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Lysophospholipids in Development: Miles Apart and Edging In

Julie D. Saba*

Children's Hospital Oakland Research Institute, 5700 Martin Luther King Jr. Way, Oakland, California 94609-1673

Abstract Sphingosine-1-phosphate (S1P) and lysophosphatidic acid (LPA) are endogenous bioactive lipids that participate in the regulation of mammalian cell proliferation, apoptosis, migration, and angiogenesis. These processes are each critical for successful embryogenesis, raising the possibility that lysophospholipid signaling may contribute to normal animal development. In fact, recent studies in developmental model systems have established that S1P and LPA are necessary for diverse developmental programs including those required for morphogenesis of vertebrate reproductive, cardiovascular and central and peripheral nervous systems (PNS), as well as the establishment of maternal-fetal circulation and the immune system. Genetic, morphological, and biochemical characterization of developmental model systems offer powerful approaches to elucidating the molecular mechanisms of lysophospholipid signaling and its contributions to animal development and postnatal physiology. In this review, the routes of S1P and LPA metabolism and our current understanding of lysophospholipid-mediated signal transduction in mammalian cells will be summarized. The evidence implicating lysophospholipid signaling in the development of specific vertebrate systems will then be reviewed, with an emphasis on signals mediated through G protein-coupled receptors of the Edg family. Lastly, recent insights derived from the study of simple metazoan models and implications regarding lysophospholipid signaling in organisms in which Edg receptors are not conserved will be explored. J. Cell. Biochem. 92: 967–992, 2004.

Key words: lysophospholipid; sphingolipid; sphingosine-1-phosphate; lysophophosphatidic acid; development; Edg receptor; angiogenesis

Embryogenesis is a highly complex event in which sequentially activated genetic programs choreograph the migration, proliferation, ablation, fusion, and differentiation of individual cells to create a functional organism composed of specific organ systems and their representative tissues [Peifer and McEwen, 2002]. A limited number of signal transduction pathways are used redundantly in different genetic programs to specify lineage, establish embryonic polarity, asymmetry and tissue patterning, and execute cell rearrangements and fate decisions that contribute to embryogenesis [Freeman and Gurdon, 2002; Wedlich, 2002]. Elucidating these signal transduction pathways can facilitate our understanding of normal and abnormal animal development. Conversely, dissecting the mechanisms that control development may lead to insights into the pathophysiology of diseases such as cancer, atherosclerosis, and degenerative neurological disorders, wherein abnormalities in the regulation of cell proliferation, migration, and death contribute to the disease state.

Sphingosine-1-phosphate (S1P) and lysophosphatidic acid (LPA) are endogenous bioactive lysophospholipids that participate in signaling pathways involved in the regulation of mammalian cell proliferation, apoptosis, and migration [Pyne and Pyne, 2000, 2002; Ye et al., 2002; Spiegel and Milstien, 2003]. LPA and S1P are structurally similar amphipathic molecules, and each serves as a ligand for a subset of G protein coupled cell surface receptors of the Edg family [Tigyi et al., 2000; Fukushima and Chun, 2001; Hla et al., 2001; Lynch and Macdonald,

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*Correspondence to: Julie D. Saba, Children's Hospital Oakland Research Institute, 5700 Martin Luther King Jr. Way, Oakland, CA 94609-1673. E-mail: jsaba@chori.org

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2001; Tigyi, 2001]. In addition, S1P and LPA appear to mediate Edg-receptor-independent signaling through as yet unknown intracellular mechanisms, and LPA was recently shown to bind to and mediate downstream signaling through a G protein coupled receptor with minimal homology to Edg receptors [Hooks et al., 2001; Noguchi et al., 2003; Olivera et al., 2003a]. Downstream targets of LPA and S1P signaling include adenylate cyclase, Ras, mitogen-activated protein kinase (MAPK), phospholipase C (PLC), c-Src tyrosine kinase, the small GTPases Rac and Rho, phospholipase D, p125FAK, matriptase, and calcium homeostasis [Meyer zu Heringdorf et al., 1998; Birchwood et al., 2001; Meacci et al., 2002; Spiegel and Milstien, 2003]. Thus far, five mammalian S1P receptors and four LPA receptors have been identified, and the highly conserved genes responsible for S1P synthesis and degradation have recently been cloned [Mao et al., 1997; Qie et al., 1997; Saba et al., 1997; Mandala et al., 1998; Chun et al., 2002]. LPA metabolism is considerably more complex than that of S1P, but most of the genes involved in LPA synthesis and degradation are also known [Pages et al., 2001]. This sequence information has made possible the use of both forward and reverse genetic approaches to elucidate the contribution of lysophospholipid signaling to animal development. Recent studies have, in fact, revealed a role for signaling mediated by each of these molecules in the developmental processes of vertebrates, as well as in simple metazoan organisms including fruitflies, nematodes, and slime mold. The identification of developmental phenotypes associated with aberrant lysophospholipid signaling is an exciting demonstration of the important role these signaling pathways play in biology.

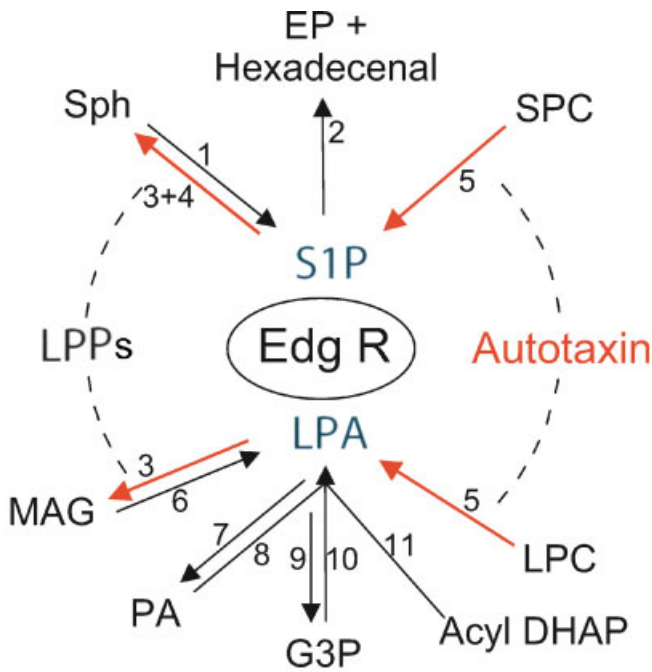
Elucidation of S1P and LPA signaling through the identification or generation of vertebrate models of development has revealed a consistent theme, that is, that both these lipid mediators exert significant influences on developmental processes in vertebrates largely by operating a complex network of signals mediated by G protein coupled receptors of the Edg family. These signals regulate cell fate and proliferation and also lead to changes in cytoskeletal proteins and adhesion molecules required for cell–cell interactions, cell–extracellular matrix interactions, cell migration, and morphogenesis in many developing organ systems, predominantly the cardiovascular and nervous systems. In contrast, invertebrate models in which Edg receptors are not conserved provide clear evidence that lysophospholipids can influence critical cellular processes and contribute to metazoan developmental programs through Edg-receptor independent (if not receptor-independent) mechanisms. This raises the intriguing possibility that Edg-receptor mediated signaling in vertebrate development is only part of an even more sophisticated system of signaling.

It should be emphasized that numerous studies indirectly implicating roles for lysophospholipid signaling in development have been performed in a wide variety of cellular systems. However, due to limitations of space, this review is focused on whole animal models and will refer to in vitro investigations only where pertinent to the interpretation of such models.

OVERVIEW OF S1P AND LPA: METABOLISM, TRANSPORT, AND SIGNALING

S1P Metabolism

The structure of S1P consists of the 18-carbon sphingoid base sphingosine and a phosphate group at the C_1 position. Phosphorylation of sphingosine, generating S1P, is catalyzed by the highly regulated enzyme sphingosine kinase (SK) (see Fig. 1) [Buehrer and Bell, 1993; Olivera and Spiegel, 2001]. The sphingosine substrate of this reaction is generated either by degradation of higher order sphingolipids or, alternatively, by the deacylation of ceramide formed through the de novo pathway of sphingolipid synthesis [Nikolova-Karakashian and Merril, 2000]. SK can also phosphorylate dihydrosphingosine generated during sphingolipid biosynthesis, yielding dihydroS1P. These phosphorylated long chain bases (LCBs) can, in turn, be dephosphorylated by a specific S1P phosphatase (S1PP) or by lipid phosphohydrolases of broad substrate specificity [Mandala, 2001; Sciorra and Morris, 2002]. Alternatively, they can be irreversibly degraded to ethanolamine phosphate and the corresponding long chain aldehyde (hexadecanal or hexadecenal) by S1P lyase [Van Veldhoven, 2000]. Each of the three genes primarily responsible for regulating S1P levels, SK, S1PP, and S1P lyase was cloned in the budding yeast, *S. cerevisiae*, where the LCBs and their phosphorylated derivatives (LCBPs) regulate calcium homeostasis and the



EP = ethanolamine phosphate
Acyl DHAP = acyl dihydroxyacetone phosphate
LPP = lipid phosphate phosphohydrolases
EdgR = Edg receptors
LPC = lysophosphatidylcholine
PA = phosphatidic acid
MAG = monoacyl glycerol
G3P = glycerol 3-phosphate
SPC = sphingosylphosphorylcholine
Sph = sphingosine
S1P = sphingosine-1-phosphate
LPA = lysophosphatidic acid

1 Sphingosine kinase
2 S1P lyase
3 LPP
4 S1P phosphatase
5 Autotaxin/lysophospholipase D
6 MAG kinase
7 monoacylglycerol phosphate acyltransferase or LPA-acyltransferase
8 Phospholipase A
9 Lysophospholipase
10 Glycerophosphate acyltransferase
11 Acyl DHAP reductase

