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MOLECULAR CLONING AND FUNCTIONAL ANALYSIS OF SCAVENGER RECEPTOR ZEBRAFISH CL-P1

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Running head: Zebrafish scavenger receptor CL-P1in embryogenesis
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ABSTRACT
Background: Scavenger receptors are generally expressed in macrophages and vascular endothelial cells and some scavenger receptors are thought to contribute to the development of atherosclerosis.
Methods: We cloned the cDNA of a zebrafish CL-P1 (collectin placenta 1) and performed a knockdown study using its antisense morpholino oligonucleotides (MO).
Results: Zebrafish CL-P1 (zCL-P1) is 51% identical to human CL-P1 in its amino acid sequence. Microbes and OxLDL bound to zCL-P1 cDNA transfected cells. zCL-P1 mRNA expression gradually increased after 6 hours post-fertilization (hpf), reached its highest level at 24 hpf, and then decreased, which is similar to the gene expression pattern of Tie-2. The knockdown of zCL-P1 led to an increase in the number of zebrafish embryos with severe morphological abnormalities such as short body lengths and defects in the dorsal aorta at 48 hpf. Simultaneous injection of both MO and synthetic zCL-P1 or zVEGF mRNA rescued the abnormal phenotype.
Conclusions: In vivo knockdown study shows that zCL-P1 is implicated in vasculogenesis and those of our *in vitro* study support its role as a scavenger receptor.
General Significance: These results suggest that zCL-P1 might be essential for vasculogenesis during the early embryonic phase in bone fish.

1. Introduction

Collectins are a family of C-type lectins harboring collagen-like sequences and carbohydrate recognition domains (CRD) [1]. They are involved in host defense through their ability to bind to carbohydrate antigens on microorganisms [2-7]. We have demonstrated recently that CL-P1 (collectin placenta 1) is a novel member of the collectin family. CL-P1 is a first membrane-type collectin which is mainly expressed in vascular endothelial cells and can bind and phagocytose bacteria as well as yeast [8]. Furthermore, it binds to oxidized low density lipoprotein (OxLDL) as a scavenger receptor [8]. Scavenger receptors are generally expressed in macrophages and vascular smooth muscle cells, in which circulating lipids have accumulated, suggesting they might contribute to the development of atherosclerosis [9, 10]. However, deletion studies using knockout mice indicate that the main role that scavenger receptors play in innate immunity is to eliminate invading microbes from the host body [11].

Angiogenesis and vasculogenesis are common biological processes that occur during embryogenesis and the menstrual cycle, and various genes are required for both. In the present study, we hypothesized that CL-P1 might be involved in the embryonic development of blood vessels in zebrafish since the CL-P1 gene in zebrafish has a high homology to its human and mice counterparts and it is produced in vascular endothelial cells [8,12]. The zebrafish embryo is excellent model for studying the genes and proteins that regulate embryonic development of blood vessels [13, 14]. In this investigation, we carried out a knockdown study of the zebrafish CL-P1 gene and found that its deletion caused a malformation of vascular vessels and subsequent developmental defects.

2. Materials and methods

2.1. Zebrafish maintenance

Zebrafish (*Danio rerio*) were maintained in a laboratory breeding colony at 28.5°C on a 12 hr light/12 hr dark cycle in accordance to the guidelines of the Ethics Committee of the Asahikawa Medical University. Embryos were collected from the natural mating of breeding pairs, maintained at 28°C, and staged according to hours post-fertilization (hpf).

2.2. *Cloning of zebrafish CL-P1 cDNA*

We observed amino acid sequences of human and mouse of CL-P1 and subsequently searched the zebrafish EST database for corresponding sequences, and found several cDNA fragments with high homology. We purified the mRNA from adult zebrafish using TRIZOL LS Reagent (Invitrogen) and a NucleoTrap mRNA Mini Kit (Macherey-Nagel) and established a cDNA library using a SMART RACE cDNA Amplification Kit (BD Biosciences). To generate a part of the zebrafish CL-P1 cDNA, primers based on the human CL-P1 (hCL-P1) cDNA (5'-TGTAAAAACGACTGGGCCCTGAG-3') were used, with the cDNA library. An amplified 106-bp PCR product containing the putative CL-P1 was sequenced. In addition, the full size cDNA was obtained by reverse transcription PCR from adult zebrafish mRNA using a SMART-RACE cDNA Amplification Kit (BD Biosciences) according to the manufacturer's protocol, was subcloned into the pT7Blue T-vector (Novagen) and was sent for sequencing.

2.3. Antibody

Expression of the in zCL-P1 (amino acids 572-733 of zCL-P1) in *E.coli* was carried out as described previously [8]. The affinity-purified zCL-P1-CRDhis rabbit antisera were also prepared by previous methods [8]. The antibody titer was measured by ELISA (data not shown). The characterization of antibody was certified by western blotting using zCL-P1cDNA transfected CHO cells and hCL-P1cDNA transfected cells. Anti-Myc murine monoclonal antibody, anti-mouse-IgG-conjugated Alexa 594, anti-mouse-IgG-conjugated Alexa 488, anti-rabbit-IgG-conjugated Alexa 488, and anti-rabbit-IgG-conjugated Alexa 594 from Molecular Probes were used at the proper concentrations as indicated in the manufacturer’s instructions.

2.4. Cell culture and isolation of a transfected cell line

Chinese hamster ovary-ldlA7 cells (CHO-ldlA7 cells) lacking functional LDL receptors was a gift of M. Krieger (MIT, USA), were maintained at 37°C in Ham's F-12 medium (Sigma) containing 5% fetal bovine serum (Invitrogen) [15]. A full-length zCL-P1 cDNA was amplified from our zebrafish cDNA library by PCR primers 5'-TCCCCGCGGATGAAGGACGACTTCAATGAT-3' and 5'-GAATCCAAAGTTCCAGTTTTACCGCGGGGA-3', subcloned into pcDNA3.1/Myc-His A vector (Invitrogen), sequenced, and transfected into CHO-ldlA7 cells using the polyethylenimine (PEI) based method. CHO/hCL-P1 was used as positive control cells [8]. Negative control cells were prepared to be transfected with above empty vector.

2.5. Analysis of biochemical properties in zCL-P1

The transient zCL-P1 transfected cells (CHO/zCL-P1) or control cells were plated at a density of $3x10^4$ cells / 0.2 ml in 14-mm wells of 35-mm plastic culture dishes and cultured in Ham's F-12 medium containing 5% fetal bovine serum. Membrane immunofluorescence analysis of CL-P1 in these CHO cells was performed. CL-P1 protein was detected using 0.24 μg/ml anti-Myc murine monoclonal antibody and anti-mouse-IgG-conjugated Alexa 594. The microorganism binding assay was performed with Zymosan A (*Saccharomyces cerevisiae*), *Staphylococcus aureus*, and *Escherichia coli* BioParticles conjugated with Alexa 488 (Molecular Probes). CHO/zCL-P1 cells or control cells were incubated at 4°C for 30 min with 50 μg/ml of these three BioParticles. After binding, cells were fixed at room temperature for 30 min with 4% paraformaldehyde in PBS and stained with anti-Myc antibody and anti-mouse-IgG-conjugated Alexa 594. In lipoprotein binding assay, cells were incubated at 4°C for 30 min with DiI-OxLDL, DiI-AcLDL, and DiI-LDL then washed with PBS, and fixed using 4% paraformaldehyde in PBS at room temperature for 20 min. Cells were washed and incubated with anti-Myc antibody and anti-mouse-IgG-conjugated Alexa 488 [8]. After washing, all cells were treated with 1 drop of Slowfade Antifade Reagent (Molecular Probes), mounted, and sealed. A fluorescence microscopy IX 70 (Olympus) was used. The monosaccharide specificities of zCL-P1-CRDhis, hCL-P1-CRDhis, and CHO/hCL-P1 soluble extracts were analyzed using sugar blot method [7,8]. Their protein solutions were dot-blotted on the membrane and incubated with sugar BP-probes: α-D-mannose, α-L-fucose, α-D-glucose, α-D-galactose, β-D-galactose, β-N-acetyl-glucosamine, α-N-acetyl-galactosamine, or β-N-acetyl-galactosamine. Then the membranes were incubated with streptavidine-biotinylated HRP (Chemicon International) and visualized using ECL reaction and were detected with an LAS 3000 [7].

