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Lysolipids in Vascular Development, Biology and Disease

Eric Engelbrecht¹, Calum A. MacRae², Timothy Hla^{1,*}

¹Vascular Biology Program, Boston Children's Hospital, Department of Surgery, Harvard Medical School, Boston, Massachusetts, USA

²Division of Cardiovascular Medicine, Brigham and Women's Hospital, Department of Medicine, Harvard Medical School, Boston, Massachusetts, USA

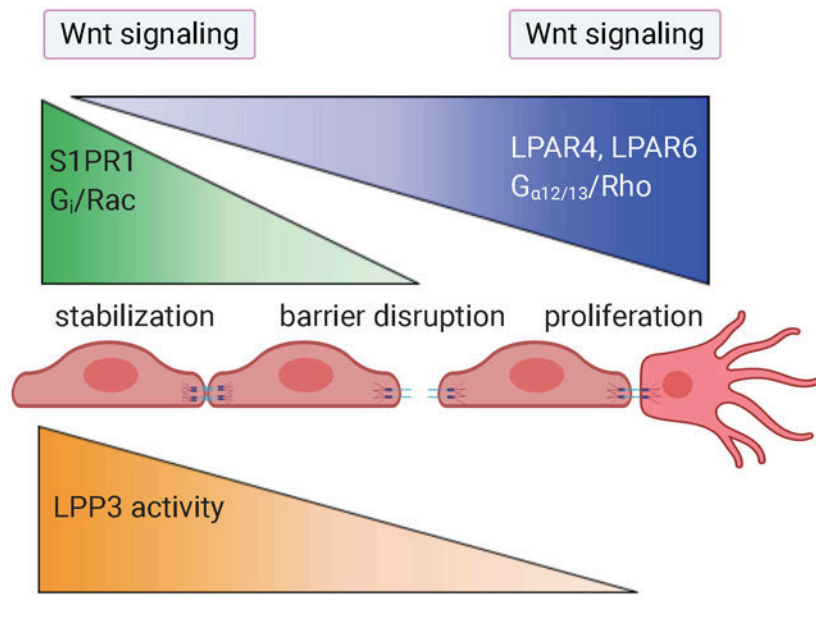
Abstract

Membrane phospholipid metabolism forms lysophospholipids, which possess unique biochemical and biophysical properties that influence membrane structure and dynamics. However, lysophospholipids also function as ligands for G protein-coupled receptors that influence embryonic development, postnatal physiology and disease. The two most well-studied species - lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P) are particularly relevant to vascular development, physiology and cardiovascular diseases. This review summarizes the role of LPA and S1P in vascular developmental processes, endothelial cell biology and their roles in cardiovascular disease processes. In addition, we also point out the apparent connections between lysophospholipid biology and the Wnt pathway, an evolutionarily-conserved fundamental developmental signaling system. The discovery that components of the lysophospholipid signaling system are key genetic determinants of cardiovascular disease has warranted current and future research in this field. As pharmacological approaches to modulate lysophospholipid signaling have entered the clinical sphere, new findings in this field promises to influence novel therapeutic strategies in cardiovascular diseases.

Graphical Abstract

*Correspondence should be addressed to: Timothy.Hla@childrens.harvard.edu.

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A. Introduction

The endothelium is a dynamic and heterogeneous organ system that responds and adapts to various stimuli during embryonic development and postnatal homeostasis. During early development of the vascular system, endothelial cells (EC) undergo vascular network formation in response to hypoxic stimuli and cooperate with other developmental events in organogenesis. Arteries, capillaries, and veins, each with their repertoire of constituent cell types, undergo vessel-specific EC differentiation. Moreover, the vasculature of each organ system exhibits heterogeneity in structure and function, a phenomenon referred to as organotypic EC specialization. Even within the same vessel segment or vascular bed there is local heterogeneity in EC structure, gene expression and function. This allows for plasticity, adaptability and resilience of the vascular system to the changing environments that the organism encounters, thus providing optimal responses for vascular barrier function, tone (contraction/relaxation), inflammation and resolution, thrombus formation, directional blood flow, and the transport of molecules and cells between blood and tissues^{1, 2}.

The diversity of endothelial subtypes is in part determined by a multitude of cell surface receptors that respond to local and/or systemic factors. Well-characterized EC receptors include receptor tyrosine kinases such as the angiopoietin (Ang) receptor TIE2, vascular endothelial growth factor (VEGF) receptors VEGFR1-3, as well as a variety of G protein-coupled receptors (GPCRs). GPCR expression in individual ECs varies depending on the vascular bed, vessel type, flow and developmental context³⁻⁶. Such GPCRs respond to a variety of ligands including small peptides (endothelins, bradykinin, neuropeptides, apelin), morphogenetic factors (Wnt proteins), proteases (thrombin, trypsin), extracellular matrix (ECM), chemokines, 17 β -estradiol, mechanical forces, metabolites, protons (pH), and bioactive lipids (lysolipids, eicosanoids, etc.). In this review, we focus on lysolipid GPCRs, in particular those that respond to metabolites of the membrane phospholipids - sphingosine 1-phosphate (S1P) and lysophosphatidic acid (LPA), as central orchestrators of vascular

development and tractable therapeutic targets in cardiovascular, autoimmune and pathogen-induced diseases.

B. Lysolipids in the vascular system

S1P and LPA metabolism

S1P and LPA are the two well-characterized bioactive lysolipid species. These molecules have hydrophobic backbones and polar phosphate head groups, which makes them impermeant to cellular membranes. S1P is less water soluble than LPA, thus necessitating carrier proteins (termed chaperones) in extracellular compartments^{7,8}. S1P circulates at concentrations of ~0.7 to 1 μM in the chaperone-associated form. Circulating LPA concentrations are much lower (estimated to be in the ten to hundreds of nM) (reviewed in^{7,8}). The majority of circulating S1P (~65%) is bound to the Apolipoprotein M (ApoM) on high density lipoprotein (HDL) particles, while the remainder is associated with albumin^{9,10}. In contrast, ~65% of LPA is associated with albumin while the remainder is predominantly HDL-bound^{11,12}. Thus, HDL and albumin are the primary chaperones for both S1P and LPA. Notably, murine development and survival proceeds in the absence of both ApoM and albumin, whereas in such situations S1P associates with other macromolecules *in vivo*, such as ApoA4¹³.

Albumin-bound S1P and LPA are short-lived in circulation ($t_{1/2} < 20$ min)^{11,14,15} and their concentrations in blood are determined by substrate availability and the activities of metabolic enzymes⁸. Two sphingosine kinase enzymes, SPHK1 and SPHK2, phosphorylate intracellular sphingosine to generate S1P¹⁶, even though a fraction of secreted SPHK1 is present in plasma¹⁷. Erythrocyte-mediated S1P export to blood by the major facilitator family transporter 2b (MFSD2B) accounts for the majority of circulatory chaperone-bound S1P¹⁸. Meanwhile, S1P synthesized in blood and lymphatic vessel endothelial cells is transported to blood or lymph by another transporter - spinster 2 (SPNS2)¹⁹⁻²². Endothelial SPNS2-mediated S1P transport accounts for ~10% of plasma S1P²³ and at least 80% of lymph S1P^{21,24,25}. Thus, S1P in lymph is primarily generated in lymphatic endothelium, whereas S1P in blood is primarily derived from red blood cells and, to a lesser extent, endothelial cells. Restricted expression of *Spns2*, *Sphk1*, and *Mfsd2b* in endothelial and/or erythroid cell types has been corroborated by single cell RNA sequencing from mouse embryos (Figure 1).

LPA synthesis is regulated by autotaxin (ATX), a secreted phospholipase D that removes the choline moiety from lysophosphatidylcholine to generate LPA²⁶. Though lysophosphatidic acids are derived from phospholipids of variable chain lengths, the most abundant circulating species in mammals is 18:1 oleoyl-LPA²⁶. A significant portion of circulating LPA is synthesized in blood upon ATX-catalyzed hydrolysis of circulating lysophospholipids, which are present in lipoproteins (LDL and VLDL) and other carriers²⁶. During embryonic development, ATX is widely expressed and exhibits particularly high expression in osteoblasts, chondrocyte progenitors, endothelium, early mesenchyme, and megakaryocytes (Figure 1).

The influence of diet and microbiome composition on sphingolipid levels and liver health is an active area of research. Dairy products and eggs are enriched with S1P precursors such as sphingomyelin²⁷. However, hydrolytic enzymes in the small intestine and colon, including sphingomyelinase, ceramidase, and glucoceramidase catabolize most ingested sphingolipids to free fatty acids and sphingosine. Nonetheless, sphingolipid-enriched diets have been shown to improve liver health, reduce lipid accumulation in tissue as well as circulating cholesterol in rodent models²⁸⁻³⁰, as well as inhibit atherosclerosis³¹. *Bacteroides*, the major genus in the human gut after weaning³², expresses serine palmitoyltransferase enzymes that participate in *de novo* sphingolipid synthesis³³. Notably, bacterial-derived sphingolipids transfer to host gut epithelium and hepatic portal vein tissue³³. Links between gut microbiota, sphingolipids, dyslipidemia, and cardiovascular health warrant further investigation.

Vascular lysolipid receptors

S1P and LPA are high-affinity ligands that bind their respective receptors with apparent nanomolar dissociation constants³⁴⁻³⁶. There are six known LPA receptors (LPAR₁₋₆)³⁷ and five known S1P receptors (S1PR₁₋₅)³⁸. Single-cell RNA-sequencing of mice at embryonic day (E)9.5 to E13.5 corroborated previous studies^{3, 39-41} demonstrating that *S1pr1* is abundantly expressed in ECs (Figure 1). *S1pr2-5* are expressed at relatively low levels in ECs both during embryogenesis and postnatally (Figure 1). Among the S1PR-deficient mice that have been generated, only *S1pr1*-knockout (KO) animals die embryonically⁴¹ (see Table 1).