Fig. 1. Intersection of LPA and S1P metabolism. S1P is generated by the actions of sphingosine kinase and catabolized by S1P phosphatase and S1P lyase. Synthesis and degradation of LPA may occur by many routes. Both S1P and LPA can be synthesized by the actions of phospholipase D/autotaxin. Both lysophospholipids can be dephosphorylated by lipid phosphate phosphohydrolases of broad substrate specificity. S1P and LPA each ligate to a different subset of G protein coupled receptors of the Edg family. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

response to heat stress and nutrient deprivation [Jenkins et al., 1997; Mao et al., 1997, 1999; Qie et al., 1997; Saba et al., 1997; Mandala et al., 1998; Birchwood et al., 2001; Jenkins and Hannun, 2001; Obeid et al., 2002]. Vertebrate and invertebrate orthologs of SK and S1P lyase have subsequently been cloned and verified in yeast mutant model systems [Kohama et al., 1998; Zhou and Saba, 1998; Liu et al., 2000a; Mandala et al., 2000; Melendez et al., 2000; Pitson et al., 2000; Van Veldhoven et al., 2000; Le Stunff et al., 2002; Mendel et al., 2003; Ogawa et al., 2003]. Lipid phosphatases specific for S1P have been identified in mice, yeast, and humans, but not in *C. elegans* or *Drosophila*, although genes with significant homology to lipid phosphohydrolases exist in the genomes of both of these organisms. The genome of the

zebrafish (*Danio rerio*) contains sequences homologous to mammalian SK and S1P lyase, but the functions of the encoded proteins have not been verified. SK, S1PP, and S1P lyase all appear subject to both transcriptional and post-transcriptional regulation [Buehrer et al., 1996; Ancellin et al., 2002; Johnson et al., 2003; Mendel et al., 2003; Pitson et al., 2003; Sukocheva et al., 2003]. Apart from the main route of S1P synthesis by SK, recent studies have shown that S1P may also be generated by catabolism of sphingosylphosphorylcholine (SPC) in a reaction catalyzed by autotaxin [Clair et al., 2003]. Autotaxin was first identified through its involvement in tumor progression and metastasis and was recently found to function as a lysophospholipase D capable of generating LPA from lysophosphatidylcholine [Stracke et al., 1992; Umezu-Goto et al., 2002]. Although the physiological relevance of autotaxin's ability to generate S1P from SPC in vitro has not been established, it is interesting to postulate that LPA and S1P may be produced at least in part by a common biochemical mechanism.

S1P is unique in its ability to mediate biological effects through two different mechanisms. S1P serves as a ligand for several members of the Edg family of G protein coupled cell surface receptors. S1P is the preferred ligand for Edg 1, 3, 5, 6, and 8 (now referred to as $S1P_{1-5}$). The downstream effects of S1P receptor signaling are mediated through different pathways determined by the subset of G proteins to which each specific receptor couples. The most clearly defined system of receptor-mediated S1P signaling is found in vascular endothelial cells, which express $S1P_1$, $S1P_2$, and $S1P_3$ [Hla and Maciag, 1990; Lee et al., 1999; Morales-Ruiz et al., 2001]. S1P signaling through its receptors has been shown to enhance endothelial cell lamellipodia formation, activation and translocation of integrins, cell spreading, migration, formation of capillary networks, homotypic and heterotypic cell–cell interactions, and invasion of collagen and fibrin matrices [Lee et al., 1999; English et al., 2000, 2001; Kimura et al., 2000; Liu et al., 2000b; Okamoto et al., 2000b; Paik et al., 2001; Tamama and Okajima, 2002; Bayless and Davis, 2003]. S1P signaling through $S1P_1$ leads to Rac-dependent cortical actin assembly, whereas signaling through $S1P_3$ leads to Rho-dependent stress fiber formation (see Fig. 2). The latter process is also regulated by Rac, as stress fiber formation in response to S1P treatment is abolished by expression of dominant negative Rac [Lee et al., 1999]. Signals mediated through both receptors coordinate to promote the recruitment of VE-cadherin and catenins to cell–cell contact sites, where they participate in the formation of junctional complexes, as determined by coimmunoprecipitation studies [Lee et al., 1999]. Both these signals appear to be required for endothelial cell morphogenesis, since microinjection of either the Rho inhibitor C3 toxin or a dominant negative Rac abolished S1P-induced adherens junction assembly. S1P-mediated stimulation of G_i and a Rac-dependent pathway is associated with recruitment of cofilin and p21-associated kinase to the cortical actin cytoskeleton and leads to acto-myosin remodeling [Garcia et al., 2001]. Ligation of $S1P_2$ and $S1P_3$ by S1P mediates Rho activation (as determined by quantitation of GTP-loaded Rho) and stress fiber formation in a dose-dependent manner [Takuwa, 2002]. Cytoskeletal changes induced by S1P signaling through its receptors on endothelial cells serve to reinforce endothelial integrity and prevent vascular permeability. In other cell systems, $S1P_2$ receptor stimulation appears to block Rac activation and inhibit cell migration, thus allowing S1P to function as a repellant signal [Okamoto et al., 2000a].

Several studies suggest coordinate or synergistic regulation of endothelial cell migration and activation by S1P and other growth factors, such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF). For example, S1P treatment has been shown to promote Akt-induced phosphorylation of both $S1P_1$ and endothelial nitric oxide synthase (eNOS) [Igarashi et al., 2001]. VEGF treatment also leads to Akt-phosphorylation and activation of eNOS, suggesting the potential for interaction between these two signaling pathways. Indeed, it was shown that S1P signaling leads to tyrosine phosphorylation and transactivation of VEGF receptor-2/Flk-1 [Tanimoto et al., 2002]. S1P signaling through G_i proteins in human umbilical vein endothelial cells was associated with phosphorylation of the adaptor protein CrkII at membrane ruffles [Endo et al., 2002]. Inhibition of VEGF receptors or Src blocked CrkII phosphorylation, and a dominant negative mutant of CrkII inhibited S1P-induced membrane ruffling, suggesting a sequential activation of S1P receptors, VEGF receptors,

