 months) and extends overall survival compared with chemotherapy alone [197, 198]. An in-depth analysis of the clinical data has shown that current angiogenic therapy effectively improves progression-free survival in some types of cancer, but that the anti-tumor effects are short-lived. Most patients who initially respond will eventually develop drug resistance, tumor recurrence and cancer metastases [199], indicating that anti-angiogenic therapy is a double-edged sword of tumor progression and metastasis. There are several reasons for this. First, current anti-angiogenic drugs reduce the oxygen supply in tumor tissue, inducing overexpression of hypoxia-inducible factor 1α (HIF-1α), which is a master transcription factor that promotes expression of a variety of tumor angiogenic and metastatic genes in tumor and stromal cells. Such tumor cells become more vasculogenic, aggressive and metastatic, resulting in tumor cell dissemination [200]. HIF-1α-dependent pro-invasive mechanisms of cancer cells involve overexpression of the HGF-receptor tyrosine kinase c-MET [201], a decrease of E-cadherin [202], and induction of MMPs [203, 204] and uPAR [205]. Second, current anti-angiogenic therapies mainly target VEGF signalling and vascular EC-mediated angiogenesis, but do not effectively affect tumor cell-mediated vascular mimicry and other tumor-specific angiogenic mechanisms, which are closely associated with tumor growth and cancer metastasis [44]. Third, the cancer vasculature and cancer microenvironment develop compensatory mechanisms when VEGF is blocked [206], including overexpression of the FGF family of ligands [207], inhibition of Notch signalling [208], increase of PlGF [209, 210], upregulation of Ang1 [211]. Work from the laboratory of Napoleone Ferrara has identified a specific myeloid cell population ($CD11b^{+}Gr1^{+}$) that migrates to tumors under the influence of granulocyte colony-stimulating factor secreted by tumors, interleukin 6 and stromal cell-derived factor 1, and mediates tumor angiogenesis and resistance to anti-VEGF therapy [212]. In the light of these limitations, researchers have explored new strategies for inhibiting angiogenesis. First, there have been many attempts to optimize combinations of anti-angiogenic drugs with chemotherapeutics. Second, researchers are investigating additional anti-angiogenic agents, beyond anti-VEGF and sprouting

angiogenesis, to target all the alternative features of cancer angiogenesis. Third, the so-called 'vascular normalization' has emerged as a new option for the control of cancer angiogenesis. Vascular normalization aims to stabilize disorganized tumor vasculature and improve blood circulation in tumor tissue [213]. Preclinical and clinical data clearly show that tumor vascular normalization by antibodies, peptides, proteins, small molecules and pericytes decreases tumor size and tumor metastasis, even if such drugs target sprouting angiogenesis and display moderate anti-cancer efficiency, similar to anti-angiogenic therapeutics [214].

Beyond cancer angiogenesis, emerging evidence indicates that the amount and structure of vessels in many non-malignant diseases are also abnormal [196, 213]. Hallmarks of altered vessels are disruption of pericyte and EC contacts, thickening of basement membranes, vasodilatation, microaneurysms, vessel tortuosity, oedema, vascular fragility, hemorrhage and hypoxia [196, 213, 215]. Ocular macular degeneration, diabetic retinopathy, wound healing, neurological disorders, haemangiomas, psoriatic skin lesions, arthritic joint disease, atherosclerotic plaques, liver disease (192) and systemic sclerosis [216, 217] are all characterized by vessel tortuosity and abnormal vascularization. Moreover, blood vessel regeneration in peripheral arterial disease [218] and ischaemic heart disease [219] leads to lower capillary density matched against vasculature defects. Pharmacological approaches used to normalize vessels in cancer can also induce vessel normalization in other angiogenic disorders in animal models and patients [220–222].

The major challenge of therapeutic approaches, based on vessel normalization, is that these strategies also stimulate the formation of immature, leaky and disorganized vessels that are poorly perfused and prone to regression once therapy has been halted [213]. We propose that an enhancement of LR activity within tumors and other diseases characterized by abnormal vascularization may promote vascular normalization; this would allow more efficient action of chemotherapeutics and cancer immunotherapy in tumors, and offer the advantage of delivering oxygen and nutrients more rapidly and efficiently to ischaemic tissue, thereby restoring tissue performance in diseases with the signature of dysfunctional angiogenesis. As discussed in this review, the presence of LRs in ECs allows proper organization of various forms of caveolin to form caveolar LRs, in which critical molecules are assembled that allow suitable angiogenesis to occur. Caveolins and caveolar LRs are therefore very relevant to maintaining EC membrane integrity in both structure and function [223, 224], indicating mutual liaison aimed at allowing normal vascular performance. The involvement of caveolae in different cardiovascular diseases makes caveolin-based therapeutic approaches an attractive possibility with which to combat myocardial ischaemia, heart failure and pulmonary hypertension [225]. All forms of caveolin (Cav-1, Cav-2 and Cav-3) are involved, according to their specific tissue distribution. In muscle cells, Cav-3 is associated with LR domains [226]. In line with these observations, the recent work of Roura and co-workers has studied the role of LRs in patients with idiopathic dilated cardiomyopathy (IDCM) [227], showing that the movement of the low-density lipoprotein receptor-related protein 1 (LRP1) to caveolar-LRs and the concomitant increase in non-LR-related ERK1/2/MMP9 activation may have crucial clinical implications in the progression of disease. Although not of ischaemic origin, progression of this disease also has the signature of marked vascular dysfunction, and myocardial LRs are conceivably new molecular actors and therapeutic targets.

Therefore, caveolins/caveolae are now considered to be therapeutic targets for a variety of diseases characterized by an excess of caveolar LR function, by targeting caveolar receptors with peptide antagonists (blockers) or agonists (activators), or with antibodies [228]. Significant alternative interventions to either increase or decrease caveolin expression are gene or cell therapy, anti-sense or siRNA approaches, the use of inhibitory peptides derived from caveolin scaffolding domains, or modulation of cellular cholesterol levels or caveolar lipid content [228].

It has been shown that dietary *n*-3 polyunsaturated fatty acids (*n*-3 PUFAs) alter the size and distribution of cell-surface LRs by forcing the segregation of cholesterol into LRs, thereby enhancing the extent of protein clustering within LRs and, subsequently, their function [229]. Moreover, *n*-3 PUFAs have an angiogenic impact [230] by stimulating cerebral angiogenesis after cerebral ischaemia [231] and reducing pathological retinal angiogenesis [232]. Overall, we believe that there is a theoretical and experimental background for further investigation of this fascinating and still unexplored field of angiogenesis control, with the aim of identifying possible new ways to support sustained angiogenesis normalization to help conventional anti-angiogenesis drugs and chemotherapeutics in tumors and angiogenic diseases characterized by the presence of dysfunctional vessels.

Figures 3 and 4 indicate LR-located molecules whose upregulation following forcing of LR cholesterol levels may promote vascular normalization.

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