Lpar1, *Lpar4*, and *Lpar6* are expressed in embryonic cardiovascular cells including endothelial, smooth muscle, and cardiomyocyte lineages (Figure 1). Among these cell types, *Lpar2* and *Lpar5* are not significantly expressed and *Lpar3* expression is limited to cardiac muscle lineages. While mice lacking any single LPAR can survive to term, only *Lpar4*^{-/-} mice exhibit partially penetrant embryonic lethality as a result of defective vasculature^{7, 42}. This phenotype is fully penetrant and more severe in *Lpar4*^{-/-}*Lpar6*^{-/-} double-KO mice⁴³, suggesting that these receptors have redundant functions during embryogenesis.

S1P and LPA regulate vascular development

Discrete functions of S1P and LPA receptors are attributable in part to their unique distribution, ligand availability, heterotrimeric G protein coupling, and activation of Rho family small GTPases (reviewed in^{7, 36}). Endothelial S1PR1 expression and signaling through G_i/Rac is restricted to perfused, S1P-containing vessels and is essential for vascular stabilization and inhibition of VEGF-induced hypersprouting^{23, 39, 40, 44, 45} (Figure 2). In the absence of endothelial S1P signaling, most, if not all, developing vascular networks exhibit excessive sprouts, branches and hemorrhagic areas that are incompatible with life after embryonic day (E)14.5. Developing capillaries and veins in S1PR-deficient retinas fail to express specialized components of neurovasculature and instead up-regulate migration- and tip cell-associated genes, such as *Esm1*, *Angpt2*, and *Apln*⁴⁵. These findings suggest that S1PR signaling is needed for vascular stability, patterning and organotypic specialization during organ development through pathways conserved from tight coevolution which integrate signaling fitness, mechanical and metabolic inputs⁴⁶.

Unlike S1P/S1PR1 signaling, which is generally perfusion-dependent for receptor expression and ligand availability, LPA appears to be more constitutively available and particularly important for LPAR signaling in ECs of non-perfused vascular sprouts^{43, 47, 48, 49} (Figure 2). LPAR4 and LPAR6-mediated activation of G_{α12/13} / Rho GTPase is required for endothelial proliferation, vascular branching and network expansion^{43, 47, 50}. These findings suggest that S1P signaling is engaged in ECs undergoing integration within the endothelium, maturation and stabilization, whereas LPA signaling occurs in ECs undergoing migration or proliferation. In addition, LPA signaling appears to be involved in vessels undergoing regression⁴⁸.

Accumulating evidence demonstrates that the integral membrane protein lipid phosphate phosphatase 3 (LPP3) inhibits endothelial LPAR signaling specifically at cell membrane regions that participate in cell-cell contact^{43, 47, 50-53}, thereby restricting LPAR signaling to non-contact sites⁵⁰. During vascular development, ECs deficient in cell-cell contacts are found primarily in blind-ended sprouts⁵⁴ and remodeling vessels⁵⁵ (Figure 2). LPP3, a lipid phosphate phosphatase with an extracellular mode of action, is likely an essential cell-autonomous regulator of EC LPAR signaling because EC-specific LPP3-KO embryos die by E11.5 with severe vascular defects in extraembryonic vasculature and in the embryo proper^{51, 56}. Some of these defects may be a consequence of LPP3 loss of function destabilizing β-catenin which has been demonstrated to vary with cell density⁵⁷. Since β-catenin is a key intracellular regulator of adherens junctions and Wnt signaling pathways, this nexus may be the point at which lysolipid and Wnt signaling pathways intersect.

S1P signaling in endothelial cells

In human umbilical vein endothelial cells (HUVECs), S1P binding to S1PR1 induces Ca²⁺ mobilization, activation of the GTPase Rac, actin polymerization of the cortical cytoskeleton, and adherens junction assembly^{58, 59}. Concomitantly, S1PR1 inhibits adenylyl cyclase activity by coupling to G_{αi}⁵⁸, thus reducing intracellular cAMP levels. Other downstream targets of S1PR1 activation include phosphoinositide 3-kinase (PI3K)/Akt activation, phospholipase C (PLC) activation and increased phosphorylated Src (p-Src)^{36, 60}.

Vascular S1P signaling results in pleiotropic yet protective endothelial cell changes. S1PR1-mediated actin rearrangement and adherens junction assembly increases endothelial barrier function *in vitro* and is likely a key mechanism by which S1PR1 enhances vascular integrity and perfusion in lung, trachea, and retina tissues^{39, 45, 61-65}. In the postnatal brain, endothelial S1PR1 limits leakage of small (< 3 kDa) molecules⁶⁶ and also functions in neural progenitor and glial cells to inhibit postnatal hemorrhage in germinal matrix⁶⁷. In large arteries, the S1PR1/G_{αi}/Akt axis regulates blood pressure and vascular tone via activation of endothelial nitric oxide synthase, which produces NO to induce vasorelaxation^{65, 68-70}. During embryonic development, the S1PR1/Rac signaling axis is essential as either S1P deficiency, EC-specific S1PR1 deficiency, or EC-specific Rac1 deficiency results in severe vascular defects and embryonic lethality^{16, 39, 40, 44, 45, 71, 72}.

S1P-deficient embryos lacking sphingosine kinases (*Sphk1*^{-/-}*Sphk2*^{-/-}) exhibit severe hemorrhage, dilated blood vessels, and do not survive after E13.5¹⁶. Similarly, global or

EC-specific *S1pr1* deletion results in severe hemorrhage, disruption of adherens junctions, and hyper-branching of distal vascular beds (e.g. brain and retinal vessels) and major proximal arteries (e.g. dorsal aorta), which is not compatible with life after E14.5^{39, 40, 44, 45}. These animals also exhibit widespread vascular hypersprouting, which is characterized by increased tip cell frequency and filopodia density in developing vasculature consistent with a failure to eliminate excess cells^{39, 40, 45}. Deletion of *Sphk1* in the erythroid lineage of *Sphk2*^{-/-} mice revealed that red blood cells generate ~95% of the S1P content in embryonic tissue, which is required for survival after E13.5²³. In addition to severe hemorrhaging and vascular malformations in the head and aorta, yolk sacs of these S1P-deficient embryos exhibited disorganized, hyper-branching capillary networks²³. Maternal administration of the S1PR1 agonist SEW2871 rescued lethality in embryos lacking RBC-derived S1P, further demonstrating the essential role of S1PR1 in developmental S1P signaling²³. However, these embryos lack *Sphk2* in platelets, and more recent analysis suggested that RBC and platelets have redundant functions as suppliers of embryonic S1P needed for proper vascular development⁷³.

Detailed insights into EC S1PR signaling have been obtained using the retina as a model of vascular network formation and maturation. Over the first 9 days of postnatal murine life, vessels grow radially and form a network of arteries, capillaries, and veins extending to the retina periphery. In perfused vessels of the nascent vascular network, S1P/S1PR1 signaling promotes endothelial maturation and adherens junction assembly^{39, 40, 45}. ECs at the angiogenic front, including tip cells of blind-ended sprouts, are very different from those in the nascent network as they are poorly perfused and lack S1PR1 expression^{39, 40, 45}. Angiogenic front ECs engage in VEGFR signaling which drives expression of JunB, c-Jun, and other “tip cell genes” that contribute to EC proliferation, migration, full integration into the endothelial monolayer and proper patterning^{6, 45, 74-76}. Evidence suggests a mechanism by which S1PR-dependent VE-cadherin assembly promotes endothelial maturation in the nascent vascular network through adherens junction assembly as well as suppression of AP-1⁴⁵ and FOXO1⁷⁷ transcription factors.

In addition, EC S1PR signaling supports vascular maturation through positive regulation of proteins critical for neurovascular specialization (discussed below)^{6, 45, 78-80}.

Concomitantly, EC S1PR signaling suppresses expression of migration-promoting genes often observed in tip cells, including *Esm1*, *Igfbp3*, and *Angpt2*^{6, 74-76, 81}. Ectopic expression of tip cell genes in the nascent network goes hand-in-hand with hypersprouting^{39, 40, 45, 75}. Inversely, inducible S1PR1 over-expression suppressed vascular sprouting and tip cell frequency, resulting in hypovascularization³⁹. Genetic mosaic studies showed that S1PR1-expressing ECs tend to incorporate into the mature regions of the vascular network rather than adopt a tip cell position⁴⁰, further supporting the notion that S1PR1 facilitates vascular maturation in a context dependent cell-autonomous manner.

Expression of Notch pathway components and target genes, which also inhibit hypersprouting⁷⁵, was unaffected in S1PR-KO retinal ECs^{39, 40, 45}. In addition, aortic hyperbranching was observed in *S1pr1*^{-/-} and EC-specific S1PR1-KO embryos but not in *Dll4*^{+/-} or EC-specific *Rbpj*-KO embryos. These distinct outcomes downstream of S1P or Notch inhibition suggest that these ligands inhibit hypersprouting through discrete

mechanisms^{39, 40, 45}. However, S1PR and Notch signaling pathways may intersect and/or cross-regulate each other in additional biological contexts, such as biomechanical signaling^{82, 83}. This framework may also be understood in the context of interactions between different cell competition pathways, but these hypotheses are only now beginning to be tested^{46, 84}.