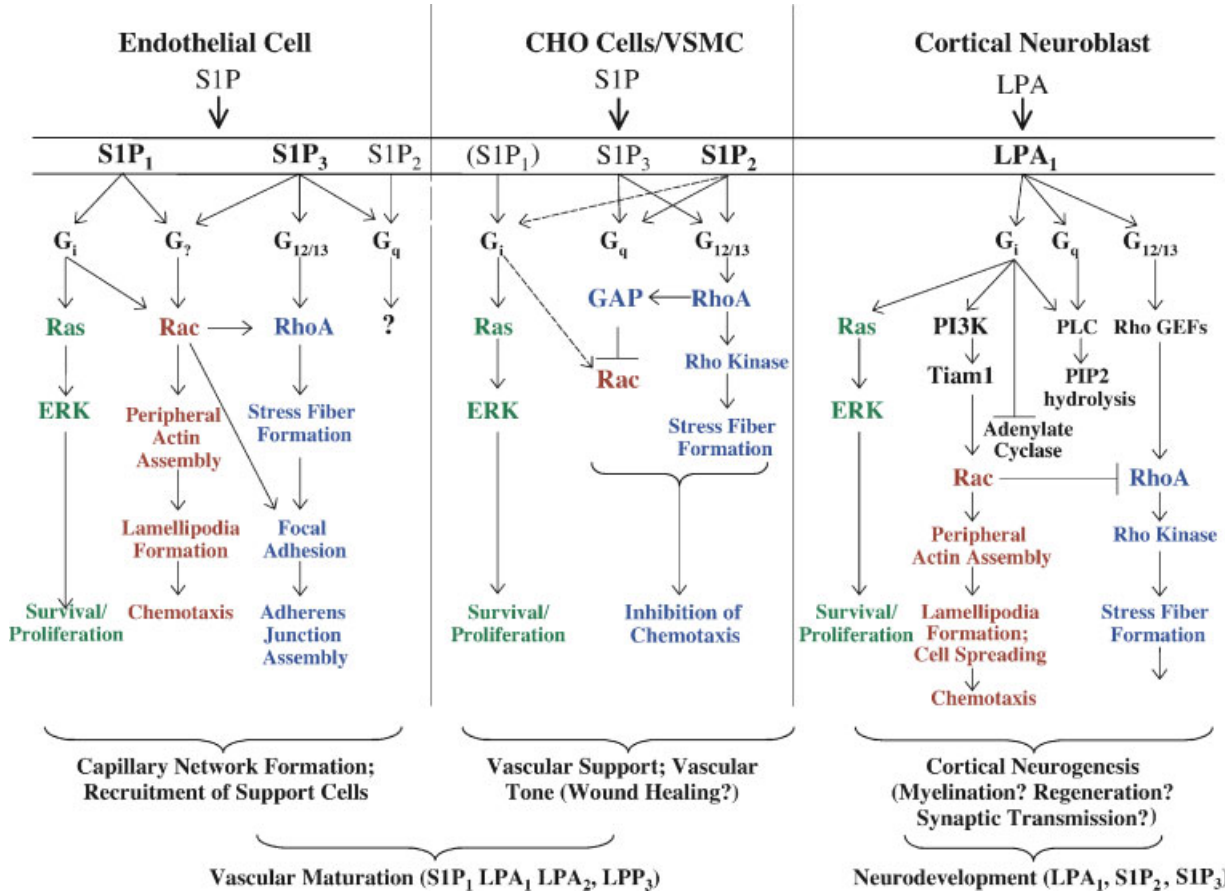


Fig. 2. S1P and LPA signaling through Rac and Rho. Three examples of S1P and LPA signaling through Edg family receptors in different mammalian cell types are shown. In each cell type, lysophospholipids have been shown to activate pathways involving the small GTPases Rac and Rho, as well as the Ras/MAPK pathway, leading to diverse effects including cell proliferation, survival, migration, inhibition of migration, and morphogenesis. These depictions are only examples of each cell type. Endothelial, smooth muscle, and neuronal cells derived from different tissues and during different developmental stages demonstrate marked differences in responsiveness to S1P and LPA. This is presumed to be due, at least in part, to differences in the combination and level of expression of specific Edg receptors. Predominant receptor pathways in each cell type are indicated by bold arrows and bold receptor designations. Rac-dependent processes are shown in red. Rho-dependent processes are shown in blue. The Ras/MAPK pathway is shown in green. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Src tyrosine kinases, and CrkII in mediating endothelial cell migration. In addition, treatment of endothelial cells with VEGF was shown to induce expression of $S1P_1$ and enhance S1P-induced eNOS phosphorylation and activation [Igarashi et al., 2003]. Both VEGF and FGF-2 promote endothelial cell migration and appear to be synergistic with S1P in mediating these effects in vitro [Harvey et al., 2002]. S1P also potentiates vascular sprout formation mediated by VEGF and FGF in an ex vivo model of angiogenesis [Licht et al., 2003]. Recently, evidence for S1P receptor-platelet derived growth factor (PDGF) receptor interactions and signaling has been reported, supporting the notion that S1P may participate in signaling crosstalk through its receptors. For example, $S1P_3$ activation by S1P in mouse embryonic fibroblasts (MEFs) and an ovarian cancer cell line led to phosphorylation of Akt, and this effect was blocked by specific inhibition of PDGF receptor signaling [Hobson et al., 2001; Baudhuin et al., 2003]. In airway smooth muscle cells, both $S1P_1$ and PDGF receptor were shown to co-immunoprecipitate using antibodies against $S1P_1$, and to be taken into endocytic vesicles together with phosphorylated p42/p44 MAPK [Waters et al., 2003]. Further, MAPK phosphorylation in response to PDGF was inhibited by blocking $S1P_1$

expression. These studies suggest a physical and functional interaction between PDGF receptor and $S1P_1$. Expression of PDGF-A and -B chains was also shown to be upregulated in vascular smooth muscle cell (VSMC) and neointimal cells in response to S1P through a mechanism involving $S1P_1$ and activation of the Ras/MAPK pathway [Usui et al., 2004]. However, MEFs derived from $S1P_1$ knockout mice were capable of responding chemotactically to PDGF, indicating that $S1P_1$ is not required for PDGF induced chemotaxis in these cells [Kluk et al., 2003]. Although the physiological relevance of these findings remains to be determined, such observations raise the possibility that S1P may be involved in additional signaling networks through crosstalk with non-Edg family receptors. Finally, $S1P_1$-dependent signaling in endothelial cells is required for the migration of supporting cells including VSMCs and pericytes to the developing vascular tree, and is therefore a critical factor in vascular maturation [Liu et al., 2000b; Allende et al., 2003].

In addition to the well-established receptor-dependent functions of S1P, there is evidence to suggest that this molecule may act independently of Edg family receptors to mediate some effects through intracellular mechanisms [Van Brocklyn et al., 1998]. Importantly, S1P's ability to mediate cell growth and survival in many cell types is dependent upon activation of SK and intracellular S1P accumulation, and pharmacological approaches suggest a receptor-independent mechanism is involved [Van Brocklyn et al., 1998; Olivera et al., 2003b]. Further, LCBPs in yeast mediate heat shock and growth regulatory responses [Kim et al., 2000; Ferguson-Yankey et al., 2002]. Since yeast contains only two G protein coupled receptor pathways, the pheromone response pathway and a cAMP/protein kinase A pathway involved in carbon and nitrogen sensing, these findings suggest that LCBPs have a receptor-independent function [Yun et al., 1998; Lorenz et al., 2000; Dohlman, 2002]. Whether LCBPs act as true second messengers or are instead producing membrane effects or influencing cellular programs by some other means has not yet been established. Regardless, significant biological effects observed in other metazoan models in which Edg receptors do not exist provide similar evidence for Edg receptor-independent S1P signaling, as will be discussed below. Recent work demonstrating that SK can be secreted to the extracellular milieu and that S1PP regulates S1P secretion from cells raise additional questions regarding the nature and location of S1P signals and the dependence or independence upon receptor stimulation [Ancellin et al., 2002; Johnson et al., 2003].

LPA Metabolism and Signaling

LPA is a simple glycerophospholipid and key intermediate in phospholipid biosynthesis consisting of a glycerol backbone, a phosphate group at the sn-3 position, a fatty acyl chain at either the sn-1 or sn-2 position, and a hydroxyl group at the remaining position. Variability in fatty acyl chain length, saturation, and type of linkage to the glycerol backbone create structural diversity among LPA molecules that may have biological import [van Corven et al., 1992; Bandoh et al., 2000; Tokumura et al., 2002]. Metabolism of LPA is considerably more complex than that of S1P, as this molecule can be formed de novo by esterification of glycerol-3-phosphate, a step catalyzed by glycerol phosphate acyltransferase (GPAT) or through several other routes, including the deacylation/reacylation of phospholipids during membrane phospholipid remodeling (see Fig. 1) [Pages et al., 2001]. LPA may be generated by the reduction of acyl dihydroxy acetone phosphate, the phosphorylation of monoacylglycerol (MAG) by monoacylglycerol kinase, deacylation of phosphatidic acid (PA) by PLA_1 or PLA_2 activity, or by hydrolysis of glycerophospholipids including lysophosphatidylcholine, lysophosphatidylserine, and lysophosphatidylinositol catalyzed by the tumor-related enzyme, autotaxin [Umezu-Goto et al., 2002]. The latter two routes of LPA production are likely to be biologically significant and occur outside the cell through ecto/exo-enzyme activity. Once formed, LPA can be dephosphorylated to MAG by lipid phosphohydrolases of the PAP type 2 family (LPP_{1-3}). Alternatively, LPA can be converted to PA by LPA acyltransferase (LPAAT) or to glycerol-3-phosphate by lysophospholipase activity.

LPA first drew attention as a bioactive molecule when it was found to induce platelet aggregation, smooth muscle contraction, and changes in blood pressure [Tokomura et al., 1978a,b, 1980; Gerrard et al., 1979; Schumacher et al., 1979]. Platelet responses to LPA (and PA) demonstrated both desensitization and

potentiation by subthreshold concentrations, suggestive of G protein coupled receptor mediated effects. Consistent with its functions as a platelet agonist, LPA is present in serum, where it circulates bound to albumin [Tigyi et al., 1991]. It later became evident that this phospholipid intermediate also stimulates proliferation, migration, DNA synthesis, and survival in a variety of cell types and organisms [Moolenaar and van Corven, 1990; van Corven et al., 1992; Jalink et al., 1993b; Van Leeuwen et al., 2003a]. Effects of LPA on fibroblasts were demonstrated to be mediated through signaling pathways initiated by activation of G protein coupled receptors, leading to multiple downstream effects (see Fig. 2) [Hordijk et al., 1993; Howe and Marshall, 1993]. Four LPA receptors have been cloned thus far: Edg-2, 4, and 7 (LPA_{1-3}) and the recently described G protein coupled receptor p2y9/GRP23, which shares minimal homology with the Edg receptors [Fukushima and Chun, 2001; Noguchi et al., 2003]. Through these receptors, LPA activates the G_q-mediated PLC/PKC/calcium signaling pathway, G_i-mediated adenylate cyclase/cAMP, Ras/MAPK and PI_3kinase/TIAM1/Rac and Akt pathways, and the $G_{12/13}$-mediated RhoA pathway (see below).

LPA produces effects on the vasculature by modulating the function of endothelial cells as well as supporting cells. Both increases and decreases in vascular permeability have been demonstrated in endothelial cells derived from different sources in response to LPA. For example, LPA treatment leads to increased permeability of tight junctions in cultured brain endothelial cells [Schulze et al., 1997]. This effect was associated with the formation of stress fibers and translocation of focal adhesion components to points of cell–cell contact. In a separate study, LPA inhibited bovine aortic endothelial permeability, indicating that LPA may produce opposite effects on the vasculature, potentially due to differences in receptor expression, adjacent cell signaling and other factors influencing the endothelial cells of different vascular beds [Alexander et al., 1998]. LPA enhances the expression of genes that promote angiogenesis, including VEGF and macrophage migration inhibitory factor, and stimulates endothelial cell migration [English et al., 1999; Sun et al., 2003]. In addition, LPA promotes migration, proliferation, calcium mobilization, and dedifferentiation of VSMCs [Hayashi et al., 2001; Boguslawski et al., 2002].

LPA is also enriched in the blood and ascitic fluid of patients with ovarian carcinoma and may be a marker for this disease, as well as a critical factor in the induction and progression of cancer [Mills and Moolenaar, 2003]. LPA was shown to stimulate cell signaling and proliferation of breast and ovarian cancer cells, as well as invasiveness of several tumor cell types [Imamura et al., 1993; Xu et al., 1995]. LPA signaling may contribute to the progression of ovarian carcinoma through multiple mechanisms, including upregulation of cyclin D1 and VEGF, thereby enhancing cell proliferation and tumor angiogenesis [Hu et al., 2001, 2003]. The finding that autotaxin produces LPA and that this may be the underlying mechanism by which autotaxin induces tumor invasion, metastasis and neovascularization underscores the importance of this molecule in both physiological and pathological processes. In contrast to its mitogenic and survival potentiating effects in some cells, LPA exerts an opposite, apoptotic effect on hippocampal neurons and several other malignant and non-malignant cell types, possibly through Rho-mediated effects on cell adhesion (see below) [Holtsberg et al., 1998; Ye et al., 2002].