2.6. Real time RT-PCR analyses

Ten zebrafish embryos each were harvested at 1-48 hpf. Total RNA was extracted using TRIZOL LS Reagent. First-strand cDNA was reverse transcribed using random hexamer primers and reverse transcriptase (TaqMan). For amplification of zCL-P1, zVEGF, zKDR, zFlt-1, zTie-2 and β-actin, we used TaqMan Gene Expression Assays and primers, and then employed an Applied Biosystems 7500 Real Time PCR System (Applied Biosystems).

2.7. Whole-mount in situ hybridization

Two antisense digoxygenin-labeled riboprobes of the zCL-P1 riboprobe corresponding to the CRD and a zKDR riboprobe were synthesized using a DIG RNA Labeling Kit (Roche Diagnostics). Whole-mount *in situ* hybridization of zebrafish embryos at 24 hpf was performed using an *In Situ* Hybridization Kit (R&D Systems) according to manufacturer's instructions. Images were observed using SZ12 stereomicroscope (Olympus).

2.8. Immunohistochemistry

Zebrafish embryos at 48 hpf and larvae at 2 months post-fertilization were extracted, fixed with 4% paraformaldehyde in PBS at 4°C for 30 min, dehydrated, embedding in paraffin and 5 μm transverse sections were prepared. Serial specimens were stained immunohistochemically or with hematoxylin-eosin [16]. After incubation with 0.01 M citrate buffer at 95°C for 20 min, sections were blocked at room

temperature for 1 hr and incubated with 10 μg/ml anti-zCL-P1 antibody or pre-immune rabbit serum as control antibody at 4°C overnight. The specimens were washed and incubated with anti-rabbit-IgG-conjugated Alexa 594 or anti-rabbit-IgG-conjugated Alexa 488 at room temperature for 40 min. Fluorescent images were observed with the confocal laser-scanning microscope FV1000 (Olympus) with/without differential interference microscopic analyses.

2.9. Preparation and injection of antisense morpholino oligonucleotides and mRNA of zCL-P1 cDNA

Antisense morpholino oligonucleotides (MO) were designed with sequences complementary to zCL-P1 cDNA starting around the initiating start codon based on the company's recommendations (Gene Tools). The MO sequences were: zCL-P1 MO 1, 5'-GTCGTCCTTCATCGTGGTCAAACTG-3'; zCL-P1 MO 2, 5'-GCACCGTTCCGTCACGAGCCAAACC-3'; zCL-P1 MO 3, 5'-ACATCAATCTCATGTCAACGCGCAG-3'; zCL-P1 5-base mismatch MO 2 (MO2-5MM), 5'-GCAgCGTTgCGTgACcAGCgAAACC-3'. An inert standard MO (5'-CCTCTTACCTCAGTTACAATTTATA-3'), which has no effect on zebrafish development, was used as a control. MOs were microinjected into 1-4 cell stage embryos as described [17]. The injected embryos were then incubated at 28°C. For preparations of 5'-capped mRNA, zCL-P1/pcDNA was linearized and transcribed with T7 RNA polymerase using an mMESSAGE mMACHINE In Vitro Transcription Kit (Ambion) according to the manufacturer's instructions. The synthesized mRNA and MO2 were co-injected into the yolks of embryos at 1-4 cell stages. The zCL-P1 mRNA sequence did not overlap that of MO2.

2.10. Microangiography and alkaline phosphatase staining

Fluorescein isothiocyanate-dextran (FITC-dextran) was injected into the sinus venosus of the anesthetized 48 hpf embryos [14]. For staining zebrafish vascular endothelial cells, some embryos were picked up at 48hpf and fixed with 4% paraformaldehyde in PBS for 30 min at room temperature, then stained with nitro-blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3'-indolylphosphatase p-toluidine salt (BCIP) as described [18,19]. Visualization and photography were performed on an SZ 12 microscope (Olympus).

2.11. Western blotting

Western blotting was performed to identify CL-P1 protein expression in CHO/zCL-P1 and CHO/hCL-P1 and the fish embryos after fertilization. Cells and ten embryos at 48 hpf were homogenized in 1x Tris-SDS, β-ME sample buffer, subjected to 4-20% SDS-PAGE and transferred to a polyvinylidene difluoride membrane using a semi-dry transfer apparatus. After blocking with blockace at 4°C overnight, the membrane was incubated with 5 μg/ml anti-zCL-P1 rabbit polyclonal antibody or anti-Myc monoclonal antibody for 1 hour. After washing with TBST, the membrane was incubated with HRP-conjugated anti-rabbit-IgG or anti-mouse-IgG for 1 hour. After washing with TBST, chemiluminescence was examined using the ECL reaction and detection with an LAS 3000 was carried out.

2.12. Rescue of zCL-P1 activity by zCL-P1 or zVEGF mRNA injection to zCL-P1 knockdown fish

For the preparation of mRNA, Capped mRNA was synthesized from the zCL-P1 or zVEGF construct using mMESSAGE mMACHINE (Ambion) according to the manufacturer's instructions. The synthesized zCL-P1 mRNA or zVEGF mRNA and MO2 were simultaneously injected into embryo yolks [20]. For the control experiment, the same amount of MO2, but without the zCL-P1 or zVEGF mRNA, was used instead.

2.13. Statistical analysis

The results are expressed as means ± SEM. Statistical analysis was performed by repeated measures ANOVA and a subsequent Fisher's LSD test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. *Isolation of a zebrafish CL-P1 cDNA*

The predicted zCL-P1 ORF encodes a 740 amino acid (aa) protein that is almost similar in size to hCL-P1 (742-aa) (Fig. 1A, B) [8]. zCL-P1 contains a 39-aa putative cytoplasmic peptide, a 23-aa transmembrane domain, and a 678-aa extracellular domain harboring a coiled-coiled region, a collagen-like domain and a carbohydrate recognition domain (CRD). An endocytosis motif (Tyr-Lys-Arg-Phe) in Fig.1A is present in the intracytoplasmic domain of zCL-P1 as in hCL-P1. The hydrophobicity plot and amino acid sequence analysis showed that the domain structure of zCL-P1 is the same as that of human CL-P1 (Fig. 1B). Overall, zCL-P1 is 51% identical to hCL-P1 and 52% identical to mouse CL-P1 in terms of its amino acid sequence (Fig. 1C). The intraplasmic and transmembrane domains in whole domain structures in human and zebrafish CL-P1 have a high homology (79 and 83%, respectively). The collagen domain has 62% homology to the human type and has 46 Gly-X-Y cycles compared with the 49 cycles of hCL-P1 collagen-specific tripeptides. zCL-P1 has two polycationic regions in the collagen domain which contains basic amino acids (arginine or lysine), although hCL-P1 has three regions and mouse SR-A has only one. The polycharge islands in the collagen-polymer structure form a strong binding site for negatively charged substances [21]. zCL-P1 has a C type lectin consisting of six cysteine residues as does hCL-P1, and its ligand specificity is of the galactose type (Gln-Pro-Asp) instead of the mannose and glucose types (Glu-Pro-Asn). CRD homology between humans and zebrafish is 52%, less than the homology of other major domains.