LPA signaling in vascular development

Endothelial responses to LPA and S1P are very different, if not in direct opposition. In HUVECs, LPA signals through LPAR6 and rapidly induces actin stress fibers that decrease cell-cell adhesion and cause intercellular gaps^{50, 85}. After binding LPA, LPAR6 couples with $G_{\alpha 12/13}$ and activates the GTPase RhoA and its target Rho kinase (ROCK1/II)^{50, 85}. This is the mechanism by which LPA reduces endothelial barrier integrity and promotes vascular leak^{50, 56, 85}. Inhibition of any individual component in LPA signaling, including LPAR6, $G_{\alpha 13}$, RhoA, or ROCK abrogates LPA induction of stress fibers^{50, 85}. Like LPAR6, LPAR4 activates the $G_{\alpha 12/13}$ /Rho/ROCK pathway³⁵, but can also couple with $G_{q/11}$, $G_{i/o}$, and G_s ³⁵. Overlapping signaling components downstream of LPAR4 and LPAR6 in embryonic endothelium may contribute to their functional redundancy during embryogenesis⁴³. Indeed, all *Lpar6*^{-/-} mice and most *Lpar4*^{-/-} mice survive to term, but all *Lpar4*^{-/-}*Lpar6*^{-/-} double-KO mice die by E10.5 due to vascular defects^{42, 43}. LPA-deficient *Atx*^{-/-} mice, EC-specific $G_{\alpha 13}$ -knockout, and *Rock1*^{-/-}*Rock2*^{-/-} mice each die embryonically with impaired vascular development⁸⁶⁻⁹⁰, suggesting that these genes encode essential components for EC LPAR signaling. Endothelial RhoA, however, is dispensable as EC-specific RhoA-knockout animals develop normally⁹¹, suggesting that EC LPARs signal through one or more of the ~18 Rho GTPases expressed in endothelium⁹².

Phenotypes associated with *Lpar4*^{-/-} embryos include pericardial effusion, severe edema, general fragility, subcutaneous hemorrhage, and lethality (~35% penetrant)⁴². *Lpar4*^{-/-}*Lpar6*^{-/-} phenotypes include pericardial effusion, severe developmental delay, poor vascular network formation in the head and intersomitic regions, absence of blood vessels in the yolk sac, and death by E10.5 (100% penetrant)⁴³. Both global and EC-specific $G_{\alpha 13}$ -KO mice die between E9.5 and E11.5 and fail to form normal yolk sac vasculature^{86, 87}. This phenotype was also seen in *Rock1*^{-/-}*Rock2*^{-/-} embryos, which die between E8.5 and E9.5⁸⁸. Thus, the LPAR/ $G_{\alpha 13}$ /ROCK signaling axis is essential for early vascular development.

In contrast to the hypersprouting phenotypes in S1PR1-deficient retinas, EC-specific LPAR4/LPAR6 double-KO (*Lpar4*:*Lpar6*^{EC}) mice exhibit hyposprouting with reduced vascular density and branching⁴³. Tip cells in *Lpar4*:*Lpar6*^{EC} retinas were few in number and exhibited defects including reduced filopodia length and frequency⁴³. This hypovascular phenotype was reproduced in *Lpar6*^{-/-} retinas, suggesting that LPAR4 and LPAR6 are not entirely redundant during sprouting angiogenesis and that LPAR6 may be the primary mediator of LPA-induced EC proliferation during retinal development⁴⁷.

ATX is widely expressed during embryogenesis and is essential for embryonic development^{89, 93}. *Atx*^{-/-} mice are LPA-deficient and have vascular defects in the embryo proper, lack yolk sac vasculature and die from circulatory failure by E10.5^{89, 90}. These phenotypes were reproduced in embryos harboring a biallelic single amino acid substitution rendering ATX

catalytically inactive, suggesting that enzymatic formation of LPA (or a related molecule) is the primary defect⁹⁴. This is in stark contrast to LPP3, for which there is substantial *in vitro* and *in vivo* evidence for an EC-autonomous LPA/LPP3/LPAR axis that controls the sub-cellular location of LPAR signaling.

Encoded by the gene *Ppap2b*, LPP3 is a glycoprotein with a channel-like structure composed of six putative transmembrane domains⁹⁵. LPP3 is best known for catalyzing dephosphorylation of phosphatidic acid, ceramide 1-phosphate (C1P), S1P, and LPA to generate diacylglycerol, ceramide, sphingosine, and monoacylglycerol, respectively⁹⁵. Biochemical analysis showed that human LPP3 has highest affinity ($1/K_m$) and catalytic efficiency (V_{max}/K_m) for LPA and PA, whereas these values were 3-4 times lower for S1P and C1P⁹⁶. Reduction of LPP3 activity can result in local and/or systemic accumulation of LPA, phosphatidic acid, C1P, and S1P⁹⁷. For example, cardiac-specific LPP3-KO mice harbor ~3-fold higher [LPA] in circulation relative to wild-type counterparts⁹⁸.

Lpp3 is expressed in many cell types and structures during embryogenesis including, but not limited to, endothelial, cardiac, vascular, nervous, and mesenchymal tissues⁹⁹ (Figure 1). Both global and EC-specific LPP3-KO mice die by E10.5 and exhibit hemorrhage of the embryo proper, defective yolk sac vasculature, and failure to form a chorioallantoic placenta^{51, 99}.

In HUVECs, LPP3 appears to partition LPAR signaling between regions of strong and weak cell-cell contact^{43, 47, 50-53}. LPP3 knockdown enhanced sensitivity to LPA-induced stress fibers⁵⁰. This LPA response was more robust in sub-confluent HUVECs, indicating that cell-cell contacts are involved in inhibition of LPAR signaling. After treatment with forskolin to enhance cell-cell adhesion, LPP3 localized to sites of cell-cell contact and the LPA-induced stress fiber response was abolished, suggesting cross talk with cAMP-regulated signaling pathways⁵⁰. However, forskolin-treated HUVECs deficient in LPP3 were sensitive to LPA, suggesting that LPP3 inhibits LPAR signaling in ECs with strong cell-cell adhesion⁵⁰. In scratch assays, LPP3 localized to sites of cell-cell contact but was absent from non-contact sites in “leader cells” at the monolayer edge⁵⁰. The leading edges (non-contact sites) of these cells showed robust stress fiber responses after LPA treatment⁵⁰, suggesting that LPAR signaling is permitted in membrane compartments that lack cell-cell contact and therefore also lack LPP3 activity. Studies using LPP3-KO mouse embryonic ECs⁵¹ or LPP3-KO human aortic ECs (HAECs)⁵² reported that LPP3 promotes adherens junction assembly^{51, 52} and inhibits intercellular gap formation⁵². LPP3-deficient HAECs showed reduced barrier function⁵³, suggesting that LPP3 promotes endothelial barrier function, perhaps by attenuating LPA-dependent Rho GTPase activation.

These results demonstrate that LPP3 can localize to sites of endothelial cell-cell contact and inhibit LPA/LPAR signaling, restricting high levels of LPAR signaling to non-cell-cell contact sites. Non-contact sites of EC membranes have important migration-associated functions in tip cells and remodeling capillaries undergoing regression⁵⁵, a process promoted by LPA/LPAR signaling⁵⁰ (Figure 2).

EC-specific LPP3-KO (LPP3-ECKO) embryos die at or before E10.5 with hemorrhagic areas in the embryo proper, abnormalities of the aortic sac, outflow tract, irregular intersomitic vasculature, defective yolk sac vasculature, failure to form a chorioallantoic placenta, as well as decreased cardiac trabeculation and growth of the compact myocardial wall^{51, 56, 99}. These findings suggest that LPP3 function leads to spatial restriction of lysolipid receptor signaling to regulate vascular development (Figure 2).

In summary, mechanistic studies that reveal the *in vitro* differences between S1P and LPA signaling explain, at least in part, the *in vivo* functions of these lysolipids. S1P signaling-deficient mice exhibit vascular hypersprouting, which is characterized by hyper-branching, disorganized vascular networks that are poorly perfused and lack barrier integrity. In contrast, LPA signaling-deficient embryos exhibit vascular hyposprouting with notable absence or apparent lack of blood vessels in some tissues, particularly in the yolk sac. Conversely, S1P1/G_i/Rac signaling promotes cell-cell contact, adherens junction assembly, and vascular stability (Figure 2).

Whether endothelial LPARs exhibit spatial expression gradients during vascular development remains to be determined. Our understanding of *in vivo* LPAR and S1PR distributions, including sub-cellular localization, has been limited by the lack of widely available high quality antibodies and the technical challenges associated with precise measurement of lysophospholipid gradients in complex tissues¹⁰⁰.

C. Developmental studies suggest lysolipid and Wnt connections

As outlined above, many of the core processes influenced by lysolipid signaling have also been observed to be regulated, to some extent, by the central pathways of early development; Notch, BMP and in particular Wnt signaling. This is interesting given the dependence of Wnt signals on lipids and lipoproteins¹⁰¹, and the intricate relationships between Wnt signals, cell polarity, intercellular junctions, cytoskeletal structure and metabolism^{84, 102, 103}. Wnt signaling's role in central decisions is illustrated by the diverse effects of mutations in Wnt pathway genes during development and in disease¹⁰⁴. Importantly, there are often parallels between the effects of Wnt gain of function and loss of function which suggest a tuning of a wide range of signaling pathways, though in many instances this may simply reflect our limited understanding of the relevant biology^{102, 105}. There are several key downstream effectors of Wnt including the β -catenin destruction complex, the planar cell polarity pathway and a complex non-canonical pathway which appears to modulate the multifaceted effects of calcium signals throughout the cell^{102, 104}.

Specific lipids and lipoproteins are required for the transmission of Wnt signals between cells, and inability to package Wnt ligands in appropriate lipoprotein essentially abolishes the downstream effects in receiving cells¹⁰¹. Classical Wnt effects are restricted to only a few cell diameters in range, but recent evidence of signaling roles for circulating lipoproteins in higher vertebrates raises the possibility that Wnt signals may also operate at a distance^{106, 107}. The lipid requirements for Wnt signal transduction are poorly understood, but specific lipids or lipoproteins may be necessary for endocytosis in the receiving cell of the ligand-receptor/co-receptor complexes and/or subsequent gating of the signals by

intraorganelle pH or other factors^{103, 108-110}. The parallels between lysolipid pathways and Wnt in development warrant further studies to elucidate underlying mechanisms. Some recently-described mechanisms are discussed in detail below.