LPA appears to play a role in neurobiology, especially in the ventricular zone (VZ) of the developing cortex, from which the first LPA receptor, Edg-2/LPA_1 was cloned and its ligand first identified [Hecht et al., 1996]. LPA was shown to induce transient rounding of neuronal cell lines, growth cone collapse, and neurite retraction [Jalink et al., 1993a]. This effect occurred through receptor activation and led to force generation through contraction of the actomyosin cytoskeleton in a RhoA-dependent process [Jalink et al., 1994]. Similarly, a requirement for Rho GTPases in the assembly of stress fibers and focal adhesions induced by serum factors including LPA was demonstrated in Swiss 3T3 cells [Ridley and Hall, 1992]. In contrast, inhibition of Rho by ADP-ribosylation using C3 toxin induced neuronal differentiation, suggesting that the LPA-RhoA pathway may prevent the differentiation of neuronal cells [Tigyi et al., 1996]. Both Rac and Rho appear to be involved in LPA signaling (see Fig. 2), and a recent study demonstrated that, upon stimulation, the LPA_1 receptor acts via Rac, PI_3kinase, and the guanine nucleotide

exchange factor Tiam1 to inactivate Rho and stimulate cell spreading and motility [Van Leeuwen et al., 2003b]. Whereas Rac appears to regulate Rho in these instances, in transfected CHO cells, $S1P_2$ activation led to Rac inhibition in a Rho-dependent manner [Sugimoto et al., 2003]. Thus, complex signaling networks are activated by lysophospholipid receptors.

Platelet responses to LPA analogs suggest Edg receptor-independent effects and indicate that a separate class of receptors on platelets may mediate LPA effects [Lynch and Macdonald, 2001]. In addition, LPA may have intracellular functions independent of receptor signaling and phospholipid synthesis/remodeling. LPA has been shown to serve (in vitro) as a substrate for endophilin, which synthesizes PA from LPA intracellularly and which may be involved in endocytosis [Schmidt et al., 1999]. LPA also was reported to bind to PPARγ, a receptor involved in regulating gene transcription related to inflammation, energy metabolism, and adipogenesis [McIntyre et al., 2003]. Whether LPA is critical to either of the latter two pathways is not yet clear. Lastly, in discussing potential intracellular roles of lysophospholipids, lysobisphosphatidic acid should be mentioned. This interesting lipid is structurally related to LPA, is enriched in late endosomes and, was recently shown to regulate endosome structure and function [Kobayashi et al., 1998]. Studies of lysobisphosphatidic acid suggest that, by participating in internal membrane subdomains, lipids may stabilize and concentrate proteins at the cytosolic interface and influence the intracellular trafficking of proteins and lipids.

DEVELOPMENTAL MODELS OF LYSOPHOSPHOLIPID SIGNALING

S1P and LPA are both bioactive lipid metabolites that are maintained at low concentrations within cells and in serum, but which may increase rapidly in the circulation upon release from activated platelets and hematopoietic cells at the platelet-endothelial cell interface [Schumacher et al., 1979; Yatomi et al., 1997; Siess et al., 2000]. Both molecules and the signaling pathways in which they participate have been implicated in cell migration and cell fate decisions, processes critical to the normal development and physiology of multi-cellular organisms. By affecting these biological endpoints, lysophospholipid metabolism and signaling could potentially affect developmental processes requiring the survival of primordial cells and their migration to sites where they are needed to establish embryonic structures and contribute to organogenesis and tissue maturation. In addition, both these molecules have been implicated in endothelial cell migration and vascular maturation during angiogenesis, which is a critical process in vertebrate development and essential for survival of the embryo beyond the first weeks of gestation. Changes in S1P and LPA signaling in the adult animal might induce diseases resulting from prolonged or shortened cell survival, pathological vascular changes, or inappropriate cellular differentiation.

Powerful model systems exist for deciphering the signal transduction pathways and other genetic components of developmental programs. Preliminary characterization of the unique array of lipid structures present in these systems, and the identification of genes encoding S1P receptors and the enzymes of lipid metabolism have allowed these models to be employed in the dissection of lipid signaling pathways and their roles in development. The basic questions addressed using developmental approaches to lysophospholipid signaling include: (1) In what tissues and during what developmental time periods are genes of S1P and LPA signaling and metabolism expressed? (expression analysis); (2) How do these gene expression patterns relate to S1P and LPA levels throughout development? (lipid structural analysis and quantitation); (3) Are the genes of S1P and LPA metabolism and signaling necessary for specific developmental processes in the living embryo? (reverse genetics); (4) How do genetic screens identifying LPA- or S1P-related genes inform us about their potential roles in development? (forward genetics); (5) How can genetic models inform us about the relationship of S1P and LPA signaling to established developmental signal transduction pathways? (phenotypic comparison to known mutants; strategic genetic crosses); (6) In which cells and tissues is the expression of genes of lysophospholipid metabolism and signaling essential to a specific developmental process? (chimerism studies). In the following sections, we will explore some examples of how developmental models have addressed the questions

above and provided specific insights regarding lysophospholipid signaling.

Lysophospholipid Signaling in Cardiac Development

Identification of factors that influence heart morphogenesis is critical to understanding congenital heart disease, which is generally caused by defects in the specific structural components of the developing heart. Vertebrate heart development involves multiple steps, each requiring changes in gene expression, cell location, and tissue morphology which together contribute to the formation of anatomical structures including atria, ventricles, septae, and valves [Collins-Nakai and McLaughlin, 2002; Brand, 2003]. This process begins with the differentiation of lateral plate mesoderm into cardiac cells, followed by migration of paired cardiac progenitor pools toward the midline, fusion of these two cellular pools to form a single heart tube, looping of the tube, chamber formation, and maturation/septation. Some of the genetic factors involved in vertebrate heart development have been elucidated by genetic studies involving zebrafish [Chen et al., 1996; Stainier et al., 1996]. The zebrafish has rapidly become an important model for the study of developmental biology. Large numbers of zebrafish mutants can be generated and screened for particular types of defects by examining their transparent embryos, which develop outside the mother.

The zebrafish gene *Miles apart* (*Mil*) was identified by two recessive mutant alleles which in the homozygous or trans-heterozygous state were found to result in cardia bifida [Kupperman et al., 2000]. This cardiac developmental defect is caused during somitogenesis by failure of the proper migration of cardiomyocyte precursors from the anterior lateral plate mesoderm to the midline, where they normally fuse to form the primitive heart tube. In *mil* mutants, these cells develop two separate, laterally placed heart structures that contain properly differentiated myocardium, display normal atrioventricular patterning and beat properly but lack a connection with the vasculature. Positional cloning and phylogenetic analysis revealed that *Mil* encodes an Edg-5/$S1P_2$ receptor ortholog, and expression of Mil in Jurkat T cells established its identity as an S1P receptor by its ability to induce calcium mobilization and MAPK activation in response to S1P and SPC but not other sphingolipid metabolites. Mil is normally expressed along the embryonic axis early in development, and in the midbrain/hindbrain boundary and tip of the tail at later stages. At the 18-somite stage, Mil is expressed just lateral to the midline, and its expression follows the myocardial precursors as they migrate to the midline. Whereas wild-type primordial cardiac cells transplanted into a *mil* mutant embryo failed to prevent cardia bifida, primordial cardiac cells of the *mil* mutant transplanted into a wild-type embryo localized to the midline properly and contributed to the development of a normal heart. This experiment suggests that Mil expression by primordial cardiac cells is not critical for heart development. Conversely, Mil expression by cells residing in the location where cardiomyocyte precursors are destined to migrate is a necessary event for successful heart development in the zebrafish. The tissues that express Mil and thereby create a permissive environment for primordial cardiac cell migration are not yet known, although anterior or pharyngeal endoderm appear likely candidates. The influence on cardiac morphogenesis of genes expressed in non-cardiac tissues is not unique to Mil, as demonstrated by GATA5 and Bonnie-and-Clyde transcription factors, both of which are expressed in the endoderm normally underlying the myocardial precursors and whose proper differentiation and ventral migration is required for subsequent myocardial precursor migration [Ober et al., 2003].

How does Mil affect cardiomyocyte migration and vertebrate heart development? One interesting hypothesis suggests that Mil facilitates cell migration by stimulating assembly of ECM molecules such as fibronectin, vitronectin, and laminin, resulting in organization of the ECM and promotion of cell–ECM interactions. This hypothesis is consistent with the finding that *mil* mutants also show blistering of their tails, which points toward a defect of epithelial integrity and abnormal integrin-mediated interactions. Sphingolipid structural analyses, and characterization of putative enzymes of sphingolipid metabolism in zebrafish have not yet been performed. In addition, the deletion of the mouse $S1P_2$ gene did not result in a cardiac defect, indicating that Mil may not be a true $S1P_2$ homolog, or that observations regarding lysophospholipid signaling in zebrafish cannot be extrapolated to mammalian development.

Nonetheless, the results of this forward genetic approach were the first to implicate a role for S1P signaling through Edg receptors in vertebrate development. The role of Mil in facilitating cell migration during cardiac morphogenesis established a theme in which lysophospholipid signaling influences development through the orchestration of embryonic cell migration events, particularly by creating a permissive environment for migrating cells. As will be discussed below, this theme is echoed by studies linking lysophospholipid signaling and cell migrations involved in vascular development, gonad formation, and embryonic patterning in both vertebrate and invertebrate organisms. Importantly, in vitro studies in homogeneous cell systems would not detect such an effect, underscoring the significance of information provided by developmental models.

