

Review

In vivo roles of lysophospholipid receptors revealed by gene targeting studies in mice

Amy H. Yang^{a,b,1}, Isao Ishii^{a,1,2}, Jerold Chun^{a,b,c,*}

^aDepartment of Pharmacology, School of Medicine, University of California, San Diego (UCSD), La Jolla, CA 92093-0636, USA

^bBiomedical Sciences Graduate Program, School of Medicine, University of California, San Diego (UCSD), La Jolla, CA 92093-0636, USA

^cNeurosciences Graduate Program, School of Medicine, University of California, San Diego (UCSD), La Jolla, CA 92093-0636, USA

Received 1 November 2001; accepted 30 January 2002

Abstract

Lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P) are extracellular ligands for a family of G protein-coupled receptors (GPCRs), LPA_{1/2/3} and S1P_{1/2/3/4/5}. Through coupling to multiple classes of G proteins and activating multiple signaling pathways, LPA/S1P receptors have been shown to be integral players for many essential cellular and physiological processes. Generation and analysis of mice deficient in each of LPA₁, LPA₂, S1P₁, S1P₂, and S1P₃ have provided valuable information on the in vivo roles of these receptors. This review is focussed on expression patterns of each receptor gene in wild-type mice, targeted deletion approaches for generating mutant animals, main phenotypes of receptor-null mice, and alterations in signaling characteristics in receptor-deficient primary cells. Altogether, these data give insights to the importance of LPA/S1P receptors at the cellular and organismal level. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Lysophospholipid; Lysophosphatidic acid; Sphingosine 1-phosphate; LPA receptor; S1P receptor; Signal transduction

1. Introduction

Lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P) are bioactive lysophospholipids (LPs) that act as mediators in various cellular processes, such as cell growth, differentiation, survival, motility, and cytoskeletal reorganization (reviewed in Refs. [1–3]). LPA and S1P are both abundant in serum and are produced by activated platelets and other cell types. From recent studies, they are recognized as extracellular ligands for a family of cognate G protein-

coupled receptors (GPCRs), which are encoded by *lpa* and *slp* genes.³ Three of these genes, *lpa*₁, *lpa*₂, and *lpa*₃, encode high-affinity LPA receptors. The remaining five genes, *slp*₁, *slp*₂, *slp*₃, *slp*₄, and *slp*₅, encode GPCRs that specifically interact with S1P or sphingosylphosphorylcholine. These LP receptors couple to multiple classes of heterotrimeric G proteins (G_{i/o}, G_{q/11/14}, G_s, and G_{12/13}) and activate various ligand-induced signal transduction pathways. Some of the signaling events that follow LP receptor activation include stimulation of serum response element and subsequent transcriptional events, activation or inhibition of adenylyl cyclase, activation of mitogen-activated protein kinase, intracellular Ca²⁺ mobilization, phospholipase C (PLC) activation, and stress fiber formation (reviewed in Refs. [2–8]).

The *lpa* and *slp* genes are widely expressed in various mammalian organ systems, such as cardiovascular, nervous and reproductive systems, and their expression patterns are regulated throughout development. The widespread existence of LPs in many tissue types (reviewed in Refs. [1,7]), the pleiotropic nature of their biological effects, and the unique spatio-temporal expression patterns of *lpa/slps* genes, combine to confer upon the LP signaling system a capability to exert diverse physiological actions in the whole organism. LPA and S1P are implicated in processes as divergent as shaping neuronal morphology, cell–cell

* Corresponding author. Present address: Merck Research Laboratories, San Diego, 3535 General Atomics Court, San Diego, CA 92121, USA. Tel.: +1-858-202-5259; fax: +1-858-202-5814.

E-mail address: jerold_chun@merck.com (J. Chun).

¹ These authors contributed equally to this work.

² Present address: Department of Molecular Genetics, National Institute of Neuroscience, 4-1-1, Ogawahigashi, Kodaira, Tokyo 187-8502, Japan.

³ This review adapts the new IUPHAR nomenclature for lysophospholipid receptor (gene) names. LPA₁ is encoded by *lpa*₁, which was formerly known as *lpA1/edg2/vzg1*. LPA₂ is encoded by *lpa*₂, which was formerly known as *lpA2/edg4*. LPA₃ is encoded by *lpa*₃, which was formerly known as *lpA3/edg7*. S1P₁ is encoded by *slp*₁, which was formerly known as *lpB1/edg1*. S1P₂ is encoded by *slp*₂, which was formerly known as *lpB2/edg5/h218/agr16*. S1P₃ is encoded by *slp*₃, which was formerly known as *lpB3/edg3*. S1P₄ is encoded by *slp*₄, which was formerly known as *lpC1/edg6*. S1P₅ is encoded by *slp*₅, which was formerly known as *lpB4/edg8/nrg-1*.

communication, tumor invasion, angiogenesis, wound healing, and embryonic development (reviewed in Refs. [1,6,9]).

