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斑馬魚水解磷酸酯受器 4 與 5 之研究

Identification of Zebrafish LPA Receptor 4 and 5

高笙詠

Milky Shen-Yung Kao

指導教授：李心予　博士

Advisor: Hsinyu Lee, PhD

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國立台灣大學碩士學位論文
口試委員審定書

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The Identification of Zebrfish LPA_4 and LPA_5

本論文係高笙詠君(R97B41031)在國立台灣大學動物學研究所完成之碩士學位論文，於民國九十九年七月十二日承下列考試委員審查通過及口試及格，特此證明

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張百恩　　陳俊彥

吳偉郁　　

所長　陳俊彥　（簽名）

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Abstract

Lysophosphatidic acid (LPA) is a simple lysophospholipid which regulates various important biological functions such as cell proliferation, migration, differentiation, and morphogenesis. The LPA receptor on cell membrane has been identified and, designated as LPA_{1-5}. LPA_{1-3} are the major members of the endothelial differentiation gene (EDG) family. LPA_{4-5}, structurally distinct from LPA_{1-3}, were first discovered as purinergic receptors in P2Y family. In zebrafish (Danio rerio), LPA_1 and LPA_3 have been identified essential for neural tube and lymphatic vessel development, while the function of other LPA receptors are not yet identified. We searched LPA receptors using the TBLASTN algorithm in the zebrafish nucleotide database, and found $zLPA_4$ and one sequence similar to both LPA_5 and P2Y5 in mice. After aligning all the known LPA receptors, the sequence showed more similarities with LPA_5 than with P2Y5. In zebrafish, $zLPA_4$ is expressed in the gills, testis and spleen after 5 dpf, and zLPA5 is expressed in most tissues after the 18 somites stage. LPA induced Ca++ mobilization in $zLPA_4$ or $zLPA_5$ over-expressed B103 rat neuroblastoma was not observed. This indicates that zebrafish LPA receptors might not trigger the conventional cascade of mammalian LPA receptors. However, down-regulation of zLPA4 leads to defections in vascular development, which needs more evidence to clarify.

Keywords: Lysophosphatidic acid, Lysophosphatidic acid receptor, blood vessel, zebrafish, development

摘要

水解磷酸酯為一種結構簡單的酯質卻同時調控多種生理功能，包括細胞增生、移動、分化、以及胚胎發育。目前已知之水解磷酸酯受器有五種，其中水解磷酸酯受器一至三屬於內皮細胞分化基因群，而水解磷酸酯受器四至五則屬於另一嘌呤受器基因群。在斑馬魚系統中，水解磷酸酯受器一已知會調控淋巴管發育，而水解磷酸酯受器三則負責神經管發育。此研究中，我們經由 TBLASTN 演算法比對斑馬魚與小鼠之基因序列，而取得斑馬魚水解磷酸酯受器四與五之序列。將斑馬魚水解磷酸酯受器四與五和所有已知之水解磷酸酯受器與嘌呤受器基因群綜合比對之後，斑馬魚水解磷酸酯受器四與五分別歸屬於水解磷酸酯受器四與五之基因群。斑馬魚水解磷酸酯受器四表現在五天大之魚鰓、睪丸以及脾臟等處。斑馬魚水解磷酸酯受器五則在十八體節發育期之後在全身表現。斑馬魚水解磷酸酯受器四與五異位表現在大鼠細胞株 B103 中無法接受水解磷酸酯調控細胞內鈣離子濃度。然而在斑馬魚胚胎中降低斑馬魚水解磷酸酯受器四之表現卻明顯的抑制斑馬魚之血管發育，導致胚胎嚴重水腫。綜合以上實驗，斑馬魚水解磷酸酯受器四應為調控斑馬魚循環發育之重要基因。

關鍵字: 水解磷酸酯、水解磷酸酯受器、斑馬魚、血管、發育

Catalog

Figures

INTRODUCTION

Lysophosphatidic acid (LPA) is a small, widespread phospholipid that acst as an extracellular signaling molecule by activating at least six known G protein-coupled receptors (GPCRs), LPA_1-LPA_6, and is able to exert physiological, phathophysiological and morphological effects on many cell types. Phospholipids are water-insoluble molecules that are most abundant on the cell membrane, but they can be further metabolized into several other forms, such as effective energy sources, molecules covalently attaching to proteins, and as intercellular and intracellular signaling molecules. Most bioactive lipids acts quickly, in a matter of seconds, on membrane proteins called GPCRs in an autocrine or paracrine manner (*1*). GPCRs act as the primary target to 50-60% of current therapeutic drugs (*2*). After the completion of human genome sequence, about 800 diverse genes were found to encode GPCRs, most of which are considered to bind various extracellular ligands such as lipids, amines and amino acids (*3*). The human genome project also revealed many GPCRs whose endogenous ligands are still unknown and are fittingly named orphan GPCRs. Many research groups are focusing on orphan GPCRs as objects of drug discovery. In fact, many GPCRs have recently been linked to their endogenous ligands (de-orphanized), yet, there are still nearly 120 GPCRs whose endogenous ligands have not been identified (*3*).

Among the GPCR binding lipid molecules, Lysophospholopids (LPLs) make up the majority of the GPCR-binding lipid molecules. LPLs consist of two major compounds, LPA and Sphingosine-1-phosphate (S1P), both of which are metabolites of membrane phospholipids and are stored in large quantities in platelets, which can be released into the blood upon being wounded. LPA and S1P are structurally similar to LPLs that have distinct receptors, but the resemblance in their tissue distribution, concentration, effector pathways and broad range of biological roles make them difficult to analyze individually. Consequently, there have been many attempts to obtain the single

receptor gene deficient animal so as to dissect the functions of each LPL. But unfortunately, due to a large number of receptors, it is difficult to observe significant phenotypes in knockout animals, for these receptors are functionally redundant to each other (*4*). Thus, investigators are working on acquiring the double or triple knockout animal (*4*). Nonetheless, we conclude that LPA plays an important role in animal physiology for the reason that autotaxin (ATX) knockout mice uniformly die at approximately embryonic day 9.5 with pronounced neural tube and vascular defects (*5-6*).

LPA is present in all eukaryotic tissues at relatively low concentrations, at higher concentrations (sub-micromolar range) in blood plasma, and even higher concentrations at wounded regions. In 1996, the first high affinity, cell surface receptor for LPA was identified as LPA_1 (*7*), this led to the discovery of two, closely related receptors, LPA_2 and LPA_3, and three rather divergent receptors, LPA_4, LPA_5 and LPA_6. Each receptor is encoded by distinct genes and named $LPAR_1$-$LPAR_6$ in humans and $Lpar_1$-$Lpar_6$ in mice. Studies utilizing cloned receptors and genetic knockout mice have presented us with notable evidence about the importance of LPA in many biological systems, such as basic cellular processes as well as multiple organ systems like the nervous system. But even 14 years after the first LPA receptor was found, there still many questions left to be answered. Which is why we are putting effort on the research of zebrafish LPA receptors. Zebrafish were used in this experiment because they are easy to handl in lage number, genetically easier to manipulate than mice and have transparent embryos that develop quickly (before the 5^{th} day of embryonic development). In addition, the large number of progeny that the fish produce allows the chance to more frequently observe embryonic lethal defects. In this research, we obtained zebrafish LPA_4 and LPA_5 sequences with bioinformatic and evolutional approaches, observed their expression and the impact after down-regulation of these genes with morpholino (MO) microinjection.

METABOLISM

The metabolism of LPA is complicated and results in numerous, chemically distinct species. Taking LPA as a signaling molecule, the term LPA generally stands for 1-acyl-2-hydroxyl-*sn*-glycero-3-phosphate, rather than other forms, such as 1-alkyl- or 2-acyl-LPA (*8-9*), the length of acyl chain and the degree of saturation vary vastly on the precursor phospholipid. As an example, 1-palmitoyl-posphotidylcholine is metabolized to 1-palmitoyl-LPA (16:0-LPA). The most abundant forms of LPA in human plasma are 16:0-, 18:2-, and 18:1-LPA (*10*). And most of the commonly available LPA in laboratory signaling studies are probably in the form of 18:1.

LPA is produced from the membrane phospholipid through two major pathways: (a) sequential activity of phospholipase D (PLD) and phospholipase A_2 (PLA_2) and (b) sequential activity of PLA_2 and lysophospholipase D (lysoPLD) (*4*), which both only in the case of producing 1-acyl-LPA. For other forms of LPA production, phospholipase A_1 (PLA_1) and diacylglyceral kinase (DGK) are involved in the more complicated metabolism of phospholipids, diacylglycerol, and other compounds more commonly observed in serum like lysophosphatidylcholine (LPC), lydophosphaticylethanolamine (LPE) and lysophosphatidylserine (LPS). LPC, LPE and LPA are first produced by at least two mechanisms. In activated platelets LPC, LPE and LPS are produced by secreted PLA_2 and phophatidylserine (PS)-specific PLA_1. In plasma, LPC is produced from phosphatidylcholine (PC) in lipoprotein by lecithincholesterol acyltransferase (LCAT) and PLA_1-like enzymes (*8*). The generated LPLs are subsequently transformed to LPA by the lysophopholipase D activity of ATX.

Autotaxin was first found to be a cancer-cell motility-stimulating factor, as a character of its assumed nucleotide phosphodiesterase activity (*11*). Enhanced expression of ATX is repeatedly observed in tumor tissues such as breast cancer (*12*), renal cancer (*13*), Hodgkin lymphoma (*14*),

hepatocellular carcinoma (*15*) and glioblastoma (*16*). Furthermore, ATX is identical to a cell motility-stimulating factor isolated from cell culture supernatant, and is implicated in metastatic and invasive potential in tumor tissues (*17*). However, the pro-migratory effect of ATX on cancer cells now known to be resulted from the autocrine signaling of LPA production. The significance of ATX was not fully appreciated until the knockout phenotype of a previous known mouse gene *Enpp2* ,encoded the ATX protein that was found to have lysoPLD activity (*5*). The *Enpp2* double null mice died uniformly at around embryonic day 9.5 with severe neural and vascular deficient (*6*). Nevertheless, $Enpp2^{+/-}$ heterozygotes survive to adulthood but have plasma LPA levels half of that of wild-type mice. This confirms that ATX is the major enzyme responsible for LPA production and LPA signaling is essential for development.

