**prox1b Activity Is Essential in Zebrafish Lymphangiogenesis**

Luca Del Giacco¹, Anna Pistocchi², Anna Ghilardi¹

1 Department of Biology, Università degli Studi di Milano, Milan, Italy, 2 Division of Regenerative Medicine, San Raffaele Scientific Institute, Milan, Italy

**Abstract**

**Background:** The lymphatic vascular system, draining interstitial fluids from most tissues and organs, exerts crucial functions in several physiological and pathological processes. Lymphatic system development depends on Prox1, the first marker to be expressed in the endothelial cells of the cardinal vein from where lymph vessels originate. Prox1 ortholog in the optimally clear, easily manipulated zebrafish model has been previously isolated and its contribution to lymphangiogenesis has been clarified. Because of a round of genome duplication occurred at the base of teleosts radiation, several zebrafish genes have been retained in duplicate through evolution. We investigated for the presence of additional prox1 genes and determined their role in zebrafish lymphangiogenesis.

**Methodology/Principal Findings:** We isolated a second ortholog, named prox1b, and analyzed its expression during development by whole mount in situ hybridization (WISH). We detected strong prox1b expression in the endothelium of the posterior cardinal vein (PCV) from where lymphatic precursors originate. To analyze prox1b involvement in lymphangiogenesis we utilized the fli1:GFP transgenics and followed the formation of the thoracic duct (TD), the primary lymph vessel in fish, after prox1b knockdown. Our findings clearly demonstrated that the absence of prox1b activity severely hampers the formation of the TD.

**Conclusions/Significance:** This work provides substantial progress toward the understanding of zebrafish lymphangiogenesis. In light of the features shared by the lymphatic systems of zebrafish and higher vertebrates, the establishment of such lymphatic model will provide a powerful tool to study, for instance, disorders of body fluid homeostasis, inflammation and cancer metastasis, and may ultimately contribute to novel therapies.

**Introduction**

The lymphatic system drains lymph away from tissues and organs back to the bloodstream, and plays a main role in the immune response and fat absorption. In addition, the lymphatic system is implicated in inflammation processes as well as cancer metastasis.

Prox1 homeogene, the vertebrate homolog of prospero in D. melanogaster [1], regulates cell proliferation, fate determination and differentiation during embryonic development, and triggers the molecular program leading to the formation of the lymphatic system. Indeed, ablation of Prox1 in mouse, Xenopus, and zebrafish impaired the formation of lymph vessels [2,3,4,5,6,7].

About 350 million years ago, a round of genome duplication occurred at the base of teleosts radiation resulting in two copies of all genes. Some of such copies have been retained in duplicate through evolution [8]. In view of this evidence, we searched for additional Prox1 orthologs in zebrafish. Interestingly, while we were conducting the experiments reported in this work, a paper describing the isolation and the expression patterns of two prox1 genes in the fish medaka has been published [9].

Here, we isolated a second zebrafish ortholog, named prox1b (to distinguish it from the first identified one, from now on designated prox1a), and showed that prox1b knockdown impedes the formation of the thoracic duct (TD), the primary fish lymph vessel [10].

**Results and Discussion**

**prox1b cloning and gene structure**

Blast analysis of the ENSEMBL zebrafish assembly version 6 (Zv6) using zebrafish prox1a full-length cDNA sequence returned two positive hits on chromosome 17, corresponding to the previously characterized prox1a [11], and prox2 [12] genes, and one hit on chromosome 7, this last one mapping at chromosome location 19,523,485–19,537,485 and never reported before. The 3,558-bp mRNA sequence is identified by the GenBank accession number FJ544314 (Figure 1). The encoded protein is related to the prospero transcription factors family, being characterized by the atypical homeodomain and two prospero domains, PD1 and PD2 (Figure 1A).

This protein represents the second zebrafish ortholog of Prox1, producing significant alignments (35% of identity) with all the Prox1 present in the GenBank database, including zebrafish prox1a. prox1b coding region is interrupted by three introns, and an additional one is located in the 5' UTR (Figure 1A), indicating that the structure of the gene has been evolutionarily conserved from zebrafish to mammals.
However, zebrafish prox1b gene does not show synteny with mammalian Prox1, as for the recently identified medaka prox1b gene [9]. Analogously to all the vertebrate Prox genes, as well as Drosophila prospero, prox1b contains the U12-dependent intron (intron 2), characterized by the unusual AT/AC splice sites, located at the beginning of the homeobox (Figure 1B) [12,13].

Role of prox1b in zebrafish lymphangiogenesis

Prox1 is a master gene controlling the processes of budding, migration and proliferation of lymphangioblasts [2,5,4,6,7]. In zebrafish, its contribution to lymphangiogenesis has been demonstrated by means of prox1a knockdown [4]. In this context, we examined the possible involvement of prox1b in lymphangiogenesis.

The first signal of gene activity appeared in the central nervous system (CNS) starting from somitogenesis (10 s stage, Figure 2A), where prox1b expression persists during development (Figure 2B,C,D,G) (a more detailed analysis of prox1b mRNA distribution in the nervous system will be discussed elsewhere). Interestingly, at 48 hours post-fertilization (hpf), we detected intense prox1b staining at the level of the posterior cardinal vein (PCV) and of the sprouts emanating from this vein. (B) prox1b contains the U12-dependent intron (intron 2), characterized by the unusual AT/AC splice sites, located at the beginning of the homeobox. doi:10.1371/journal.pone.0013170.g001

Figure 1. Nucleotide and deduced amino acid sequences of zebrafish prox1b. (A) arrowheads show the positions of the introns. The asterisk indicates the stop codon at the end of the open reading frame. The wave and solid lines mark PD1 and PD2, respectively. The homeodomain is boxed. The sequence has been submitted to the GenBank/EMBL database under accession number FJ544314.
Interestingly, the lack of the TD determined by the injection of prox1b in 70% of injected embryos (n = 70) (Figure 3J). (Figure 3A,E,I), fish [15] at 5 dpf (Figure 3), the developmental stage at which the TD searching for the presence/absence of the TD in fli1:GFP transgenic knockdown produced a lymphatic phenotypes that we evaluated the possible functional role of the gene in lymphangiogenesis.