Despite a growing understanding of LP receptors and their signaling systems, there has been a lack of direct evidence for their physiological roles in intact animals until recently. The generation of receptor-null mice allows direct examination of the systemic roles of LP receptors *in vivo* as well as further elucidation of LP receptor-specific signaling pathways in receptor-null primary cells. This review focuses on analyses carried out in $LPA_1^{-/-}$, $LPA_2^{-/-}$, $S1P_1^{-/-}$, $S1P_2^{-/-}$, and $S1P_3^{-/-}$ null mice (Refs. [10–13] and Contos et al., submitted for publication), detailing gene expression patterns of each LP receptor gene in wild-type mice, gene targeting strategies, phenotypes of receptor-null animals, and signaling properties in receptor-null primary cells.

2. *lpa*₁

2.1. Expression patterns

The 3.8 kb mRNA transcript of *lpa*₁ is highly expressed in the embryonic cerebral cortical ventricular zone (VZ) in the dorsal telencephalon during the period of neurogenesis [14–16]. The expression disappears in the VZ right before birth. The *lpa*₁ transcript reemerges within the postnatal murine nervous system in oligodendrocytes and Schwann cells, the myelinating cells of the central and peripheral nervous systems, respectively [17–20]. The postnatal spatio-temporal expression patterns of *lpa*₁ in the nervous system closely parallel those of myelination, implicating LPA_1 in this process [17,19,20]. The *lpa*₁ transcript can also be detected at significant levels in the adult by Northern blot analysis in brain, heart, lung, and testis, and at lower levels in other organs examined, but not in liver (Fig. 1 and Ref. [5]).

2.2. *lpa*₁ gene targeting

The LPA_1 -null mice were generated by our group [10]. Of the five exons constituting mouse *lpa*₁, exon 3 contains transmembrane domains I–VI, accounting for 68% of the *lpa*₁ open reading frame (ORF). Exon 3 was therefore targeted for deletion, using the *Cre-loxP* system. This approach allowed the removal of selectable markers (neomycin-resistant and thymidine kinase genes) and their associated constitutive promoters, in addition to the deletion of exon 3, upon homologous recombination [10].

2.3. Knockout phenotypes and signaling characteristics

The heterozygous *lpa*₁^(+/-) males and females were bred to produce homozygous *lpa*₁^(-/-) mice without sexual bias [10]. A small percentage of *lpa*₁^(-/-) embryos had frontal hematomas, and approximately 50% of *lpa*₁^(-/-) neonates

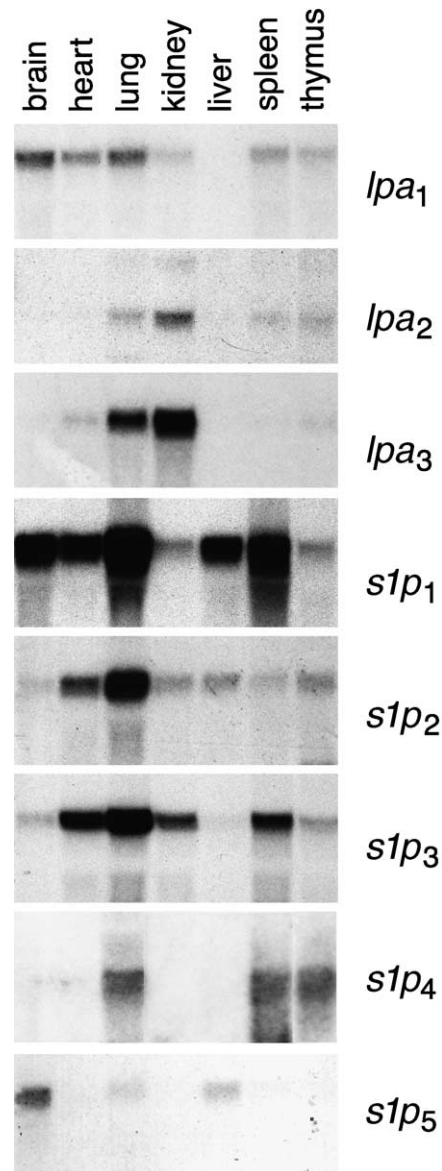


Fig. 1. Expression patterns of the eight LP receptor genes in adult mouse tissues. Total RNA (20 μ g per lane) of various tissues was examined by high-stringency Northern blot analysis using specific probes to mouse *lpa*/*s1p* genes. Partially adapted from Refs. [5,12,27].

in both sexes died before 3 weeks of age, most in the first few days of life. Nearly half of the *lpa*₁^(-/-) pups survived to adulthood and were fertile.

The *lpa*₁^(-/-) adults displayed phenotypic abnormalities [10], such as craniofacial dysmorphism (shorter snouts and more widely spaced eyes) and up to 30% reduction in average body weight relative to control siblings. Further investigation revealed a suckling defect amongst the majority of *lpa*₁^(-/-) pups, manifested by little or no milk in their stomachs, thus explaining their partial neonatal lethality and decreased size. The suckling defect observed amongst *lpa*₁^(-/-) pups may be attributable to a lack of olfactory detection and/or processing, which requires the functions of olfactory epithelium, bulb, and possibly cortex. These

nervous system structures, however, appeared indistinguishable between wild-type and $lpa_1^{(-/-)}$ animals upon histological examination.