3.2. Expression and characterization of zCL-P1 in CHO cells

The Fig. 2A showed pcDNA3.1/Myc-His A vector using the transfection vector of zCL-P1 for CHO cells. Western blotting study in Fig. 2B using anti-myc and anti-zCL-P1 antibodies indicates that zCL-P1 has an approximate molecular mass of 120 kDa band and 90kDa band in these cells as hCL-P1 [8]. This affinity-purified zCL-P1 antibody could recognize only zCL-P1 specifically. Membrane immunofluorescence analysis showed that zCL-P1 was expressed as a type II membrane protein since it was detected by an anti-C-terminal tag antibody (anti-Myc monoclonal antibody) (Fig. 2C). Microbe binding studies demonstrated yeast (Zymosan A) and *S.aureus* bound to CHO/zCL-P1 cells as well as CHO/hCL-P1 cells with high specificity. However, *E. coli* could not bind to CHO/zCL-P1 cells, which was different from those specific binding to CHO/hCL-P1 cells [8] (Fig. 2D). AcLDL as modified LDL or LDL as control failed to bind to CHO/zCL-P1 cells but only OxLDL could bind to them (Fig. 2E). Sugar blot analyses demonstrated that CHO/hCL-P1 soluble protein was specifically stained with α-D-galactose, α-N-acetyl-galactosamine, and β-N-acetyl-galactosamine. However, zCL-P1-CRD and hCL-P1-CRD was weakly stained with α-L-fucose, α-D-glucose, β-D-galactose, but not stained with α-D-mannose, α-D-galactose, or α/β-N-acetyl-galactosamine (Fig. 2F). The sugar binding activity between CL-P1-CRDs and CHO/hCL-P1 protein was completely different. These results indicated two possibilities that CL-P1-CRDs produced by E.coli were not well refolding or the whole extracellular molecule of CL-P1 was important for sugar binding activity.

3.3. Gene expression of zCL-P1 in zebrafish

A quantitative PCR examination of CL-P1, VEGF, and VEGF receptor activity showed that the transcript of zCL-P1 was present from an early embryonic stage prior to the appearance of a vascular organ (Fig. 3A). The expression peak of zCL-P1 mRNA was found at 24 hpf, after a gradual rise from the 6 hpf stage. This expression pattern of zCL-P1 mRNA was similar to that of zTie-2 mRNA. However, the expression pattern of zVEGF mRNA was similar to mRNAs of zVEGF receptor/zKDR and /zFlt-1 [22,23]. Whole-mount *in situ* hybridization of embryos at the 24 hpf stage using a zCL-P1 RNA probe showed that zCL-P1 mRNA was mainly localized in the dorsal aorta (DA) but slightly in the intersegmental vessels (ISV) (Fig. 3B). zKDR mRNA was observed in ISV as well as in DA (Fig. 3B). Immunohistochemical staining with/without differential interference contrast (DIC) microscopic analyses and hematoxylin-eosin staining of fish embryos at the 48 hpf stage showed that the affinity-purified

antibody was able to stain fish specifically (Fig. 3C) and zCL-P1 was partially localized in the dorsal aorta and dorsal vein due to with DIC (Fig. 3D). Another immunohistochemical study using adult fish at 2 months post-fertilization showed that dorsal longitudinal anastomotic vessels harboring CL-P1 form the network structure (Fig. 3E).

3.4. Defects in blood vessel and body development detected in the zCL-P1 knockdown study

We constructed three different CL-P1-antisense morpholino oligonucleotides (MO1, 2, and 3) whose designs were based on sequences at different sites of the 5'-untranslated region of zCL-P1 mRNA. To evaluate the potency and specificity of MO1-3, we used a five-base-mismatched (against MO2) oligonucleotide (5MM) and a control oligonucleotide (control). Using the above oligonucleotides, we then performed a zCL-P1 gene knockdown study (Fig. 4A–D, Table 1). At 48 hpf, we found various malformations such as body trunk formation collapse, shortness in length, severe trunk winding, and pericardial edema (Fig. 4B, C). We classified the strong phenotype (strong) as having severe developmental deficiencies and the weak phenotype (weak) as having features between the strong phenotype and the normal in Fig. 4B. Embryos with the strong phenotype showed less activity, and fewer heartbeats and contractions. The three MOs caused similar malformations and the rates of their respective phenotypes were MO-dose-dependent and oligonucleotide-sequence-specific (Fig. 4A, D, Table 1, 2). Western blotting analysis using whole fish embryos revealed the complete depletion of CL-P1 protein by MO2 but not by 5MM or the control (Fig. 4A). With alkaline phosphatase staining, we detected endogenous alkaline phosphatase in vascular endothelial cells (Fig. 4C1). The weak phenotype fish retain DA but the strong phenotype fish lose DA or ISV. By means of microangiography with an injection of FITC-dextran, we were able to visualize functional blood flow in the fish vessels and observed the abnormal blood flows in the weak and strong phenotype fish (Fig. 4C2). These two types of staining of MO-treated fish revealed vascular defects affecting the dorsal aorta and /or intersegmental vessels. MO2 at 5 ng caused 65.7% of fish to have a dorsal aorta defect phenotype but 2.5 ng of MO2 caused only 17.6% of fish to have this phenotype (Fig. 4D). The extent of the severe defects in vessels was MO dose-dependent.

3.5. Co-injection of exogenous zCL-P1 or zVEGF mRNA and MO2 induces recovery from vascular defects in zCL-P1 gene knockdown fish

The synthesized zCL-P1 or zVEGF mRNA and MO2 were simultaneously co-injected into one-cell stage embryos, which caused the strongest phenotype. The 57% of fish with a defective phenotype was also lowered to 28% after co-injection of 250 pg zCL-P1 mRNA and this reduction was dependent on the zCL-P1 mRNA dose (Fig. 5A). The additional co-injection of 1000 pg zCL-P1 mRNA caused to clear recovery effect in the fish with strong and weak phenotypes (Fig. 5B).

The expression kinetics of zVEGF, zKDR, zFlt-1, and zTie-2 mRNAs in MO injected fish were observed at 24, 36, and 48 hpf (Fig. 6A). We found that zVEGF mRNA was reduced and zKDR mRNA was slightly increased by MO injection at all three times. The expression of zTie-2 mRNA was not affected by MO injection. In Fig. 6B, the 73% of fish with a total defective phenotype was lowered to 52% after co-injection of 250 pg zVEGF mRNA and the reduction of abnormal proportion with strong phenotype was also dependent on the dose of zVEGF mRNA. However, we could not observe the complete recovery in this dose. We failed to give the higher dose as zCL-P1 mRNA because only 250 pg zVEGF mRNA caused to the pericardial edema (Fig. 6B).

4. Discussion

Our work revealed *in vitro* characteristics of the zebrafish CL-P1 gene product as well as the manner in which CL-P1 functions in the vascular development of the zebrafish embryo. zCL-P1 has about fifity % homology to its human and mice counterparts in amino acid sequences and its activity *in vitro* is also similar. In addition, zCL-P1 is expressed in vascular tissue at the mRNA and protein level at both early embryonic and adult stages. Knockdown of endogenous CL-P1 expression using three distinct MOs during embryogenesis resulted in temporal and spatial disruption of embryonic vascular development. However, the vascular defects induced by a loss of CL-P1 expression were rescued in a dose-dependent

manner by treating these fish with additional CL-P1 mRNA.

We analyzed the time-course expression of CL-P1 mRNA in zebrafish. A previous report demonstrated that vasculogenesis in this fish starts from around 6 hpf [24]. The expression pattern of zCL-P1 mRNA was similar to that of zTie-2 mRNA as were those of two markers of vasculogenesis, the zVEGF and zVEGF receptors of zKDR and zFlt-1 mRNAs. The time-course change in zVEGF mRNA seen in this study was quite similar to that of previous reports [24, 25]. Furthermore, it was demonstrated by *in situ* hybridization and immunohistochemistry that zCL-P1 mRNA and protein expression is partially localized in the dorsal aorta in normal zebrafish embryos. These results suggest that zCL-P1 expression in zebrafish might be involved in vasculogenesis. Previous studies have reported that KDR is expressed in angioblasts at 14 hpf, and KDR-expressed angioblasts migrate medially to form dorsal aorta (so-called vascularization) [26]. We therefore hypothesized that CL-P1 might be expressed in angioblasts as is KDR, and may play a role in vasculogenesis.

