

Chapter 14

Zebrafish as a Model Animal for Studying Lysophosphatidic Acid Signaling

Junken Aoki and Hiroshi Yukiura

Abstract Lysophosphatidic acid (LPA) is a second-generation lysophospholipid mediator that exerts multiple biological functions through its own cognate receptors. LPA is produced by specific enzymatic reactions and activates receptors with similar structures (Edg receptors and P2Y receptors), which results in a variety of actions from embryonic blood vessel formation to development of cancer. LPA-related genes are highly conserved in vertebrates. In the zebrafish genome, three LPA-producing enzymes and nine LPA receptors are present. In vitro experiments have shown that LPA-related genes in zebrafish are conserved biochemically. LPA-related genes can be up- and downregulated by injecting morpholino antisense oligonucleotides (MOs) specific to LPA-related genes or mRNAs in zebrafish embryos. Such tools help to assess the biological function of these genes. For example, knockdown of the LPA-produced enzyme autotaxin (ATX) in zebrafish embryos resulted in malformation of embryonic blood vessel formation, which has also been observed in embryos from ATX-knockout mice. Simultaneous inactivation of multiple genes is possible by injecting more than one MO in zebrafish embryos, which makes it possible to identify the LPA receptors responsible for embryonic blood vessel formation. Gene functions can be also eliminated in zebrafish embryos by pharmacological tools such as enzyme inhibitors or receptor antagonists. Interestingly, overexpression of ATX in zebrafish embryos resulted in duplication of the heart (two-heart phenotype) and the phenotype was canceled by treating the embryos with LPA receptor antagonists. The zebrafish system is a powerful tool not only for identification of gene functions but also for development of drugs against enzymes and receptors.

Keywords Lysophosphatidic acid • G protein-coupled receptor • Autotaxin • Zebrafish • Blood vessel formation • Embryo

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Abbreviations

ATX	Autotaxin
Edg	Endothelial differentiation gene
hpf	Hours post fertilization
LPA	Lysophosphatidic acid
LPC	Lysophosphatidylcholine
MO	Morpholino antisense oligonucleotide
S1P	Sphingosine-1-phosphate

14.1 Introduction

Lysophosphatidic acid (LPA; 1- or 2-acyl-*sn*-glycerol-3-phosphate) is a small glycerophospholipid that has a wide range of actions through its receptors. Most of the actions of LPA were mediated by six G protein-coupled receptors (GPCRs) named LPA₁₋₆. Through the six LPA receptors, LPA has been shown to be involved in several physiological processes including neuronal development (LPA₁) [1], implantation (LPA₃) [2], blood vessel formation (LPA₄) [3], and hair follicle formation (LPA₆) [4]. LPA also has pathological roles such as progression of lung fibrosis (LPA₁) [5], cancer development (LPA₂) [6, 7], and drug- or irradiation-induced cell death in the intestine (LPA₂) [8]. LPA is produced extracellularly by two enzymes, autotaxin and PA-PLA₁α [9]. Studies of LPA synthetic pathways have revealed that ATX is involved in embryonic blood vessel formation [10, 11] whereas PA-PLA₁α is involved in hair follicle formation [12, 13]. The level of extracellularly produced LPA has been suggested to be downregulated by LPA-degrading enzymes called lipid phosphate phosphatases (LPPs) [14]. LPPs are membrane-bound enzymes that efficiently remove phosphate by their phosphatase activity.

14.2 LPA-Related Genes in Zebrafish

14.2.1 *Zebrafish as a Model for Elucidating the Role of Human Genes*

Zebrafish are widely used for studies of vertebrate gene function. Approximately 70 % of human genes have at least one obvious zebrafish orthologue [15]. The virtually transparent embryos of this species, and the ability to accelerate genetic studies by gene knockdown or overexpression, have led to the widespread use of zebrafish

in the detailed investigation of vertebrate gene function and, increasingly, the study of human genetic disease. Fluorescent markers can be used *in vivo* to tag specific cell types and visualize their location and migration during embryogenesis. Moreover, well-developed videomicroscopic techniques have been available for detailed analyses of developmental stages. Analyses of vascular formation using mutants and antisense morpholino oligos (MOs), for example, have identified a number of molecules involved in vasculature development, including growth factors, cell adhesion molecules, and transcription factors [16]. These analyses have shown that the basic mechanisms of embryonic blood vessel formation are conserved in vertebrates. In addition to the traditional forward genetics, injection of morpholino oligonucleotides allows us to target gene knockdown more rapidly [17]. Moreover, new genome-editing tools such as TALENs (transcription activator-like effector nucleases) [18] and CRISPR (clustered regularly interspaced short palindromic repeats)/Cas [19] have also been applied to the zebrafish model, providing exciting new opportunities for high-efficiency mutagenesis.