Since LPA has been shown to be a survival factor for Schwann cells in vitro, probably by signaling through the LPA₁ receptor [19], the effect of lpa_1 deficiency on Schwann cell survival was examined. Young adult sciatic nerve sections were analyzed for the incidence of apoptotic cells by in situ end labeling (ISEL⁺), a method that labels fragmented DNA ends [21]. Not only was there an 80% increase in the percentage of ISEL⁺-labeled cells in $lpa_1^{(-/-)}$ vs. wild-type mice, but there were also fewer total cells [10]. However, no gross movement/locomotion abnormality was evident in $lpa_1^{(-/-)}$ mice, reflecting a relatively normal myelination process. This was possibly due to a low incidence of Schwann cell apoptosis (18%) in $lpa_1^{(-/-)}$ mice, which might have been insufficient to impair myelination.

Other cellular effects of LPA exposure include cell rounding and migration, which are mediated by the small GTPase Rho pathway [16,22,23]. When embryonic day (E)12–E13 cerebral cortical cluster cultures were treated with LPA, a concentration-dependent reduction in cluster area was observed [10,16]. This reflected both cell migration towards and cell rounding within clusters. In $lpa_1^{(-/-)}$ cells, there was a decrease in cluster compaction in response to LPA, compared to wild-type cells.

Cells within the $lpa_1^{(-/-)}$ clusters also responded to LPA with decreased bromodeoxyuridine incorporation relative to those in wild-type clusters. In contrast, there was an increase in the proliferative responsiveness to basic fibroblast growth factor (bFGF) in $lpa_1^{(-/-)}$ clusters, suggesting a compensatory mechanism for the loss of the LPA₁-mediated proliferative response [10].

Finally, LPA-induced PLC activation, adenylyl cyclase inhibition and Rho activation were examined in mouse embryonic fibroblasts (MEFs) established from wild-type and $lpa_1^{(-/-)}$ E14 embryos (Contos et al., submitted for publication). MEFs express lpa_1 and lpa_2 genes, and respond to LPA with PLC activation, adenylyl cyclase inhibition and Rho activation (Ref. [12] and Contos et al., submitted for publication). In $lpa_1^{(-/-)}$ MEFs, PLC activation was moderately reduced and adenylyl cyclase inhibition was severely reduced while Rho activation was unchanged, indicating a selective loss of LPA signaling in $lpa_1^{(-/-)}$ MEFs (Contos et al., submitted for publication).

Thus, the perhaps surprising phenotypes of craniofacial deformity and impaired neonatal suckling in LPA₁-null mice add another layer of complexity to a myriad of biological activities attributed to receptor-mediated LPA signaling. In Schwann cells and embryonic cerebral cortical neuroblasts where LPA₁ is normally enriched, lack of the receptor gene led to significant perturbations of cellular processes, such as apoptosis, proliferation, and cytoskeletal reorganization. Thus, LPA₁ signaling is not completely redundant, and is required for normal organismal development.

3. lpa_2

3.1. Expression patterns

The lpa_2 transcript is detected in the embryonic brain, and declines shortly after birth [5,24]. Major loci of expression in the adult include kidney, lung and testis, and lower levels of expression are found in other organs examined (Fig. 1 and Ref. [5]).

3.2. lpa_2 gene targeting

The LPA₂-null mice were generated by our group (Contos et al., submitted for publication). The mouse lpa_2 genomic locus consists of three exons. Exon 2 encodes for the majority of transmembrane domains, and was therefore targeted for deletion. A construct was made in which the second half of exon 2 was replaced with the neomycin-resistant gene (Contos et al., submitted for publication).

3.3. Knockout phenotype and signaling characteristics

The $lpa_2^{(-/-)}$ mice were obtained at the expected Mendelian frequency without sexual bias and developed normally without obvious phenotypic abnormalities. They were grossly indistinguishable from their non- $lpa_2^{(-/-)}$ siblings in appearance, size, general behavior, and longevity, and were fertile (Contos et al., submitted for publication).

LPA-induced PLC activation, adenylyl cyclase inhibition, and Rho activation were examined in MEFs established from $lpa_2^{(-/-)}$ E14 embryos. In $lpa_2^{(-/-)}$ MEFs, LPA-induced PLC activation was severely reduced (20% of that in wild-type cells at 1 μM LPA), whereas LPA-induced adenylyl cyclase inhibition and Rho activation were almost unchanged (Contos et al., submitted for publication). These data indicate that the selective loss of LPA signaling in $lpa_2^{(-/-)}$ MEFs is different from that in $lpa_1^{(-/-)}$ cells, with $lpa_1^{(-/-)}$ MEFs showing an additional decrease in adenylyl cyclase inhibition.