In the intertangled pathways of LPA production, there is an uprising bright spot that we cannot leave about, the novel synthetic pathway of LPA mediated by mPA-$PLA_1\alpha$/LIPH. Both mPA-$PLA_1\alpha$ and β, also called LIPH and LIPI, respectively, belong to the lipase gene family (*18*-*19*). They have about 50% amino acid similarity with each other. Most of the time, lipase hydrolyze fatty acids of triacylglycerol (TG) at the *sn*-1 and *sn*-3 positions. There are six extracullar and three intracellular PLA_1 isozymes are known in mammals. In most of the studies, the physiological substrates of these isozymes are not known. However, some of the extracellular PLA_1 isozymes such as hepatic lipase and endothelial lipase are known to hydrolyze phospholipids in addition to TG. In the phylogenetic tree of the lipase of the lipase gene family, PS- PLA_1, mPA-$PLA_1\alpha$ and mPA-$PLA_1\beta$ cluster and form a subfamily (*20*), in which "m" stands for "membrane-bound" and "PA" represents "phosphatidic acid-specific" and "PS" for "phosphatidylserine-specific".

Recent research on genetics disorders of human hair loss revealed that mPA-$PLA_1\alpha$/LIPH could be important for hair growth through its LPA-producing activity. Two independent groups presented

that human hair growth deficiency is linked to a genetic defect in the phospholipase gene mPA-PLA$_1$α/LIPH (*21-22*). Kazantseva et al. identified mPA-PLA$_1$α/LIPH as a causal gene of hair loss and hair growth defect through screening about 350,000 individuals in two populations from the Volga-Ural region of Russia (*22*). Ali et al. also discovered mutations in the gene for mPA-PLA$_1$α/LIPH among four generations of a Pakistani family with hereditary human hair deficiency (*21*). In both of the studies, affected individuals were homozygous for a deletion or a mutation in the mPA-PLA$_1$α/LIPH gene on chromosome 3q27. LIPH is expressed in hair follicle. Malformation of hair follicles is always associated to a hereditary disease, the results from these studies indicate that mPA-PLA$_1$α/LIPH regulates the formation and development of hair follicles and furthermore hair growth.

Moreover, two latter studies (*23-24*) showed yet another causative gene for human hair loss. By mapping the causative gene, they found homozygous truncated mutations in P2Y5, which encodes an orphan GPCR. P2Y5 is the closest homologue of LPA$_4$, with an amino acid identity of 56%. Consequently, Pasternack et al. showed that LPA act as a ligand for P2Y5 in reporter gene and radio-labeled ligand binding experiments. Interestingly, the expression pattern of P2Y5 resemble to that of mPA-PLA$_1$α/LIPH where mostly in inner root sheaths of hair follicles. These results of two independent results on human genetic defects indicates that LPA produced by mPA-PLA$_1$α/LIPH activates P2Y5 in the inner root sheaths. Although the specific pathways how LPA regulates hair follicles growth are not clearly known. These studies showed that in hair follicles, LPA signaling through mPA-PLA$_1$α/LIPH and P2Y5 , latter known as LPA$_6$, has a critical role in hair growth. Furthermore, mPA-PLA$_1$α/LIPH is a actual LPA-producing enzyme *in vivo*.

EDG FAMILY LPA RECEPTORS

Since the early twentieth century, lysophospholipids have been known to have biological activity. But the effects were thought to be the consequence of nonspecific detergent-like structure of lysophospholipids that disrupts plasma membrane. The mystery stays until a receptor was found in 1996, in a search for the ventricular zone (VZ) specific expressing gene in the cerebral cortex. Which leads to the identification of ventricular zone gene 1 (vzg-1) or endothelial differentiation gene 2 (edg-2), that was proved to be a high-affinity GPCR for LPA, latter called as LPA_1 (*7*). Thereafter, sequence similarities granted the identification of few more S1P receptors, and two more LPA receptors, LPA_2 and LPA_3.

LPA_1

LPA_1 is the first high-affinity, wide expressing receptor identified for LPA (*7*). The mammalian *LPAR1* gene (human chromosomal locus 9q31.3) encodes an approximately 41-kDa protein consisting of 364 amino acids with 7 transmembrane domains. Extensively expression of *Lpar1* is observed in adult mice, with significant presence in at least brain, uterus, testis, lung, small intestine, heart, stomach, kidney, spleen, thymus, placenta, and skeletal muscle (*25-26*), which is quite similar in humans (*27*). LPA_1 couples with and activates several types of G proteins such as $G_{\alpha i/o}$, $G_{\alpha q/11}$ and $G_{\alpha 12/13}$ (*28-29*). Most of the cellular effects of LPA_1 activation are through these G proteins, such as cell proliferation and survival, cell migration, and cytoskeletal changes; altered cell-cell contact through serum-response element activation, Ca2+ mobilization, and adenylyl cyclase inhibition; and activation of mitogen-activated protein kinase, phopholipase C, Akt, and Rho pathways (*25*).

In the *Lpar1* knockout mice, unexpected in vivo functions of this receptor were unveiled. *Lpar1*$^{-/-}$ mice show 50% reduction of perinatal survival in a mixed (C57Bl/6J × 129) genetic background and further decreased in pure (C57Bl/6J or Balb/cByJ) genetic backgrounds (J. Chun,

unpublished observations). The survived mice have a reduced body size, craniofacial dysmorphism with blunted snouts, and increased apoptosis in sciatic nerve Schwann cells (*30-31*). Deficient in suckling, attributed to olfactory defects, likely leads to perinatal lethality.

LPA_2

Lpar2 was identified from GeneBank searches of orphan GPCR genes for the 60% amino acids identity to *Lpar1*. In humans, *LPAR2* (chromosomal locus 19q12) encodes a protein that has as 348 residues, with calculated molecular mass of around 39kDa (*32*). The expression of *Lpar2* is relatively restricted compared to that of *Lpar1* (*25, 27*). In mouse, *Lpar2* is clearly present in kidney, uterus, and testis and moderately expressed in lung; and with lower levels of expression in stomach, spleen, thymus, brain, and heart (*25*). In mouse embryo, *Lpar2* is also observed in embryonic brain but decline within a week after birth (*25*). In human, *Lpar2* is highly expressed in testis and leukocytes, with moderate expression in prostate, spleen, thymus, and pancreas (*27*). In the case of tumor cells, abnormal expression of *Lpar2* has been reported, pairing the receptor to tumor-promoting activities.

LPA_2 activates the $G_{\alpha i/o}$, $G_{\alpha q/11}$ and $G_{\alpha 12/13}$, which leads to downstream activation of Ras, mitogen-activated protein kinase, phosphatidylinositol 3-kinale, Rac, phospholipase C, diacylglycerol, and Rho, which is similar to LPA_1 (*30*). LPA_2 activation is generally associated with cell survival (*33-34*) and cell migration (*35-37*), which makes it a stimulating factor for cancer metastasis (*38-39*).

Moreover, LPA_2 has been reported to have interaction with proteins other than G proteins. For example, LPA_2 promotes cell migration through interactions with focal adhesion molecule TRIP6 (*40-41*), and several PDZ proteins and zinc finger proteins are also reported to interact directly with the carboxyl-terminal tail of LPA_2 (*42*). Furthermore, LPA_2 mediated signaling can plays as an inhibitory effects against the EGF induced migration and invasion of pancreatic cancer cells through

the $G_{\alpha 12/13}$/Rho pathway (*43*). These evidence indicates that LPA_2 signaling cross-regulates between classical G protein signaling cascades and other signaling pathways to regulate the efficiency and specificity of signal transduction.

Lpar2$^{-/-}$ mice are viable, roughly normal, and born at normal Mendelian rations without sexual bias. But in double knockout animal of *Lpar1* and *Lpar2*, frontal hematomas is worse than that observed in *Lpar1*$^{-/-}$, suggesting that LPA_2 is probably developmentally redundant to LPA_1.

LPA_3

Lpar3 was revealed with degenerate PCR-based cloning and homology searches as an orphan GPCR gene. *LPAR3* (human chromosomal locus 1p22.3-p31.1) encodes a around 40kDa GPCR which has ~50% of amino acid sequence similarity with LPA_1 and LPA_2. *LPAR3* is expressed in human heart, testis, prostate, pancreas, lung, ovary, and brain (*44-45*). In mouse, expression is observed in testis, kidney, lung, small intestine, heart, stomach, spleen, brain and thymus (*25*). Interestingly, *Lpar3* mRNA was shown to specifically expressing in the luminal endometrial epithelium at ovum implantation (*46*), and the expression is regulated by progesterone and estrogen (*47*).

LPA_3 couples with $G_{\alpha i/o}$ and $G_{\alpha q}$ but not $G\alpha_{12/13}$, through which it stimulates LPA mediated phopholipase C activation and calcium mobilization, adenylyl cyclase inhibition and activation, and mitogen-activated protein kinase activation but not cell rounding in neuronal cells. Furthermore, LPA_3 is less sensitive to saturated acyl LPA like other EDG receptors do, but has a relative higher preference for 2-acyl-LPA unsaturated fatty acids. Taking the fact that we commonly use 18:1 LPA for signaling experiments into consideration, the differences of ligand affinity between cognate LPA receptors might affect our understanding of the physiological roles of LPA receptors *in vivo*, unless we have further information on the natural occurrence of LPA with different isoforms.

Lpar3-/- mice are grossly normal and viable, but recent report showed that in female nulls, it is important for embryo implantation, spacing and prenatal development as well (*46*). However, on the basis of LPA_3 expression in the frontal cortex, hippocampus, and amygdala (*44-45*), no significant neuronal morphology were observed in the *Lpar3* null mice.