14.2.2 Structure, Sequence Homology, and Biochemical Properties of LPA-Related Genes in Zebrafish

LPA-related genes such as LPA receptors, LPA-producing enzymes, and LPA-degrading enzymes are highly conserved in zebrafish (Table 14.1). The zebrafish genome has homologous genes for autotaxin (2 genes), PA-PLA α (2 genes), LPA receptors (9 genes), and LPP (6 genes) (Table 14.1; Fig. 14.1). As is often the case with zebrafish genes, there are two close homologues for ATX, PA-PLA α , LPA $_2$, LPA $_5$, LPA $_6$, LPP1, LPP2, and LPP3, which might be generated by gene duplication (Table 14.1). Thus, nine genes for LPA receptors (*lpa1*, *lpa2a*, *lpa2b*, *lpa3*, *lpa4*, *lpa5a*, *lpa5b*, *lpa6a*, *lpa6b*), two genes for ATX (*atxa*, *atxb*), two genes for PA-PLA α (*papla1aa*, *papla1ab*), and six genes for LPPs (*lpp1a*, *lpp1b*, *lpp2a*, *lpp2b*, *lpp3a*, *lpp3b*) are present. The amino acid sequences of these zebrafish LPA-related genes are similar to their mammalian homologues (Table 14.1; Fig. 14.1). These LPA-related genes are highly conserved between zebrafish and mammals, usually with 50–70 % amino acid sequence homology. It is noted that among the LPA-related genes, zebrafish LPA $_1$ shows approximately 90 % amino acid identity to mammalian LPA $_1$, suggesting that its role is conserved in a wide range of vertebrates. Most of these LPA-related genes in zebrafish were shown to conserve their biochemical functions. Indeed, seven LPA receptors (except for *lpa5a* and *lpa5b*) were activated by LPA to induce downstream G-protein signaling [20]. Two ATXs (*atxa* and *atxb*) also hydrolyzed lysophosphatidylcholine to produce LPA *in vitro* [20].

Table 14.1 Amino acid sequence homology between zebrafish and mammalian (human and mouse) lysophosphatidic acid (LPA)-related genes

Zebrafish	<i>Homo sapiens</i> (human)		<i>Mus musculus</i> (mouse)	
	Genes	Amino acid sequence homology (%)	Genes	Amino acid sequence homology (%)
<i>atxa</i>	ATX	66.7	Atx	66.1
<i>atxb</i>		66.6		66.1
<i>papla1aa</i>	PAPLA1a	49.3	Papla1a	47.3
<i>papla1ab</i>		48.7		47.9
<i>lpa1</i>	LPAR1	89.3	Lpar1	90.1
<i>lpa2a</i>	LPAR2	49.5	Lpar2	50.8
<i>lpa2b</i>		55.3		55.6
<i>lpa3</i>	LPAR3	62.0	Lpar3	61.1
<i>lpa4</i>	LPAR4	63.7	Lpar4	64.2
<i>lpa5a</i>	LPAR5	32.0	Lpar5	33.5
<i>lpa5b</i>		28.5		29.2
<i>lpa6a</i>	LPAR6	63.0	Lpar6	63.6
<i>lpa6b</i>		55.5		56.4
<i>lpp1a</i>	LPP1	69.2	Lpp1	65.5
<i>lpp1b</i>		62.5		60.2
<i>lpp2a</i>	LPP2	63.0	Lpp2	67.0
<i>lpp2b</i>		69.0		68.2
<i>lpp3a</i>	LPP3	73.2	Lpp3	71.3
<i>lpp3b</i>		47.8		47.3

14.2.3 Functional Aspects of LPA-Related Gene in Zebrafish

14.2.3.1 Embryonic Blood Vessel Formation

Autotaxin (ATX)-null mice die around embryonic day 9.5–10.5 with profound vascular defects in both the yolk sac and embryo, and with aberrant neural tube formation [10, 11, 21]. A number of mutants and knockout mice have shown phenotypes similar to those in ATX-knockout mice [22, 23]. However, the precise phenotypes of these mice have not been determined because real-time observation of blood vessel formation is impossible for mice. Introduction of mutant ATX, in which Thr²¹⁰, an amino acid responsible for the catalytic activity of ATX, was replaced with alanine, could not rescue the phenotype, indicating that the product of ATX, that is, LPA, is involved in embryonic vascular formation [24]. In addition, none of the LPA receptor knockout mice has shown a similar phenotype [1–3, 25, 26], and thus, it remains to be solved which LPA receptors are involved and how ATX regulates embryonic vasculature in the early developmental stages.