Considering previous studies that have shown somewhat similar intracellular signaling pathways evoked by activating LPA₁ and LPA₂ receptors [23,25], it is rather surprising not to see obvious phenotypes. It is worth noting that lpa_1 and lpa_2 transcripts colocalize in some adult mouse tissues, such as kidney, lung and testis, suggesting possible redundant in vivo roles of LPA₂.

4. slp_1

4.1. Expression patterns

In situ hybridization studies revealed that, during mouse embryonic development, the slp_1 gene is weakly and diffusely expressed beginning at E8.5 [26], and subsequently expressed in various cardiovascular and nervous

system loci [11,27]. Northern blot analysis showed that the ~3.0 kb transcript of *sIp1* is highly expressed during E14–18 in the developing mouse brain. From E15.5 on, intense *sIp1* signals were found localized to centers of ossification [26]. The embryonic expression patterns of *sIp1* thus implicate the gene in embryonic cardiovascular, brain, and skeletal development. In adult mice, brain, heart, lung, liver, and spleen express abundant levels of the *sIp1* transcript (Fig. 1 and Refs. [12,26,27]).

4.2. *sIp1* gene targeting

SIP₁-null mice were generated by Liu et al. [11]. The mouse *sIp1* gene is composed of 2 exons, with exon 2 encompassing the entire *sIp1* coding region [26]. To disrupt the *sIp1* allele, a replacement vector containing a *LacZ* marker preceded by an internal ribosomal entry sequence was introduced into embryonic stem cells. Upon homologous recombination, the *sIp1* ORF was disrupted after the first 42 amino acids and a bicistronic *sIp1*–*LacZ* transcript under the control of the endogenous *sIp1* promoter was generated. The expression of the β-galactosidase reporter protein was used as a marker for where *sIp1* expression would normally occur in mice with the disrupted *sIp1* allele [11].

4.3. Knockout phenotype and signaling characteristics

Genotyping analysis of offspring from *sIp1*^(+/-) intercrosses revealed that no *sIp1*^(-/-) embryos survived beyond E14.5, indicating embryonic lethality [11]. The *sIp1*^(-/-) embryos appeared phenotypically normal up to E11.5. During E12.5–13.5, however, there was less blood in the vasculature of the yolk sac, which grew progressively more edematous. During the same period, embryonic hemorrhages and edema were evident in the body and limbs of *sIp1*^(-/-) embryos. However, overall morphology of the vasculature, and expression patterns of molecules involved in adherens junction assembly and early endothelial cell development appeared largely indistinguishable between wild-type and *sIp1*^(-/-) embryos, indicating normal vasculogenesis and angiogenesis [11].

In contrast, the organization of vascular smooth muscle cells (VSMCs) and morphology of the endothelial cells surrounding the arterial vasculature appeared defective [11]. During normal embryonic development, by E11.5 the aorta is completely sheathed by VSMCs. In longitudinal and transverse sections of E12.5 *sIp1*^(-/-) aortae, however, there was a lack of smooth muscle α actin (SMαA)-labeled VSMCs on the dorsal surface. In addition, VSMCs on the ventral surface of aorta were disorganized and the endothelial cells were discontinuous. The endothelial cells also adapted an abnormal cuboidal morphology. Blood cells were often seen leaking through the abnormal vasculature of the *sIp1*^(-/-) embryos. Similar defects were observed in mutant cranial arteries and capillaries, where abnormally rounded nuclei were found in some endothelial cells.

Electron microscopy further confirmed a reduction of VSMCs/pericytes adjacent to endothelial cells in capillaries of E12.5 mutant embryos. The endothelial cell junction, however, developed normally in the absence of *sIp1*. Taken together, these data suggest that vascular maturation was incomplete in *sIp1*^(-/-) embryos, due to deficient recruitment of VSMCs/pericytes to vessel walls [11].

SIP₁ has been implicated in SIP-induced cell migration [28–32]. The chemotactic response was examined in MEFs derived from *sIp1*^(-/-) embryos [11]. Wild-type MEFs express *sIp1*, *sIp2* and *sIp3*, whereas *sIp1*^(-/-) MEFs lack the *sIp1* expression, with their *sIp2* and *sIp3* expression levels unchanged compared to those in wild-type MEFs. In contrast to wild-type MEFs that responded to 100 nM SIP with an approximately four-fold increase in chemotactic response, *sIp1*^(-/-) MEFs displayed a much diminished migration response to the same concentration of SIP [11]. In addition, *sIp1*^(-/-) MEFs did not respond to SIP with Rac activation, which has been shown to be important for SIP-induced chemotaxis [31,33]. These observations suggest that SIP₁ is crucial for SIP-mediated cell migration and Rac activation.

The major phenotype of *sIp1*^(-/-) mice, impaired vascular maturation, was perhaps not unexpected, given that SIP₁ has been postulated to play a role in morphogenetic differentiation of vascular endothelial cells into capillary-like tubules and that it has a critical role in SIP-mediated migration [33,34]. Based on the phenotypes and diminished Rac activation in *sIp1*^(-/-) mice, the authors proposed a model where SIP naturally present in blood stimulates the migration of VSMCs toward developing vessel walls through SIP₁-mediated Rac activation pathway(s).