DISCOVERING THE "NON-EDG" LPA RECEPTOR $LPAR_4$

LPA_4 was originally identified as an orphan GPCR from an exploration of expressed sequence tag database, and was further found to be a specific receptor for LPA through ligand screening (*48*). In this research, Noguchi K et al. stably expressed orphan GPCRs in Chinese hamster ovary (CHO) cells membrane, with N-terminus hemagglutinin (HA)-tagging, and screened each of the clones with 198 lipids. By flow cytometry with anti-HA antibody, they not only confirmed significant membrane expression of each orphan GPCR but also found 1-olelyl-LPA induced both intracellular calcium mobilization and cyclic adenosine monophosphate (cAMP) formation through P2y9, also called GPR23. Competitive radioligand binding assays revealed that p2y9/GPR23 bound various LPA species but with highest affinity to 18:1-LPA. Ectopically stable expression of p2y9/GPR23 in B103 cells, a rat neuroblastoma cell line with very low endogenous LPA receptor expression, further explained LPA dependent cellular response through p2y9/GPR23 (*49-50*), hereafter known as LPA_4.

Unlike the other three LPA receptors, LPA_4 belongs to another evolutionary branch called P2Y purinergic receptors, and is encoded by a single exon. The P2Y family consists of mainly G protein coupling receptors for nucleotides and almost exists in all human tissues. However, LPA_4 cannot be activated by any nucleotides or nucleosides tested, but only by LPA. The evolutionary basis of how a structurally specified purinergic receptor can binds and responds to a long chain glyceric phospholipid is still unknown, which is one of the questions we are trying to approach in this research.

LPAR4 gene is located on human chromosome X region q13-q21.1, with a intronless single exon encoding 370 amino acids protein of molecular weight around 42 kDa (*51-52*). Intriguingly, LPA_4 specifically binds to 18:1-LPA and with a structural analogs preference order of 18:1->18:0->16:0->14:0->1-akyl->1-alkenyl-LPA (*48*), which is similar to that reported in EDG LPA receptors. Nevertheless, in the case of human LPA_4, it only shares 20-24% amino acid identity with three other EDG family LPA receptors.

In LPA_4-overexpressing cells, LPA induces cellular responses such as cell rounding and stress fiber formation through the $G\alpha_{12/13}$ and Rho/Rho-kinase pathways (*49-50*), as observed in LPA_1, LPA_2 and LPA_5. LPA_4 also triggers intracellular cAMP accumulation through $G_{\alpha s}$, and calcium mobilization through $G_{\alpha q/11}$ and $G_{\alpha i}$ (*49-50*), which may be a cell type dependent activity (*50*). Interestingly, LPA_4 is currently the only known LPA receptor that couples to $G_{\alpha s}$.

LPA_4 RNA is expressed in mouse heart, skin, thymus, ovary, developing brain and embryonic fibroblasts (*49, 53*). In human, similar expression pattern was reported except *LPAR4* is massively expressed in the ovary (*48*), which may related to its genomic location of X chromosome, and refers to its role in embryonic development we will discuss later.

Recently, LPA_4 double null mice have been reported, though the animal showed no apparent abnormalities. However, *Lpar4*$^{-/-}$ fibroblasts are hypersensitized to LPA induced migration response, indicating that LPA_4 plays an inhibitory role in LPA induced migration (*53*). Which further confirmed in LPA_1 expressing B103 cells, that heterologous expressing LPA_4 suppresses LPA_1-dependent migration and LPA-induced migration and invasion of colon cancer cells.

LPA_5

GPR92/GPR93 was an orphan GPCR closely related to LPA_4, with 35% amino acid identity, which also belongs to P2Y family. Human *LPAR5* is located on chromosome 12q13.31 and encodes

372 amino acids with around 41 kDa molecular weight. Chun and colleagues showed that heterologous expression of LPA_5 exerted LPA dependent activity in B103 and RH7777 cells, both with very low or no endogenous LPA response. LPA_5 couples to $G_{\alpha q/11}$ and $G_{\alpha 12/13}$, which further stimulates calcium mobilization, cAMP production and inositol phosphate elevation as well (*54-55*). Moreover, LPA_5 was shown to be related to LPA stimulated neurite retraction, receptor internalization and phosphatidylinositol hydrolysis (*55*). In murine tissue, *Lpar5* is broadly expressed in embryonic brain, small intestine, skin, spleen, stomach, thymus, lung, heart, liver, and embryonic stem cells (*54-55*).

In a calcium mobilization assay, Fujuwara et al. screened several lipid or non-lipid compounds with site-directed mutagenesis of LPA_5 residues or computational binding pocket fitting of LPA_5 protein structure (*56-57*). They showed the rank order of LPA_5 agonists is alkyl glycerol phosphate > LPA > farnesyl phosphates >> N-arachidonoylglycine. In the research, they confirmed LPA_5 to be a *bona fide* LPA receptor, and also found several LPA_5 agonists and two non-lipid antagonists as well.

Although LPA_5 has been proved to be activated by 18:1-LPA, subsequent studies also indicates that LPA_5 can response to farnesyl pyrophosphate at a much higher concentrations relative to 18:1-LPA (*57-58*). The fact that LPAR5 has varied affinity to varied isoforms of LPA brings the question up-front: Whether distinct ligand affinity related to the different localization and expression pattern of LPA receptors, and furthermore, the interconnected regulation of one LPA receptor to each other?

BIOLOGICAL FUNCTIONS OF LPA

LPA was first known as a blood pressure regulator in 1978 (*59-60*). Whereas, the physiological regulative function of LPA was attributed to its role as a metabolic intermediate. Most of the physiological functions of LPA have since been discovered to be mediated by specific interaction

with its six cognate receptors (LPA1-6) (*29, 61*). Numerous gain- and loss-of-function studies have revealed a variety of biological, receptor dependent functions, such as mitogenic effects, cell differentiation, cell survival, cytoskeletal reorganization, process retraction, and cell migration (*29, 61-62*). These LPA-mediated activities take parts into nervous system function, vascular development, immune system function, cancer, reproduction, fibrosis, and obesity. In this paragraph, we will have an abstract idea on those discoveries.

Nervous system

The nervous system is one of the major loci for LPA receptor expression, and LPA presents in the brain at relatively high concentrations (*9*). LPA_1 was first found to be specifically expressed within the proliferative cortical VZ of the embryonic brain (*7, 28*), suggesting the importance of LPA signaling in the development of VZ cells. Moreover, LPA receptors are expressed in almost all cell types of the nervous system. And LPA signaling were known to affect several neuronal development process, such as cortical development and function (*63-64*), growth and folding of the cerebral cortex (*65*), growth cone and process retraction (*66-68*), survival (*65*), migration (*69*), adhesion (*31*), and proliferation (*30, 65*). Furthermore, LPA signaling may be involved in the pathophysiology of schizophrenia and neuropathic pain (*70-72*)

Vascular system

The regulation of vascular system involved several aspects, from the proliferation, migration, adhesion, differentiation, and assembly of vascular endothelial cells and vascular smooth muscle cells (VSMCs). Recent analysis of knockout mice showed the frequency of frontal cephalic hemorrhages in *Lpar1*$^{-/-}$ mice, increased in *Lpar1*$^{-/-}$/ *Lpar2*$^{-/-}$ double null mice (*30, 73*). Moreover, ATX-null mice embryo dies with neuronal and vascular deficit at 9.5 embryonic day. These *in vivo* results pinpoint the value of LPA signaling in vascular development. Besides vessel structure, LPA_1

and LPA_3 were found to be the primary mediators of LPA-induced platelet activation in a pharmacological study (*74*). However opposing results were also reported that LPA may inhibit platelet activation in mice (*75*). On the regulation of VSMCs, LPA_1 and LPA_2 were shown to exhibit opposing effects on the migration of primary VSMCs derived from knockout mice (*35*). In concert with numerous bioactive mediators, including growth factors and cytokines, LPA was known to mediates wound healing (*29, 61, 76-77*).

Immune system

Undoubtedly, LPA is the one of the major constituent of serum that represents blood plasma upon wound. Base on the well established knowledge that LPA takes parts in wound healing, it is inevitable that people would have an interest in analyzing its relationship with the immune system. LPA receptors are expressed in immune cells, such as lymphocytes (*34, 37, 78-79*), dendritic cells (DCs) (*80-81*), and lymphoid organs such as the spleen and thymus (*29, 54, 56*). In the modulation of T cells and DCs activation, LPA was shown to excite or attenuate T cell activity under different cell activation-state and varied LPA receptor combinations. Moreover, in a recent study, ATX was presented to regulate lymphocyte trafficking (*82*). Additional research utilizing multiple receptor deficient mice will certainly help on the detailed understanding LPA signaling in immune system

Cancer

Since LPA is known to regulate angiogenesis and lymphangiogenesis in the case of wound healing, the vessel regulating role of LPA is further tested in cancer, for elevated vessel structure is generally observed in tumors. The first clue of this correlation was reported in 1964 that LPA's metabolic precursor LPC (lysolecithin) was found to be massively increased in the serum of ovarian cancer patient (*83*). In a latter study, ATX was identified as a motility-stimulating factor for cancer cells (*17*), and which further found as a result for its lysoPLD activity in 2002 (*84-85*). The

involvement of LPA in the cancer progression and metastasis is generally due to its vessel regulating activity, which appears to be modulated at the level of receptor expression rather than ligand concentration (*39, 86*). Furthermore, the tumorigenic activity of LPA were shown to mostly resulted from hypoxia, which is generally observed in tumor (*87*).

Reproduction

Lately, numerous studies have furnished considerable evidence for the involvement of LPA signaling in the process of reproduction, such as spermatogenesis, male sexual function, ovarian function, fertilization, embryo spacing, implantation, decidualization, pregnancy maintenance, parturition, and related diseases (*26*). LPA is present in the follicular fluid of healthy individuals (*88*), and ATX activity is elevated in serum of normal pregnant women at the third trimester of pregnancy, which is further enhanced in patients at risk for preterm delivery (*84, 89*). In a triple gene deleted animal of $LPA_{1\text{-}3}$, significant defects in germ cell survival and azoospermia were reported (*90*). Moreover, LPA_3-deficient female mice showed delayed implantation, embryo crowding, and reduced litter size (*46*), which is similar to those reported in cyclooxygenase-2 (prostaglandin producing enzyme) deficient mice. And the LPA_3-dependent phenotype can be rescued by exogenous administration of prostaglandins (*46*), indicating that separable LPA_3 signaling occur during implantation upstream of prostglandin. The specific mechanism of LPA_3 regulating spacing remain to be clarified but may involve cytosolic phospholipase A_2 ($cPLA_{2\alpha}$) or Wnt/β-catenin signaling for the deficient female mice show similar embryo-crowding phenotypes as that observed in *Lpar3*$^{-/-}$ mice (*91-92*).

