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斑馬魚水解磷酸酯受器 4 功能之研究

The Investigation of LPA Receptor 4 Functions in Zebrafish

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本論文係 林予農君（學號 R99B41010）在國立臺灣大學動物學研究所、所完成之碩士學位論文，於民國 100 年 7 月 27 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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大學畢業正式加入 Lab504 後，我從大學部的身份轉為研究生。睽違了四年後我仍舊選擇了一樣的方向，著實感謝陳擎霞老師，崔文慧老師與曾婉芳老師在我大學時期帶領我瞭解這個領域的美，尤其謝謝吳書平老師每每提醒我前方道路與方向，如果沒有老師的慷慨指引，一切將困難許多。

從最初進行研究，開始面對科學的未知起，非常謝謝李心予老師耐心的引導我積極思考，老師的激勵一直鼓舞著我對研究的熱忱。感謝張百恩老師在實驗操作上詳細的指導，老師嚴謹的實驗態度讓我受益良多，同時，感謝大方給我實驗上建議與協助的黃銓珍老師與蕭崇德老師。另外，在我身為初學者時，給予無限包容，教我理解實驗原理、細心地一步一步帶我熟悉實驗、逐步引導我學會欣賞期刊論文中的科學奧妙與解答我所有疑惑的高笙詠學長，誠心感謝學長的傾囊相授。

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摘要

水解磷酸酯 (lysophosphatidic acid) 是一種結構簡單的水解磷酯 (lysophospholipid)，它與許多生理作用如傷口癒合、癌症惡化、心血管活動、神經調節運作、生殖系統調節、血小板活化與毛髮生長等過程皆密切相關。水解磷酸酯主要經由一具有水解磷酯酶 (lysophospholipase D) 活性的酵素 (autotaxin) 催化磷酯 (phospholipid) 分解而成，並進一步作為膜外訊息傳遞分子，以活化不同受器，如水解磷酸酯受器 1-6。為了解不同受器的功能，前人的研究利用催化水解磷酸酯生成酵素 (autotaxin) 基因被抑制的小鼠，分析在水解磷酸酯缺少的情況下小鼠所受到的影響，進而發現由於血管發育缺陷所導致的早期胚胎死亡。然而，在其他研究中，水解磷酸酯受器 1-3 基因被抑制的小鼠中並沒有類似的症狀出現。本研究探討水解磷酸酯受器 4，以期發現其可能活化的下游途徑。藉由斑馬魚平台，在注射抑制基因表現分子而導致水解磷酸酯受器 4 缺少的早期斑馬魚胚胎中，發現不正常的發育現象。除了可能由血管滲漏與淋巴管回收體液不完全所導致的心臟水腫，也同時觀察到心跳速率與血液流速的明顯降低。另外，體節間縱向血管數目的減少及異常的生長方向，也顯示出血管發育的異常。但是，在運用注射大分子葡聚糖 (dextran, tetramethylrhodamine) 觀察循環系統的結果中，淋巴管的發育並沒有受到明顯影響。總結以上，我們認為在斑馬魚中，水解磷酸酯 4 有調控血管發育的可能性。

關鍵字：水解磷酸酯，水解磷酸酯受器，血管，發育，斑馬魚

ABSTRACT

Lysophosphatidic acid, LPA, is a structurally simple lysophospholipid. Derived from phospholipids through autotaxin catalyzation, LPA acts as an extracellular signaling molecule that activates various receptors, LPA_{1-6}. Activation of these receptors induces wound healing, cancer progression, cardiovascular function, nervous system regulation, reproduction, platelet activation, hair growth, etc. To clarify the function of different LPA receptors, several knockout animals have been obtained. While the autotaxin deficient mice expressed the phenotype of embryonic lethality due to vascular defects, none of the single or double deficient LPA_{1-3} mice expressed similar phenotypes. In this study, I investigate the physiological roles of LPA_4, which is structurally distinct from LPA_{1-3}, using zebrafish as a model. LPA_4 morpholino was microinjected into one-cell stage zebrafish embryo to knock down the expression level of LPA_4 in embryos. In day two, edema around the pericardial region was observed, suggesting a vascular leakage or failure of lymphatic vessels to absorb body fluids in the LPA_4 deficient embryos, which was similar to the result of a previous study where LPA_1 was found to be essential for lymphatic vessel development in zebrafish. In addition, a significant decrease in heart rate and blood flow was observed in some individuals. Furthermore, the number of intersegmental vessels decreased, suggesting abnormal blood vessel development. However, the dextran uptake was not affected in

the LPA_4 knock down embryos, suggesting that the development of lymphatic vessels was normal. We conclude that LPA_4 may regulate the vascular development in zebrafish.

Key Words: lysophosphatidic acid, G-protein coupled receptor, blood vessel, development, zebrafish

CATALOG

INTRODUCTION

Metabolism of LPA

LPA, known as lysophosphatidic acid, is a lysophospholipid derived from cell membrane phospholipids. There are different species of LPA composed of glycerophosphate backbones with varying acyl chain lengths and degrees of saturation, but generally, LPA stands for 1-acyl-2-hydroxyl-sn-glycero-3-phosphate (1,2). It is generally detected in all eukaryotic cells at relatively low concentrations and in some biological fluids, with especially high concentrations in plasma. The most abundant forms of LPA in human plasma are 16:0, 18:2, and 18:1 LPA (3), and most of the commonly used LPA in signaling studies are in the form of 18:1 (8).