Lysophospholipid Signaling in Vascular Development

Congenital vascular neonatal diseases are rare, with the exception of large vessel anomalies including arteriovenous malformations, coarctation of the aorta, and ductus arteriosus [Marchuk et al., 2003]. Nonetheless, understanding the genetic programs that direct vascular development and maturation are relevant to the pathophysiology of a number of common diseases marked by abnormalities of vascular growth, including cancer, coronary artery disease, diabetes, peripheral ischemic disease, hypertension and rheumatoid arthritis [Harry and Paleolog, 2003]. The vascular system is the earliest organ to develop, and its function is required throughout embryogenesis, providing oxygen, nutrients, and signals to developing tissues. Blood vessels derive from hemangioblasts, which give rise to both blood cell and vascular cell precursors called angioblasts [Ema and Rossant, 2003]. Angioblasts migrate, fuse together and become polarized to form neovessels containing a lumen and surrounded by a basal lamina during vasculogenesis. These vessels subsequently develop sprouts, a process called angiogenesis. Vascular remodeling allows additional branching during development, leading to the complexity of the mature vascular tree. In addition, nascent vessels become surrounded by mural cells including pericytes and VSMCs, a process called vascular maturation [Hellstrom et al., 2001; Jain, 2003]. The importance of S1P signaling in mammalian vascular development was established when Proia and colleagues created $S1P_1$ null mice that lack the major S1P receptor expressed on the surface of endothelial cells [Liu et al., 2000b]. During normal embryogenesis in the mouse, $S1P_1$ is expressed as early as E9.5 and can be found in the cardiomyocytes of the common ventricular chamber of the heart, endothelial cells of the vasculature including dorsal aorta, intersomitic arteries and capillaries, VSMCs, ossifying bones, and the central nervous system (CNS). Homozygous $S1P_1$ null mice exhibited embryonic lethality at 12.5–14.5 days of gestation, with notable limb shortening and widespread hemorrhage. Although the developing vascular tree appeared largely intact, careful histological examination of neovessels showed an absence of pericytes, which are supporting cells related to VSMCs. It was suggested that pericytes may require S1P/$S1P_1$ interactions for proper migration to the vascular tree, and that loss of $S1P_1$ expression led to the observed failure of vascular maturation and resulting in lethal hemorrhage. Both pericytes and VSMCs are recruited to endothelial tubes formed during vasculogenesis, where they provide structural stability and regulatable contractile forces that modulate vessel aperture [Hellstrom et al., 2001; Allende and Proia, 2002]. Pericyte migration to microvascular structures requires signaling through the PDGF-B/PDGF receptor-β, whereas VSMC recruitment and coverage of large vessels are controlled by the TGF-β/endoglin/SMAD5 signaling pathway (which interestingly was recently shown to be modulated by S1PP and ceramide) [Oshima et al., 1996; Yang et al., 1999; Sato et al., 2003]. Mouse knockout models in which the PDGF pathway is disrupted die during the perinatal period due to microvascular hemorrhage and edema, whereas mutants in the TGF-β pathway die much earlier due to failure of proper angiogenesis at E11.5-12.5. $S1P_1$ knockout mice die at an intermediate stage between these two events and demonstrate prominent lack of VSMC coverage on the dorsal surface of the aorta. This distinguishes these mutants from TGF-β pathway mutants, which lack VSMC coverage of the entire aorta and from PDGF pathway mutants, which lack only capillary supporting structures. Fibroblasts derived from $S1P_1$ knockout embryos were incapable of activating Rac and failed to migrate toward S1P in vitro. Further, targeted

disruption of $S1P_1$ in mouse endothelial cells phenocopies the generalized $S1P_1$ knockout, indicating that receptor signaling in endothelial cells is necessary for vessel ensheathment by VSMCs [Allende et al., 2003].

A second phenotype that was appreciated in the $S1P_1$ knockout embryos was abnormal limb bud development. Further investigation of the role of $S1P_1$ in this process recently revealed that $S1P_1$ is highly expressed in both blood vessels and interdigital mesenchymal cells of the developing mouse limb and is required for proper chondrocyte condensation and digit morphogenesis [Chae et al., 2004]. Loss of $S1P_1$ expression in the knockout model led to multiple specific abnormalities including hyperplastic vasculature with abnormal morphology and induction of hypoxia-inducible factor-1α and VEGF gene expression. As similar limb bud defects were observed in endothelium-specific $S1P_1$ knockout mice, it would appear that signaling through $S1P_1$ in endothelial cells contributes to normal limb development. In summary, these studies suggest an important role for S1P in mediating vascular maturation during embryonic development. This role involves S1P/$S1P_1$ interactions and is similar to but distinct from other signaling pathways implicated in vasculogenesis. Uncovering the specific mechanisms by which S1P signaling through $S1P_1$ facilitates pericyte and VSMC migration and ensheathment of neovessels will be an important contribution to our understanding of developmental and postnatal angiogenesis.

In contrast to the severe phenotype of $S1P_1$ knockout mice, the limited phenotypes associated with loss of expression of other S1P receptors suggest more subtle involvement of these signaling pathways in development. For example, $S1P_3$ was found to be expressed in a wide variety of tissues in the E14.5 embryo, with the exception of liver [Ishii et al., 2001]. $S1P_3$ knockout mice demonstrated no appreciable defects, although MEFs derived from homozygous knockout embryos demonstrated a significant decrease in PLC activation and modest decrease in adenylyl cyclase inhibition in response to S1P. Upregulation of $S1P_2$ was found in the brain and heart tissues of the $S1P_3$ knockout mouse, suggesting that a compensatory mechanism may exist in some tissues. $S1P_2$ expression pattern was similar to that of $S1P_3$ and, as with the $S1P_3$ knockout, $S1P_2$ knockout mice were fully viable, fertile and demonstrated no obvious abnormalities of development. Since $S1P_2$ and $S1P_3$ appear to have similar or overlapping expression, function, and mechanisms of action including downstream activation of G_q, G_{13}, and G_i proteins, it was postulated that these receptors might be redundant in function. To evaluate this possibility, a double knockout lacking expression of $S1P_2$ and $S1P_3$ was generated [Ishii et al., 2002]. The double knockout model was remarkable for poor survival in the perinatal period and substantially reduced litters resulting from double-null crosses. Although surviving double-null animals displayed no anatomical or physiological defects, maternal negligence of pups was a frequent finding (see below). Fibroblasts from $S1P_2$ knockout embryos were deficient only in Rho activation, whereas $S1P_2$/$S1P_3$ double knockout fibroblasts were devoid of Rho responses and demonstrated additional deficiencies in PLC activation and calcium mobilization; adenylyl cyclase responses remained intact. These studies indicate that $S1P_2$ and $S1P_3$ coordinately provide an essential function mediated through G_q or $G_{12/13}$ pathways.

Like S1P, LPA signaling contributes to vascular stability, regeneration, angiogenesis, and platelet–endothelial interactions. To evaluate the potential role of LPA receptor-mediated signaling events in animal development, mouse models lacking expression of individual LPA receptors were generated. Homozygous LPA_1 knockout mice were uniformly notable for their short snouts, widely spaced eyes, and small size compared to heterozygous littermates [Contos et al., 2000]. A small percentage of these mice developed frontal hematomas either pre- or postnatally, and mice exhibiting hematomas were found to have more pronounced facial dysmorphism, suggesting that these two phenotypes may be related. LPA_1 is normally expressed in the developing facial bones, and the knockout phenotype, though mild, suggests a localized role in angiogenesis and/or vascular stability during embryogenesis. Neurodevelopmental defects characteristics of the LPA_1 knockout and contributing to their poor survival will be discussed below.

LPA_1 and LPA_2 share some downstream signaling targets, including PLC, calcium, and Rho, indicating that they may serve overlapping roles in vivo. Therefore, an LPA_2 knockout mouse and combination LPA_1/LPA_2 double null

mice were generated and analyzed [Contos et al., 2002]. LPA_2 homozygous mice were indistinguishable from heterozygous and wild-type littermates, and the only appreciable phenotype in LPA_1/LPA_2 double knockout mice was an increase in incidence of frontal hematomas. Despite the lack of developmental phenotype associated with loss of LPA_2 expression, LPA-induced responses that remained intact in LPA_1 and LPA_2 knockout fibroblasts (including PLC activation, calcium mobilization, JNK activation, Akt activation, and stress fiber formation) were diminished or lost in embryonic fibroblasts derived from LPA_1/LPA_2 knockout fibroblasts. These findings demonstrate that LPA_2 does function redundantly with LPA_1 in mediating signaling responses. Thus, the signals lost in the double knockout compared to the LPA_1 knockout may not be necessary during fetal development, although they could be required for maximal response to stress or injury in the adult animal.

In addition to LPA and S1P receptor knockouts, two mouse models with the potential to alter normal lysophospholipid metabolism have been generated. In these models, expression of two different PAP type 2 genes were disrupted. The PAP type 2 enzymes, also called lipid phosphate phosphohydrolases (LPP) due to their ability to hydrolyze a range of lipid phosphate substrates including S1P, LPA, PA, and ceramide phosphate, are membrane-bound glycoproteins involved in regulating the ratio of phosphorylated to unphosphorylated bioactive lipids [Sciorra and Morris, 2002]. LPP1 and LPP2 are ubiquitously and uniformly expressed throughout the developing mouse embryo, whereas LPP3 is expressed in a temporally regulated and tissue-specific manner [Escalante-Alcalde et al., 2003]. LPP3 expression is restricted to the extra-embryonic ectoderm of early (E6.5) embryos, in the anterior domain of the embryo and in the extra-embryonic membranes at E7.5, subsequently in the chorion, allantois, paraxial mesoderm, somites, developing gut and pericardio-peritoneal canal, and eventually in limb buds, peripheral nervous system (PNS), cranial nerves, mammary gland primordia, and the umbilical cord and placenta at later developmental stages. A still different pattern of expression is observed in adults, with LPP3 being expressed in lung, cerebellum, and cardiac atrium. Loss of PAP2c/LPP2 expression had no identifiable effect on mouse development, survival, or fertility [Zhang et al., 2000]. In contrast, disruption of the PAP2b/LPP3 gene led to uniform embryonic death prior to E10.5 associated with gastrulation defects and developmental delay [Escalante-Alcalde et al., 2003]. Two major defects appeared responsible for this lethality. First, null embryos failed to establish a chorio-allantoic placenta, a process which requires LPP3 expression in both chorion and allantois, based on chimeric experiments. VCAM1 and $\alpha 4$ integrin are known factors involved in mediating chorio-allantoic fusion [Yang et al., 1995]. As both were correctly expressed in LPP3 null embryos, other as yet unidentified effectors are likely to interact with LPP3 in mediating this process. Second, null embryos exhibited abnormal vascularization of the yolk sac, in which endothelial cells failed to form an organized vascular plexus, resulting in hemorrhage. Capillary formation in explants of LPP3 null allantoises or wild-type allantoises treated with the LPP3 inhibitor propranolol were severely compromised, indicating a prominent role for LPP3 in vasculogenesis of this organ. In addition, axis development was affected in null mutants (see below).