5. *sIp2*

5.1. Expression patterns

During embryonic development, *sIp2* expression is localized to the embryonic brain, with the strongest level appearing right before birth [13,27,35,36]. The expression level in brain decreases postnatally with time, and is almost undetectable in the adult. In addition to brain, adult mice also express the 2.8 kb *sIp2* transcript strongly in heart and lung (Fig. 1 and Refs. [12,27]).

5.2. *sIp2* gene targeting

The SIP₂-null mice were generated by MacLennan et al. [13]. The genomic organization of the murine *sIp2* gene is similar to that of *sIp1*. The ORF resides completely within exon 2 of the two known exons. To generate mice with *sIp2* mutation, a replacement vector was constructed that contains a *LacZ* marker gene in addition to typical selectable markers and homologous regions. The targeted disruption resulted in deletion of the entire *sIp2* ORF [13].

5.3. Knockout phenotypes

Intercrosses between $sIp_2^{(+/-)}$ mice gave a rise to $sIp_2^{(-/-)}$ mice at the expected Mendelian frequency. When embryonic and postnatal $sIp_2^{(-/-)}$ mice were examined, no obvious abnormalities were found in appearance, gross anatomy, and wound healing. In particular, with regards to the nervous system effects of sIp_2 deficiency, normal peripheral axon guidance, neuronal cell development, motor/sensory function, and hippocampal and neocortical development were observed [13].

The most apparent phenotype of $sIp_2^{(-/-)}$ mice, spontaneous seizures during 3–7 weeks of age, was observed while performing neurological tests on these mice [13]. A typical seizure in $sIp_2^{(-/-)}$ mice consisted of a 2–10 s wild running episode, immediately followed by either up to 1 min of freezing or tonic–clonic convulsion, which occasionally led to death. These symptoms are characteristic of many forms of epilepsy. Live monitoring as well as video-recording showed that only $sIp_2^{(-/-)}$ but not wild-type mice experienced such seizures. The penetrance of this phenotype was difficult to assess due to the sporadic nature of seizures observed in $sIp_2^{(-/-)}$ mice. However, based on the percentage of deaths amongst $sIp_2^{(-/-)}$ mice that experienced seizures in a sampling population, the authors concluded that almost all $sIp_2^{(-/-)}$ mice in the colony displayed this phenotype during the 3–7-week period [13].

When underlying electrophysiological causes of the seizures were explored by electroencephalographic recordings, continuous or intermittent, high amplitude wave discharges were observed in $sIp_2^{(-/-)}$ mice. By employing whole cell patch clamp on brain slices, significant increases were detected in both the frequency and amplitude of spontaneous, as well as electrically evoked, excitatory postsynaptic currents in $sIp_2^{(-/-)}$ neocortical pyramidal neurons, relative to those recorded in wild-type cells at basal physiological conditions. Current clamp experiments also revealed spontaneous bursts of action potentials accompanied by transient shifts of depolarization in $sIp_2^{(-/-)}$ pyramidal neurons, with the frequency of both measures increased by the GABA_A receptor antagonist bicuculine. Moreover, electrically evoked epileptiform depolarization responses in the presence of bicuculine were larger in $sIp_2^{(-/-)}$ than in wild-type pyramidal neurons. Taken together, these results suggested the hyperexcitable nature of $sIp_2^{(-/-)}$ neurons.

Recently the *Mil* gene, a zebrafish homologue of sIp_2 , has been isolated by positional cloning [37]. Mutation(s) in *Mil* resulted in defective myocardial precursor migration in zebrafish. When expressed in mammalian cells, mutant *Mil* diminished responses in S1P-induced signal transduction pathways [37]. *Mil* is thus thought to be an integral player during vertebrate heart development. In view of this finding, it is somewhat surprising that $sIp_2^{(-/-)}$ mice appeared anatomically normal. This discrepancy in phenotypes between two species might reflect evolutionary divergence

of S1P signaling via the S1P₂ receptor. Finally, the observation of spontaneous seizures in $sIp_2^{(-/-)}$ mice is intriguing, and determining signaling mechanisms underlying this phenotype represents important future work.

6. sIp_3

6.1. Expression patterns

At E14, the sIp_3 transcript is highly expressed in lung, kidney, intestine, diaphragm, and certain cartilaginous regions, but not in liver [12]. The strong expression of the transcript in embryonic lung and brain persists through E18 [27]. Adult tissues examined by Northern blot analysis showed abundant expression of the 3.8 kb sIp_3 transcript in heart, lung, kidney, and spleen, with no signal in liver (Fig. 1 and Refs. [12,27]).

6.2. sIp_3 gene targeting

The S1P₃-null mice were generated by our group [12]. Similar to both sIp_1 and sIp_2 genomic structures, the sIp_3 gene also consists of 2 exons, with the ORF completely contained within exon 2. A replacement vector utilizing the *Cre-loxP* system was designed, which, upon homologous recombination, deleted the entire ORF and allowed subsequent removal of selectable marker genes and their associated constitutive promoters [12].