Two major pathways can produce LPA: the sequential activity of phospholipase D and phospholipase A2 and also by the activity of autotaxin (13, 19). Autotaxin was first found to be a cancer-cell motility-stimulating factor (14, 15, 16, 17) due to its assumed nucleotide phosphodiesterase activity (7). The significance of autotaxin was not fully understood until the knockout phenotype of a mouse gene, Enpp2 was observed; the gene was found to encode the autotaxin protein that was found to have lysoPLD activity (5, 18, 19, 20). The Enpp2 double null mice died with severe neural and vascular deficiency at early embryonic stages (6). Nevertheless, Enpp2

heterozygotes mice having plasma LPA levels lower than those of wild type mice survived to adulthood, which suggests that autotaxin is the major enzyme related to LPA production and signaling essential for development (20, 21).

Also, LPA acts as an extracellular signaling molecule through at least six known G protein-coupled receptors (32, 61), LPA_{1-6}, and is able to exert physiological, pathophysiological and morphological effects on many cell types (25, 26). LPA goes through diverse down stream signaling pathways, such as cell proliferation, survival (43, 54), migration (40, 41), and calcium mobilization (25, 42).

LPA receptors

Since the first LPA receptor were identified in 1996 (8), it has been reported that the effects of LPA are mediated through at least six LPA receptors, with all of them being G protein-coupled receptors (27, 57). At first, in the early twentieth century, LPA was thought to elicit from LPA1-3 (28, 29, 44, 45, 47), the classic LPA receptors that belong to the Edg family. But in 2003, the fourth LPA receptor was identified, which lead to the discovery of the fifth and sixth receptor (51). These three receptors are structurally distinct from Edg family and reveal different regulations (22, 23, 24, 48).

Functions of LPA receptors

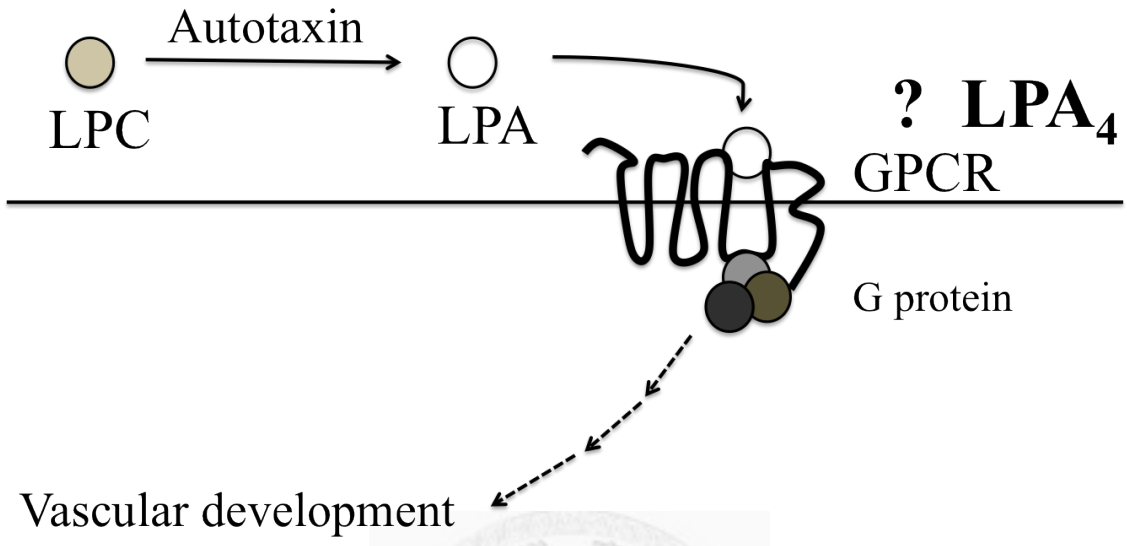
As a bioactive phospholipid, LPA has been implicated for important roles in many organ systems, such as wound healing (21), cancer progression (33), nervous system regulation (34), cardiovascular function (36, 60) and reproduction (37, 39). In order to investigate the function, which is difficult for the redundant function of various receptors, deficient animals have been acquired. Previous studies have used autotaxin deficient mice to discern the functions of autotaxin. The deficiency caused embryonic lethality due to vascular defects (5,6). Also, LPA receptor deficient mice have been obtained. There are single and double deficient mice of LPA_{1-3} (4). However, none of these strains exhibit a phenotype similar to autotaxin deficient mice (30, 35, 38, 46, 62). Since then, we have focused on zebrafish LPA receptors. Zebrafish are used in various projects because they are easy to collect large amounts of data from, are genetically easier to manipulate than mice, have transparent embryos and quickly develop into adulthood, yielding numerous advantages, such as the frequent and fast detections of lethal defects. The previous study published by our laboratory found that LPA_1 deficient zebrafish show abnormal lymphatic patterns (31). Unlike wild type fish that have thoracic duct beneath dorsal aorta, deficient fish have no formation of thoracic duct. Therefore, we presume that different LPA receptors, especially non-Edg receptors, might be necessary to recapitulate the phenotype of LPA deficiency. By

using morpholino microinjection, we intend to find out the precise regulation of LPA_4 in zebrafish.

RATIONALE

In order to clarify the downstream signaling pathway of autotaxin regulated vascular development in early developmental stage, we analyzed the phenotype of LPA_4 knock down zebrafish to investigate whether the structurally distinct non-EDG family LPA receptor 4 is the one that participates in it. For the strong correlation between autotaxin and production of lysophosphatidic acid, we think there is a possibility that LPA_4 deficient zebrafish may show similar symptoms as autotaxin deficient mice.