S1P and Wnt signaling in blood-retina-barrier development

Like lysolipid signaling, Wnt signaling is transduced by a family of receptors with seven transmembrane domains, some of which have been shown to couple to heterotrimeric G proteins¹¹¹. The protein Norrin (*Ndp*), a TGF- β family member produced by glia, is a high-affinity Wnt-like ligand for its EC receptor Frizzled4 (FZD4)¹¹². Norrin/FZD4 signaling increases the activity of β -catenin and TCF/LEF transcription factors (TFs)¹¹³, which leads to induction of proteins that promote a functional blood-retina-barrier (BRB) and suppression of proteins that cause vascular leakage or fenestration^{113, 114}. The developing BRB becomes dysfunctional upon loss of Norrin, or EC-specific deletion of *Fzd4*, *Ctnnb1* and *S1pr1*^{39, 40, 45, 113, 114}. There are shared albeit distinct outcomes of endothelial S1P/S1PR and Norrin/FZD4/ β -catenin signaling in BRB development, which suggests both convergent and divergent signaling, possibly as a function of underlying metabolic states, microenvironmental or humoral factors¹⁰³.

Wnt-deficient retinas are hypovascular¹¹³⁻¹¹⁶ whereas S1PR1 signaling-deficient retinas are hypervascular. Thus, Wnt signaling promotes EC proliferation and vascular branching¹¹⁶ while S1P signaling stabilizes blood vessels^{39, 40, 45}. An *in vivo* reporter of canonical Wnt signaling was active throughout the developing retinal vascular ECs⁸⁰, suggesting that β -catenin and TCF/LEF factors are active in blind-ended vascular sprouts as well as in ECs undergoing maturation. Whether Wnt signaling is involved in expression of tip cell genes at the vascular front is not known. However, there is evidence suggesting that both Wnt and S1P signaling suppress a tip cell gene expression program in the nascent vascular network.

Deficiency of either Wnt or S1P signaling results in vascular leakage and hemorrhage^{39, 45, 113}, which can increase the concentration of VEGF and activation of VEGFRs. In fact, EC VEGFR signaling is required for expression of the prototypical tip cell genes *Esm1*, *Apln*, and *Igfbp3*⁷⁴, which are induced in retinal ECs that lack Wnt or S1P signaling (Figure 3). While tissue hypoxia and high VEGF might contribute to ectopic expression of tip cell genes in *Ndp*-KO and *S1pr*-KO retinal ECs, it is also possible that VEGFR-independent mechanisms contribute to S1P- and Wnt signaling-mediated suppression of these genes in the nascent vascular network. Indeed, tissue hypoxia might be anticipated to interrupt Wnt signaling, possibly explaining some of the selective phenomena observed^{103, 109}.

Yanagida et al. (2020) reported that 97 neurovasculature-enriched transcripts were down-regulated in S1PR-deficient retinal ECs⁴⁵. *Ndp*-KO retinal ECs also down-regulated many of these transcripts (Figure 3A,B)¹¹⁷. For example, both Wnt and S1P signaling are required for normal expression of tight junction components (*Lsr*, *Ocln*), transporters (*Mfsd2a*, *Tfrc*), and transcription factors (*Lef1*, *Tcf7*, *Zic3*) that are enriched in the vasculature of the central nervous system (CNS)⁴⁵ (Figure 3). Expression of *Cldn5* and *Plvap*, well-characterized targets of Wnt signaling in the BRB, were not significantly affected in S1PR signaling-deficient retinal ECs (Figure 3A,D)⁴⁵. Therefore, S1P and Wnt signaling regulate common and distinct gene sets that determine neurovascular structure and function. For example,

suppression of *Plvap* (fenestrae) and induction of *Cldn5* (tight junctions) are likely two mechanisms by which Wnt signaling, but not S1P signaling, promote BRB integrity (45 and Figure 3). However, S1PR1 signaling is upstream of CLDN5 expression in lymphatic ECs during developmental lymphangiogenesis¹¹⁸. Thus, S1PR1 and other EC surface receptors do not exhibit a “one-size-fits-all” model of signaling and transcriptional outputs, but rather have unique functions according to developmental stage, vascular bed, microenvironmental context, and EC subtype.

Any epistatic relationship between endothelial Wnt and S1P signaling is anticipated to be multifaceted and insights will likely require analysis of novel mouse strains¹⁰², such as *Ctnnb1^{flex3/+};S1p1^{f/f};Cdh5-Cre^{ERT2}*, which would address whether increasing β -catenin activity is sufficient to rescue maturation defects in S1PR1-KO retinal ECs. The *Ctnnb1^{flex3}* allele encodes a β -catenin protein that resists degradation and is sufficient for induction of a BRB-like endothelial phenotype (MFSD2A+, LEF1+, CLDN5+, PLVAP-) in the vasculature of circumventricular (e.g. neuroendocrine) organs⁸⁰. Additionally, *R26-8xTCF/LEF-LSL-H2B-GFP;S1p1^{f/f};Cdh5-Cre^{ERT2}* mice would likely address the effect of EC-specific *S1pr1*-knockout on β -catenin and TCF/LEF transcriptional activity in retinal ECs⁸⁰.

Mosaic deletion of *Fzd4* in BRB and BBB vasculature showed that induction of CLDN5 and suppression of PLVAP occurs in a FZD4-dependent, cell-autonomous fashion and is unlikely secondary to hypoxia or high VEGF levels⁸⁰. Similar mosaic experiments in S1PR signaling-deficient mice might reveal cell-autonomous S1PR versus hypoxia-dependent effects on expression of BRB-enriched genes such as *Mfsd2a*, *Tfrc*, and *Lef1*. Alternatively, fms-like tyrosine kinase-1 (sFlt1) that blocks VEGF signaling maybe a useful reagent to investigate the role of this pathway in Wnt- and S1P signaling reporter mice. Because VEGF itself can induce vascular leak¹¹⁹, adherens junctions (VE-cadherin) should also be examined to determine the role of aberrant VEGF signaling in junctional breakdown observed in S1PR-KO retinal vasculature^{39, 40, 45}. These experiments may provide novel mechanistic insights into S1PR- versus VEGFR-mediated gene expression and vascular function during BRB development in the context of Wnt signaling.

LPP3, Lysolipids and Wnt signaling in gastrulation and axial patterning

Some *Lpp3^{-/-}* embryos exhibited defective gastrulation with axis duplication at E7.5 (30% penetrance), a phenotype reminiscent of ectopic Wnt signaling⁹⁹. Over-expression of *Xwnt3a* or *Xwnt8* induces axis duplication in *Xenopus* embryos⁹⁹, as does over-expression of *Cwnt8C*¹²⁰ or ablation of the Wnt signaling inhibitor *Axin*¹²¹ in mouse embryos. At E7.0, expression of *brachyury*, a WNT3 target gene, is restricted to the primitive streak⁹⁹; however, *Lpp3^{-/-}* embryos with axis duplication have two *brachyury*-expressing primitive streak structures⁹⁹. In addition, expression of the Wnt signaling antagonist *Dkk1* is markedly reduced in *Lpp3^{-/-}* embryos⁹⁹. Importantly, axis duplication induced by injection of *Xwnt3a* or *Xwnt8* mRNA was inhibited by co-injection with mouse *Lpp3* mRNA, demonstrating that LPP3 can inhibit signaling by these Wnt ligands⁹⁹.

There are several open questions related to LPP3-mediated axial patterning, including whether specific lysolipid receptors are involved. To date, axis duplication has not been reported in S1PR- or LPAR-deficient mice, suggesting that LPP3-mediated axial patterning

does not require signaling by individual lysolipid receptors. This notion is supported by insights from *Drosophila*, which lack orthologs of vertebrate G protein-coupled lysolipid receptors but express two LPP3 homologs, *wun* and *wun2* (Wunens) that are essential for gastrulation^{95, 99}. In somatic cells, *wun* and *wun2* produce phospholipid metabolites that serve as guidance cues by repelling germ cells (GCs), a process that is required for bilateral sorting of GCs away from the ventral midline^{95, 122}. Wunen-deficient embryos have scattered GCs and high frequency of GC death¹²³. Interestingly, a *Drosophila* GPCR called Tre1 is required for GC migration towards high concentrations of Wunen-generated phospholipids in a G_{αo}-dependent manner¹²⁴. In support of the notion that Tre1 binds Wunen metabolites, the human Tre1 homolog, GPR84, binds medium chain fatty acids¹²⁵. Thus, Tre1 might be the primary receptor for Wunen-generated phospholipids, though this would require confirmation by receptor signaling assays.

Tre1, in addition to guiding GC migration, is required for Rho-mediated protein polarization in GCs¹²⁴. *Drosophila* Tre1 may have conserved functions that are split among multiple proteins in vertebrates¹²⁴. The presence of compensatory mechanisms in vertebrates may explain why defective gastrulation is ~30% penetrant in *Lpp3*^{-/-} mice but 100% penetrant in Wunen-null *Drosophila* embryos. In addition, the phosphatase activity of *wun2* is essential for GC survival¹²⁶. Human LPP3 can rescue GC death in *wun2*-deficient embryos, suggesting an evolutionarily conserved phosphatase function for LPP3 in gastrulation¹²⁶.

Evidence to date strongly suggests that the phospholipids metabolized by LPP3 must be spatially compartmentalized to ensure normal axial patterning. Given that lipoprotein-derived precursors feed into lysolipid metabolic pathways, LDL, VLDL and other lipoproteins may be involved in spatial control of lysolipid signaling. However, identification of the specific lipids, the means of compartmentalization and clarification of downstream signaling mechanisms all remain a major challenge. Isolation of these molecules will provide mechanistic insight into Wunen and LPP3-mediated axial patterning, cell survival, and may also reveal how phospholipids regulate Wnt signaling in vertebrates and/or invertebrates¹²⁷. A detailed mechanistic understanding of embryonic Wunen and LPP3 metabolites and cognate receptors may inform exploration of LPP3-mediated endothelial functions during development, postnatal homeostasis and disease.