6.3. Knockout phenotypes and signaling characteristics

Mice homozygous for the sIp_3 -null allele were born at the expected Mendelian ratios without sexual bias, and were fertile and healthy [12]. The average litter size for $sIp_3^{(-/-)}$ intercrosses was modestly but significantly smaller than that from $sIp_3^{(+/-)}$ male × $sIp_3^{(+/+)}$ female crosses. The reason for this observation is not known. Further histological and hematological analyses, however, failed to detect any obvious differences between $sIp_3^{(-/-)}$ and their wild-type littermates.

Despite a lack of phenotypic abnormality of the $sIp_3^{(-/-)}$ mice, selective losses of S1P signaling were observed in $sIp_3^{(-/-)}$ MEFs [12]. Northern blot analysis of wild-type MEFs revealed expression of sIp_1 , sIp_2 and sIp_3 , but neither sIp_4 nor sIp_5 . No compensatory increases in sIp_1 and sIp_2 transcript signals were detected in S1P₃-null MEFs [12]. When stimulated with S1P, wild-type MEFs responded with PLC activation, inhibition of adenylyl cyclase, and Rho activation. On the other hand, $sIp_3^{(-/-)}$ MEFs, in response to S1P, exhibited a marked decrease in PLC activation and a slight decrease in adenylyl cyclase inhibition. In contrast to the demonstration of S1P-mediated Rac activation in wild-type MEFs by Liu et al. [11] discussed above, wild-type MEFs generated in this report responded to S1P with Rho, but not Rac, activation. S1P₃ deficiency in MEFs did not alter the

S1P-mediated Rho response, indicating that S1P₃ does not play a major role in activation of this signaling pathway.

Analyses of S1P-mediated signaling properties in S1P₃-null MEFs allowed contributions from various S1P receptors to be assessed in a more physiologically relevant setting than in many in vitro LP receptor overexpression studies. The alterations in S1P-induced signaling responses in S1P₃-null MEFs also indicated the unique role(s) S1P₃ plays during embryonic development. Amongst S1P receptor-null mice, S1P₃-null mice are the only one without obvious phenotypes at the basal state; however, they may show phenotypes different from wild-type when challenged with ligands such as S1P.

7. Concluding remarks

The past decade has seen a rapid growth of LP-related research and a wealth of data on signaling pathways through LP receptors. Physiologically relevant functions of these receptors deduced from various in vitro studies have motivated the generation of receptor-null mice to examine these functions in vivo. The results from such analyses are summarized in Table 1. While some of these roles were verified in LP receptor-null animals, such as the importance of LPA₁ and S1P₁ in Schwann cell survival and vascular development, respectively [10,11], other effects were absent

from their respective receptor-null mice. For instance, S1P₂ and S1P₃ have both been implicated in S1P-induced proliferation, survival, migration, and morphogenesis of several cell types; however, both *s1p2*^(-/-) and *s1p3*^(-/-) mice appeared free of gross anatomical defects [12,13]. In addition, given the prominent expression of *s1p3* in gonadal tissues [12], and previous studies implicating S1P in oocyte function [38], the reproductive tissues in *s1p3*^(-/-) mice appeared unexpectedly normal [12]. The discrepancies between in vitro- and in vivo-based functions could be explained by the overlapping expression patterns of a subset of LP receptors that could potentially be upregulated to compensate for the loss of one receptor. This compensatory upregulation was observed for *s1p2* transcript levels in brain and heart in S1P₃-null mice [12]. It is also likely that other, non-LP-mediated, functions could be enhanced to compensate for the loss of an LP receptor. The observation that *lpa1*^(-/-) embryonic cerebral cortical cells responded to bFGF with greater proliferative responses than those in wild-type cells was an example of this compensatory mechanism [10]. In short, results from LP receptor-null animals, as well as signaling properties of primary cells from these mice, demonstrate the intricate interplay of diverse LP signaling pathways in vivo, and provided invaluable tools for future LPA and S1P studies.

It is obvious that several lines of investigation await further pursuit. First is to generate mice deficient for the

Table 1
Summary of *lpa* and *s1p* gene targeting approaches, mutant mice phenotypes, and effects of *lpa/s1p* deficiency on signaling properties