MODEL


Autotaxin
LPC
LPA
? LPA4
GPCR
G protein
Vascular development

MATERIALS AND METHODS

Maintenance of zebrafish

Wild-type AB strain and Tg(Fli: EGFP) transgenic strain were maintained at 27-28° C under a controlled 14-h light/ 10-h dark cycle. Embryos were staged according to morphology of Kimmel et al. (9).

The expression profiling of LPA4 in zebrafish by RT-PCR

Zebrafish RNA of different organs was collected in a similar procedure. In order to preserve as much RNA as possible, fish were sacrificed by being laid on ice for 5 minutes and dissected under binomial microscope as soon as the fish's gills stopped moving. Separated organs were homogenized in 1 ml TRIzol® (Invitrogen) using syringes with different tip (from 23G, 27G to 30G needles) openings as soon as possible. The homogenized organs were collected in TRIzol® and stored in -80°C until the time of RNA extraction. Upon RNA extraction, each vial of 1ml TRIzol® was added to 100 μl chloroform and vortexed for 30 seconds before being held on ice for 5 minutes. Then, it underwent phase separation with 15 minutes of 12000 rpm centrifugation at 4°C. The upper aquarius phase was then transferred to a new vial and added to 2× volume of 100% Ethanol and held for 15 minutes at -20°C followed by 15

minutes 12000 rpm centrifugation to precipitate RNA. Then the aquarius ethanol was carefully discarded, and the RNA pallet was washed with 70% ethanol and centrifuged at 3000 rpm for 5 minutes. Again, the upper aquarius phase was discarded and the RNA pallet air dried in a laminar flow for 15 minutes at room temperature and suspended in 50 μl DEPC water. The suspended RNA was heated on a hotplate at 55°C for 10 minutes before its density was measured with Nano Drop (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 liters of embryo cDNA were used as a template to undergo real-time PCR by using ABsolute™ QPCR SYBR® Green Mixes (Thermo Scientific) and iCycler Thermal Cycler (BioRad). LPA4 primer was as follow: intF' ATTGCTTCTTTGACCCGGTG, R' TCAGAACTGAGTCTCACCAA.

Microinjection procedures

At the night before mating morning, pairing a female zebrafish with a male in a single tank with mesh bottom to allow embryos to pass through and avoid being eaten. A transparent acrylic plate separates the pair from one another to help them get acquainted and is removed the next morning after the lights turn on. Embryos are collected after mating and transferred into petri dishes. Microinjection of LPA_4

morpholino into one-cell stage embryos is conducted. The injection volume is kept at 3nl within 4ng morpholino by measuring concentration and controlling exact time to avoid extending the injection point. After injection, embryos were cultured in tank water.

Design of morpholino oligonucleotides

Antisense morpholino were custom made by Gene Tools (Philomath). LPA_4 expression were knocked down by translation blocking morpholino (abbreviated as MO) of zebrafish LPA_4 designed as 5'-TCTCATTAAGAACAAGACTGGCC-3' and mis-paired control oligos of zebrafish LPA_4 morpholino (abbreviated as mis-MO) designed as 5'-TCTgAaTAAcAACAAcACTcGCCAT-3'. The morpholino was dissolved in 0.1% phenol red and buffer, and a final quantity of 4ng was microinjected into embryos.

Edema examination

In order to exclude abnormality caused by difference of diffusion. Homeostasis balanced environment was provided for embryos by using Danieau' s buffer (composed of 58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO3)_2$, 5 mM HEPES) during the maintenance.

Rhodamine-dextran injection

Fresh prepared dextran, tetramethylrhodamine (Invitrogen) were diluted with buffer to appropriate working concentrations. Microinjection was conducted carefully into zebrafish muscle under homeostasis environment. The dextran was given time to assimilate into the circulatory system. Then, permeability was assessed, along with length of thoracic duct, to evaluate lymphatic vessel development by observing the red fluorescence of dextran.

Microscopic analysis and live imaging

Zebrafish juveniles were first examined under dissecting microscope to check whole mount appearance, edema severity, heartbeats and blood flow. With limited water drop or 0.01% tricaine (Sigma) to restrict the movement of juveniles, further fluorescent image captures were implemented under LEICA DM13000B microscope.

RESULTS

Expression profile of LPA4 in zebrafish

In order to dissect the expression in various tissues of LPA_4 in zebrafish, the technique of real-time PCR was used to reveal the expression profile. The expression appeared at the gill, testis and spleen, tissues related with circulation and reproduction (Fig. 1).

Edema under zebrafish LPA4 morpholino microinjection

In order to certify the function of LPA_4 in zebrafish, we knocked down LPA_4 by using translation morpholino through early embryo stage zebrafish. Compared to the normal development of no injection wild type zebrafish (Fig. 2A) and the mis-MO control zebrafish (Fig. 2B), the LPA_4 deficient zebrafish showed edema around pericardial region (Fig. 2C and 2D) over half of the deficient zebrafish (Table 1). The severance of edema increased as the dosage of morpholino increased, different levels of phenotype appeared, ranging from fairly normal to moderate (showed slightly swelling at the edema region) and to severe (showed nearly doubled swollen to wild type). The final dosage of morpholino used was 4ng/μl to show the most obvious phenotype in the lowest number of fatalities. The edema indicates an accumulation of

interstitial fluids, which might result from vascular leakage or failure of lymphatic vessels to absorb fluids. Furthermore, the embryos were being examined in Danieau's buffer. While edema remain in balanced homeostasis fluid condition (Fig. 3B), it was certain that the edema wasn't caused by diffusion difference between zebrafish trunk and surrounding environment.