D. Lysolipids in cardiovascular disease

Insights from human studies

In the 1970s, landmark studies reported negative correlations between coronary artery disease (CAD) severity and circulating HDL levels¹²⁸⁻¹³⁰. Subsequent biochemical analyses of HDL particles have uncovered significant heterogeneity¹³¹. For example, on a stoichiometric basis, 1 in 10 HDL particles contains S1P¹³². Even though only a minority of studies in the HDL field have focused on lysolipids, recent work has linked HDL-S1P to CAD. For example, HDL-S1P, 1) correlates inversely with the severity of coronary atherosclerosis¹³³, 2) is an independent predictor of coronary in-stent restenosis¹³⁴, 3) is lower in patients with stable CAD than in healthy individuals^{135,136}, and 4) correlates negatively with the occurrence of CAD independently of HDL-cholesterol¹³⁶ (reviewed in¹³⁷). In HUVECs, HDL isolated from CAD patients was ineffective at stimulating S1PR1-

dependent vasoprotective signaling events, including vasodilation, which was rescued by providing exogenous S1P¹³⁸.

Lipoprotein(a), which is highly predictive of cardiovascular diseases in humans, was shown to supply autotaxin and therefore involved in local signaling of LPA via its receptors. In calcific aortic valve disease, lipoprotein(a)-derived autotaxin directly induces valve fibrosis and calcification, presumably via a Rho GTPase signaling pathway^{139, 140}. Inhibitors of this signaling axis may be useful in the medical management of aortic valve diseases.

S1PR1 genomic heterogeneity may contribute to cardiovascular disease risk. For example, SNPs in the N-terminal cap region of S1PR1 were associated with multivessel cardiovascular disease in a patient cohort, suggesting a potential regulatory function of this receptor¹⁴¹. Asthma is characterized by a chronic inflammatory process with increased vascular permeability. Several SNPs (rs2038366, rs3753194, rs59317557) in the putative enhancer and promoter regions of *S1pr1* associate with increased risk of asthma development¹⁴². The SNP rs2038366 was notable for conferring *S1pr1* downregulation by luciferase assay in human pulmonary artery ECs¹⁴².

S1PR1: Pharmacologic considerations

The FDA-approved S1PR functional antagonist FTY720 (fingolimod) is prescribed as an immunomodulatory agent to treat relapse-remitting multiple sclerosis¹⁴³. This drug's mechanism of action is down-regulation of lymphocyte S1PR1 in lymphoid tissue, which sequesters lymphocytes by preventing chemotaxis towards high [S1P] in circulation¹⁴⁴. While this lymphocyte-targeted drug has clear immunological benefits, endothelial S1PR1 is also being explored as a therapeutic target in autoimmune and fibrotic conditions. Studies using experimental disease models (discussed below) suggest that endothelial S1PR1 agonism mitigates inflammation through at least two mechanisms: 1) enhancing the vascular barrier and 2) attenuating endothelial inflammatory responses, each limiting leukocyte recruitment to tissue parenchyma¹⁴⁵.

The notion that pharmacologic or endogenous agents elicit varying degrees of “biased” engagement of S1PR1 with either G_i/Rac or β-arrestin pathways has both clarified and added complexity to our understanding of S1P signaling^{38, 106, 146}. For example, arterial ECs of the thoracic aorta express S1PR1 mRNA¹⁴⁷ and protein¹⁰⁶ in a homogenous manner and are exposed to circulatory S1P. However, S1PR1/β-arrestin coupling is heterogeneous in aortic arterial endothelium of adult mice^{106, 147}, and the frequency of S1PR1/β-arrestin coupling increases with temporal transition from early postnatal to young adult, which coincides with upregulation of thrombospondin-1^{106, 147}. Despite high expression and the functional importance of S1PR1 in early postnatal mouse retinal ECs, we observe relatively low levels of S1PR1/β-arrestin coupling in these cells (unpublished observation). In contrast, developing embryonic lymphatic vessels show high levels of S1PR1/β-arrestin coupling¹¹⁸. While these data suggest a spectrum of S1PR1/β-arrestin signaling among ECs, we lack *in vivo* evidence for such a spectrum of S1PR1/G_i signaling. We can hypothesize that cells highly engaged in β-arrestin signaling are relatively low in S1PR1/G_i activity. Endogenous mechanisms that skew S1PR1 towards β-arrestin versus G_i are unclear, but may involve concomitant signaling pathways, such as LPARs¹⁴⁸, VEGFRs,

junctional signals, shear force responses, or S1PR1 association with presently unknown cofactors. A prototypical example of cofactor-dependent signaling in vascular endothelium occurs in the CNS, when ECs respond to WNT7 ligands with the multi-protein complex of FZD4/GPR124/RECK/LRP6¹⁴⁹¹⁵⁰¹⁵¹¹⁵².

Patients taking FTY720 risk complications from lymphopenia, which is a direct result of β -arrestin-mediated S1PR1 internalization in lymphocytes^{144, 153}. A similar mechanism in ocular endothelium may underpin macular edema that occurs in a small subset (0.8-1.5%) of patients¹⁵⁴. Vascular development is apparently unaffected in mice expressing two mutant S1PR1 alleles (*S1pr1^{S5A/S5A}*) that encode a β -arrestin coupling- and internalization-defective receptor¹⁵³. Furthermore, *S1pr1^{S5A/S5A}* mice are more resistant to FTY720-induced lung vascular leakage and S1PR1 degradation⁶¹. Therefore, endothelium-targeted S1PR1 agonists would likely provide maximal therapeutic benefit if biased towards activation of G_{α_i} /Rac to limit β -arrestin recruitment (i.e. mimic *S1pr1^{S5A/S5A}*) and avoid receptor degradation and lymphopenia. A compound matching these criteria was recently described and showed therapeutic efficacy in preclinical models of coronary endothelial damage and renal ischemia/reperfusion injury¹⁴⁶. In a recent phase 1 clinical study of diabetics, this compound, SAR247799 stimulated myocardial perfusion without inducing lymphopenia in diabetics¹⁵⁵.

Taken together, these studies of S1PR1 signaling highlight an important consideration for drug development and remind us that S1P measurement in patient fluids or tissue does not inform on S1PR1 expression or the extent of β -arrestin versus G_i /Rac signaling, which may have more functional significance than S1P levels alone.

S1P in experimental disease models

Rheumatoid arthritis and systemic lupus erythematosus, though etiologically complex, share the pathophysiologic mechanisms of neutrophil activation and immune complex (IC) deposition in tissues with resultant end-organ damage. S1PR1 agonism limits vascular barrier leakage associated with IC deposition⁶⁴. Inversely, genetic inactivation of EC S1PR1 or pharmacologic S1PR1 antagonism resulted in more vascular leak and pulmonary neutrophil accumulation relative to control animals⁶⁴.

After organ damage, endothelial S1PR1 promotes recovery, regeneration and limits fibrosis. In a hydrochloric acid-induced model of lung injury, EC S1PR1 protected against vascular leak and limited fibrosis¹⁵⁶. Following partial hepatectomy, EC S1PR1 protected against fibrosis and improved liver vascular function, perfusibility, tissue regeneration, and animal survival¹⁵⁷. Perhaps it is not surprising that S1PR1 is important for tissue regeneration as the vasculature is a critical component of most major organ systems and S1PR1 is a central regulator of vascular network formation, but other roles for lysolipid signals in endothelial or epithelial biology may be involved.

In the murine *Apoe*^{-/-} high-fat diet (HFD) model, EC-specific S1PR1 deficiency exacerbated disease severity and macrophage infiltration into atherosclerotic plaques¹⁰⁶. While S1P signaling in ECs seems protective in the context of atherosclerosis, S1P regulation of macrophage phenotypes is more complex. S1PR2¹⁵⁸ or S1PR3¹⁵⁹ deficiency

attenuated foam cell accumulation into lesions – an effect that is myeloid cell-intrinsic, as evidenced by bone marrow chimera experiments. In contrast, S1PR1-specific agonists confer an anti-inflammatory macrophage phenotype *in vitro*¹⁶⁰. Thus, we propose a model describing pro- and anti-inflammatory effects of S1P as first compartmentalized among cell types (vascular vs myeloid)¹⁶¹, secondarily compartmentalized between different S1P receptors (S1PR1 vs S1PR2/3), and at a tertiary level when considering S1PR1 association with G_i/Rac versus β -arrestin pathways.

S1PR1 signaling is engaged in arterial ECs of the aorta intima as well as in adventitial lymphatic endothelium¹⁴⁷. In homeostasis, S1PR1 attenuates expression of pro-inflammatory transcripts in arterial (e.g. *Cx3cl1*/fractalkine, *Vcam1*, *Ptgs2*) and lymphatic ECs (*Ccl21*, *Irf8*, *Il7*). Furthermore, S1PR1 is a critical regulator of developmental lymphangiogenesis¹¹⁸. Therefore, future studies might parse out arterial versus lymphatic S1PR1 signaling in mitigation of inflammation.

Consistent with an anti-inflammatory S1PR1 function, Teijara et al. demonstrated that the S1PR1 agonist CYM-5442 mitigates influenza virus-induced pulmonary cytokine storm and leukocyte infiltration¹⁶². These effects of CYM-5442 were observed in *Rag2*^{-/-} mice, which lack mature B and T cells, suggesting a minor role for lymphocyte S1PR1¹⁶². Lung ECs from influenza virus-infected mice showed reduced CCL2 and CXCL10 expression in response to CYM-5442¹⁶², suggesting that EC S1PR1 attenuates cytokine amplification during influenza virus infection. The expression of cytokines has been linked to Wnt-Ca2+ signaling in ECs, and there is initial evidence of bidirectional cross-talk between TLR2/4, inflammasome activation and canonical Wnt signals¹⁶³⁻¹⁶⁵, though the detailed molecular mechanisms have not been studied.