As stated earlier, zebrafish has two ATX orthologues, both of which have been shown to have lysophospholipase D activity to produce LPA. To examine the development of embryonic blood vessels called intersegmental vessels (ISVs) in zebrafish

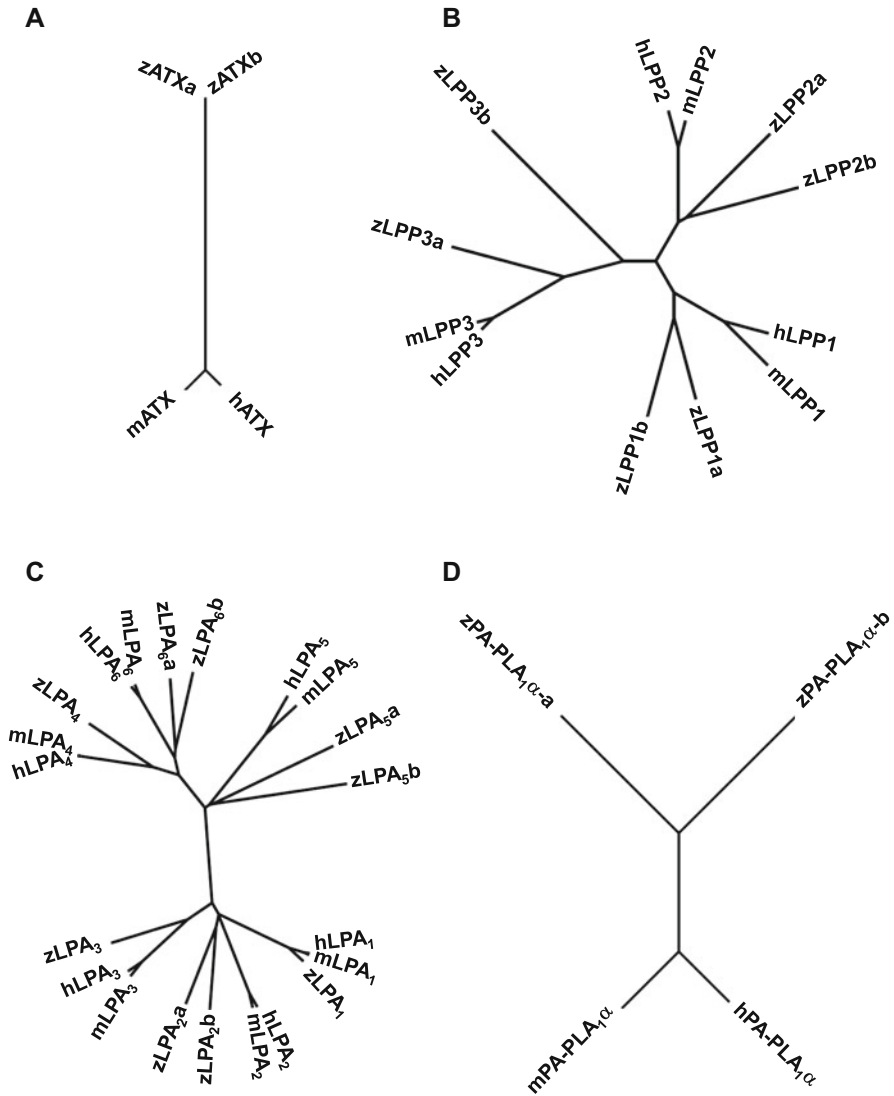


Fig. 14.1 Phylogenetic relationships of lysophosphatidic (LPA)-related genes [ATX (a), LPP (b), LPAR (c), and PAPLAIA (d)] based on analyses of individual genes from zebrafish, mouse, and human. Trees were all inferred using GENETYC-MAC Ver. 13.1.1

embryos, a transgenic line was used in which endothelial cells were labeled with EGFP [27]. Injection of embryos with ATX MO caused ISVs to stall in mid-course and to aberrantly connect to neighboring ISVs. The aberrant vascular network in ATX-downregulated embryos is not caused by abnormal proliferation of endothelial cells, because endothelial cells are differentiated and the number of the cells was normal. It should be stressed here that the zebrafish system makes it possible to

precisely analyze blood vessel formation, which is very difficult in mice. Another important point was that the ISV phenotype has not been reported so far, indicating that the ATX–LPA system was a novel axis that regulates the embryonic blood vessel formation.