A small percentage of the injected embryos displayed (Figure 3M,N), resembling the M wall always associated with miol to severe cardiac edema (Figure 3M,N), resembling the prox1a-lymphatic phenotype previously described [4]. A small percentage of the injected embryos displayed impaired circulation, and heart and trunk defects that did not allowed the observation of the vasculature, and for this reason was not included in the final analysis. The specificity of the lymphatic phenotype induced by prox1b knockdown was verified through the coinjection of a prox1b full-length mRNA properly mutated in the region targeted by the morpholino, which rescued the normal phenotype (Fig 3C,G,K). In order to corroborate the role of prox1b in lymphangiogenesis, the expression of lyve1, a specific molecular marker for lymphatic endothelial cells [14,16], has been assayed (Figure 4). In comparison to control embryos (Figure 4A,C), lyve1 expression in the trunk of prox1b morphants was weak or completely absent at 48h (80%, n = 30; Figure 4B) and 72 hpf (82%, n = 25; Figure 4D), indicating an impairment of lymphatic development.

To ascertain whether prox1b might synergize with prox1a to promote lymphangiogenesis, we injected the two orthologs specific MOs. The simultaneous knockdown of the two genes did not determine more severe phenotypes or the increase of the number of affected embryos in comparison to the prox1b-MO single morphants (data not shown). The attempt to use higher MOs concentrations resulted in acute generalized defects that impeded the observation of the vasculature, and for this reason was not included in the final analysis. The specificity of the lymphatic phenotype induced by prox1b knockdown was verified through the coinjection of a prox1b full-length mRNA properly mutated in the region targeted by the morpholino, which rescued the normal phenotype (Fig 3C,G,K). In order to corroborate the role of prox1b in lymphangiogenesis, the expression of lyve1, a specific molecular marker for lymphatic endothelial cells [14,16], has been assayed (Figure 4). In comparison to control embryos (Figure 4A,C), lyve1 expression in the trunk of prox1b morphants was weak or completely absent at 48h (80%, n = 30; Figure 4B) and 72 hpf (82%, n = 25; Figure 4D), indicating an impairment of lymphatic development.

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Methods

Ethics Statement

All embryos were handled according to relevant national and international guidelines.

Fish and Embryos Maintenance

Fish of the AB strain and transgenics for fli:GFP [15] were maintained at 28°C on a 14-hr light/10-hr dark cycle. Embryos were collected by natural spawning and staged according to Kimmel and colleagues [17].

prox1b identification and cDNA cloning

Zebrafish prox1b gene was identified through in-silico search using prox1a full-length cDNA as a bait. Two gene specific primers (prox1bF: 5’-CACCGCCCATATAAGACCACA-3’, and prox1bR: 5’-TTAATCTGCGGCGCTCCCCGTG-3’) have been used to amplify the full-length cDNA. The 3’ UTR have been obtained through RACE technique while the 5’ UTR has been identified searching the GenBank ESTs database.

Whole mount in-situ hybridization (WISH)

WISH was carried out as previously described [18,19]. Histological analysis of previously hybridized embryos was carried out on 8 μm sections.
Injections were carried out on 1- to 2-cell stage embryos. To repress prox1b mRNAs translation, an ATG-targeting morpholino (MO) was synthesized (Gene Tools); 5'-GGGAATCCA-TAGCCTCTTTTTCTGT-3'. prox1b-specific MO was used at the concentration of 6 ng per embryo in 1X Danieau buffer (pH 7.6). To repress prox1a mRNA translation, a prox1a-specific MO was used at the concentration of 8 ng per embryo, as previously reported [4,12,20,21]. As control, we injected 8 ng per embryo of a standard control MO (stdr-MO). MO-mRNA double injection experiments were conducted using 300 pg of capped mRNA per embryo together with the above reported amount of the MO of interest. For live microscopy observation, 5 dpf fli1:GFP transgenic embryo together with the above reported amount of the MO of experiments were conducted using 300 pg of capped mRNA per embryo under a fluorescent microscope. For the larvae have been anesthetized and monitored under a fluorescent microscope from 24 to 48 hpf. The presence/absence of the GFP signal has been monitored under a fluorescent microscope from 24 to 48 hpf.

Supporting Information

Figure S1 prox1b-MO specifically reduces prox1b mRNA translation. For the in-vivo test of the specificity of prox1b-MO, a prox1b-GFP sensor has been generated. (A) The construct contains 96 bp of the 5’ UTR, and the first 432 bp of the prox1b coding sequence (Nprox1b) fused with the GFP open reading frame. The blue bar indicates the region of the mRNA targeted by the prox1b-MO. The construct, obtained by PCR, has been cloned into the pTargetT expression vector (Promega) and used for injection experiments. (B) GFP-positive cells in the trunk (inset and arrowheads) and (C) in the yolk epithelium (arrowheads) are visible following coinjection of the sensor and the stdr-MO. (D) The complete absence of fusion protein expression when the sensor is coinjected with prox1b-MO confirms the specificity of action of the morpholino. Scale bar represents 100 μm.

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Author Contributions

Conceived and designed the experiments: LDG. Performed the experiments: LDG AP Ag. Analyzed the data: LDG. Wrote the paper: LDG.

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