GENE EXPRESSION AND PHYSIOLOGICAL FUNCTION OF LPA_4 AND LPA_5

Although LPA_4 was originally found in 1997 (*51-52*), the physiological functions of LPA_4 and LPA_5 were still unclear, after 12 years of their discovery (*4*). However, as our knowledge of the EDG

LPA receptors mounting up, more and more groups are presenting striking results of P2Y LPA receptors, with corresponding comparison between LPA receptors.

EXPRESSION

In an expression profiling research paper, Noji et al. presented the expression patterns of LPA receptors during mouse early development in a whole-mount *in situ* hybridization experiment (*93*). LPA_4 was expressed in the prospective midbrain-hindbrain boundary (MHB), with a parallel pattern reported for ATX. At a later stage, around E12.5, the MHB expression diminished to MHB vicinity, but elevated expression was observed at hindbrain, maxilllary processes, and first bonchial arches, limb buds, developing liver and somites. Along with time, the limb bud LPA_4 expression became restricted to its proximal region, and then prospective zeugopod region and the tips of the developing digits as well. On the other hand, LPA_5 was shown to express in the forebrain, rostral midbrain and margins of the neural folds, just before they were to close at the midline (*93*). LPA_5 was reported to be ubiquitously expressed at E12.5, and relatively elevated in the craniofacial region. These reports further underscored the importance of LPA_4 and LPA_5 in embryonic development, especially their involvement in neural folding and midbrain formation, and the digits growth.

REPRODUCTION

As Yanagida et al. presented in a review article recently (*94*), the notion of LPA_4 and LPA_5 physiological function were brought to the surface. In adult mice, LPA_4 was observed in brain, heart, lung, thymus, kidney, and skeletal muscle, and intriguingly, highly expressed in ovary, uterus and placenta of female mice. The ovary centered LPA_4 expression was also reported in human (*48*). It is therefore expected that LPA_4 might plays a role in the female reproduction. Although no notable change was observed during menstrual cycle, LPA_4 mRNA expression levels increased along with pregnancy at the uterus implantation site but not ovary (*94*). At 12.5 days post coitum (dpc), embryos

showed approximately 4.5-fold higher expression of LPA_4 mRNA than the placenta. In combination of the previous expression profile reports, LPA_4 may involve in several developmental process such as implantation and embryonic development of brain and digits. Nevertheless, the physiological functions of elevated LPA_4 expression in female ovary are still unknown.

NEURAL PHYSIOLOGY

The expressed sequence tag cDNA encoding LPA_4 was originally isolated from human brain (*52*). As discussed above, LPA was shown to stimulate neurite retraction and cell aggragation of LPA_4 stably expressing B103 cells in a $G_{\alpha 12/13}$-Rho-ROCK dependent pathway (*49*). Moreover, LPA_4 mRNA was consistently observed both in undifferentiated human embryonic stem cells and during their differentiation toward neural stem/progenitor cells (*95*). On the other hand, LPA_5 was reported to express in the dorsal root ganglia (DRG) (*55-56*), which predominantly expressing transient receptor potential vanilloid type 1 (TRPV1) channel. In this research Moon MJ et al. presented that the DRG calcium mobilization stimulated farnesyl pyrophosphate (FPP) can be inhibited by transfection of siRNA of LPA_5 (*56*). Taken together, LPA_4 may be implicated in neurodevelopmental process, whereas LPA_5 may play a role in the modulation of pain and nociceptive transmission.

PLATELETS

Low-density lipoprotein (LDL) with oxidative modification, which exists in the circulation or exposed after rupture of atherosclerotic plaques to blood cells, was known to activate platelets (*96*). LPA is one of the main platelet-activating lipid of oxidized LDL and human atherosclerotic lesions (*97*), and was known to induce platelet shape change (*98*) or platelet aggregation at higher concentration (*96*). However, the platelet stimulating activity of LPA requires the synergism of adenosine diphosphate (ADP), which mediates the positive feedback of platelet activation (*96*). Since platelet is the main source of LPA, it is plausible that LPA may play a role as autocrine signal that

triggers and amplifies platelet activation response. Nevertheless, platelets act differently with varied isoforms of LPA (*99*), and LPA receptors expressing in platelets are mostly LPA_4 and LPA_5. Combined with the singular ligand affinity of LPA_5, which actually parallel to those of platelets, and the fact that LPA_5 agonistic activity consistently associated with the platelet shape change, LPA_5 might take parts in the activation of platelets. On the contrary, platelets in approximately 20% of individuals failed to aggregate upon LPA, which is coincident with increased LPA_4 mRNA (*100*). These findings are potentially of clinical interest because LPA non-responders were less likely to have coronary artery disease, and the differences of biased coagulating activity may results from diverse LPA receptor expression.

INTERCONNECTED MUTUAL REGULATION

As mentioned above, LPA_4 was known to negatively regulates LPA_1-dependent fibroblast migration, which was the very first evidence that indicates LPA_4 may plays as a brake against other LPA receptor (*53*). Recently, Yaworsky et al. presented that osteoblast differentiate 4 times faster with down-regulated LPA_4 (*101*), which is also a LPA_1-dependent mechanism. Moreover, LPA_4 deficient mice have increased trabecular bone volume, number and thickness as well. On the other hand, more recently, Sims et al. reported LPA may also activate osteoclasts cyotosolic calcium concentration, evoke retraction and promote cell survival in a LPA_1-dependent fashion (J Biol Chem. 2010 Jun 15. Epub ahead of print). However, the retraction activity of osteoclasts require the collaboration of another LPA receptor that stimulates $G_{\alpha 12/13}$, which is yet unknown, despite LPA_2, LPA_4 and LPA_5 are known to exist in osteoclasts. Collectively, these evidences support the impression that cognate LPA receptors may regulate each other in order to exhibit delicate control of LPA activities.

ZEBRAFISH AS A MODEL FOR LPA RECEPTOR RESEARCH

To date, four of the known LPA receptors (LPA_1-LPA_4) have been separately disrupted in mice (*30, 46, 53, 64, 70, 73*). LPA_1/LPA_2 double-deficient and LPA_1/LPA_2/LPA_3 triple-deficient strains have also been generated and analyzed (*73, 90*). However, none of these deficient mice exhibited a phenotype similar to ATX-deficient mice, which with embryonic lethality due to vascular defects (*5-6*). Therefore, different combinations of LPA receptors, especially non-Edg receptors might be necessary to recapitulate the phenotype of LPA deficient, and possibly, other subtypes of LPA receptors may remain to be involved, such as GPR87 and P2Y10 (*102-103*).

Basing on the knowledge of these evidences mainly observed in mammals, there still a lot yet left unknown, especially for P2Y family LPA receptors. The main obstacle in mammal LPA research is that the screening of deficient phenotypes is limited to the offspring number of null mice, despite the LPA receptor deficient may affect on the progeny survival and the difficulty of mouse *in vivo* assay system. Therefore, here in this study, we aim to establish a zebrafish animal model that can be genetically manipulated with large amount and analyzed *in vivo*, which also as a tool to reveal developmental defects that cannot be seen in the mouse uterus.

As Lee et al. presented earlier (*104*), zebrafish LPA_1 is known to be essential for lymphatic vessel formation, and $zLPA_3$ is critical for neural tube development (unpublished result from Lee). Previous reports indicate that the phenotype of LPA receptor deficient in zebrafish may not necessarily parallel to those observed in mammals, which suggest that the signaling pathway might differ from fish to mice. However, the physiological function observed in zebrafish actually match those presented in ATX deficient mice, but with a much longer observation window (5 days), and outnumbered experimental repeats

In this study, we intended to identify and analyze the developmental and physiological function of zebrafish LPA_4 and LPA_5. In combination of bioinformatics and evolutionary analysis, we identified $zLPA_4$ and $zLPA_5$. The mRNA expression of these genes were monitored with real-time PCR and whole-mount *in situ* hybridization techniques. By morpholino (MO) microinjection of each gene, we observed developmental defects in $zLPA_4$ or $zLPA_5$ down-regulated embryos.

MATERIAL AND METHODS

PROTEIN SEQUENCE ALIGNMENT

By using mouse LPA receptor 4 and 5 protein sequence as bait, we search for zebrafish LPA receptor 4 and 5 with TBLASTN analysis in the zebrafish nucleotide collection database. Zebrafish LPA_4 is found as XP_001334713.1, and $zLPA_5$ is found as NP_955900.1. The latter one have low similarity of about 30% with both mouse LPA_5 and LPA_6, but after aligning with all known the LPA, S1P receptors and P2RY receptors of different species, the sequence has highest similarity to the LPA_5 family. Also, zebrafish LPA_6 is identified by another group with morpholino attenuation experiment (*105*).

PHYLOGENETIC TREE AND PERCENTAGE SIMILARITY ANALYSIS

All the known LPA, S1P receptors and P2RY receptors are aligned with ClustalX2, and tree was drew by bootstrapping analysis with one thousand random trial. Confident reference are sited beside each branch as number of percentage. Each receptors of different species was clustered into separated group of each receptor family. The analysis is done in both protein and nucleotide sequences, and reveals similar structure that the two identified sequences belonged to LPA_4 and LPA_5 family. Percentage similarity of LPA_4, LPA_5 and LPA_6 between human, mouse and zebrafish are collected and presented into chart.

EXPRESSION CONSTRUCT OF $zLPA_4$ AND $zLPA_5$

Zebrafish LPA_4 is amplified from 3 dpf zebrafish WT cDNA with Phusion DNA polymerase (New England Biolab) by using forward primer: ATGGCCAGTCTTGTTCTTAA and reverse primer: TCAGAACTGAGTCTCACCAA. And $zLPA_5$ is amplified with forward primer: ATGACTTCAAACAACACTAC and reverse primer: TTATTGTCCAGTCCAACTCG. Both CDS

were ligated into pGEM T easy vector with additional adenosine to each end of the blunt end PCR product. Construct were selected and sequenced to obtain full-length CDS without mutation.