To prove the biological functions of CL-P1 in zebrafish, we performed a series of experiments employing zCL-P1 gene knockdown. The gene knockdown study revealed that the loss of CL-P1 resulted in markedly abnormal phenotypes such as a defective dorsal aorta and an overly short trunk. The vascular development of zebrafish consists of vasculogenesis of the axial vessels and angiogenesis of ISV sprouting. Two reports have demonstrated that lack of the dorsal aorta in the developing stage causes a short body length in zebrafish VEGF and KDR morphants [27-29]. A small-mount injection of MO not only decreased the frequency of the DA deficiency but also increased the ISV loss. These defective phenotypes were also found in VEGF and VEGF receptor knockdown fish [29]. Pericardial edema also occurred in CL-P1 knockdown embryos as it had with VEGF. It is therefore speculated that zCL-P1 gene knockdown suppresses normal vasculogenesis, thereby inducing shortness of the body length. This speculation is supported by our results that zCL-P1 mRNA simultaneously rescued both the dorsal aorta defect and the shorter body length. We therefore conclude that CL-P1 might be involved in zebrafish vasculogenesis. Further investigation is needed to clarify the relationship between CL-P1 and VEGF, its receptors or Tie-2.

The fact that amino acid sequences of CL-P1 are highly conserved in humans and mice, strongly suggests that CL-P1 has a very important role in maintaining homeostasis and immune system [30, 31]. Furthermore, Fig. 1 shows that the intraplasmic domain is especially highly conserved, compared with the coiled-coil domain, collagen-like domain, and the CRD. There are a di-leucine motif, an acid cluster, and an endocytosis motif of YKRF sequences in its cytoplasmic domain and its 16th tyrosine residue might be phosphorylated, and could be activated as sorting signal of YTRF sequences in the endocytosis receptor [32]. CL-P1 may bind to any particular ligands during zebrafish embryogenesis so that certain signals can be transmitted downstream through the CL-P1 cytoplasmic site. Further experiments are required to demonstrate any ligands and cytoplasmic signal pathways with vasculogenesis and angiogenesis.

It has already been demonstrated that several molecules including VEGF, KDR, and NRP1 play important roles in vasculogenesis [33]. We observed the reduction of zVEGF mRNA in zebrafish embryos after MO injection in Fig .6. We detected the recovery by the additional injection of zVEGF mRNA inferior to the effect by zCL-P1 mRNA in CL-P1 knockdown fish. These results indicate that there is any relationship between zCL-P1 and zVEGF with fish vasculogenesis.

We demonstrated here that CL-P1 is involved in zebrafish vasculogenesis, and that among members of the collectin and scavenger receptor families, its role in vasculogenesis appears to be unique. Therefore, we suggest that the scavenger receptor CL-P1 would be considered as a candidate molecule with involvement in vasculogenesis. We consider this study can provide a glimpse into the functional role of the CL-P1 molecule *in vivo* in vertebrates. The mechanism underlying its function in the vasculature, its significance in pathological angiogenesis and vasculogenesis, and its functional conservation in mammalian development remain to be determined.

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FIGURE LEGENDS

Fig. 1. Deduced amino acid sequences and cDNA sequence, of zebrafish CL-P1 (A). The nucleotide sequence is numbered from 5' to 3'. The hydrophobicity plot and amino acid sequences analysis showed that the domain structure of zCL-P1 is the same as that of human CL-P1 (A, B). A comparison of homology between human, mouse and zebrafish CL-P1 amino acids is shown in Figure 1C.

Fig. 2. Expression and characterization of zCL-P1. The zCL-P1 ORF was subcloned into pcDNA3.1/Myc-His (+) vector, followed by transfection into CHO cells (A). CHO/zCL-P1, CHO/hCL-P1, and control cells were analyzed by Western blotting analyses using an anti-Myc monoclonal antibody and anti-zCL-P1 rabbit antibody (B). Membrane immunofluorescence analysis of CL-P1 in CHO/zCL-P1 cells (zCL-P1) or control cells (vector), using anti-Myc antibody and anti-mouse-IgG-conjugated Alexa 594 (C). The binding of microorganisms in CHO/zCL-P1 cells (zCL-P1), CHO/hCL-P1 cells (hCL-P1), or vector transfected cells (vector) was assayed with Zymosan A (*Saccharomyces cerevisiae*) BioParticles, *Staphylococcus aureus* BioParticles, and *Escherichia coli* BioParticles conjugated with Alexa 488 (Molecular Probes). After binding, cells were stained with anti-Myc antibody and anti-mouse-IgG-conjugated Alexa 594 (D). CHO/zCL-P1 cells (zCL-P1) and vector transfected cells (vector) were incubated with DiI-OxLDL, DiI-AcLDL, and DiI-LDL and incubated with anti-Myc antibody. After 30 min incubation, cells were incubated with anti-mouse-IgG-conjugated Alexa 488 (E). Comparison of the binding of several sugar-biotin probes to zCL-P1CRDhis (zCL-P1CRD), hCL-P1CRDhis (hCL-P1CRD), and CHO/hCL-P1 cells extracts (hCL-P1) (F).

Fig. 3. CL-P1 mRNA and protein expression in zebrafish embryos. The time-course sequence of CL-P1, KDR, VEGF, Flt-1, and Tie-2 mRNA expression in zebrafish (A). Whole-mount *in situ* hybridization using zCL-P1 or zKDR riboprobe was performed in zebrafish embryos at the 24 hpf stage (B). Each photo on the right shows a high power view of the area in the corresponding dorsal aorta. The arrows or arrowhead indicate the dorsal aorta or the intersegmental vessels, respectively. Cross-sections of 48 hpf stage embryos were stained with H&E, anti-CL-P1 antibody, or with/without DIC (C, D).The arrow or arrowhead indicates the dorsal aorta or the dorsal vein, respectively (D). The cutting position, H&E staining (low power view), and FITC staining of CL-P1 (high power view) in the transverse section of a two-month-old zebrafish (E). The right photo shows a high power view of the area in the corresponding network vessels in the center photo. Arrowhead indicates the dorsal blood vessel.

Fig. 4. Gene knockdown study using MOs. MOs were microinjected into embryos with various concentrations of zCL-P1 MO2, 5MM or the control (A). Western blot analysis was carried out in 48 hpf zebrafish embryos that had been injected with the standard oligonucleotide, 5MM or MO2 (A). Vascular formation was evaluated by alkaline phosphatase (ALP) staining (C1). Microangiography was performed using fluorescein isothiocyanate-dextran (FITC-dextran) (C2). Unstained zebrafish embryos at 48 hpf (B). Embryos at 48 hpf that had received the standard oligonucleotide or MO2 (B, C). The terms control, severe, and weak indicate the level of abnormality in the gross appearance of embryos. Each (B, C2) shows a high power view of the corresponding upper photograph. DA indicates dorsal aorta (arrow) and ISV represents intersegmental vessels (arrowhead) (C1, 2, D). The vascular defects of embryos injected with 2.5 or 5 ng of MO2 (D).