Knocking out LPA receptors in mice revealed the cellular processes specific to each of six LPA receptors, from brain development (LPA₁) to hair follicle formation (LPA₆). None of the individual knockouts was lethal. As already stated, ATX downregulation resulted in embryonic lethality and impaired blood vessel formation in both mice and zebrafish. Thus, it is possible that multiple LPA receptors redundantly regulate the embryonic blood vessel formation, or that novel LPA receptor(s) are involved. To suppress multiple LPA genes at a time in mice by crossing mice in which different LPA receptors are knocked out would require much time and labor. However, injecting zebrafish with MOs makes it possible to suppress multiple genes at a time. Simultaneous downregulation of multiple LPA receptors in zebrafish embryos revealed that LPA receptors have a redundant function in embryonic blood vessel formation. Downregulation of *lpa1* and *lpa4* caused abnormalities of blood vessel formation similar to those caused by *atx* downregulation. The phenotypic similarity strongly suggests that the LPA receptors and ATX act in the same axis governing embryonic blood vessel formation.

14.2.3.2 Neural Development and Regulation of Left–Right Asymmetry

Because zebrafish embryos with a partially established vascular system can develop for 7 days, other roles of ATX were uncovered by gene knockdown experiments using MOs. ATX is secreted by cells from the floor plate of the hindbrain and stimulates olig2-positive progenitor cells to differentiate into oligodendrocyte progenitors [28]. Dorsal forerunner cells (DFCs) regulate the formation of the central organ for establishing L-R asymmetry in zebrafish, called Kupffer's vesicle (KV). ATX–LPA₃ receptor signaling was found to induce calcium fluxes in DFCs, indicating that LPA is a regulator of L-R asymmetry in zebrafish embryos [29]. Our preliminary data suggest that ATX is also involved in the development of cartilage as ATX knockdown results in malformation of cartilage in zebrafish embryos.

14.2.3.3 Crosstalk Between LPA and S1P Signaling Revealed by Overexpression of Autotaxin in Zebrafish Embryos

Nakanaga et al. accidentally found that when ATX was overexpressed in zebrafish embryos by injecting *atx* mRNA, the embryos showed cardia bifida, a phenotype induced by downregulation of S1P signaling [30]. A similar cardiac phenotype was not induced when catalytically inactive ATX was introduced. The cardiac phenotype was synergistically enhanced when MOs against S1P receptor (*s1pr2/mil*) or S1P transporter (*spns2*) were introduced together with *atx* mRNA. The *Atx*-induced cardia bifida was prominently suppressed when embryos were treated with an MO

against LPA₁. Thus, the study provided the first *in vivo* evidence of crosstalk between LPA and S1P signaling.

14.2.3.4 Zebrafish as a Tool for Drug Development

We have also tried to use the zebrafish system to evaluate small compounds for drug development. When zebrafish embryos injected with ATX mRNA were treated with an LPA receptor antagonist (Ki16425) (by just adding the compound to water in 96-well plates in which the embryos develop), it dramatically suppressed the cardia bifida phenotype [30]. The LPA antagonist was found to be active against zebrafish LPA receptors. However, our compounds that had ATX-inhibitory activity did not inhibit zebrafish ATX. Interestingly, overexpression of mammalian ATX instead of zebrafish ATX in zebrafish embryos induced a similar cardia bifida phenotype, and the phenotypes were efficiently suppressed by some of our ATX inhibitors specific for mammalian ATX. It should be noted that only a small fraction of such compounds suppressed the phenotype, even though all the compounds efficiently suppressed the ATX activity in a test tube. Such compounds were also found to be effective *in vivo* in mice. Thus our preliminary trial indicated that the zebrafish system is a powerful tool for *in vivo* evaluation of small compounds. Because the evaluation can be performed in 96-well plates, only small amounts of compounds are required, which makes it possible to evaluate the compounds in a chemical library in a first or second screening.

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