STAGE AND TISSUE DEPENDENT RNA EXPRESSION

At the night before mating morning, each pair of female and male zebrafish were transferred to a single tank with mesh bottom to allow egg passing through and keeps fishes away from eating the eggs. Females and males are separated from each other by a transparent acrylic plate which avoids fighting and helps fishes getting acquainted with each other. At the morning of mating, acrylic plate is removed as long as the lights turned on, and eggs are collected after the observation of tail chasing activity. Eggs are cultivated under 28.5℃ in fish water, malformed and dead embryo are removed daily so as to keep the water clean. At the time of each developmental stage, embryo are observed under binomial microscope before picking 30 embryos for RNA extraction. Each 30 embryos are transferred into a 1.5 ml vial and left with 100 μl fish water, added 1 ml of **TRIzol**® (Invitrogen) and homogenized with 1 ml syringe with needles of different tip size, from 23G, 27G to 30G needles. The homogenized embryo were collected in **TRIzol**® and stored in -80℃ till the time of RNA extraction. Upon RNA extraction, each vial of 1ml **TRIzol**® is added with 100 μl chloroform and vortex for 30 seconds before standing on ice for 5 minutes, then undergo phase separation with 15 minutes of 12000 rpm centrifugation at 4℃. The upper aquarius phase is then transferred to a new vial and added with 2× volume of 100% Ethanol and stand for 15 minutes at -20℃ followed by 15 minutes 12000 rpm centrifugation to precipitate RNA. Then carefully discarded the aquarius ethanol, and wash the RNA pallet with 70% ethanol, centrifugated 3000 rpm for 5 minutes. Again, discard the upper aquarius phase and air dry the RNA pallet in a laminar flow for 15 minutes at room temperature, and resuspended with 50 μl DEPC water. The resuspended RNA were heated on a hotplate at 55°C for 10 minutes before density observed with NanoDrop (Thermo Scientific). Two micro grams of embryo RNA were reverse-transcribed into cDNA with ReverTra Ace (TOYOBO), and the resulting 20 μl

cDNA were diluted 2× with DEPC water. Two micro liter of embryo cDNA were used as template to undergo real-time PCR by using ABsolute™ QPCR SYBR® Green Mixes (Thermo Scientific) and iCycler Thermal Cycler (BioRad). Gene specific primer for $zLPA_4$, $zLPA_5$, z beta Actin and zEF1α were designed by using pDraw32. And primer sequences as follows: $zLPA_4$ intF' ATTGCTTCTTTGACCCGGTG, $zLPA_4$ R' TCAGAACTGAGTCTCACCAA, $zLPA_5$ F' ATGACTTCAAACAACACTAC, $zLPA_5$ intR' CCATGTAGATCACTGGAACA, zbActin RT F' CCAGCTGTCTTCCCATCCA, zbActin_RT_R' TCACCAACGTAGCTGTCTTTCTG, zEF1α_RT_F' CTGGAGGCCAGCTCAAACAT, zEF1α_RT_R' ATCAAGAAGAGTAGTACCGCTAGCATTAC. Acquired fluorescent reading were analyzed with iQ5 (BioRad), and CT values of reference genes were used to generalize deviation between samples. $ZLPA_4$ and $zLPA_5$ RNA expression level were presented as relative fold to the average amount of zbActin and zEF1α.

Zebrafish RNA of different organs were collected in a similar procedure. In order to preserve as much RNA as possible, fish were sacrificed by laying on ice for 5 minutes and dissected under binomial microscope as long as fish's gill stop moving. Separated organs were homogenized in 1 ml **TRIzol**® with syringe with different tip opening as soon as possible. Collected organ RNA were then extracted with chloroform and precipitated by 100% ethanol, washed with 70% ethanol, air dried and resuspended in 50 μl DEPC treated water, followed by density determination. And revers transcribed into cDNA then used as template for real-time PCR, with zbActin and zEF1α as internal control.

WHOLE MOUNT IN SITU HYBRIDIZATION

The in situ hybridization experiment were carried out as in (*106*). In brief, probe were synthesized by using $zLPA_4$ pGEM T and $zLPA_5$ pGEM T vectors as template and undergo PCR

amplification by using VAS Taq Blue DNA polymerase (BIONOVAS) with primers containing 5' T7 promoter sequence: TAATACGACTCACTATAGGG. Each gene were amplified in two sets of primers in order to obtain sense and antisense template with 5' or 3' T7 promoter sequence. Primer sequences as follows, Sense: $zLPR_4$ T7F' TAATACGACTCACTATAGGGgtggcattgacgactccttc, $zLPA_4$ R' TCAGAACTGAGTCTCACCAA, $zLPA_5$ T7F' TAATACGACTCACTATAGGGatgacttcaaacaacactac, $zLPA_5$ R' TTATTGTCCAGTCCAACTCG, Antisense: $zLPA_4$ F' ATGGCCAGTCTTGTTCTTAA, $zLPA_4$ T7R' TAATACGACTCACTATAGGGtcagaactgagtctcaccaa, $zLPA_5$ F' ATGACTTCAAACAACACTAC, $zLPA_5$ T7R' TAATACGACTCACTATAGGGttattgtccagtccaactcg. (Capitalized letters represent the T7 promoter sequence.) The amplified template with opposite T7 promoter orientation are subjected to T7 RNA polymerase (Promega) with DIG RNA labeling mix (UTP) (Promega) so as to produce each sense and antisense strand RNA probe of each gene.

Embryo are collected at different developmental stages with 4% freshly prepared paraformaldehyde 4℃ overnight, and then switch into 100% methanol and stored in -20℃ till needed. Before hybridization, the embryos are hybridized in PBS, permeabilized by proteinase K, washed in PBT and prehybridized in Hybridization Mix (HM) 70°C for 5 hours. For each 200 μl of HM, 40 ng of DIG-labeled RNA probe were added, and hybridized overnight at 70℃. After probe hybridization, embryos were washed through serial concentration of 2×SSC, 0.2×SSC, and then PBT. After 4 hours or blocking, sheep anti-digoxigenin-AP Fab fragments (Roche Diagnostics)(alkaline phosphatase conjugated) were added into blocking buffer at 1/10,000 dilution overnight at 4℃. For staining, the embryos were washed with PBT, changed into alkaline Tris buffer, and stained with NBT and BCIP in dark. The staining reaction were terminated by stop solution and mounted in 100% glycerol.

LPA INDUCED CA++ MOBILIZATION

The $zLPA_4$ and $zLPA_5$ over-expression construct were made by transferring each gene from pGEM T vecters into pIRES2 EGFP vectors through EcoRI site, the constructs were sequenced to confirm preferred orientation. Rat neuroblastoma cell line B103 were known to express very low level of intrinsic LPA receptors, which were transfected with $zLPA_4$ pIRES, $zLPA_5$ pIRES, or pIRES2 EGFP vector only by Lipofectamine™ 2000 (Invitrogene) for 24 hours with 500ng DNA/1.5µl Lipofectamine ratio per 90,000 cells in 96 wells plate. Fluorescence were observed 24 hours after transfection in order to confirm gene expression. About 48 hours after transfection, cells were stained with 4 uM Fluor 3-AM 37℃ for 40 minutes, washed with PBS. The calcium mobilization were measured by Flexstation® 3 (Molecular Devices) by comparing time-laps fluorescent readings with different stimulations, such as 5µM LPA, 5µM S1P, vector controls or 1% Triton X-100.

BONE AND CARTILAGE DIFFERENTIAL STAINING

Embryos were anaesthetized in 0.08% Tricaine® , and fixed in 4% paraformaldehyde/ PBS for 24 hours. Then washed in PBS and stained for cartilage in 0.1mg/ml Alcian Blue (Sigma) in ethanol/acetic acid (4:1), then rehydrated through ethanol series from 90%, 50% to 30%. The rehydrated embryos were digested overnight in 50mg/ml trypsin in 30% sodium tetraborate in water, and stained for bone with 0.4 ml of Alizarin Red S (Sigma) solution in 10 ml 0.5% KOH. At desired staining stage, embryos were destained in 1% KOH/glycerol series and stored in glycerol.

RESULTS

HIERARCHICAL TREE OF ALL KNOWN LPA, S1P, P2RY RECEPTORS

In order to discover the zebrafish LPA receptor 4 and 5, we used the known mouse receptor protein sequences as bait and searched in zebrafish nucleotide sequence database. At the first glance, zebrafish LPA_4 analogue XP_001334713.1 was found to has relatively high similarity to it's mammal parallel receptor. Whether the other sequence NP_955900.1 has an equally low similarity to both mouse LPA_5 and LPA_6, along with another zebrafish gene NP_001073524.1. Since both S1P/LPA are lysophospholipids, and LPA receptors can be divided into two families, that LPA_{1-3} are in the EDG family and LPA_{4-6} are in the P2RY family, we aligned all the known LPA, S1P and P2RY receptors protein sequences between species, and drew a hierarchical tree with random bootstrapping (**Fig. 1**). In the resulting figure, each receptor of different species were clustered into it's own family, with confidence reference sited at the branch. In accordance with our knowledge, S1PR1-5 are closely related to LPA_{1-3} for they all belong to EDG family, and $LPA_{4/5}$ are similar to most other P2RY receptors. But each of the LPA receptors gathered in their own clusters, that XP_001334713.1 stays with other species' LPA_4, and NP_955900.1 stays with other species' LPA_5, but NP_001073524.1 were found to cluster with LPA_6. The sequence NP_001073524.1 was further confirmed as $zLPA_6$ by another group (*105*), and was found to be important for zebrafish embryo vessel development in a morpholino knock-down experiment. In this figure, we further verified the identity of zebrafish LPA receptor 4 and 5.