In summary, $S1P_1$ has a major and essential role in embryonic vascular maturation, and LPP3 expression is essential for vascular developmental processes required for establishment of the fetal-maternal circulation. In contrast, the frontal hematomas observed rarely in LPA_1 knockout mice and more frequently in LPA_1/LPA_2 double knockout models suggest a localized role for LPA signaling through these receptors in vascular development. $S1P_2$ and $S1P_3$ do not seem to be essential for vascular formation or remodeling during embryogenesis, although analysis of MEFs from these knockouts confirm the linkage of these receptors to G_q and $G_{12/13}$. The effects of gene disruption on wound healing, vascular remodeling, and development of vasculopathy in the postnatal period have not yet been examined in surviving mutant models or chimeras. There are no reports describing $S1P_4$ or $S1P_5$ knockout mice or mammalian models of S1P metabolism. In addition, controversies regarding the contribution of $S1P_2$ signaling to development remain, since others have found that loss of $S1P_2$ expression alone leads to increased neuronal excitability and seizures [MacLennan et al., 2001]. The distinct phenotypes observed in ostensibly identical developmental models

serve to illustrate the complexity of these model systems. Different genetic backgrounds, modifier genes, knockout constructs, location of genomic insertions, and many other factors are likely to influence phenotypic outcomes. Recognition and follow-up of subtle discrepancies between model systems may provide opportunities to identify biochemical network partners and signaling crosstalk, revealing potentially important insights regarding the heterogeneity of disease manifestations [Paigen, 2002].

Lysophospholipids in Development of the Immune System

Development of the human immune system begins with the onset of hematopoiesis and requires T- and B-lineage specification, lymphocyte and dendritic cell homing and differentiation, thymic development and the elimination of self-reacting T-cells by apoptosis, and mobilization of mature, naïve immune cells [Fischer, 2004]. Immune maturation continues postnatally as the immune system is continually challenged and activated with new antigenic stimuli. Primary immunodeficiency diseases predispose individuals to infections, allergy, autoimmunity and cancer, and immune modulation is an important goal in organ tissue transplantation and vaccine development [Huang et al., 2002; Cinque et al., 2003]. Lysophospholipids have been implicated in various aspects of T-, B- and dendritic cell function and activation, allergic responses, and inflammation [Huang et al., 2002; Xu et al., 2003]. In that light, the phosphorylated form of FTY720, an immune modulatory drug and S1P receptor agonist, induces T-cell sequestration in lymphoid tissues leading to lymphopenia [Brinkmann et al., 2002; Mandala et al., 2002]. Thus, manipulation of S1P signaling may provide an important new approach to immune modulation in the transplantation setting. While a discussion of the broad area of immune function and inflammation is beyond the scope of this review, three recent studies exploring the involvement of S1P signaling in lymphocyte trafficking and the early development of the immune system will be discussed.

To better understand the mechanism by which FTY720 causes sequestration of naïve lymphocytes to secondary lymphoid organs including peripheral lymph nodes and Peyer's patches, Goetzl and colleagues evaluated S1P receptor expression on human and mouse B- and T-lymphocytes [Graler and Goetzl, 2004]. They found that $S1P_1$ and $S1P_4$ were the most highly expressed S1P receptors in mouse spleen CD4 and CD8 T-cells and CD19 B cells, as well as on human blood CD4 and CD8 T-cells. Human naïve CD8 T-cells also expressed $S1P_5$. In a series of experiments performed on both human and mouse lymphocytes, low nanomolar concentrations of FTY720 were shown to reversibly and non-competitively inhibit S1P-induced lymphocyte responses including mobilization and calcium influx. FTY720 was capable of downregulating $S1P_1$, $S1P_2$, and $S1P_5$ receptor expression by internalization (without activation) and presumably by inhibition of receptor recycling. These studies suggest that the unphosphorylated drug induces lymphopenia by desensitizing lymphocytes to S1P-induced responses, especially through the $S1P_1$ receptor, which is highly expressed on lymphocytes and responsive to FTY720-mediated downregulation. This contrasts with the effects of the phosphorylated form of the drug, which appears to function as a receptor agonist against four of the five S1P receptors.

In order to investigate S1P signaling in immune system development, a T-cell-specific $S1P_1$ knockout mouse was generated using the Cre/loxP system [Allende et al., 2004]. While homozygous knockout pups appeared normal at birth and grew to become fertile adults, examination of the peripheral blood demonstrated a marked reduction in circulating T-cells, resulting in a lower percentage of T-cells and higher percentage of B-cells compared to control animals. When both spleen and peripheral lymph nodes of knockout mice were examined, T-cells were proportionately missing, whereas B-cells appeared to be unaffected by $S1P_1$ disruption. In contrast, mature T-cells were substantially increased in thymic tissue of $S1P_1$ knockout mice compared to controls, although the overall architecture of thymi and maturation of T-cells both appeared normal. To measure egress of T-cells from the thymus, intrathymic injection of fluorescein isothiocyanate was performed, followed by quantitation of labeled T-cells in the peripheral blood. A markedly reduced number of labeled T-cells in the periphery of knockout mice compared to controls demonstrated that the depletion of circulating T-cells in these animals was due to a defect in emigration of mature T-cells from the thymus. $S1P_1$ expres-

sion was found to be upregulated during T-cell maturation, and this correlated with an increase in responsiveness to S1P in a chemotaxis assay.

In a parallel study, these same $S1P_1$ knockout mice demonstrated T-cell depletion in the periphery, a lowering of B-cells in the blood and lymph, and a requirement for $S1P_1$ expression in both T- and B-cells for normal egress of lymphocytes from lymphoid organs [Matloubian et al., 2004]. $S1P_1$-dependent S1P signaling was upregulated during T-cell development prior to exit from the thymus, enhancing egress. In addition, this responsiveness was downregulated during the activation of mature T-cells in the periphery, enhancing retention in lymph nodes. Finally, as in the in vitro study mentioned above, FTY720 was found to downregulate $S1P_1$ expression, leading to a similar pattern of lymphocyte retention in lymphoid tissues. Thus, while S1P signaling through $S1P_1$ does not appear crucial for hematopoietic differentiation, localization of T-cells to thymus, or thymocyte maturation, it is necessary for the establishment and maintenance of normal hematological parameters via T-cell mobilization from the thymus. Further, regulation of S1P signaling through $S1P_1$ is required for proper lymphocyte trafficking in response to immunogenic challenge, as exemplified by the exaggerated state of downregulation induced by FTY720 treatment.

Lysophospholipids and Neurodevelopment

Understanding the signaling pathways involved in the establishment of neural architecture, function and remodeling may uncover the etiology of congenital diseases including anencephaly and neural tube defects. It may also lead to new approaches to achieve neuronal regeneration/repair in diseases marked by neuronal death, including stroke, Alzheimer's disease, multiple sclerosis, and trauma [Compston, 1998; Gurgo et al., 2002]. Mammalian nervous system development begins with formation of the notochord and its stimulation of adjacent ectoderm to become the neural plate. The neural plate folds to become the neural tube that will eventually develop into the entire nervous system. The anterior end of the tube gives rise to the major anatomical structures of the brain (cerebral hemispheres, diencephalon, midbrain, pons and cerebellum, and medulla oblongata), whereas the rest becomes the spinal cord. Cell differentiation and division within the neural tube lead to the formation of different cell populations, including neurons and glial cells, as well as neural crest cells that will form the PNS. The development of the cerebral cortex is itself a highly complex process involving the generation of neurons from neuroblast precursors in the VZ overlying the lateral ventricle. Neurogenesis is followed by the migration of cortical postmitotic neurons toward the cortical plate, branching of dendrites and extension of axons (neuritogenesis), and synapse formation. Cell migration, proliferation, removal by apoptosis, tissue patterning, morphological changes, and myelination of cortical axons all ultimately contribute to the final organization and function of the cortex and other nervous system tissues.

LPA signaling has been implicated in brain development, as high levels of LPA have been demonstrated in the brain, possibly derived from membrane-bound and soluble autotaxin activity and from postmitotic cortical neurons that have been shown to synthesize and secrete LPA [Das and Hajra, 1989; Kawagoe et al., 1995; Fukushima et al., 2000]. In addition, LPA levels are induced in response to injury [Steiner et al., 2002]. Not unexpectedly, LPA receptors are enriched in the nervous system, and their expression patterns have suggested potential involvement in developmental processes including neurogenesis, neuronal migration, axon extension, and myelination [Cervera et al., 2002; McGiffert et al., 2002]. The first LPA receptor, LPA_1, was identified in a screen for G protein-coupled receptors demonstrating high expression in the cerebral cortex [Hecht et al., 1996]. LPA_1 is expressed in a restricted fashion within the neuroblasts of the neuroproliferative VZ of the developing cortex, in the dorsal olfactory bulb, along the pial surface of the cerebral wall, possibly in pial cells of neural crest origin, and in developing facial bone tissue. Expression is observed during E11-E18, corresponding to a time period during which neurogenesis occurs. LPA_1 expression is undetectable in the VZ after this point, but reappears during the first postnatal week within oligodendrocytes, glial cells of the CNS involved in myelination of developing fiber tracts during this time period. Schwann cells of the PNS, which correspond roughly to oligodendrocytes in their myelinating function, express high levels of LPA_1 early in development and persistently throughout life.