Gene deleted	Gene targeting approach	Phenotypes	Alterations in signaling characteristics (compared to wild-type controls)	References
<i>lpa1</i>	<ul style="list-style-type: none"> • Cre-loxP-based replacement vector • Deletion of exon 3 (containing the majority of transmembrane domains) 	<ul style="list-style-type: none"> • Fifty percent neonatal lethality • Impaired suckling in neonatal pups • Smaller size • Craniofacial dysmorphism • Increased apoptosis in sciatic nerve Schwann cells 	<ul style="list-style-type: none"> • Embryonic cerebral cortical cells stimulated with LPA • Loss of cell cluster compaction • Decrease in BrdU incorporation • Embryonic fibroblasts stimulated with LPA • Decrease in PLC activation and adenylyl cyclase inhibition 	[10] and Contos et al., submitted
<i>lpa2</i>	<ul style="list-style-type: none"> • Replacement vector • Deletion of half of exon 2 (containing transmembrane domains IV–VI) 	<ul style="list-style-type: none"> • No obvious phenotypes 	<ul style="list-style-type: none"> • Embryonic fibroblasts stimulated with LPA • Decrease in PLC activation 	Contos et al., submitted
<i>s1p1</i>	<ul style="list-style-type: none"> • Replacement vector with <i>LacZ</i> marker gene • Deletion of ORF after the first 42 amino acids • Creation of <i>s1p1-LacZ</i> hybrid transcript under the control by endogenous <i>s1p1</i> promoter 	<ul style="list-style-type: none"> • Embryonic lethality • Defective vascular maturation due to a deficiency of vascular smooth muscle cells/pericytes 	<ul style="list-style-type: none"> • Embryonic fibroblasts stimulated with S1P • Decrease in Rac-mediated chemotaxis 	[11]
<i>s1p2</i>	<ul style="list-style-type: none"> • Replacement vector with <i>LacZ</i> marker gene • Deletion of entire ORF 	<ul style="list-style-type: none"> • No gross anatomical abnormalities • Seizures during 3–7 weeks of age • Neuronal hyperexcitability 	No data	[13]
<i>s1p3</i>	<ul style="list-style-type: none"> • Cre-loxP-based replacement vector • Deletion of entire ORF 	<ul style="list-style-type: none"> • No obvious phenotypes 	<ul style="list-style-type: none"> • Embryonic fibroblasts stimulated with S1P • Decrease in PLC activation • Slight decrease in adenylyl cyclase inhibition 	[12]

remaining receptor genes, *lpa₃*, *sIp₄* and *sIp₅*. The characterization of these mice will provide more information on the physiological roles of each of the LP receptors. Second, it will be of interest to crossbreed different lines of *lpa/sIp* mutant mice to obtain mice lacking multiple *lpa/sIp* genes. This approach could potentially circumvent the complication of overlapping role/distribution of LP receptors in vivo, expose phenotype(s) masked in mice homozygous null for a single *lpa/sIp* gene, and amplify physiological defects due to loss of LPA/S1P signaling. Finally, for receptor-null mice with an embryonic lethal phenotype (such as S1P₁-null animals), conditional gene disruption by employing the *Cre-loxP* system will enable studies on the effects of *lpa/sIp* gene disruption at a later developmental stage and in selected tissues where expression is normally enriched. In the case of *sIp₁^(-/-)* mice, embryonic lethality occurred at E14.5; however, the nervous system expression of *sIp₁* increases significantly from E14 on and is sustained throughout adulthood. Specifically, the *sIp₁* transcript is highly concentrated in polymorphic cell layer of the dentate gyrus in the hippocampal region of the neonatal cerebrum, whereas the signal is abundant in the Purkinje cell layer of adult cerebellum [26]. The conditional *sIp₁* deletion would therefore allow an examination of the role of S1P₁ in brain development beyond E14.5.

Studies on LP receptor-null mice have emerged within the past 2 years, and will continue to provide new insights to the in vivo roles of LP receptors.

Acknowledgements

We thank past and current members of the Chun laboratory at UCSD who have been involved in the generation, crossbreeding and analyses of the *lpa/sIp*-mutant mice.