PROTEIN SEQUENCE ALIGNMENT OF HUMAN, MOUSE AND ZEBRAFISH LPA_4 AND LPA_5

All of the LPA receptors are GPCRs, thus as in **Fig. 2a**, we aligned the human, mouse and zebrafish protein sequences of LPA_4 and LPA_5, both alignment show conserved GPCR's seven transmembrane domains. But in zebrafish receptors, C terminus sequences are highly variated from

their mammalian relatives, in which cytosolic domains were known to be responsible for the binding of down stream signaling proteins such as the trimeric G protein complex (*4*) and PDZ scaffold proteins (*42*) such as NHERF2. These results suggest that the zebrafish LPA receptors 4 and 5 might have different cytosolic binding affinities compared to mammal's. In addition to cytosolic domain, the N terminus of $zLPA_4$ is also 20 amino acids shorter than mammalian receptors, which their physiological function were not yet known. These finding reveals the conserved and variated domains of $zLPA_4$ and $zLPA_5$, which sheds light on the evolutionary research of LPA signalling.

PERCENTAGE SIMILARITY OF LPA_4 AND LPA_5 BETWEEN SPECIES

In the alignment of LPA receptors between different species, we collected the identical percentage of the same receptor, and arranged into this chart (**Fig.2b**). Compared to zebrafish LPA receptor 1 (*104*), LPA_4 and LPA_5 are much less conserved from fish to mammal, despite most of the LPA receptors are conserved between mouse and human. Especially in $zLPA_5$, in contrast with the 60% similarity of $zLPA_4$ and $zLPA_6$, the zebrafish analogue has a low similarity to it's mammalian relatives for about 30%. In comparison with **Fig. 2a**, the LPA_5 family is diverted from other R2RY LPA receptors such as LPA_4 and LPA_6, which indicates LPA_5 might plays quite different roles in fishes and mammals, and it's physiological function may also differs from LPA_4 due to it's broad ligand affinity (*107*).

LPA INDUCED CALCIUM MOBILIZATION IN B103 CELLS THROUGH $zLPA_4$ AND $zLPA_5$

All mammalian LPA receptors were known to trigger cytosolic calcium mobilization upon LPA stimulation (*4*). Therefore we over-expressed $zLPA_4$ and $zLPA_5$ in rat neuroblastoma cell line B103 along with a downstream IRES sequence and EGFP marker gene to monitor exogenous gene expression. The cells were subjected to calcium fluorophore staining, and observed for LPA stimulated calcium mobilization. But unfortunately, we are not able to observe any LPA specific

calcium deposition in this assay, by taking vector only transfection and vehicle treatment as negative control, which may due to the C terminus differences of $zLPA_4$ and $zLPA_5$ between fish and mammal.

DEVELOPMENTAL STAGE DEPENDENT zLPA_4 AND zLPA_5 RNA EXPRESSION

In order to dissect the developmental roles of $zLPA_4$ and $zLPA_5$, first, we analyzed the expression timing and amount of these two genes. With the technique of real-time PCR, we used two reference genes zβActin and zEF1α as internal control, but we were surprised by the variation of reference gene expression along with fish development. Both genes transcription level increased with time, but zEF1α RNA peaks at 6 somites stage, however zβActin RNA level increases in a slower rate, which peak at 96 hpf stage. Taking these variations into account, the increased RNA of $zLPA_4$ and $zLPA_5$ before gastrulation can be seen as maternal RNA deposition. Thus, embryonic $zLPA_4$ RNA is expressed at 5^{th} day of development, and $zLPA_5$ RNA is expressed since 24 somites stage. Both gene's RNA expression significantly increased after 5 dpf (data no shown).

WHOLE MOUNT *IN SITU* HYBRIDIZATION OF zLPA_4 AND zLPA_5

With the knowledge of the expression timing of these two genes, we further analyzed the spatial pattern of RNA expression at different developmental stages. With zHSP70 antisense probe as positive control, which zHSP70 is known to be highly expressed in 2 dpf embryo eyes, we are not able to observe any specific pattern between the comparison of antisense and sense probes of $zLPA_4$ and $zLPA_5$. Which suggest these two may not be specifically expressed in certain organ that can be observed under binomial microscope, however, we cannot exclude the possibility that these two LPA receptors may expressed at a smaller scale such as microvascular system or skin.

DISCUSSION

Lysophophatidic acid is readily known to play important roles in several physiological, developmental and phathophysiological processes (*4*). However, the recent expansion of P2Y family LPA receptors further pinpoint the magnitude of LPA signaling, which it requires this large number of receptors to confer harmonious control of LPA activity. In the present study, we seek to identify the physiological and developmental role of $zLPA_4$ and $zLPA_5$. With evolutionary consistent sequence resemblance, $zLPA_4$ and $zLPA_5$ were identified and analyzed for their mRNA expression in different developmental stages and adult tissues. Zebrafish LPA_4 and LPA_5 mRNA were expressed at 4 dpf and 18 somites respectively. Functional analysis of LPA-dependent response were assayed for calcium mobilization, but no ligand-specific activity were observed. However, vascular structure significantly defected in the $zLPA_4$ MO microinjected morphant.

On an evolutionary aspect, as the nucleotide sequence mutate with time, most of the information on DNA will wither except those functionally needed. Speaking from fish to human, it took nearly 250 million years to expand from the first vertebrate to mammals, and another 250 million years for *Homo sapiens* to come. As a signaling mechanism to evolve, in the case of LPA receptors, only the functionally essential characters can be conserved, such as the protein conformation, ligand binding sites, signaling protein binding domains and the hydrophobic transmembrane domains. Through protein sequence TBLASTN analysis, we identified zebrafish cognate entry of LPA_4 and LPA_5, on the basis that function reserved in amino acid sequences. However, mounting results of zebrafish genomic analysis have indicated that the fish genome duplicated after they split from tetrapod lineage (four-footed) (*108*). The excessive genetic material may have facilitated the vast radiation of teleosts (bony fish) (*108*). Nevertheless, only a subset of the duplicates have been retained in modern teleost genomes, that similar amount of duplicated genes were reported in zebrafish and *Tetraodon*

(pufferfish) (*108*), despite the location of reserved duplicates differed. In the percentage similarity of LPA receptors, $zLPA_4$ and $zLPA_6$ showed a reasonable identity with their mammal analogues, whereas $zLPA_5$ has low sequence relationships. The low conservation of LPA_5 sequence may due to the elevated variation after genome duplication, or the differentiated need of LPA_5 signaling. Taking LPA_6 as an example, being a gene partially essential for zebrafish vessel development, the mutant gene of *LPAR6* in human was shown to wilt only hair follicle rather than cardiovascular system, not to mention that sequence conservation is much higher in $zLPA_6$ than in $zLPA_5$. However, the $zLPA_5$ morphant were generally normal, without significant malformation, suggesting that the physiological function of $zLPA_5$ may lies in a more delicate or developmentally matured aspect.

G protein-coupled receptors (GPCRs) constitiute one of the largest and most extensively studied gene family of mammalian genomes (*109*). All GPCRs share a common functional conformation of seven α-helical transmembrane regions but many with various function domains, especially in their highly diverse N-terminus. The main role of GPCRs is to recognize a diversity of extracellular ligands such as hormones, proteins and lipids and to transduce their signals into the cell (*110*). Virtually all types of cells express certain number of GPCRs, whereas the types of GPCRs expressed are usually tissue specific, a character that makes it an important target for pharmacological study. The family of GPCRs can be divided into seven subfamilies, base on their distinct nature of ligand binding, from Glutamate, Rhodopsin, Adhesion, Frizzled, Taste type2, Secretin to Olfactory receptors. All of the known LPA receptors belong to the rhodopsin family, in which some of the receptors take photon sensing molecules as their ligand. However, each of the EDG and P2Y family LPA receptors belongs to different group of rhodopsin family, α and γ group respectively (*109*).

As presented in the hierarchical tree of LPA receptors, the $zLPA_4$ and $zLPA_5$ clustered with their own cognate groups. Which separated away from the EDG family receptors, despite they all belong

to the rhodopsin family. On how that two sequential distant family can each evolved their own receptors that recognize the same ligand remains to be the most intriguing question in the research of LPA receptors. In 2009, Zhang et al. have presented an in-depth comparison of ground-state and activated-state LPA_4 crystal structure and found the plausible ligand binding loci of LPA_4. Based on their analysis, LPA was predicted to bind LPA_4 in an orientation similar to that reported for LPA_{1-3}, but through a different network of hydrogen bonds (*107*). On the other hand, as the protein sequence of zLPA5 verified in the evolutionary test, the receptor was found to be relatively more distant from its mammal analogues than other LPA receptors. Several causes may lead to the diversification of $zLPA_5$, either it play different signaling roles in zebrafish and mammals as in the case of LPA_6, or the function of $zLPA_5$ have been changed to meet the variated need of fish physiology after the splitting of tetrapods. But currently we do not have enough proof to support any of the theories.

After the name of G protein-coupled receptor, GPCRs signaling are majorly transduced through the binding of trimeric-heteromer G proteins, such as $G_{\alpha i/o}$, $G_{\alpha q/11}$,$G_{\alpha 12/13}$, and in the case of LPA_4, $G_{\alpha s}$. The trimeric G protein complex bind to the C terminus of GPCRs and dissociate upon ligand stimulation, thus the signaling property is largely depending on the C terminal sequence diversity of GPCRs (*4*). As previously presented (*4*), LPA_4 is the only LPA receptor that acitvates $G_{\alpha s}$ and thus has a significantly different C terminal sequence. In our protein sequence analysis of $zLPA_4$ and $zLPA_5$, most of the sequence variation were also centered at the cytosolic domain when compared to mammal parallels, which suggests that the intracellular binding property of $zLPA_4$ and $zLPA_5$ may not necessarily take after that of mammals. To approach this question, the binding preference of zebrafish G proteins must be studied, nevertheless little was known about zebrafish cognate G proteins.

The embryonic $zLPA_4$ mRNA expression begins at late development, which is similar as reported in mouse LPA_4 expression. However, LPA_5 was reported to be universally expressed at E12.5, which resembles the case we observed in $zLPA_5$. The resemblance of expression timing might contributes to a parallel developmental regulation, but more detailed information should be gathered both form mouse and zebrafish.

Functional verification of $zLPA_4$ and $zLPA_5$ has been tested *in vitro*, but no LPA specific intracellular calcium mobilization were observed in the exogenous overexpressed B103 and HEK293T cells. Combining the sequence variation of LPA_4 and LPA_5 between mammals and zebrafish, the zebrafish cognate LPA receptors might not capable of triggering conventional mammalian signaling cascades, which is observed in the study of $zLPA_1$.