Expression studies and in vitro analysis of LPA_1/LPA interactions in cells of VZ origin and in myelinating cells of the PNS and CNS have implicated this signaling pathway in neuronal cell function and Schwann cell survival [Weiner and Chun, 1999; Weiner et al., 2001; Yang et al., 2002]. For example, LPA treatment of cell lines derived from the VZ stimulates a program of morphological changes accompanying cell cycle progression and characterized by cell rounding and process retraction, collectively termed "interkinetic nuclear migration" [Fukushima et al., 2000]. In addition, ionic changes and cellular depolarization occur in response to LPA treatment, suggesting that LPA provides an early extracellular stimulus resulting in electrophysiological changes in developing neurons [Dubin et al., 1999]. LPA/LPA_1 interactions may also be involved in the function of myelinating cells such as Schwann cells of the PNS, which express LPA_1 and demonstrate enhanced survival and diminished serum deprivation-induced apoptosis when pretreated with LPA (but not S1P). The effect of LPA treatment appears to be mediated through LPA_1 and activation of the $G_{i/o}$/PI_3kinase/Akt signaling pathway, and survival is enhanced by activation of the Rho pathway and resulting increases in ECM-mediated cell adhesion [Weiner and Chun, 1999; Ye et al., 2002].

To evaluate whether these functions are critical to normal development and survival, an LPA_1 knockout mouse was generated, as described above [Contos et al., 2000]. Interestingly, loss of LPA_1 expression resulted in a number of notable phenotypes in addition to craniofacial dysmorphism and occasional frontal hemorrhage. These included 50% neonatal lethality and reduced animal size (appreciated shortly after birth and into adulthood in surviving mice and associated with reduction in fat stores). The neonatal lethality was explained by failure of the pups to exhibit normal suckling behavior, resulting in starvation or stunted growth. This was postulated to be a result of changes in the olfactory bulb and cerebral cortex, preventing normal olfaction required for suckling, since the affected pups rooted normally and thus had normal motor responses, yet often could not locate the mother's nipples. Although no anatomical defects in the PNS (or CNS) were observed, LPA_1 null mice demonstrated an increase in apoptosis of sciatic nerve Schwann cells from 10% in wild type to 18% in the knockout mice. Myelination of the sciatic nerve was not compromised, presumably because of sufficient remaining Schwann cells. As expected, neuroblasts derived from null embryos failed to respond to LPA stimuli in vitro. Interestingly, LPA_1 deficient mice exhibited behavioral and neurological findings associated with psychiatric diseases, including alterations in levels of amino acids, the levels and turnover of the neurotransmitter 5-hydroxytryptamine, and a deficit in prepulse inhibition, which is a model of sensorimotor gating mechanisms in the brain affected in schizophrenia [Harrison et al., 2003]. These studies are exciting, in that they indicate the potential to uncover subtle neurological defects using similar approaches. Further, they suggest that alterations of lysophospholipid signaling may be implicated in the pathophysiology of neuropsychiatric disease.

In addition to LPA_1 knockout mice, $S1P_2$/$S1P_3$ double knockout mice demonstrated a high perinatal mortality rate in the absence of obvious anatomical defects. An attempt was made to elucidate the reason for poor survival of double mutant pups by evaluating the outcomes of mutant embryos transplanted into wild-type mothers and wild-type embryos transplanted into mutant mothers. The results (neither scenario led to completely normal growth and survival) suggest that a combination of defects in prenatal development and parent–offspring interactions may account for the high perinatal mortality of double null pups from double null crosses. The exact etiology of the defect and its ramifications for human development remain unclear. It is hoped that the identification of subtle pathological indicators, cortical functional deficits, and/or defects of synaptic transmission will provide clues regarding the exact role of $S1P_2$ and $S1P_3$ in development.

Elucidation of the role of LPA in development has focused not only on LPA receptors but also on enzymes of LPA metabolism. The tumor-related protein autotaxin is responsible for LPA synthesis and more recently was shown to catalyze formation of S1P as well. One recent study found that rat optic nerve oligodendrocytes (the glial cells responsible for myelination of axons in the CNS) express and export autotaxin to the extracellular environment concomitant with the onset of myelination [Fox et al., 2003]. Autotaxin expression remains high during the peak of myelination but is low in the

adult CNS. In an in vitro assay, recombinant autotaxin inhibited adhesive interactions between oligodendrocytes and extracellular matrix proteins normally present in the developing CNS. Interestingly, although this process was abolished by autotaxin-specific antibody treatment and was also inhibited by pertussis toxin, suggesting the requirement of a G protein coupled receptor, autotaxin's effect was found to be independent of its lysophospholipase D activity. Taken together, these results suggest that autotaxin may act as an autocrine factor to facilitate oligodendrocyte motility, process outgrowth, and/or myelin membrane formation through a non-enzymatic function of the polypeptide.

Neuronal plasticity is the ability of neurons to undergo regenerative axon sprouting and navigation to a specific locale. Nitsch and colleagues identified plasticity-related gene 1 (PRG1) by rat cDNA screening [Brauer et al., 2003]. This gene encodes a neuron-restricted LPP ectophosphatase capable of degrading LPA in the extracellular space. PRG1 is one member of a unique family of vertebrate-specific LPPs, which differ structurally from other LPPs in having a carboxy-terminal 400 amino acid cytoplasmic extension of unknown function. PRG1 is first expressed in the rat hippocampus and entorhinal cortex (but not other cortical tissues) at late embryonic stages and is upregulated after experimental denervation of the hippocampus. Early embryonic explants from the entorhinal cortex, which do not express PRG1, retract their neurites upon exposure to LPA, whereas postnatal explants expressing PRG1 are insensitive to LPA treatment. Further, expression of PRG1 in a neuronal cell line known to respond to LPA with growth cone collapse led to LPA resistance, whereas a catalytically inactive mutant PRG1 did not. These results are consistent with the notion that PRG1 facilitates axonal outgrowth and attenuates LPA-induced neurite retraction and cell rounding by hydrolyzing LPA. Three additional PRG family members, PRG2-4, have been identified by sequence homology. PRG2 is expressed in neuronal cell layers of the developing brain and in hippocampus and cerebellum postnatally, although its function has not been reported. PRG3 is expressed in hippocampus, thalamus, cortex, and olfactory bulb by E16. Expression peaks perinatally in hippocampus and cortex, and is transiently downregulated in response to neuronal overstimulation [Savaskan et al., 2004]. Interestingly, PRG3 is capable of stimulating neurite outgrowth and changes in cell morphology when transfected into a neuronal cell line, despite the fact that it displays no LPA ecto-phosphatase activity. The ability of PRG3 and autotaxin (as presented above) to mediate biological effects independent of LPA hydrolysis emphasizes a recurring finding, that is, that enzymes of S1P and LPA metabolism may act on developmental pathways through more than one mechanism. Possible explanations include the ability of a single enzyme to promote distinct biological endpoints via the metabolism of different lipid substrates, the ability of a catalytically inactive protein to interfere with lipid signaling through binding of a bioactive lipid, or functions of the polypeptide which are altogether independent of lysophospholipid signaling. Further investigation of these unexpected observations may provide new and interesting insights regarding bioactive lysophospholipids and the enzymes that regulate them.

Two recent studies suggest that both LPA and S1P may be directly involved in the neuronal plasticity of an unusual tissue with significant regenerative function. The olfactory mucosa contains neurons that sense odorants in the external environment. Due to its vulnerability to toxic exposures and resulting apoptotic cell death, this tissue appears to have evolved more extensive regenerative capacity than other vertebrate neuronal tissues. An expression analysis approach was taken to learn more about the genes involved in neuronal repair and plasticity. Interestingly, one of the most highly expressed genes in mouse olfactory mucosa compared to other tissues was S1P lyase [Genter et al., 2003]. High expression of a functional enzyme was confirmed by in situ hybridization and activity assays, indicating that S1P metabolism could be involved in the unusual characteristics of this neuronal tissue. Although the mechanism for this is not yet known, increased S1P lyase expression and decreased S1P could affect the viability, localization, and/or differentiation of neurons or supporting cells of the olfactory mucosa. Interestingly, the olfactory ensheathing cells, which provide an environment supportive of axonal regeneration, were found to be affected by LPA treatment, which acts via Rho-GTPase, MAPK, and PI_3kinase signaling pathways to influence

proliferation, migration, and cytoskeleton assembly of these cells [Yan et al., 2003]. Since both Schwann cells and olfactory ensheathing cells have been shown to enhance axonal regeneration when injected into injured spinal cords, the potential for lysophospholipid signaling in this unique tissue is a promising new area of research with the potential for therapeutic benefit [Li et al., 1997].

Lysophospholipids in Reproduction

Apoptosis is a normal feature of the developing mammalian reproductive tract, affecting both somatic and germ cell constituents of female gonads. Recent studies have demonstrated that S1P and ceramide regulate gonadal cell apoptosis during prenatal development and also after exposure to damaging agents such as irradiation and chemotherapy used in cancer treatment [Morita and Tilly, 2000]. Whereas sphingomyelinase and ceramide appear to induce apoptosis in oocytes, S1P promotes oocyte survival. Sphingomyelinase-deficient mice demonstrated substantially greater numbers of oocyte-containing primordial follicles in the ovaries at birth and throughout life, due to attenuation of apoptosis [Morita et al., 2000]. This effect was recapitulated by administering S1P to ovaries in culture, which suppressed oocyte and DNA fragmentation. Interestingly, LPA protected against cellular fragmentation but did not abrogate DNA fragmentation, indicating that protection afforded by S1P is specific. Although no vertebrate models demonstrating deficient S1P catabolism are available, it would be predicted that oocyte survival would be enhanced by S1P accumulation. These studies are of great importance in that they illustrate how a relatively subtle observation in a developmental model may potentially lead to the ability to influence "physiological" human processes such as menopause and provide therapeutic interventions in genetic, environmentally induced or iatrogenic disease.