The primary Mendelian forms of atherosclerosis result from mutations in a small number of genes which cause familial dyslipidemias and premature vasculopathy. These genes (*Ldlr*, *ApoB*, *Lrp6*, *Ldlrap*, *Pcsk9*) not only all share the defining vascular phenotypes but also participate in different aspects of Wnt signaling, implying some commonality to the underlying mechanisms of atherosclerosis, but to date defining any shared mechanism has proven elusive^{108, 166, 167}. Activation of canonical Wnt signaling has been observed in the endothelium of murine models prior to the emergence of focal atherosclerotic lesions and has been attributed to flow effects¹⁶⁸. Disruption of physiological endothelial-smooth muscle interactions with proliferation of subjacent smooth muscle is associated with local canonical Wnt activation in reporter mice¹⁶⁹. In later stages of the atherosclerotic process, several aspects of Wnt signaling have been directly implicated in vascular calcification, including evidence that LRP6 mitigates calcification in *Ldlr*^{-/-} diabetic mice¹⁷⁰. Ongoing work exploring the role of Wnt and lysolipids in the pathophysiology of atherosclerosis spans the full repertoire of Wnt signaling, but unifying generalizable insights have yet to emerge¹⁷¹.

LPA and vascular disease

In patients with acute coronary syndromes, culprit coronary arteries showed elevated LPA levels relative to the peripheral circulation¹⁷². LPA accumulates in the lipid core region of

human and mouse atherosclerotic lesions¹⁷³⁻¹⁷⁵, and unstable plaques show high frequency of ATX immunostaining in the necrotic core¹⁷⁶.

In addition to disruption of endothelial junctions (discussed above), LPA also induces NF κ B signaling and expression of downstream pro-inflammatory molecules in ECs¹⁷⁷.

Furthermore, LPA has been shown to positively regulate monocyte/macrophage uptake of oxidized-LDL^{178, 179}, expression of the pro-inflammatory molecule IL-1 β ¹⁷⁹, and inhibit apoptosis¹⁸⁰, which may inhibit macrophage clearance from subendothelial space. These effects are likely mediated by LPAR1 and/or LPAR2^{181,182}. Importantly, the LPA/LPAR5 signaling axis induces platelet activation, which may contribute to atherothrombosis^{183, 184}. Finally, a pharmacologic inhibitor of LPAR1/3 reduced plaque burden and myeloid infiltrate^{185, 186} in two different models of murine atherosclerosis, whereas injection with LPA20:4 increased plaque burden and myeloid infiltrate¹⁸⁶. Collectively, these data imply that LPA enhances atherothrombosis and subendothelial foam cell accumulation, though careful studies of signaling events intrinsic to specific cell types are lacking in this field.

Consistent with human genetic studies suggesting a protective role for LPP3 in endothelium^{53, 187-189}, EC LPP3 expression protects against lung vascular leakage in homeostatic and endotoxemic conditions in mice⁵⁶. Global reduction of LPP3 in the postnatal period accelerates atherosclerosis development in a mouse model, which is largely attributable to the role of LPP3 in smooth muscles cells but unlikely related to LPP3 function in myeloid cells¹⁹⁰. Inhibition of LPA signaling by an ATX inhibitor or pan-LPAR inhibitor rescued the exacerbated lung vascular leakage in LPP3-ECKO animals. Conversely, mice with low levels of circulating ATX (and likely reduced LPA content) were resistant to LPS-induced lung vascular leakage⁵⁶. Intradermal LPA injection induced permeability of skin vasculature in a dose-dependent fashion⁵⁶, consistent with HUVEC responses to LPA^{50, 85}. In endotoxemia models, LPP3-ECKO mice showed ~3-fold increases plasma [IL-6] and peritoneal leukocyte recruitment after thioglycolate injection, suggesting that EC LPP3 attenuates inflammatory responses⁵⁶. Collectively, these data suggest that endothelial LPP3 attenuates LPA/LPAR-mediated vascular permeability and inflammation.

Several multi-ethnic genome-wide association studies (GWAS) found evidence of a strong association between CAD and a SNP (rs17114036) in intron 5 of *Ppap2b* (which encodes the LPP3 protein)¹⁸⁷⁻¹⁸⁹. In HAECs, rs17114036 is within a ~1.2 kb peak of high chromatin accessibility and histone modification (H3K27ac, H3K4me2) associated with active enhancers⁵³. Other cell types (K562, GM12878, NHEK) lacked indications of active chromatin, suggesting a unique role for this *cis*-element in ECs⁵³. Luciferase assays demonstrated significant enhancer activity for the ~1.2 kb region at rs17114036⁵³. CRISPR-Cas9-mediated deletion of a ~66 bp region enclosing rs17114036 attenuated LPP3 expression⁵³. Consistently, mutagenesis of the risk allele (T/T) to the protective genotype (T/C) resulted in an ~6-fold increase in enhancer activity, establishing a causal relationship between the T/T variant and attenuated LPP3 expression⁵³. Taken together with information from GWAS studies, these experiments suggest that strategies which promote endothelial LPP3 activity or expression may have therapeutic benefit in cardiovascular disease (CVD).

E. Outstanding questions and perspectives for Future Research

S1P and LPA receptors, and molecules that regulate lysolipid bioavailability, are emerging as tractable targets for a range of pathologies that stem from autoimmune diseases, tissue injury, and pathogen infection. Animal models have yielded insights regarding downstream outcomes of single and collective receptor signaling, namely, S1PR1 as the primary endothelial S1PR promoting stabilization and maturation, while LPAR4 and LPAR6 promote EC proliferation and vascular front expansion. Further studies of sub-cellular location-specific LPAR activation, such as LPP3-regulated restriction of signaling to regions devoid of cell-cell contacts, warrant further study. Similarly, we lack mechanisms to describe S1PR1 biased signaling towards G_i/Rac or β -arrestin pathways. Understanding of these pathways may facilitate rational drug design as well as development of assays that, in addition to lipid measurements, will more precisely inform on the lysolipid signaling status of patients.

We lack a mechanism to explain why the rs17114036 SNP, which appears to regulate EC LPP3 expression, is strongly correlated with CVD risk. Is the mechanism as straightforward as aberrant LPAR signaling in coronary arteries downstream of increased local LPA levels? Or do individuals with this genetic variant have developmental defects, perhaps involving Wnt signaling, that compound with other factors and manifest as CVD? As we learn more about the basic science of lysolipid signaling using biochemical, cell culture, and animal models, we will be better prepared for rational design of therapeutic agents and evaluation of their efficacy.

Post-transcriptional regulation of lysolipid receptors in cell type-specific contexts is an active area of investigation. Several studies have identified microRNAs (miRNAs) that directly or indirectly downregulate *S1pr1* expression in human cancer cell lines, including miR-148a in ovarian cancer¹⁹¹, miR-149 in hepatocellular carcinoma¹⁹², and miR-133b in nasopharyngeal carcinoma¹⁹³. At least two studies have shown that miR-155 targets *S1pr1* in lymphocytes^{194,195}. miR-24 downregulates *S1pr1* expression in HUVECs and human kidney epithelial cells¹⁹⁶, miR-24 antagonism improved survival and tissue vascularization in a model of renal ischemia/reperfusion injury¹⁹⁶. Similarly, the miR-17~92 cluster negatively regulates normal and ischemia-responsive arteriogenesis around mouse limbs, likely via inhibition of Wnt signaling components including FZD4 and LRP6¹⁹⁷. As compared to vascular S1P receptors, less is known about miRNA regulation of *Lpar4* and *Lpar6*, though one study showed miR-139-5p targets *Lpar4* in human umbilical cord mesenchymal stem cells¹⁹⁸. miRNA signaling in vascular biology has been reviewed elsewhere^{199,200,201}, and additional insights are likely to arise from unbiased profiling in a human 3D-culture angiogenesis model²⁰². Currently, data indicate that antagonism of endothelial miRNAs that downregulate *S1pr1* may improve vascular function after tissue injury.

The enzyme S1P lyase (SPL) irreversibly catabolizes S1P to phosphoethanolamine and hexadecenal²⁰³. Along with LPP3²⁰⁴ and SPNS2²⁰, SPL is critical for T cell egress from lymphoid organs to circulation²⁰⁵. Mechanistically, SPL expressed by thymic dendritic cells causes parenchymal S1P “sinks” that promote T cell chemotaxis towards relatively high

[S1P] in the bloodstream²⁰⁶. Ongoing research aims to determine whether SPL-targeted therapies may provide immunomodulatory benefits similar to those of FTY720 (fingolimod) without inhibiting vascular-protective S1P signaling²⁰⁶²⁰⁷²⁰⁸²⁰⁹.

Finally, understanding the complex and often contradictory effects of Wnt and lysolipid signaling in vascular biology is likely to require a much deeper exploration of the developmental effects of these convergent pathways on endothelial biology. Accumulating evidence suggests that the potential to connect mechanotransduction, metabolism, cell polarity and intercellular communication or competition with the fundamental processes of aging remains high, but mechanistic molecular models will require unraveling many of the most complex temporal and regional signaling hierarchies in development^{84, 102, 104}.