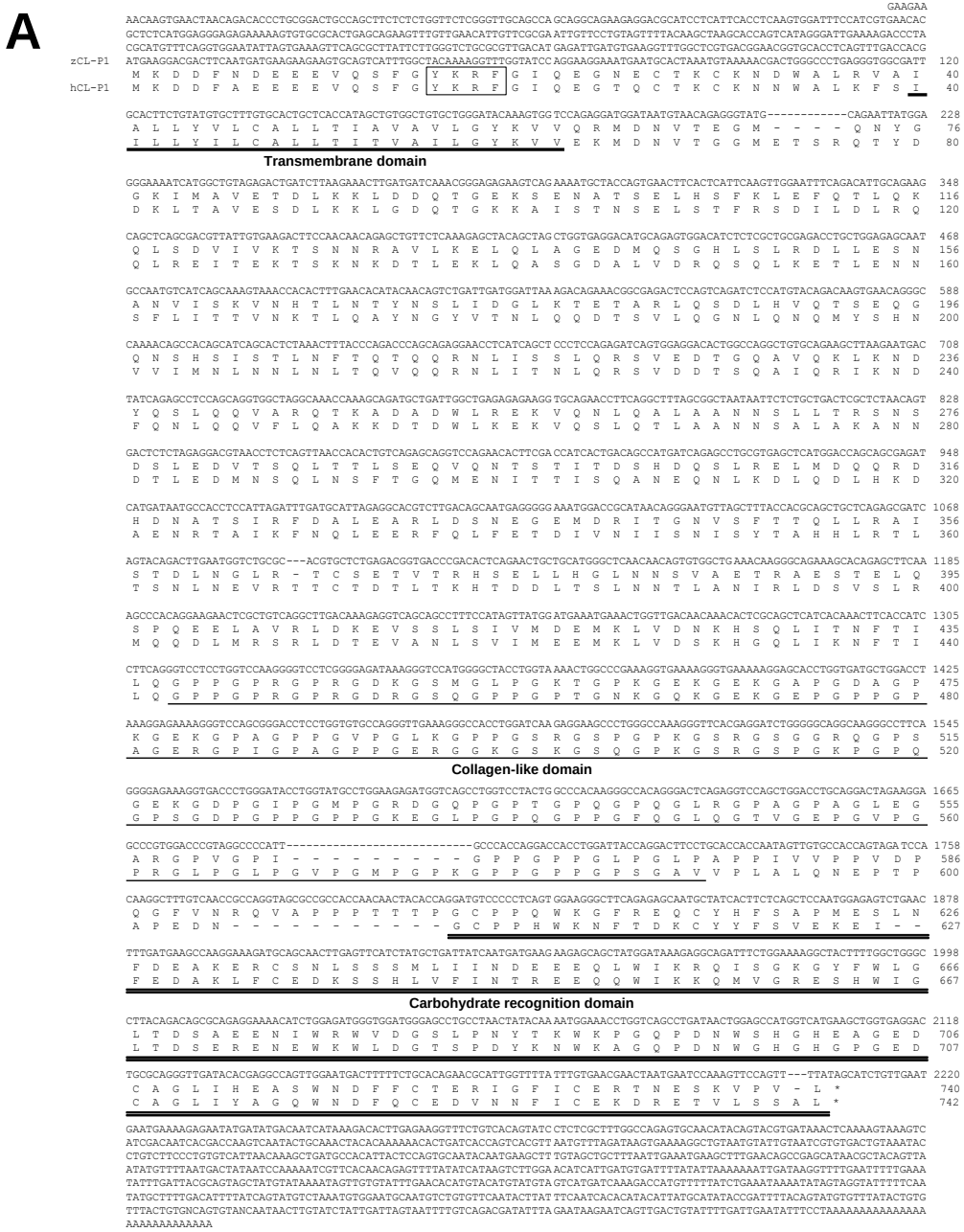
Fig. 5. Effect of the synthesized zCL-P1 mRNA on the abnormal phenotype frequency induced by MO2. The synthesized zCL-P1 mRNA and MO2 were simultaneously injected into embryos and the phenotype was evaluated at 48 hpf. Each bar represents the means ± SEM of 111 - 139 zebrafish embryos. *$p < 0.01$ and **$p< 0.05$ when compared with MO2 (5 ng) alone (A). The additional co-injection of 100 and 1000 pg zCL-P1 mRNA caused to clear recovery effect on the level (normal, weak, strong) of abnormality in the gross appearance of embryos (B).

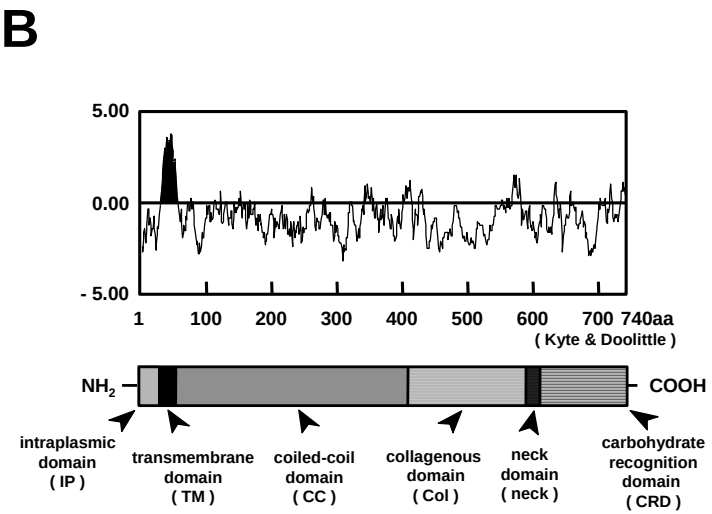
Fig. 6. The expression and role of zVEGF mRNA in the abnormal phenotype induced by MO2. The expression kinetics of zCL-P1, zKDR, zVEGF, zFlt-1, and zTie-2 mRNA in MO2, 5MM MO2 injected zebrafish, or control zebrafish at 24, 36, 48 hpf (A). The co-injection of 10, 100, and 250 pg zVEGF

mRNA caused to some recovery effect on the level of abnormality (B).

Figure

Fig. 1


A
zCL-P1
hCL-P1
Transmembrane domain
Collagen-like domain
Carbohydrate recognition domain


B
5.00
0.00
- 5.00
1
100
200
300
400
500
600
700 740aa
(Kyte & Doolittle)
NH2
COOH
intraplasmic domain (IP)
transmembrane domain (TM)
coiled-coil domain (CC)
collagenous domain (Col)
neck domain (neck)
carbohydrate recognition domain (CRD)

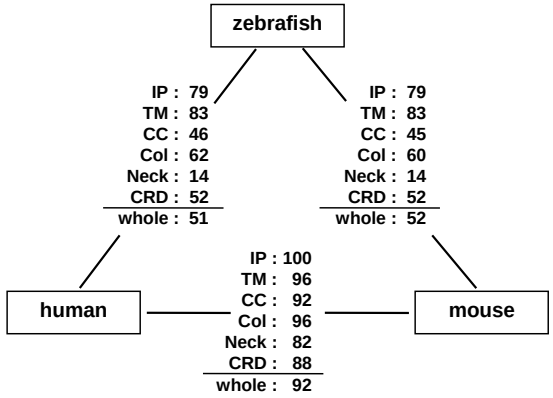

C
zebrafish
IP : 79
TM : 83
CC : 46
Col : 62
Neck : 14
CRD : 52
whole : 51
IP : 79
TM : 83
CC : 45
Col : 60
Neck : 14
CRD : 52
whole : 52
IP : 100
TM : 96
CC : 92
Col : 96
Neck : 82
CRD : 88
whole : 92
human
mouse