Abnormal heartbeat and circulation in LPA_4 deficient zebrafish

As embryos developed, slowing down of heartbeat (Fig. 4B), with almost forty percent of the LPA_4 knock down zebrafish (Table 2), was clearly observed in deficient zebrafish when compared to wild type (Fig. 4A). The heart rate difference between wild type and severe deficient was observed to be up to 60 beats per minute, dropping from 158 beat/ min in wild type to 127 beat/ min in severe deficient in average (videos are available at http://lab504.lifescience.ntu.edu.tw/3-林予農.htm). Also, the speed of blood flow decreased conspicuously in deficient fish. While the moderate ones show notable slowing down, some of the severe ones can easily see the scrolling of erythrocyte or even no blood flowing with very faint heartbeats.

Abnormal vascular pattern in LPA_4 knock down zebrafish

By observing Tg(Fli: EGFP) zebrafish, vascular pattern was easily revealed. As wild type (Fig. 5A and 5C) show orderly-arrangement and parallel intersegmental vessel, it was easy to position the abnormal deficient. Comparing the normal development of mis-MO injected zebrafish (Fig. 5B and 5D) to wild type (Fig. 5A and 5C), disarranged vascular pattern with partially developed intersegmental vessels were seen in MO injected juveniles (Fig. 6 and 7). As about forty percent of examined (Table 3) showed irregular vascular patterns at middle of trunk (Fig. 6), over twenty percent of examined (Table 3) were accompanied with disappearance of intersegmental vessel (Fig. 7) around the anterior (Fig. 7B) and posterior (Fig. 7C). As for the abnormal pattern, some of the abnormal vessels stopped at the middle of ventricle, some of which merged with another vessel nearby in an abnormal direction, and some of which missed the entire single intersegmental vessel (Fig. 6C).

Lymphatic development in LPA4 deficient zebrafish

In an attempt to find possible reasons for pericardial edema, we investigated the permeability in circular environment. By microinjecting dextran, hydrophilic polysaccharides absorbed by the circulatory system, we clarified the pattern and

development of lymph vessels. Unexpectedly, comparing with wild type (Fig. 8A), there was no apparent deviance of lymphatic system in LPA_4 deficient zebrafish (Fig. 8B). Indicating other reason then lymphatic abnormal causing pericardial edema.

DISCUSSION

Since the discovery of vascular development regulation by autotaxin in 2006 (5, 6), numerous studies have been exploring various possible pathways in detail. In our study, similar abnormality related to blood vessel development in LPA_4 deficient zebrafish were observed.

LPA_4 is classified with its own cognate groups, the purinergic family receptors, in compare with Edg family receptors, in compare with Edg family receptors (50, 52, 53). From the beginning of LPA_4 identification in 2003 that lead to the further verification of LPA_5 (55, 58) and LPA_6 (22), reasonable answers have appeared for previously unanswered questions. For instance, distinct ligand specificity induced by LPA was not accounted for by classic LPA receptors, and also, responses existed to LPA or its analogs without any EDF family receptors being detected. Thence, the exploration of purinergic family LPA receptors has been developed in recent studies.

In this study, all of the results presented were related to the circulatory system, with most of the evidence being related to blood vessel system. Because the pericardial edema positioning resembled our previous research about LPA_1 regulating lymphatic vessel development (31), we expected to find similar regulation in this study. However,

there was no significant evidence to further support the regulation of LPA_4 to lymphatic system.

As more results appear, concepts of the whole picture and of the details are revealed. Especially on LPA_4, studies published by Hayakazu, et al. (10) and Yukiura, et al. (11) have furthered the investigation in this field; this former research has shown credible results indicating the regulation of blood and lymphatic vessel formation by LPA_4. Generally similar to the phenotypes observed in autotaxin deficient mouse, the LPA_4 deficient mice in the former study showed various embryonic abnormalities including dilated blood and lymphatic vessels with bleeding to edema.

In addition, the later research focused on the regulation of autotaxin with vascular development, so the deficient zebrafish of single knock down or double knock down of different LPA receptors have been obtained. Results show dissociation between dorsal aorta and segmental arteries on intersegmental vessel under co-injection of LPA_1 and LPA_4 (11). As for our results, the irregular intersegmental vessel development has been curtained under LPA_4 knock down only.

As our research continues, previous studies have shown results agreeing with our prediction and hypothesis. During mouse embryogenesis, LPA_4 might regulate blood and lymphatic vessel formation for the dilate blood vessels with bleeding and dilated lymphatic vessels with edema, which match our discovery of abnormal vascular pattern. While the study used splice-inhibiting morpholino that inhibit translation, we used translation-blocking morpholino that directed against the initiation codon of target mRNA. The treatments of multiple LPA receptors morpholino results show that LPA_1 and LPA_4 double knock down zebrafish have abnormal segmental artery development, which agrees with our LPA_4 single knock down result of intersegmental vessel deficiency. As more and more clues come out, it is clearer to picture the function of LPA receptor 4.

Furthermore, certain studies about the relationship of nervous and vascular development (12) have shown guidance conjunction in between. After nervous development, several signs indicate that lymphendothelial cells were guided by the growth of RoP axon and associated axons, which yields another insight into the involvement of nervous system in upstream pathway.

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FIGURES

Fig. 1 Expression profile of LPA_4 in zebrafish

LPA_4 mRNA expression were specifically detected at gill, testis and spleen among adult zebrafish organs.

Fig. 2 Edema under zebrafish LPA4 morpholino microinjection

At 2dpf, moderate (C) and severe (D) edema around pericardial region was observed in LPA_4 knock down in zebrafish, while no injection wild type juvenile (A) and mis-morpholino injected control zebrafish (B) showed normal development.