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Nonstandard Abbreviations and Acronyms

EC	Endothelial cells
VEGF	Vascular endothelial growth factor
GPCR's	G protein-coupled receptors
S1P	Sphingosine 1-phosphate
LPA	Lysophosphatidic acid
HDL	High density lipoprotein
MFSD2B	Major facilitator family transporter 2b
SPNS2	Spinster 2
ATX	Autotaxin
HUVECs	Human umbilical vein endothelial cells
GCs	Germ cells
SPL	S1P lyase
CAD	Coronary artery disease
BRB	Blood-retina-barrier

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Highlights

- Sphingosine 1-phosphate (S1P) and lysophosphatidic acid (LPA) are secreted lipid mediators produced by metabolism of membrane phospholipids
- S1P and LPA interact with specific G protein-coupled receptors to regulate vascular development, physiology and cardiovascular diseases
- S1P/ LPA signaling axis intersects with fundamental developmental systems such as Wnt / β -catenin
- Metabolic and signaling genes in the S1P/ LPA system show cardiovascular disease-specific heterogeneity

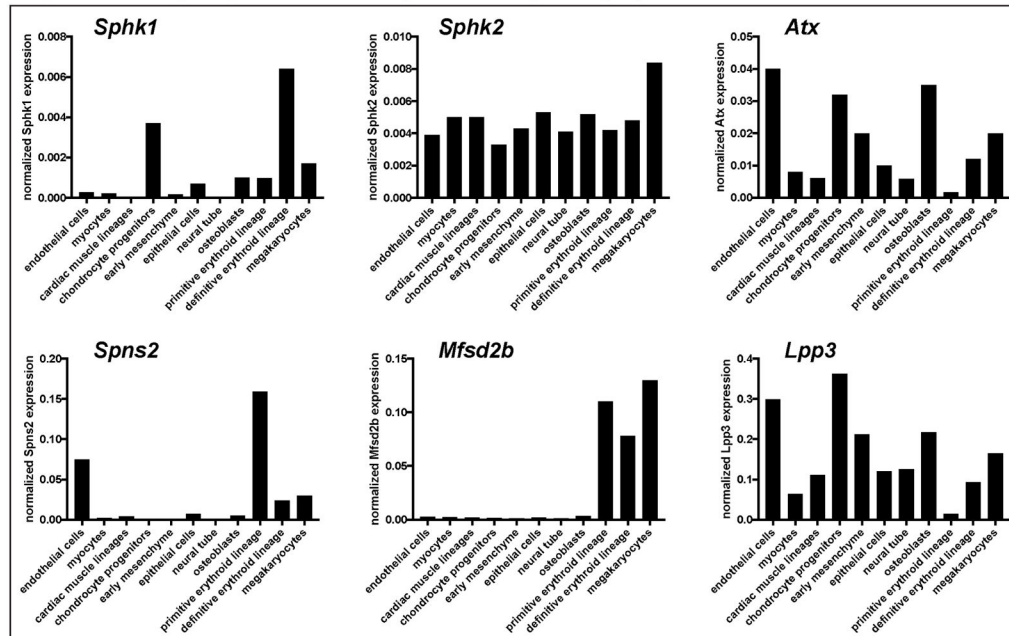
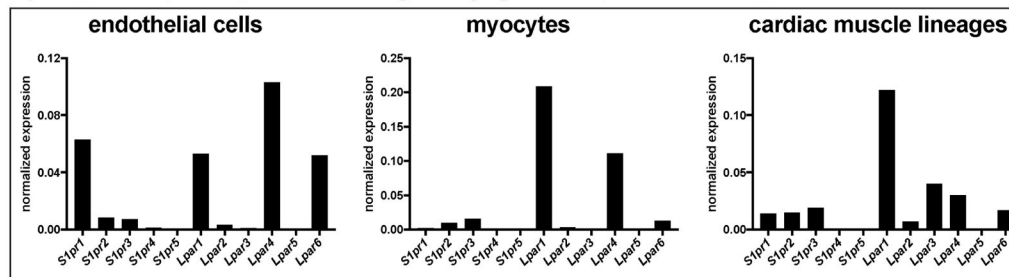
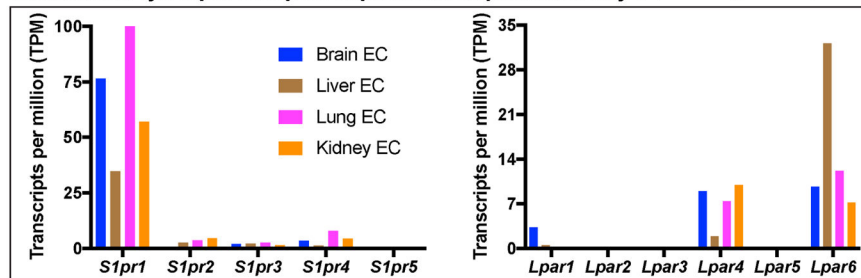
A Expression of lysolipid metabolism genes during embryogenesis (Cao et al., 2019)**B Lysolipid receptor expression during embryogenesis (Cao et al., 2019)****C Endothelial lysolipid receptor expression at postnatal day 7 (Sabbagh et al., 2018)**

Figure 1. Expression of lysolipid metabolic and signaling genes during embryogenesis and in postnatal endothelium.

(A-B) Expression of genes encoding lysolipid metabolic enzymes or transporters (*Sphk1*, *Sphk2*, *Lpp3*, *Mfsd2b*, *Spns2*) (A) or receptors (*S1pr1-5*, *Lpar1-6*) (B) in selected cell types and embryonic structures. Single-cell (sc)RNA-seq data are from a publicly available database (<https://oncoscape.v3.sttrcancer.org/atlas.gs.washington.edu.mouse.rna/landing>) provided by the authors²¹⁰. (C) Expression of S1P and LPA receptors from RNA-seq of freshly-isolated EC from postnatal day 7 mouse brain, liver, lung, or kidney⁶.

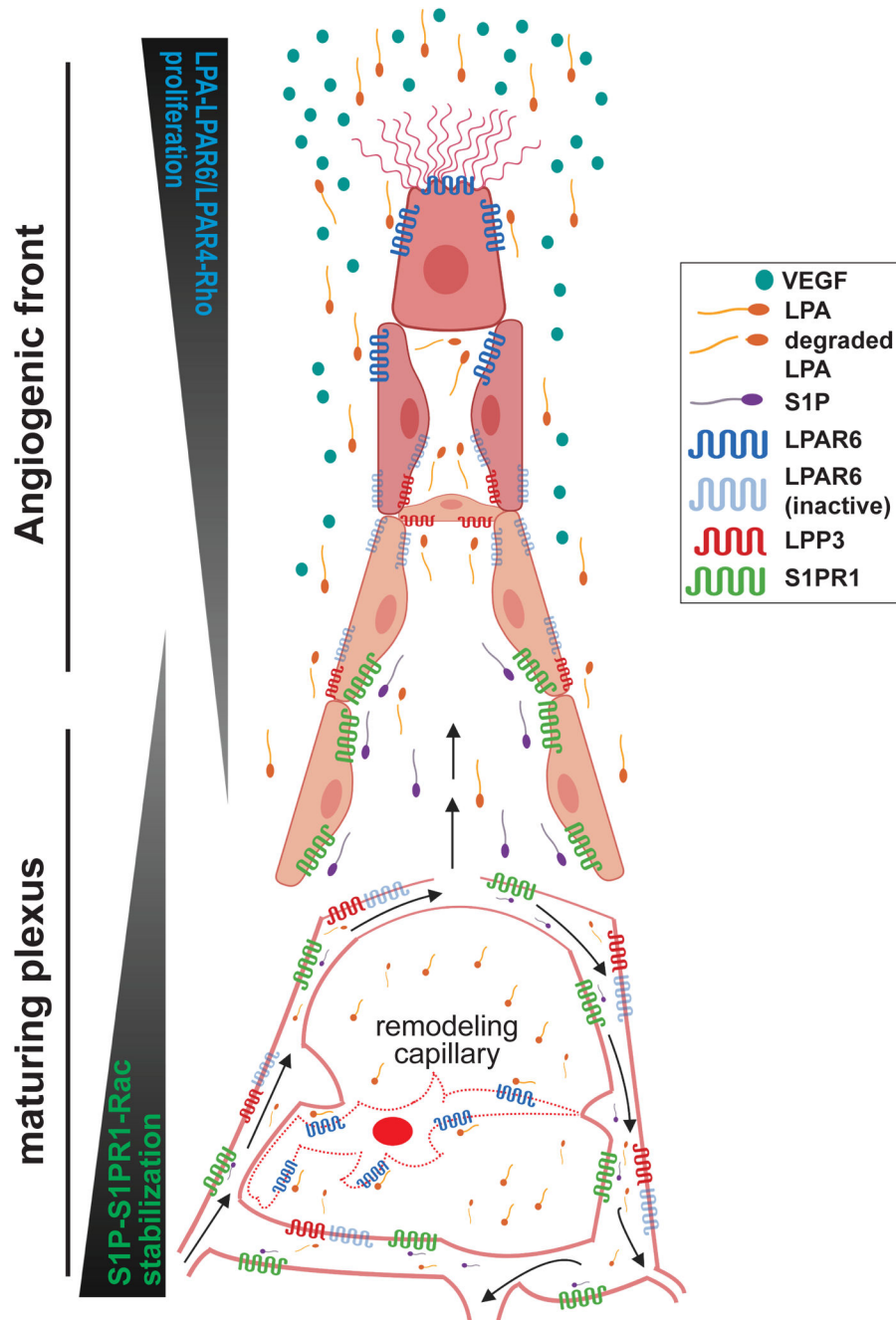


Figure 2. Schema of LPA and S1P signaling during retinal sprouting angiogenesis.

The angiogenic front of a developing vascular plexus, composed of ~6-10 “rows” of endothelial cells (EC), is surrounded by high levels of VEGF¹. Here, LPA activates EC LPAR6 (and possibly LPAR4), promoting proliferation and migration downstream of $G_{\alpha 12/13}$ and Rho family GTPases in cooperation with VEGF receptor signaling. S1PR1 expression is relatively low in these EC.