However, by the microinjection of $zLPA_4$ and $zLPA_5$ specific morpholino, the down-regulation of $zLPA_4$ protein levels leads to severe defects in zebrafish vascular development, which was further confirmed through the negative control of five base mismatched $zLPA_4$ morpholino. The observation of developmental regulation of $zLPA_4$ is still under process, with the recently gained transgenic fish fli:EGFP that carry fluorescent blood vessels, and cardiovascular injection of Dextran-rhodamine.

Recently, several reports have revealed the regulative role of LPA in osteoblasts (*101*), osteoclasts (J Biol Chem. 2010 Jun 15. Epub ahead of print) and chondroprogenitor cells (J Bone Miner Metab. 2010 May 11. Epub ahead of print). Besides the inhibitory effect of LPA_4 in the LPA_1-dependent osteoblast differentiation, Sims et al. also presented that LPA is critical for the activation and survival of osteoclasts, which is collaborated by LPA_1 and a yet unknown LPA receptor. Furthermore, Hiraki et al. reported chondroprogenitor cells stimulation is modulated by LPA as well, which is majorly through LPA_{1-3}. In the bone development, chondrocytes usually comes

before osteoblasts and osteoclasts. Since LPA have been known to regulate chondrocytes through LPA1-3, whether LPA4 may also plays an inhibitory role against LPA_1 in chondrocytes remains to be our first question to be answered. On the other hand, Lee et al. had discovered several novel LPA_4 receptor agonists and inverse agonists through a clever screening strategy (ASSAY and Drug Development Technologies. 2010. Epub ahead of print), which may leads to a new page for the exploration of LPA_4 biological roles, especially when we could treat the zebrafish embryos with different agonists.

After all, there still many to be done to refine the arguments. First of all, the specificity and efficacy of the morpholino anti-sense binding should be clarified. Since 5 base mismatched morpholino could make a good negative control for the $zLPA_4$ morpholino, we need a better way to prove the level of protein down-regulation. For example, by transfecting each morpholino into cell lines with overexpressioned $zLPA_4$ protein C-termini fused with three tandem EYFP, each with different reading frames. Therefore, we can measure the knock-down level of $zLPA_4$ MO by the reduction ratio of EYFP fluorescence. Furthermore, the $zLPA_4$ MO microinjection must be done in the fli:EGFP transgenic zebrafish to show the exact level of vascular defects, and the defected vessel should be further analyzed to reveal whether they are blood vessels or lymphatic vessels. However, in this article, we identified the $zLPA_4$ and $zLPA_5$ genes, collected their expression profile in zebrafish, and found that $zLPA_4$ is essential for zebrafish vascular development.

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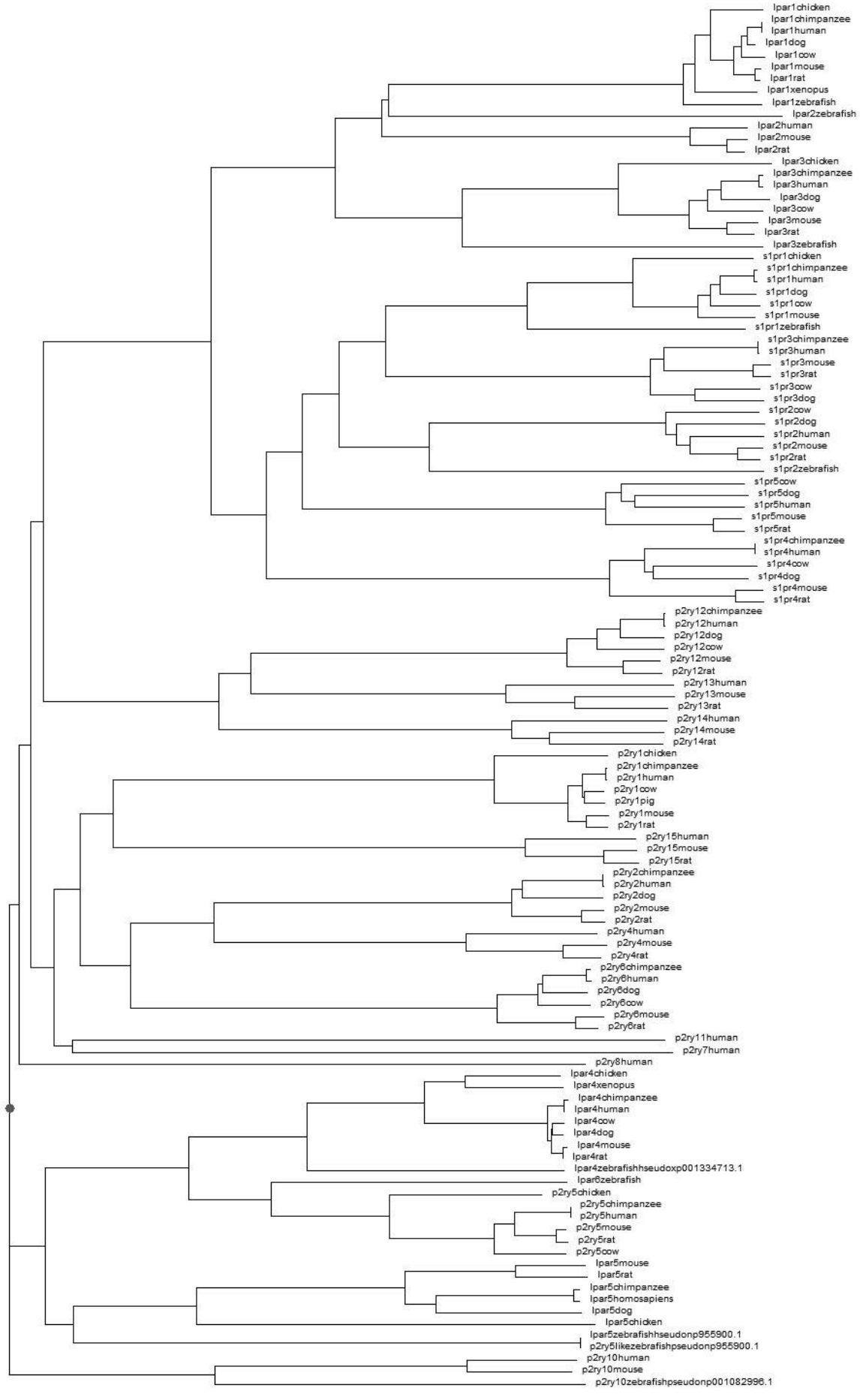
FIGURES

FIGURE.1 HEIRARCHICAL TREE OF LPA4 AND LPA5 (UPCOMING PAGE)

All available referenced LPA,S1P, and P2Y receptors protein sequence were retrieved from NCBI, and aligned by using ClustalX2. By taking S1P and other P2Y receptors as outgroup, we are able to identify two sequences as $zLPA_4$ and $zLPA_5$.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p2ry10mouse
p2ry10zebrafishpseudonp001082996.1

FIGURE.2A PHYLOGENETIC ANALYSIS OF LPA_4 AND LPA_5 IN DIFFERENT SPECIES.

a. protein sequence conserved alignment of human, mouse and zebrafish LPA4 and LPA5

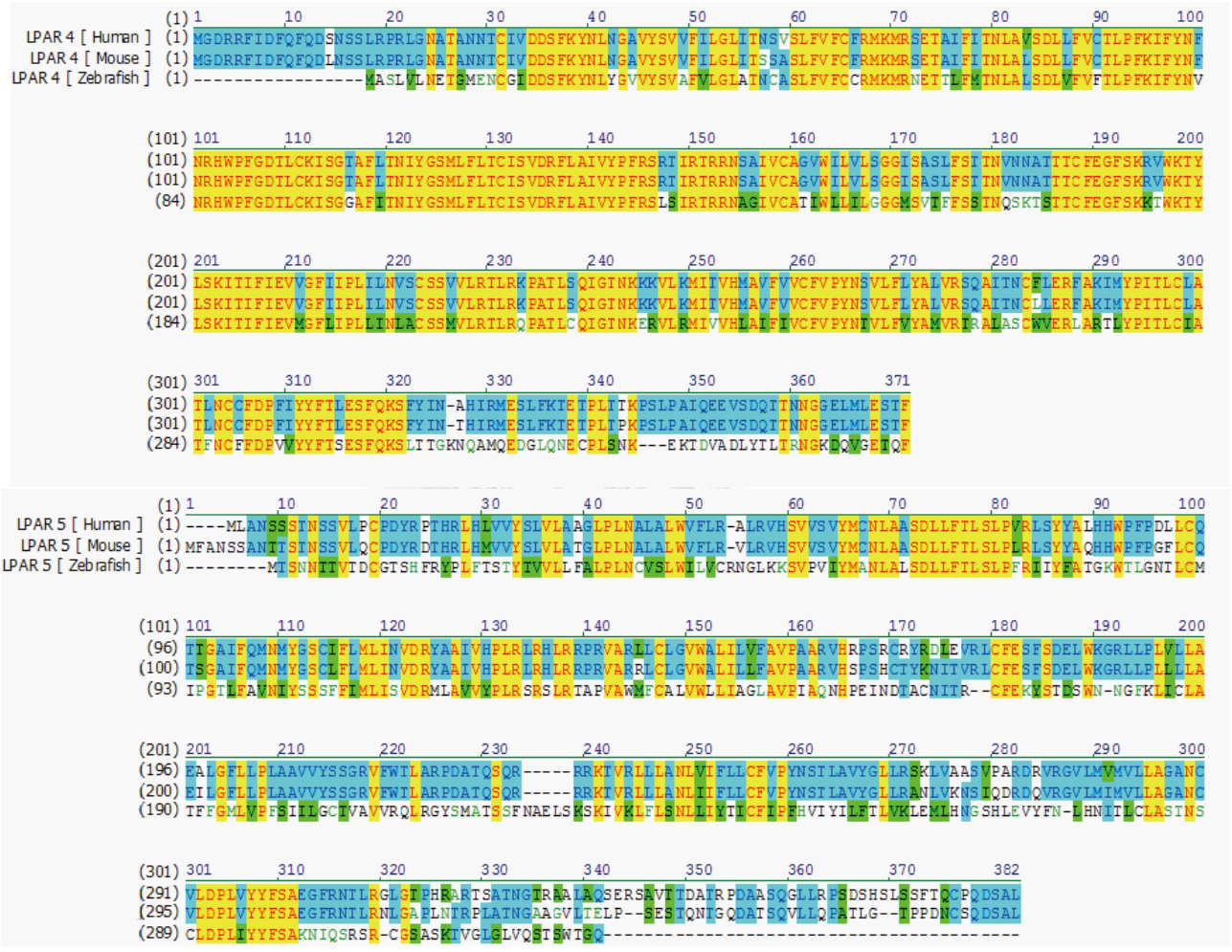


FIGURE.2B PHYLOGENETIC ANALYSIS OF LPA_4 AND LPA_5 IN DIFFERENT SPECIES.