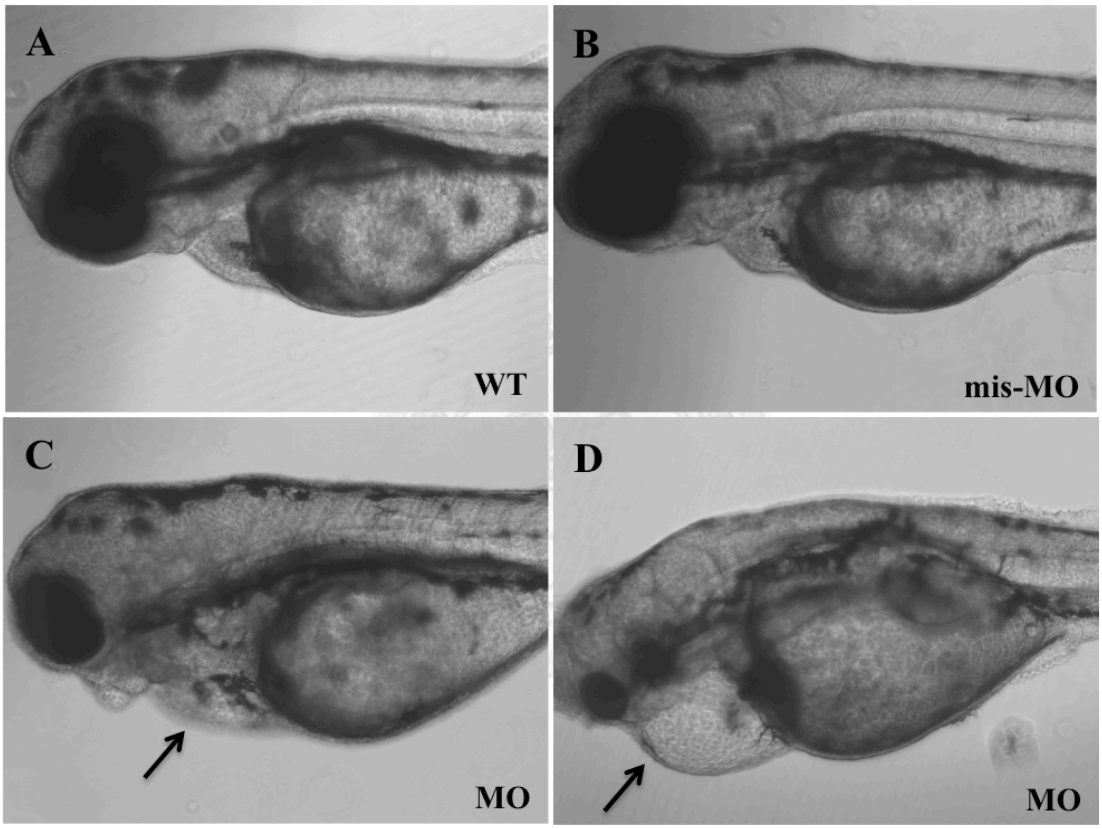


Fig. 3 Edema reamain under homeostasis condition

Comparing to the previous environment which juvenile maintained in tank water (A), edema around pericardial region still existed with Danieau's buffer (B).

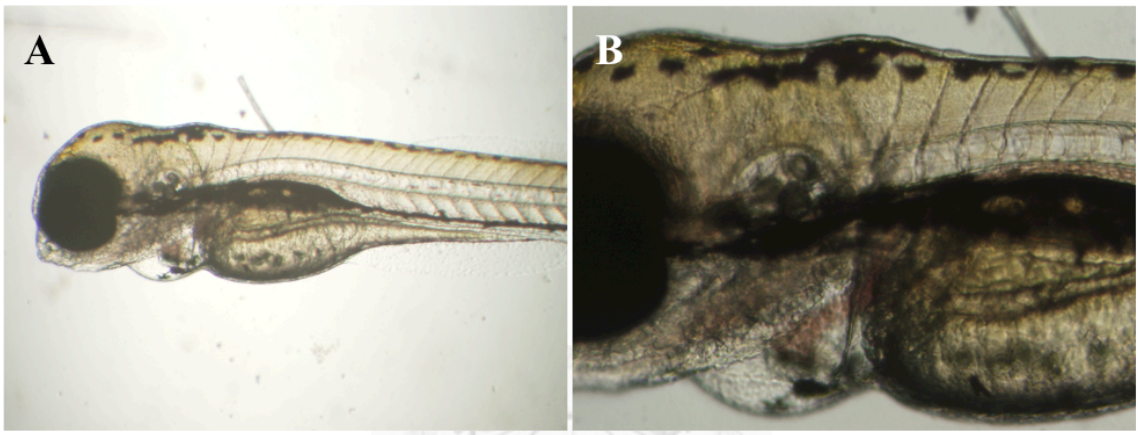


Fig. 4 Heartbeat and blood flow decrease under zebrafish LPA_4 morpholino microinjection

Video recordings of heartbeat of wild type (A) and LPA_4 knock down (B) zebrafish. As heart rate of wild type zebrafish is around 160 beat/min, LPA_4 deficient zebrafish has a conspicuously slower average heart rate of 130 beat/min (C). The videos are available at http://lab504.lifescience.ntu.edu.tw/3-林予農.htm.