Cell-cell contacts are stronger in more mature vascular regions and are speculated to be enriched with active LPP3, which degrades LPA and limits EC LPAR signaling. Meanwhile, as these maturing vascular regions acquire S1P from flowing blood (black arrows), EC

express high levels of S1PR1. S1P-S1PR1 signaling contributes to vascular stability (e.g. stabilization of adherens junctions) and permits organotypic vascular specialization in the retina. These S1PR1-mediated events are likely downstream of RAC1 activation. Growing vascular networks undergo remodeling to optimize tissue perfusion (black arrows). We speculate that poorly perfused EC are “selected” for pruning in part because they become deficient in S1P-S1PR1 signaling. Resultant vascular instability decreases cell-cell adhesions and reduces LPP3 activity, thereby creating micro-domains of LPA that activate LPAR6 on remodeling EC to promote incorporation into well-perfused capillaries.

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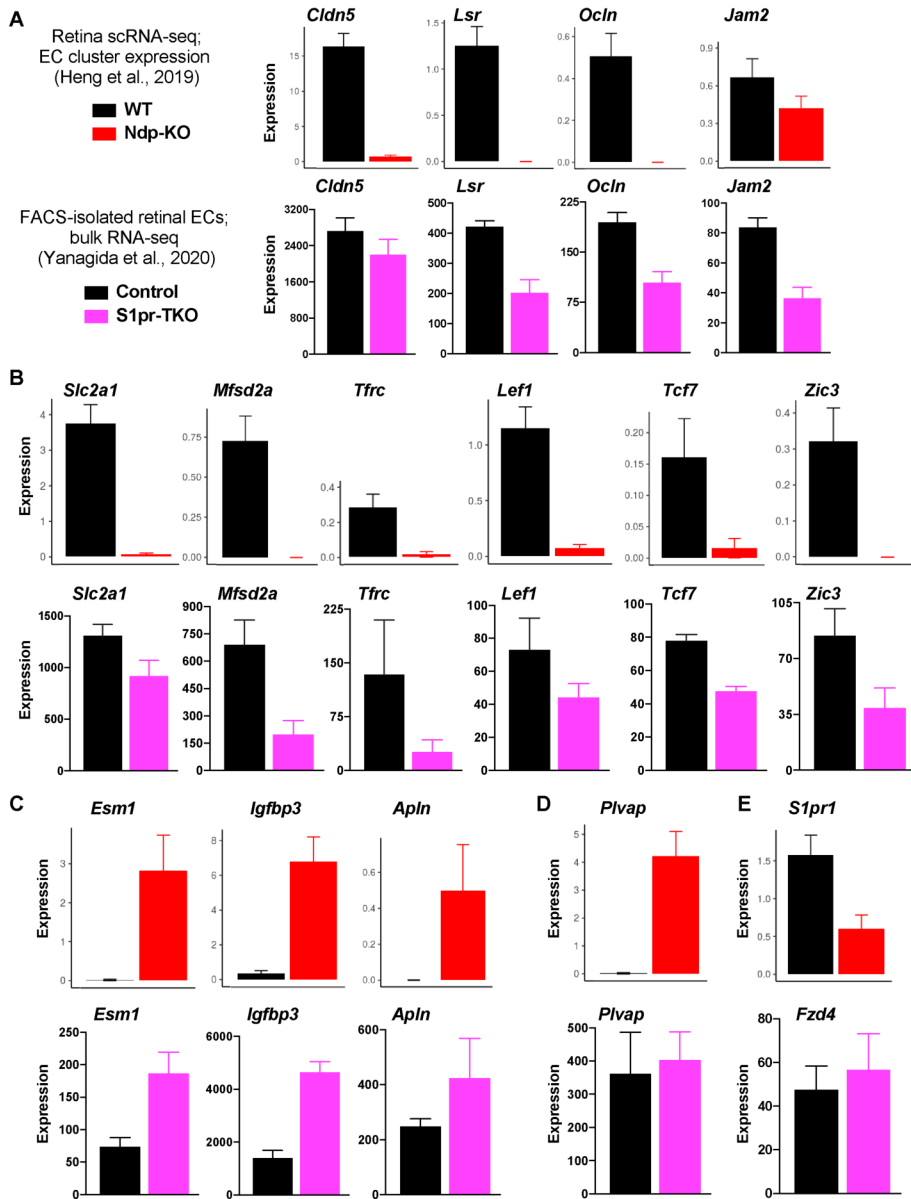


Figure 3. Gene expression in Norrin-deficient and S1PR-deficient retinal endothelial cells. (A-E) Expression of selected genes in Norrin knockout (Ndp-KO) and WT retinal EC were acquired from <https://jacobheng.shinyapps.io/cnshypoxia/>¹¹⁷. For control and S1PR-deficient retinal EC, data were acquired from GEO accession GSE141440⁴⁵. (A-B) Expression of neurovascular-enriched transcripts that encode tight junction components (A), transporters or transcription factors (B). (C) Expression of genes that are enriched in tip cells. (D) Expression of *Plvap*. (E) *S1pr1* or *Fzd4* expression in Ndp-KO or S1PR-deficient retinal EC, respectively.

Table 1.

Vascular phenotypes observed in mice with altered lysolipid signaling

Mouse strain	allele description	lethality	yolk sac	embryo proper	developing retina vasculature	Reference PMID
SIP receptors						
S1pr1-KO	null allele	E14.5	lacks blood, edematous	hemorrhage, pericardial cavity edema	-	11032855
EC S1pr1-KO	Tie2-Cre	E14.5	lacks blood, edematous	hemorrhage, enlarged pericardial cavity	-	12869509
EC S1pr1-KO	Cdh5-Cre ^{ERT2}	-	-	-	hypersprouting	22975328 22975327
EC S1pr1-Tg over-expression	Cdh5-Cre ^{ERT2}	-	-	-	hyposprouting	22975328
S1pr2-KO	null allele	-	-	grossly normal	-	15138255
S1pr3-KO	null allele	-	-	grossly normal	-	15138255
S1pr1,2-KO	null alleles	E12.5	-	hemorrhage, poor vascular networks	-	15138255
S1pr1,2,3-KO	null alleles	E11.5	-	hemorrhage, poor vascular networks	-	15138255
S1pr1,2,3-KO	Rosa-Cre ^{ERT2}	-	-	-	hypersprouting	32059774
EC S1pr1,2,3-KO	Cdh5-Cre ^{ERT2}	-	-	-	hypersprouting	32059774
LPA receptors						
Lpar1-KO	null allele	-	-	grossly normal	-	14697676
Lpar2-KO	null allele	-	-	grossly normal	-	12215548
Lpar3-KO	null allele	-	-	grossly normal	-	15875025
Lpar4-KO	null allele	E10.5-E18.5 (35%)	poor vascular network	hemorrhage	-	20713964 31335323
Lpar5-KO	null allele	-	-	grossly normal	-	23039190
Lpar6-KO	null allele	-	-	grossly normal	hyposprouting	30804442 31335323
Lpar4,6-KO	null alleles	E11.5	poor vascular network, large vessels absent	poor vascular networks, pericardial effusion, developmental delay, axial turning defect	-	31335323
EC Lpar4,6-KO	Cdh5-Cre ^{ERT2}	-	-	-	hyposprouting	31335323
SIP or LPA metabolic enzymes						
Sphk1-KO	null allele	-	-	grossly normal	-	15459201
Sphk2-KO	null allele	-	-	grossly normal	-	16314531
Sphk1,2-KO	null alleles	E13.5	-	hemorrhage	-	16314531
RBC Sphk1,2-DKO	EpoR-Cre	E13.5	hypersprouting	hemorrhage	-	25250575
Atx-KO	null allele	E10.5	poor vascular network, large vessels absent	poor cranial and cardiac vascular networks, neural tube defects, axial turning defect	-	16829511 16782887
EC Atx-KO	Tie2-Cre	-	grossly normal	grossly normal	grossly normal	Thesis link

Mouse strain	allele description	lethality	yolk sac	embryo proper	developing retina vasculature	Reference PMID
Atx-Tg over-expression	CAG-Cre	E10.5	poor vascular network, large vessels absent	developmental delay, neural tube defects	-	25992708
Atx-Tg over-expression	CAG-Cre ^{ERT2}		-	-	hyposprouting	25992708
Lpp3-KO	null allele	E6.5 (30%), E10.5 (70%)	poor vascular network, large vessels absent	axis duplication, hemorrhage	-	12925589
EC Lpp3-KO	Tie2-Cre	E8.5-E13.5	poor vascular network	hemorrhage, cranial vascular defects, malformed branchial arch arteries, endocardial defects, trabeculation defects	-	27125875 24504738
GPCR signaling components						
EC Rac1-KO	Tie2-Cre	E9.5	poor vascular network, large vessels absent	numerous vascular abnormalities, branchial arch arteries absent	-	18245172
EC Rac1-KO	Cdh5-Cre ^{ERT2}	-	-	hemorrhage	EC dense at vascular front, hypovascular nascent vascular network	26872874
EC RhoA-KO	Tie2-Cre	-	grossly normal	grossly normal	-	31406143
EC RhoA-KO	Cdh5-Cre ^{ERT2}	-	-	-	grossly normal	31406143
EC Cdc42-KO	Tie2-Cre	E9.5	poor vascular network, large vessels absent	hemorrhage, defective vascular lumens, EC polarity defects	-	26253403
EC Cdc42-KO	Cdh5-Cre ^{ERT2}	inducible mid-gestation	-	hemorrhage, defective vascular lumens, EC polarity defects	EC dense vascular network, reduced tip cell filopodia	26253403
EC G _{α13} -KO	Tie2-Cre	E9.5-E11.5	poor vascular network, large vessels absent	hemorrhage, developmental delay, pericardial dilatation, cranial vascular defects	-	15919816
Rock1,2-KO	null alleles	E8.5-E9.5	poor vascular network, large vessels absent	axial turning defect, developmental delay	-	21895889