A potential link between LPA and reproductive function was revealed by a recent study reporting the generation of transgenic mice in which a lipid phosphate phosphatase-1 transgene was ubiquitously expressed under regulation of a chicken β-actin promoter [Yue et al., 2004]. Interestingly, the male transgenic mice demonstrated atrophic testes, severe disruption of spermatogenesis, and Leydig cell hypoplasia. In contrast, female reproductive organs were not histologically different from control animals, although female transgenics had consistently smaller litters; the etiology of this finding is not clear. Importantly, despite an increase in LPA ectophosphatase activity in the fibroblasts of transgenic mice, plasma LPA levels were unaffected. This could be explained if phospholipid metabolism is differentially affected in specific tissues, if LPP1 has a role in reproductive function and development independent of its catalytic activity, or if the regulation of other phospholipids by LPP1 contributes to reproductive function.

Lysophospholipids and Axis Development

The vertebrate body plan contains anteroposterior (A/P) and dorso-ventral axes. The organization of the basic axes early in embryogenesis and of bilateral symmetries of tissues and appendages around these axes are critical to animal development. The LPP3 knockout mouse, described above with respect to vascular development, also demonstrated infrequent but severe effects on embryonic axis formation, including short A/P axis, anterior truncation, embryonic development external to the yolk sac membranes, and duplication of axial structures including the notochord [Escalante-Alcalde et al., 2003]. These latter defects appear to be due to alterations of the Wnt/β-catenin signaling pathway, as demonstrated by increased expression of *Wnt* regulated genes in vivo and by the finding of an inhibitory effect of LPP3 expression on β-catenin-mediated gene transcription in vitro. LPP3 expression induced a ventralizing effect on embryonic development that was recapitulated by injection of murine LPP3 mRNA into *Xenopus* embryos. Embryonic fibroblasts from LPP3 null embryos lacked LPP3-related enzymatic activity and exhibited predictable changes in phospholipids including diminished diacylglycerol and increased PA. Although cellular S1P and LPA levels were below the limits of detection, extracellular LPA was increased in the culture medium of LPP3 null cells compared to wild type, indicating that LPA metabolism was perturbed. Interestingly, the effects of LPP3 on *Wnt* signaling were independent of LPP3 catalytic function, as they were induced by a catalytically inactive mutant as well as a wild-type LPP3, uncovering a previously unidentified role for LPP3 independent of its phosphohydrolase activity.

Simple Metazoan Models of Lysophospholipid Signaling

Considering the information gained from vertebrate models, one might question the utility of invertebrate organisms such as *Drosophila* and *C. elegans*, whose genomes do not contain recognizable Edg receptors, toward the elucidation of lysophospholipid signaling. However, these simple metazoan models have provided new insights into the mechanisms by which lysophospholipids and the enzymes that modify them may affect animal development. A few examples will serve to illustrate this point.

In *Drosophila*, the *wunen* genes encode enzymes that function as phospholipid phosphatases and are members of the family of LPP genes described above [Zhang et al., 1996]. Wunen proteins have been shown to regulate germ cell migration and survival through a mechanism that involves phosphohydrolase activity, creating a chemical gradient in the embryo that guides germ cells to their proper final location in the developing gonad [Zhang et al., 1997; Starz-Gaiano et al., 2001]. Although it is still not known which phospholipid substrate is depleted by the actions of Wunen, it is likely to be either LPA or S1P, both of which have been directly or indirectly implicated in the regulation of cellular migrations in developing organs of other species [Burnett and Howard, 2003]. Interestingly, mutations in Wunen were also found to influence left-right axis formation in the developing gut of *Drosophila* embryos, reminiscent of the effect of LPP3 mutation on mouse axis formation [Ligoxygakis et al., 2001].

Like *Wunen*, another enzyme involved in lysophospholipid catabolism, sphingosine phosphate lyase (S1P lyase) has been shown to influence various aspects of *Drosophila* development, reproduction, and survival [Herr et al., 2003]. This enzyme catalyzes the irreversible cleavage of S1P at the $C_{2\text{-}3}$ carbon-carbon bond, yielding ethanolamine phosphate and a long chain aldehyde. *Sply* is the *Drosophila* S1P lyase homolog, and its expression is restricted to the developing gut of *Drosophila* embryos. Loss of *Sply* expression leads to defects of muscle development and muscle cell survival in the adult fly, as well as reproductive phenotypes including egg retention and ovarian degeneration. At least some of these effects appear to be due to dysregulation of developmental and postnatal apoptosis, as determined by TUNEL staining in the developing embryo and by genetic complementation analysis. Although the mechanism underlying the influence of *Sply* expression on cell fate is not fully elucidated, many of the characteristics of the *Sply* mutant phenotype are reversed by inhibiting de novo synthesis of sphingolipids and accumulation of sphingolipid intermediates, including phosphorylated LCBs. Deletion of one of two SK genes present in the fly genome and which is expressed, like *Sply*, primarily in the developing gut, leads to a phenotype marked primarily by an increase in lethality and diminished egg laying [Herr et al., 2004].

A role for LCBP metabolism has been implicated in the developmental programs of other simple metazoan models including *C. elegans*, where S1P lyase expression is essential for maximal survival and reproduction and in *Dictyostelium*, where S1P and S1P lyase expression have been shown to influence cell motility and spore formation, as well as resistance to cytotoxic reagents [Li et al., 2000, 2001; Mendel et al., 2003]. The mechanisms by which LCBP metabolism contributes to these processes have not yet been fully elucidated, and important questions remain regarding how genes demonstrating gut-restricted expression patterns affect the development of distant organs. Despite questions raised by these studies in simple metazoans, they demonstrate the importance of lysophospholipid metabolism in development throughout the animal kingdom and suggest that lysophospholipids can function independently of Edg receptors. Whether lysophospholipids are activating members of an as yet unrecognized family of receptors, or whether these interesting lipids can act in an entirely receptor-independent manner remains to be determined. However, precedent for a receptor-independent mode of action is found in budding yeast, in which LCBPs have been shown to regulate stress responses, calcium homeostasis, and endocytosis in the absence of specific receptors.

SUMMARY

The characterization of strategic mutant models has begun to provide insight into the influence of lysophospholipids on animal development and maintenance of tissue integrity. These studies indicate that S1P and LPA signaling pathways are used extensively in a

variety of developmental programs in both vertebrate and invertebrate organisms. LPA and S1P receptor knockout mice provide an array of complementary model systems in which to dissect and differentiate specific developmental pathologies associated with the loss of lysophospholipid signaling pathways and thus identify their individual and shared contributions to prenatal and perinatal mammalian development. $S1P_1$ and LPA_1 play fundamental roles in embryogenesis, as loss of their expression is associated with high or uniform lethality. The $S1P_1$ knockout mouse uncovered a critical role for S1P signaling in embryonic vascular maturation, whereas the LPA_1 knockout mouse phenotype suggests a limited role for LPA signaling in developmental angiogenesis but a critical role in neurodevelopment. The early lethality of $S1P_1$ and LPP3 knockout embryos precludes the identification of phenotypes in later developmental stages. The generation of conditional and tissue-specific knockouts of these genes may reveal additional roles for lysophospholipid signaling in development. LPA_2 null mice, like $S1P_2$ and $S1P_3$ null mice, are without developmental defects, although a role in stress or wound healing in the adult animal cannot be discounted.

Important questions regarding the extent, diversity, mechanisms of action, and indirect consequences of lysophospholipid signaling in development remain to be answered. For example, which cells produce signaling S1P and LPA, and how are these molecules transported throughout the organism? What is the substrate of the *wunen* gene product? Does autotaxin regulate the synthesis of S1P in vivo, and is the role of autotaxin in cancer and angiogenesis mediated at least in part through S1P? Could the effects of this and other enzymes of lipid metabolism be dictated by which substrates are available to the enzyme at a given time and location? What role might the many genes involved in LPA and S1P metabolism play in development? How parallel are the mechanisms of S1P and LPA signaling? For example, might S1P be acylated to ceramide phosphate and induce vesicle formation and/or endocytosis, in a manner similar to that proposed for the conversion of LPA to PA by endophilin? Is there a role for intracellular LPA signaling, similar to that proposed for S1P? Do receptor families other than the Edg receptors participate in developmental signaling? Is there a binding partner to FTY720 in simple metazoans? What are the intracellular targets of receptor-independent S1P signaling? Is there a role for lysophospholipids in critical membrane fusion events in development? How are the genes of lysophospholipid signaling and metabolism regulated? How does lysophospholipid signaling integrate with conserved developmental pathways such as Notch, Wnt, and Hedghog? Do mutations affecting lysophospholipid metabolism affect human development? In addition to these specific questions, it remains an enigma why two such similar signaling pathways as those mediated by LPA and S1P evolved and have remained functional in vertebrates.

Finally, it should be emphasized that the ultimate result of changes in intracellular, pericellular, and circulating levels of S1P and LPA depend upon many factors, including but not limited to Edg receptor expression. The lysophospholipid pathways discussed here do not operate in isolation and are, in fact, details within the panorama of phospholipid and sphingolipid biosynthetic and signaling pathways. As integral parts of these complex metabolic networks, S1P and LPA may influence steps outside our immediate view. In addition, similarities between LPA and S1P structure, signaling and metabolism suggest at least the possibility that biochemical competition between these two molecules in the interaction with cell surface receptors, enzymes, intracellular channels, or transport proteins could influence biological outcome and vascular development. Additional studies should help to provide a more comprehensive understanding of the molecular and biochemical mechanisms by which lysophospholipids and the enzymes of lysophospholipid metabolism affect lipid signaling at large. Genomic, proteomic, metabolomic, and lipidomic approaches will paint a broad picture of this dynamic process; strategic genetic model systems will provide critical tools with which to explore the roles of specific enzymes and effector proteins. With effort, the biochemical characterization of lysophospholipids and pertinent enzymes in these model systems should allow us to take full advantage of these genetic systems, by verifying or refuting the expected biochemical consequences of knockout mutations. The efficacy of this strategy is exemplified by *S. cerevisiae*, previously considered an impenetrable biochemical system due to its large number of proteases. This system

has added substantially to our understanding of the metabolism and functional significance of sphingolipids and other lipid signaling pathways, due to the dual power of performing biochemical analyses in a genetically tractable organism.

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