References

- [1] W.H. Moolenaar, *Exp. Cell Res.* 253 (1999) 230–238.
- [2] S. Spiegel, S. Milstien, *Biochim. Biophys. Acta* 1484 (2000) 107–116.
- [3] N. Fukushima, I. Ishii, J.J. Contos, J.A. Weiner, J. Chun, *Annu. Rev. Pharmacol. Toxicol.* 41 (2001) 507–534.
- [4] W.H. Moolenaar, *Ann. N. Y. Acad. Sci.* 905 (2000) 1–10.
- [5] J.J. Contos, I. Ishii, J. Chun, *Mol. Pharmacol.* 58 (2000) 1188–1196.
- [6] S. Pyne, N. Pyne, *Pharmacol. Ther.* 88 (2000) 115–131.
- [7] S. Pyne, N.J. Pyne, *Biochem. J.* 349 (2000) 385–402.
- [8] Y. Takuwa, H. Okamoto, N. Takuwa, K. Gonda, N. Sugimoto, S. Sakurada, *Mol. Cell Endocrinol.* 177 (2001) 3–11.
- [9] J. Chun, *Crit. Rev. Neurobiol.* 13 (1999) 151–168.
- [10] J.J. Contos, N. Fukushima, J.A. Weiner, D. Kaushal, J. Chun, *Proc. Natl. Acad. Sci. U.S.A.* 97 (2000) 13384–13389.
- [11] Y. Liu, R. Wada, T. Yamashita, Y. Mi, C.X. Deng, J.P. Hobson, H.M. Rosenfeldt, V.E. Nava, S.S. Chae, M.J. Lee, C.H. Liu, T. Hla, S. Spiegel, R.L. Proia, *J. Clin. Invest.* 106 (2000) 951–961.
- [12] I. Ishii, B. Friedman, X. Ye, S. Kawamura, C. McGiffert, J.J. Contos, M.A. Kingsbury, G. Zhang, J.H. Brown, J. Chun, *J. Biol. Chem.* 276 (2001) 33697–33704.
- [13] A.J. MacLennan, P.R. Carney, W.J. Zhu, A.H. Chaves, J. Garcia, J.R. Grimes, K.J. Anderson, S.N. Roper, N. Lee, *Eur. J. Neurosci.* 14 (2001) 203–209.
- [14] J.H. Hecht, J.A. Weiner, S.R. Post, J. Chun, *J. Cell Biol.* 135 (1996) 1071–1083.
- [15] A.E. Dubin, T. Bahnson, J.A. Weiner, N. Fukushima, J. Chun, *J. Neurosci.* 19 (1999) 1371–1381.
- [16] N. Fukushima, J.A. Weiner, J. Chun, *Dev. Biol.* 228 (2000) 6–18.
- [17] J. Allard, S. Barron, J. Diaz, C. Lubetzki, B. Zalc, J.C. Schwartz, P. Sokoloff, *Eur. J. Neurosci.* 10 (1998) 1045–1053.
- [18] J.A. Weiner, J.H. Hecht, J. Chun, *J. Comp. Neurol.* 398 (1998) 587–598.
- [19] J.A. Weiner, J. Chun, *Proc. Natl. Acad. Sci. U.S.A.* 96 (1999) 5233–5238.
- [20] J.A. Weiner, N. Fukushima, J.J. Contos, S.S. Scherer, J. Chun, *J. Neurosci.* 21 (2001) 7069–7078.
- [21] A.J. Blaschke, K. Staley, J. Chun, *Development* 122 (1996) 1165–1174.
- [22] N. Fukushima, Y. Kimura, J. Chun, *Proc. Natl. Acad. Sci. U.S.A.* 95 (1998) 6151–6156.
- [23] I. Ishii, J.J. Contos, N. Fukushima, J. Chun, *Mol. Pharmacol.* 58 (2000) 895–902.
- [24] J.J. Contos, J. Chun, *Genomics* 64 (2000) 155–169.
- [25] S. An, T. Bleu, Y. Zheng, E.J. Goetzl, *Mol. Pharmacol.* 54 (1998) 881–888.
- [26] C.H. Liu, T. Hla, *Genomics* 43 (1997) 15–24.
- [27] G. Zhang, J.J. Contos, J.A. Weiner, N. Fukushima, J. Chun, *Gene* 227 (1999) 89–99.
- [28] J. Kon, K. Sato, T. Watanabe, H. Tomura, A. Kuwabara, T. Kimura, K. Tamama, T. Ishizuka, N. Murata, T. Kanda, I. Kobayashi, H. Ohta, M. Ui, F. Okajima, *J. Biol. Chem.* 274 (1999) 23940–23947.
- [29] F. Wang, J.R. Van Brocklyn, J.P. Hobson, S. Movafagh, Z. Zukowska-Grojec, S. Milstien, S. Spiegel, *J. Biol. Chem.* 274 (1999) 35343–35350.
- [30] D. English, A.T. Kovala, Z. Welch, K.A. Harvey, R.A. Siddiqui, D.N. Brindley, J.G. Garcia, *J. Hematother. Stem Cell Res.* 8 (1999) 627–634.
- [31] H. Okamoto, N. Takuwa, T. Yokomizo, N. Sugimoto, S. Sakurada, H. Shigematsu, Y. Takuwa, *Mol. Cell. Biol.* 20 (2000) 9247–9261.
- [32] J.H. Paik, S. Chae, M.J. Lee, S. Thangada, T. Hla, *J. Biol. Chem.* 276 (2001) 11830–11837.
- [33] M.J. Lee, S. Thangada, K.P. Claffey, N. Ancellin, C.H. Liu, M. Kluk, M. Volpi, R.I. Sha'afi, T. Hla, *Cell* 99 (1999) 301–312.
- [34] T. Hla, T. Maciag, *J. Biol. Chem.* 265 (1990) 9308–9313.
- [35] A.J. MacLennan, C.S. Browe, A.A. Gaskin, D.C. Lado, G. Shaw, *Mol. Cell. Neurosci.* 5 (1994) 201–209.
- [36] A.J. MacLennan, L. Marks, A.A. Gaskin, N. Lee, *Neuroscience* 79 (1997) 217–224.
- [37] E. Kupperman, S. An, N. Osborne, S. Waldron, D.Y. Stainier, *Nature* 406 (2000) 192–195.
- [38] Y. Morita, G.I. Perez, F. Paris, S.R. Miranda, D. Ehleiter, A. Haimovitz-Friedman, Z. Fuks, Z. Xie, J.C. Reed, E.H. Schuchman, R.N. Kolesnick, J.L. Tilly, *Nat. Med.* 6 (2000) 1109–1114.