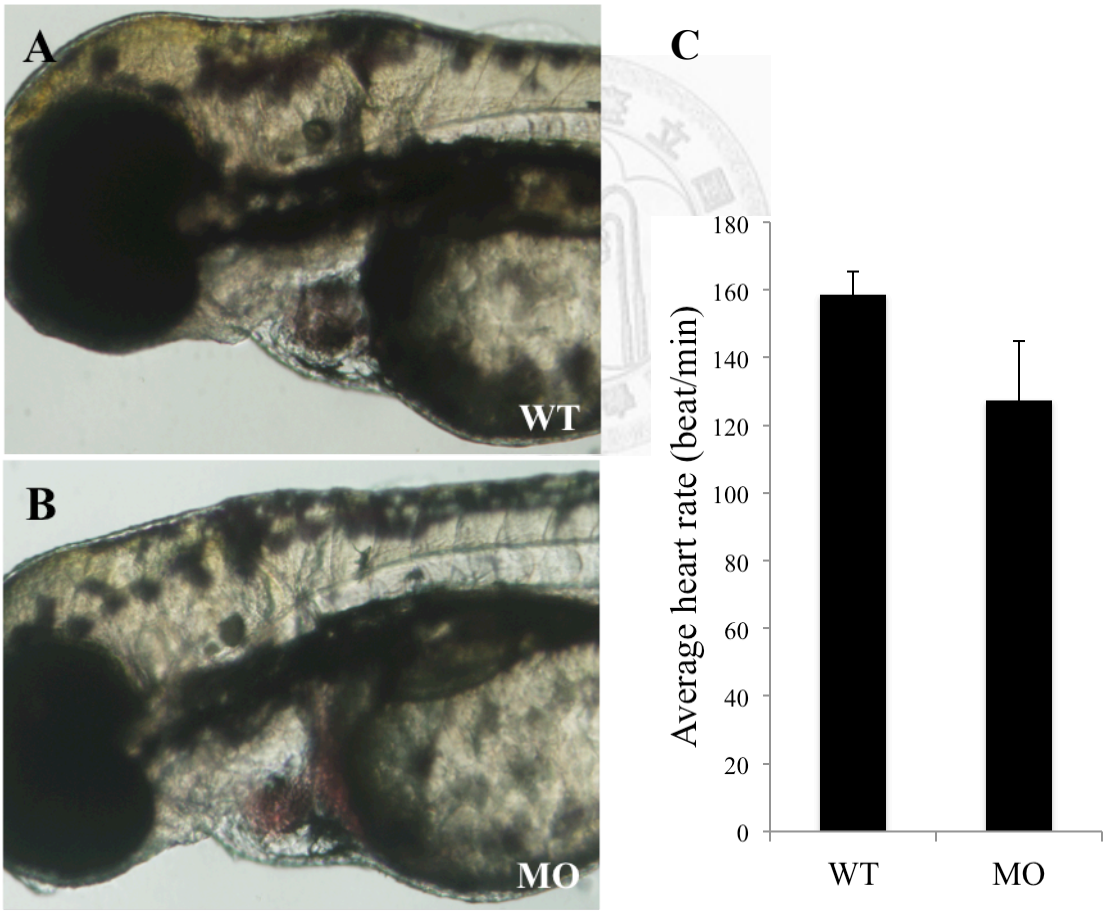


Fig. 5 Normal developed vascular pattern

In Tg(Fli: EGFP) zebrafish line, vascular pattern is clearly shown at 2dpf. It is obvious that both wild type (A) and mis-MO injection (B) zebrafish show orderly distributed vascular pattern. Also the intersegmental vessels of wild type (C) and mis-MO (D) are completely developed.