b. percent similarity of LPA4, LPA5 and P2RY5

	Human	Mouse	Chicken	Zebrafish	(%)
$zLPA_4$	**100**	**98.1**	**79.7**	**60.9**	**Human**
		100	**79.7**	**61.2**	**Mouse**
			100	**68.6**	**Chicken**
				100	**Zebrafish**

	Human	Mouse	Chicken	Zebrafish	(%)
	100	**79**	**47.7**	**29.6**	**Human**
$zLPA_5$		**100**	**48.3**	**31.7**	**Mouse**
			100	**30.2**	**Chicken**
				100	**Zebrafish**

	Human	Mouse	Chicken	Zebrafish	(%)
zLPA6	**100**	**93**	**70.3**	**60.8**	**Human**
		100	**78.8**	**61.1**	**Mouse**
			100	**53.5**	**Chicken**
				100	**Zebrafish**

FIGURE.3 LPA INDUCED CALCIUM MOBILIZATION IN zLPA$_4$ OR zLPA$_5$ OVEREXPRESSED B103 CELLS.

LPA induced calcium mobilization were observed in zLPA$_4$ or zLPA$_5$ transiently expressed B103 cells, with S1P and Triton X-100 as positive controls. However, no significant LPA-dependant calcium mobilization were observed.

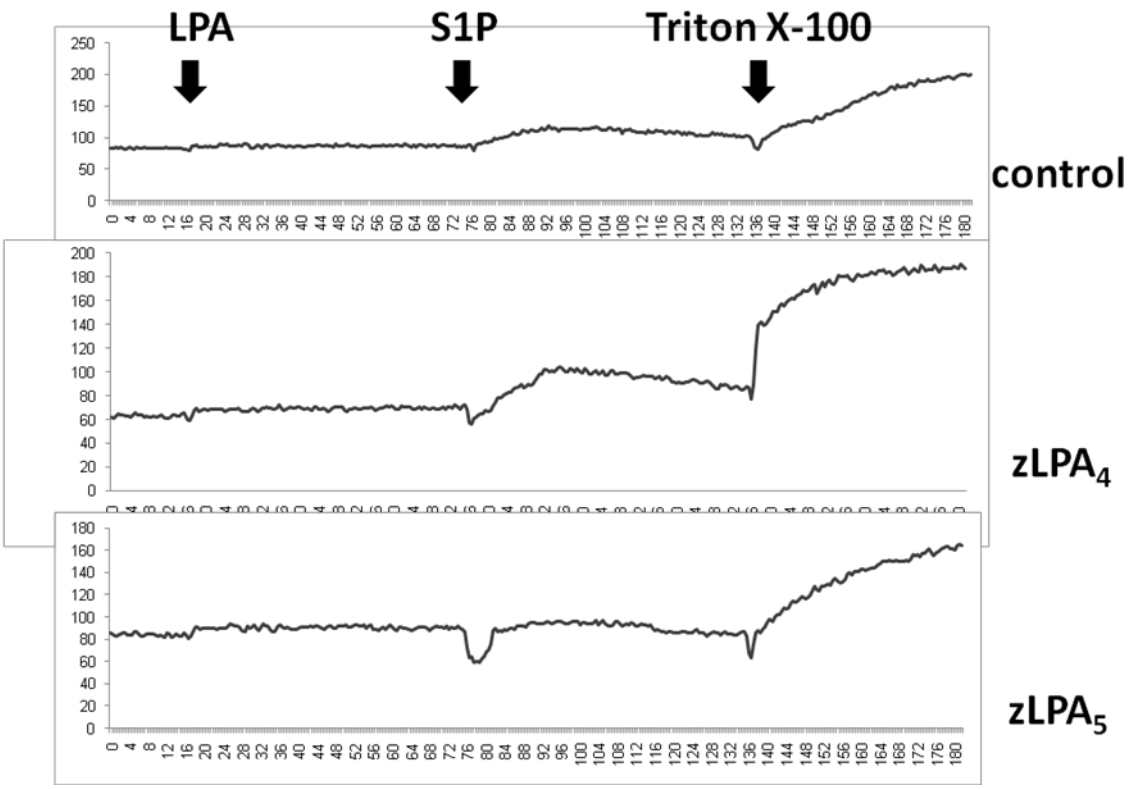
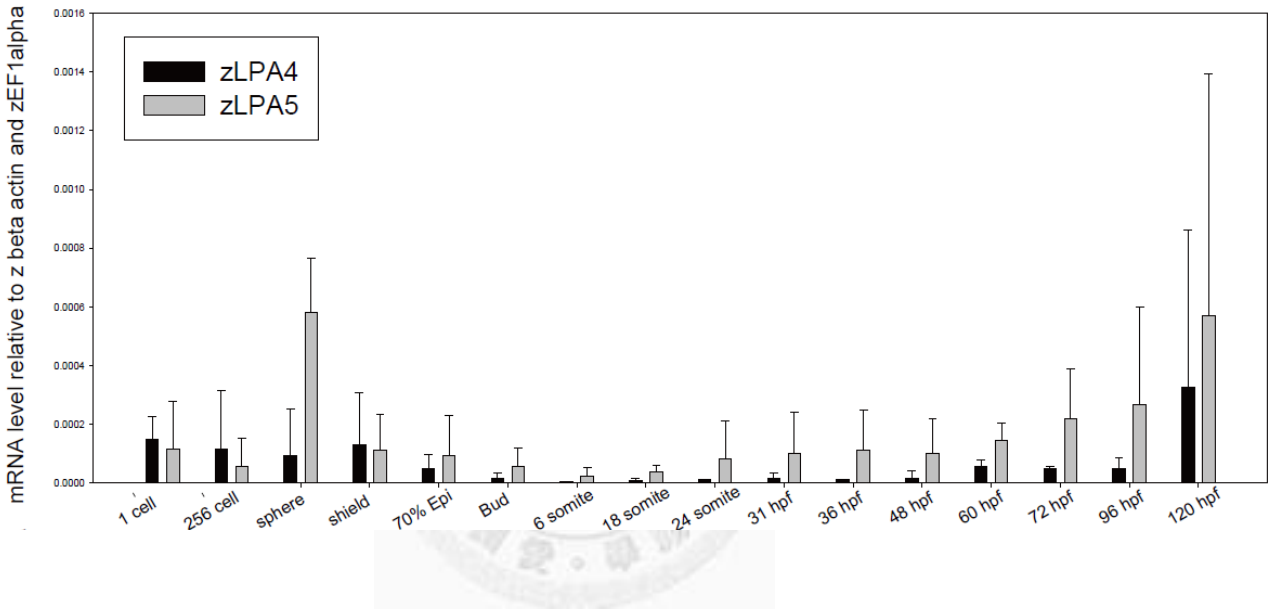


FIGURE.4 DEVELOPMENTAL STAGE DEPENDANT MRNA EXPRESSION OF ZLPA$_4$ AND ZLPA$_5$

zLPA$_4$ mRNA expression were observed at 120 hpf, and zLPA$_5$ mRNA were observed after 18 somites. The expression levels of reference genes along different stages were also presented, which shows that the expression levels of reference genes significantly increased after shield. The unexpected low level of reference gene expression explains the higher expression of zLPA$_4$ and zLPA$_5$ before shield, which might be maternal originated.



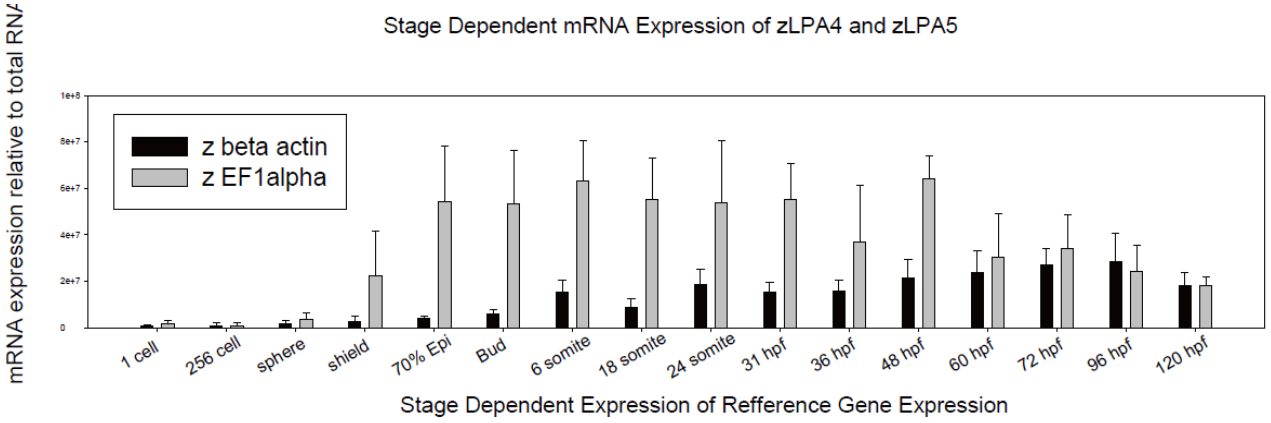


FIGURE.5 WHOLE MOUNT IN SITU HYBRIDIZATION OF zLPA$_4$ AND zLPA$_5$

By using antisense probe specifically binding of target mRNA, the dark blue region represent the expression pattern of target genes. However, no significant differences were observed in the comparison of antisense and sense probe staining.