Fig. 2

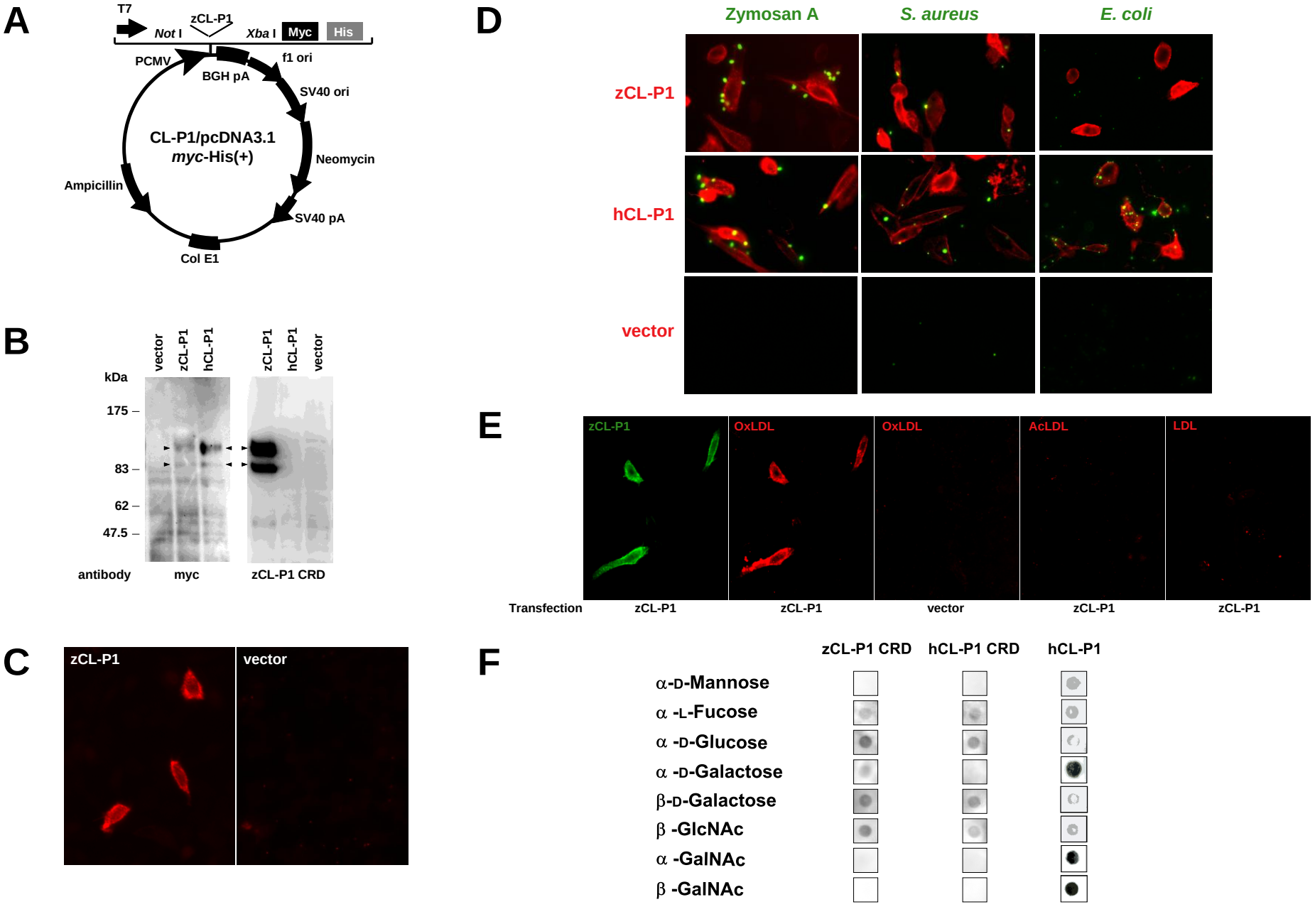

A
T7
zCL-P1
Not I
Xba I
Myc
His
PCMV
BGH pA
f1 ori
SV40 ori
CL-P1/pcDNA3.1
myc-His(+)
Neomycin
Ampicillin
SV40 pA
Col E1
B
vector
zCL-P1
hCL-P1
zCL-P1
hCL-P1
vector
kDa
175
83
62
47.5
antibody
myc
zCL-P1 CRD
C
zCL-P1
vector
D
Zymosan A
S. aureus
E. coli
zCL-P1
hCL-P1
vector
E
zCL-P1
OxLDL
OxLDL
AcLDL
LDL
Transfection
zCL-P1
zCL-P1
vector
zCL-P1
zCL-P1
F
zCL-P1 CRD
hCL-P1 CRD
hCL-P1
α-D-Mannose
α -L-Fucose
α -D-Glucose
α -D-Galactose
β-D-Galactose
β -GlcNAc
α -GalNAc
β -GalNAc

Fig. 3

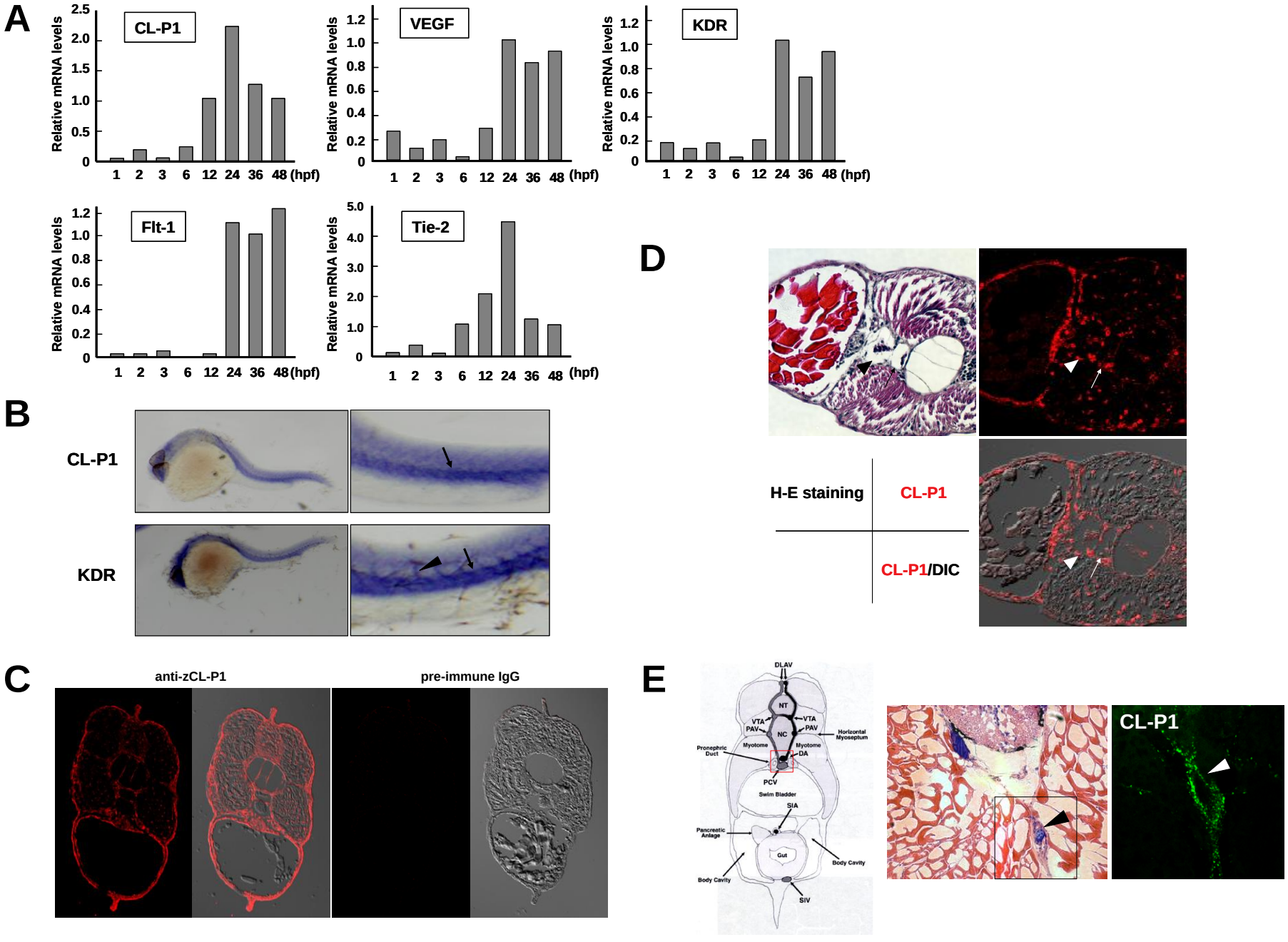

A
CL-P1
VEGF
KDR
Flt-1
Tie-2
Relative mRNA levels
1 2 3 6 12 24 36 48 (hpf)
B
CL-P1
KDR
C
anti-zCL-P1
pre-immune IgG
D
H-E staining
CL-P1
CL-P1/DIC
E
DLAV
NT
VTA
PAV
NC
Horizontal Myoseptum
Myotome
Pronephric Duct
DA
PCV
Swim Bladder
SIA
Pancreatic Anlage
Gut
Body Cavity
SIV
CL-P1