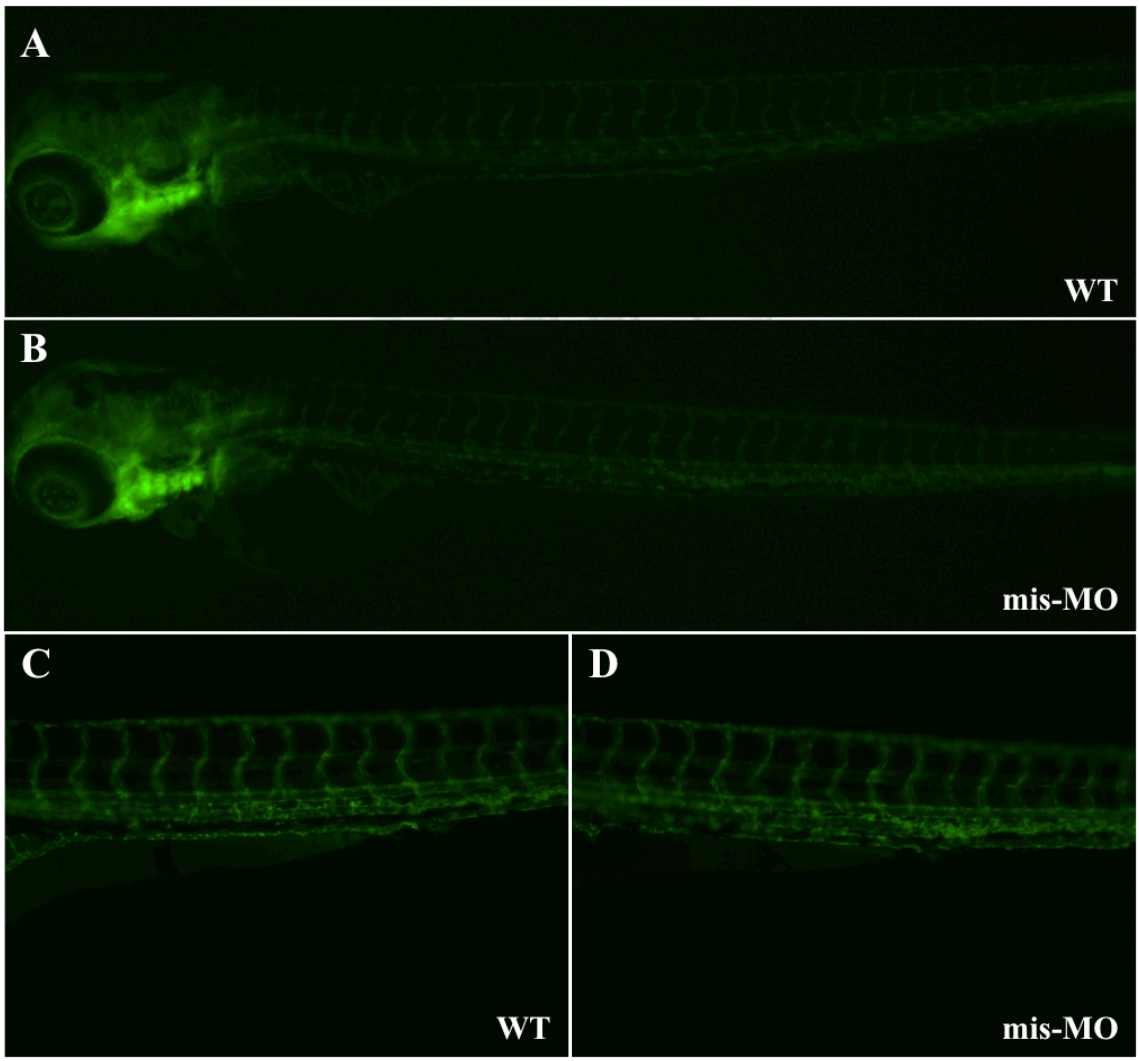


Fig. 6 Abnormal of vascular pattern in knock down LPA4 zebrafish

LPA_4 MO injection zebrafish (A) show abnormal development in vascular pattern. The intersegmental vessels grow in unparalleled direction (B, an enlarged image of square region of A) and some even merged with other vessels (C, an enlarged image of square region of B) indicate abnormal development with irregular vessel structure.

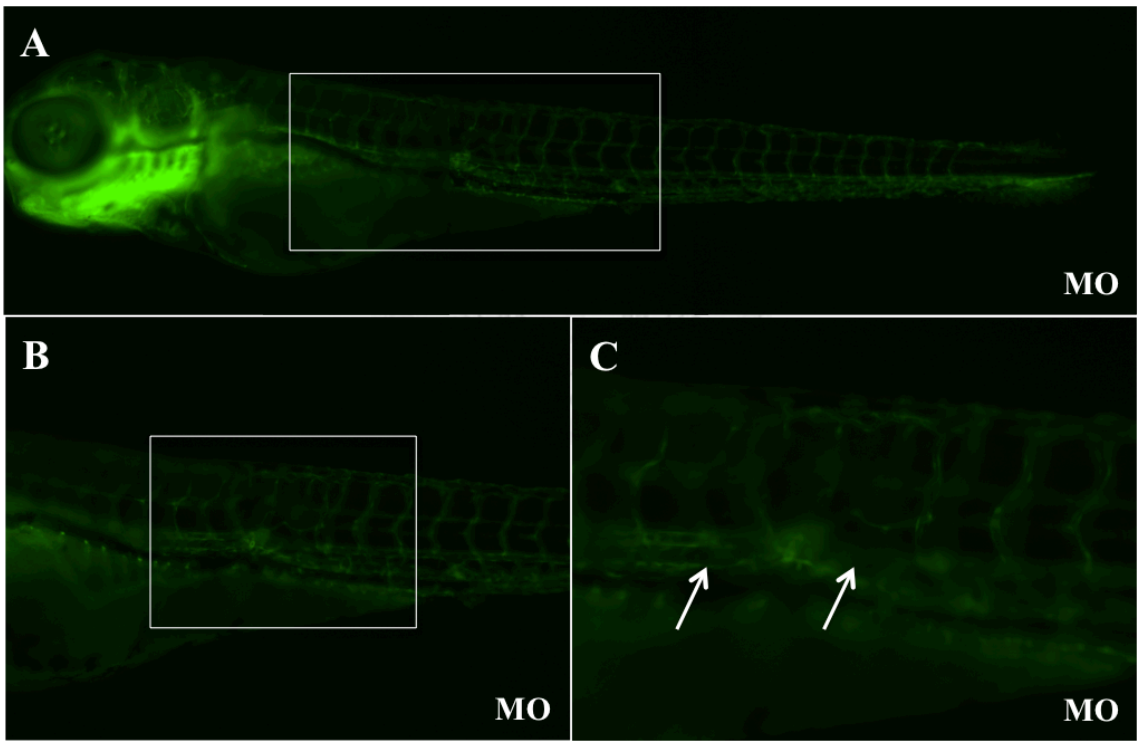


Fig. 7 Absence of intersegmental vessels in knock down LPA4 zebrafish

Some of the deficient zebrafish (A) show absence of intersegmental vessels. With some absences located near anterior region (B, an enlarge image of the upper square region of A) and some of others located around posterior region (C, an enlarge image of the lower square region of A).