Fig. 4

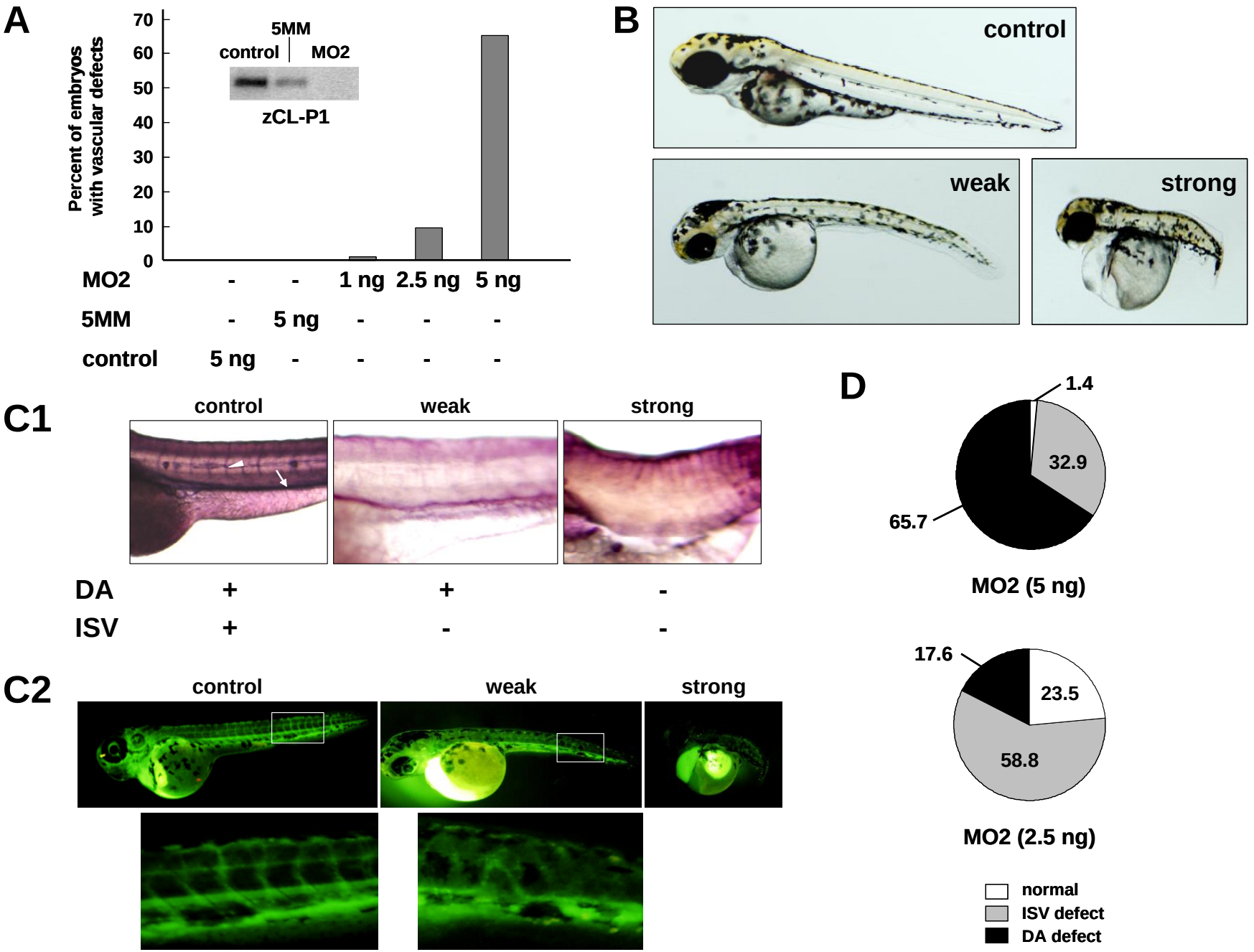
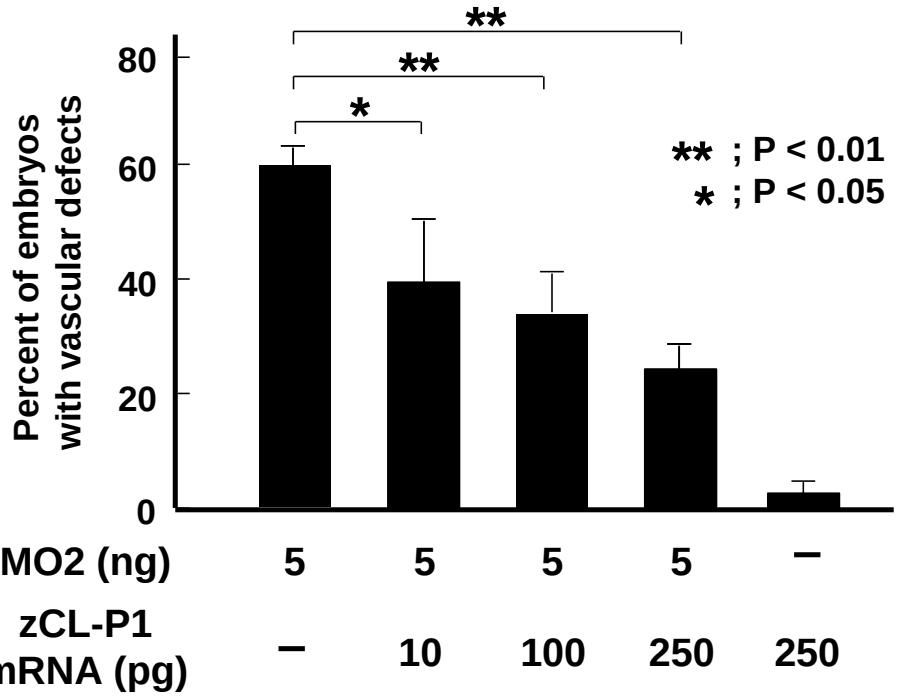

A
Percent of embryos with vascular defects
70
60
50
40
30
20
10
0
5MM
control
MO2
zCL-P1
MO2 - - 1 ng 2.5 ng 5 ng
5MM - 5 ng - - -
control 5 ng - - - -
B
control
weak
strong
C1
control
weak
strong
DA + + -
ISV + - -
C2
control
weak
strong
D
1.4
32.9
65.7
MO2 (5 ng)
17.6
23.5
58.8
MO2 (2.5 ng)
normal
ISV defect
DA defect

Fig. 5

A


**
**
*
80
60
40
20
0
Percent of embryos with vascular defects
** ; P < 0.01
* ; P < 0.05
MO2 (ng)
5
5
5
5
–
zCL-P1 mRNA (pg)
–
10
100
250
250