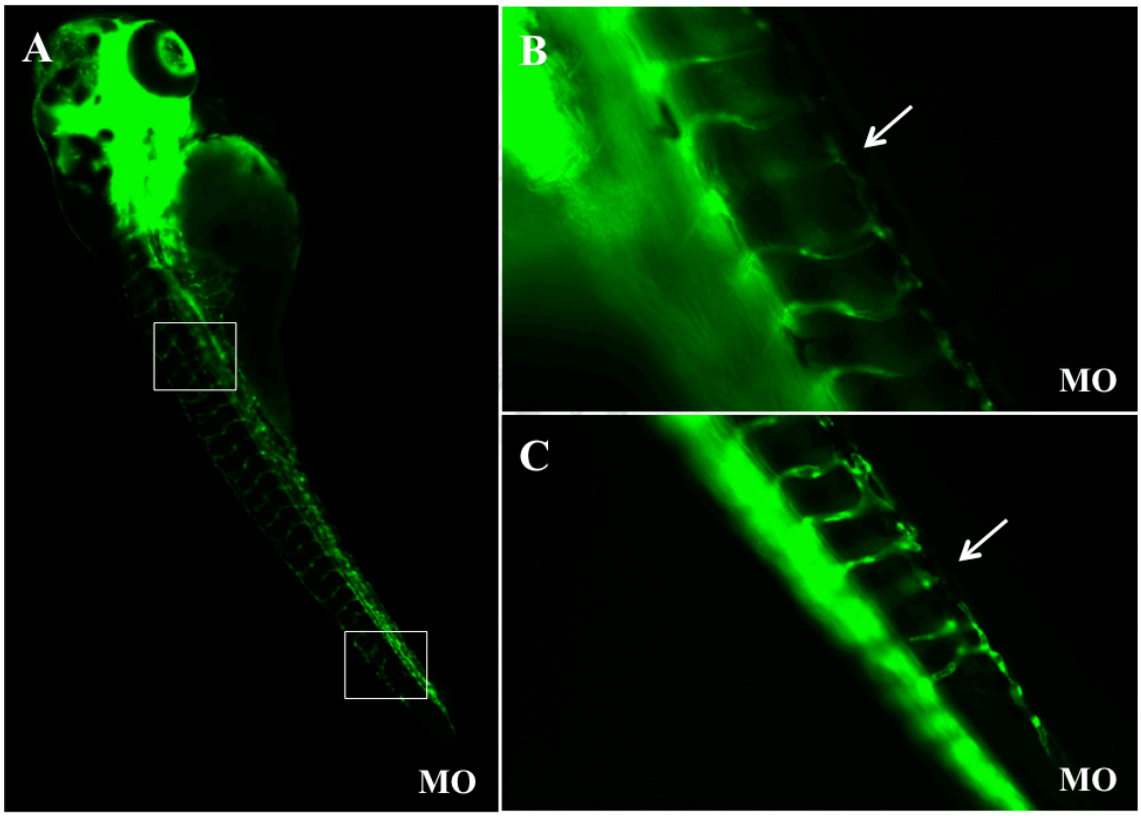
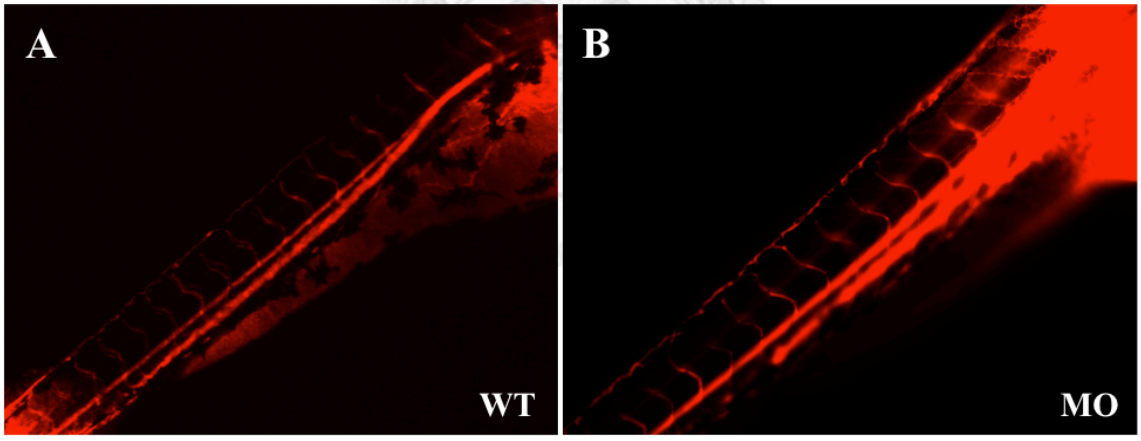


Fig. 8 Lymphatic development under zebrafish LPA_4 morpholino microinjection

Lymphatic development was analyzed by dextran, which will be absorbed into the circulatory system upon microinjection and provide a clear image of permeability in vascular vessels. As the injection was conducted at 3dpf, there was no apparent defect in the main blood vessel or the lymphatic vessel in both wild type (A) and LPA_4 knock down zebrafish (B). There was also no visible decrease of permeability in intersegmental vessels.



TABLES

Table 1 Analysis of pericardial abnormality under knock down of LPA_4 in zebrafish

		Pericardial edema				
condition	dose	%	n	total	moderate %	severe %
mis-MO	4ng	12%	6	49	4%	0%
MO	4ng	51%	35	68	17%	34%

Table 2 Analysis of blood circulation slowdown under knock down of LPA_4 in zebrafish

		Heart rate decrease		
condition	dose	%	n	total
mis-MO	4ng	2%	1	43
MO	4ng	39%	12	30

Table 3 Analysis of vascular defects under knock down of LPA_4 in zebrafish

		Intersegmental vessel (ISV) defects					
		Abnormal ISV			Absence ISV		
condition	dose	%	n	total	%	n	total
mis-MO	4ng	9%	4	44	0%	0	0
MO	4ng	42%	28	65	23%	15	64