B

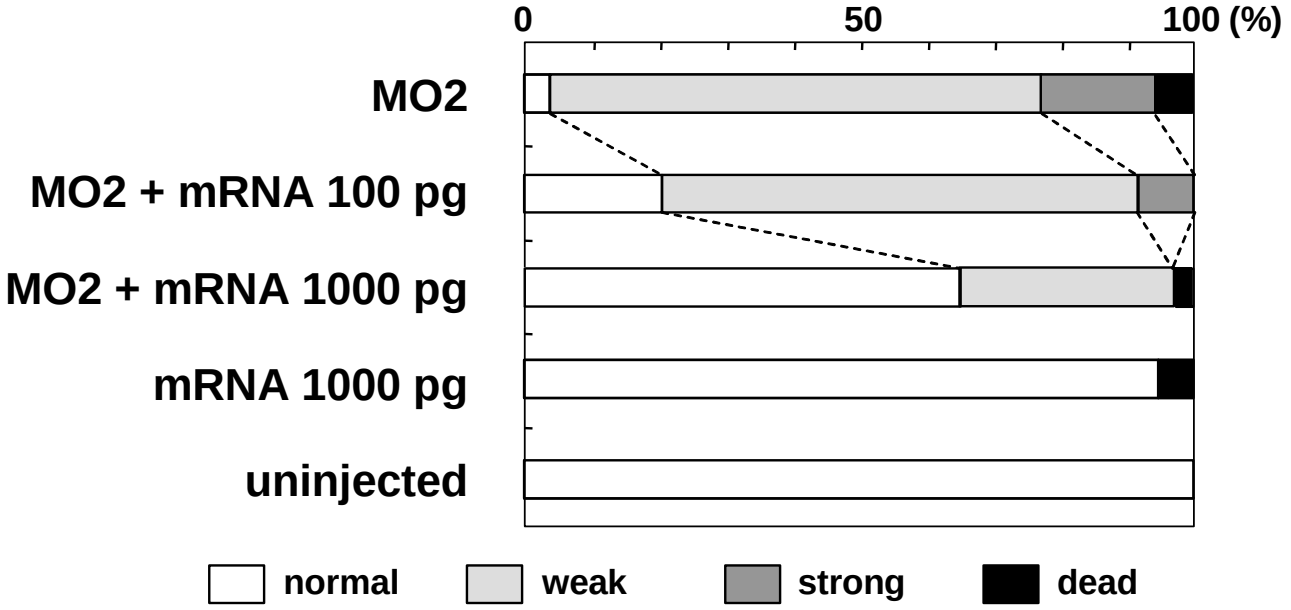

0
50
100 (%)
MO2
MO2 + mRNA 100 pg
MO2 + mRNA 1000 pg
mRNA 1000 pg
uninjected
normal
weak
strong
dead

Fig. 6

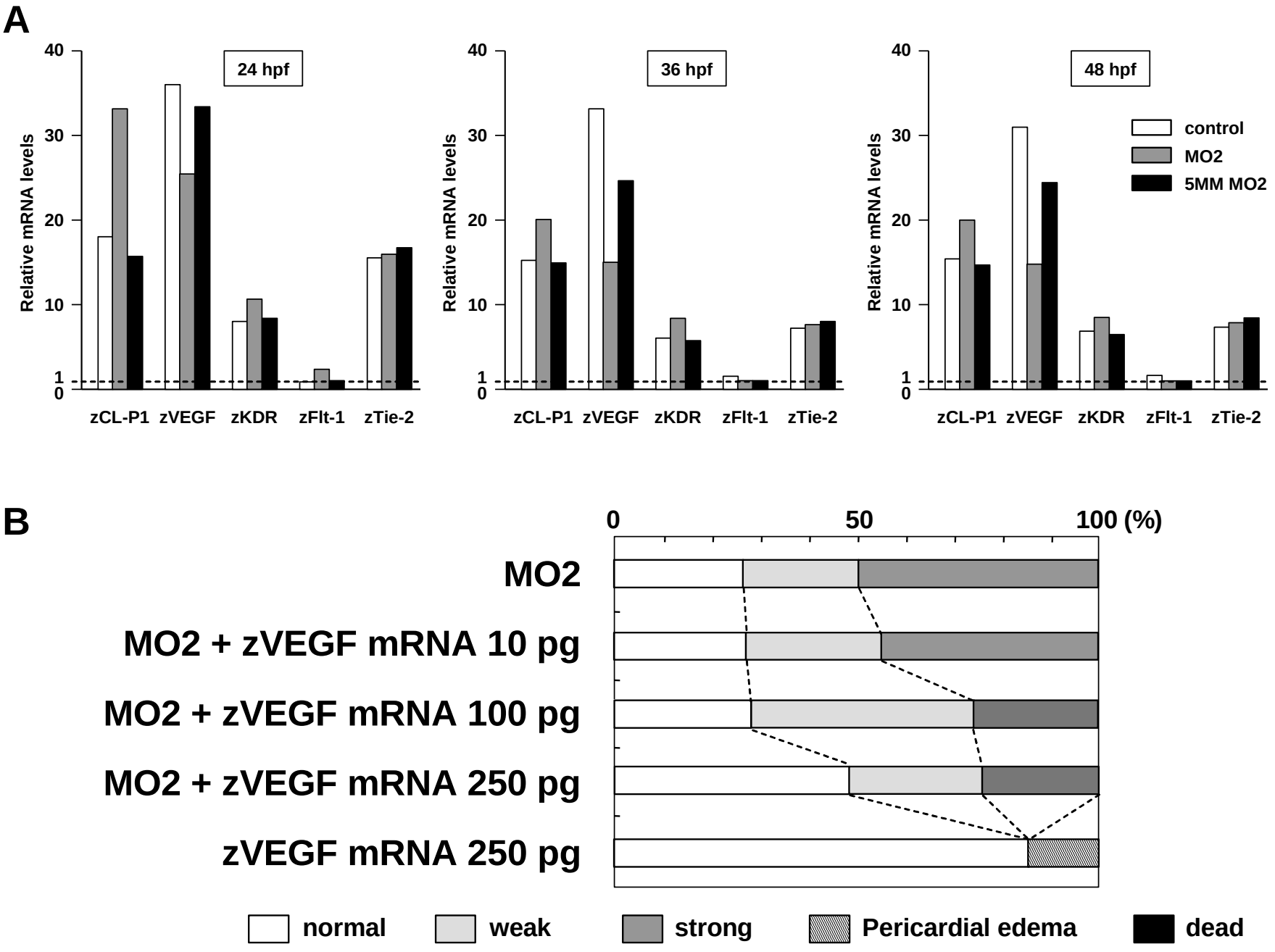

A
24 hpf
36 hpf
48 hpf
Relative mRNA levels
40
30
20
10
1
0
zCL-P1
zVEGF
zKDR
zFlt-1
zTie-2
control
MO2
5MM MO2
B
0
50
100 (%)
MO2
MO2 + zVEGF mRNA 10 pg
MO2 + zVEGF mRNA 100 pg
MO2 + zVEGF mRNA 250 pg
zVEGF mRNA 250 pg
normal
weak
strong
Pericardial edema
dead

Table 1. Dose response effect of the antisense morpholino oligonucleotide MO2 on abnormal phenotype frequency in zebrafish embryos

		Abnormal phenotype frequency (%)	
	Dose (ng)	Experiment 1	Experiment 2
control	5	0	0
MO2	1	1.0	0
	2.5	3.6	0
	5	39.8	43.3
5MM	5	1.0	0

The design and sequences of the antisense morpholino oligonucleotide (MO2) and a five-base-mismatched MO against MO2 are shown in Materials and methods. MOs were microinjected into 1-4 cell stage embryos, and phenotypes were observed at 48 hpf. Microscopic observation of embryos revealed that evident defects, such as shortness in body length and inadequate blood circulation, were frequently observed and classified as abnormal phenotypes. Each value represents the frequency (%) of malformation in 52 - 118 zebrafish embryos.

Table 2. Gene knockdown using MO2 caused the abnormal phenotype and death in zebrafish.

			standard	MO2 (1ng)	MO2 (2.5ng)	MO2 (5ng)	uninjected
total number			52	76	60	60	160
normal (%)			48 (92.3)	70 (92.1)	53 (88.3)	15 (25.0)	155 (96.9)
phenotype (%)	48 hpf	weak	0	1 (1.3)	7 (11.7)	15 (25.0)	0
		strong	0	0	0	26 (43.3)	0
		p. edema	0	0	1 (1.7)	29 (48.3)	0
		unknown	0	0	0	0	0
		total	0	1 (1.3)	7 (11.7)	41 (68.3)	0
dead (%)	24 hpf		2 (3.8)	1 (1.3)	0	3 (5.0)	5 (3.1)
	48 hpf	weak	0	2 (2.6)	0	0	0
		strong	0	0	0	0	0
		p. edema	0	0	0	0	0
		unknown	2 (3.8)	2 (2.6)	0	1 (1.7)	0
		total	4 (7.7)	5 (6.6)	0	4 (6.7)	5 (3.1)

Embryos at 48 hpf that had received the standard oligonucleotide or MO2 (1, 2.5, 5ng). The terms strong and weak indicate the level of abnormality in the gross appearance of embryos (Fig. 4B). The p. edema means the fish having pericardial edema. The rates of their respective phenotypes were MO2-dose-dependent and oligonucleotide-sequence-specific.