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Immunohistochemical Demonstration of Lymphatic Vessels in Adult Zebrafish

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Morphological profiles of lymphatic vessels in adult zebrafish trunk and ovary were studied by immunohistochemistry and electron microscopy. The present immunohistochemistry for Prox1 was successful in demonstrating lymphatic vessels in zebrafish. The zebrafish trunk revealed two types of bilateral longitudinal lymphatic trunks draining lymph centripetally along dorsal aorta and posterior cardinal veins. Large honeycomb lymphatic sinus was further shown around common cardinal veins. In the zebrafish ovary, the lymphatic vessels, comprising endothelial cells only, encompassed arterioles in their lumen. This peculiar structure appeared to be conserved in vertebrates including mammals and might serve for control of blood temperature and tissue homeostasis. The present study is first to delineate lymphatic vessels in adult zebrafish by immunohistochemistry. Our immunohistochemical results showed usefulness of immunostaining for Prox1 not only for demonstration of lymphatic vessels in zebrafish, but also for examination of their function and dynamics in pathophysiological condition.

Key words: lymphatic vessel, zebrafish, immunohistochemistry, Prox1

I. Introduction

The lymphatic system plays an important role in transport of tissue fluid, cellular elements and nutrients such as rich emulsion of fat, to the blood stream to regulate homeostasis [2]. While the structure and distribution of lymphatic vessels have been described in various mammals, recent studies have documented lymphatic vessels and their development in zebrafish (*Danio rerio*) by injection of dyes [10, 12, 21] and transgenic techniques [5, 6, 8, 15, 18]. However, structural organization of the lymphatic system in zebrafish has not been fully clarified, and no information is available regarding lymphatic vessels within visceral organs. Furthermore, immunohistochemical analysis of the lymphatic system in zebrafish has not been virtually executed.

Prospero homeobox-1 (Prox1) has been shown to induce lymphatic transdifferentiation of embryonic venous endothelial cells as a master molecule of lymphatic lineage by reprogramming endothelial cell transcriptome [20]. The expression of Prox1 is restricted in the cellular nuclei of both lymphatic endothelial cells and their precursor cells within embryonic cardinal veins among the vascular system [20]. It has been also reported that Prox1 displays highly conserved amino acid sequences across the species including mammals, chicken, frog and zebrafish and plays similar roles in vertebrate organisms [12, 14, 20]. Therefore, Prox1 is thought to be an excellent lymphatic endothelial marker also in zebrafish. Hence, the present study depicts fine morphological profiles of lymphatic vessels in somatic trunk and ovary as a visceral organ in adult zebrafish by immunohistochemistry for Prox1.

II. Materials and Methods

Animals

Twelve adult zebrafishes (wild type, over 12 weeks after fertilization) and two adult female BALB/cN sea mice weighing 40–50 g were used in this study. The fishes and

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mice were maintained under standard husbandry and laboratory conditions, respectively. The mice were allowed standard animal chow and water *ad libitum*. All animal experiments were conducted in accordance with the Guidelines for Animal Experimentation of Oita University. The fishes and mice were sacrificed under deep anesthesia in water containing Tricaine (4 mg/ml; Sigma, St. Louis, MO, USA) and with an intraperitoneal injection of sodium pentobarbital (>150 mg/kg), respectively. Some anesthetized fishes were injected with 20 nl of India ink into the ovarian parenchyma before sacrifice. After ovaries were collected from the mice in a cold phosphate buffer (pH 7.4), several fishes and the murine ovaries were immediately frozen in liquid nitrogen for light and fluorescence microscopy. Ovaries of some fishes were fixed in Karnovsky's fixative for longer than 24 hr at 4°C for conventional transmission electron microscopy (TEM). Frozen specimens were cut into 10 μm thick sections and processed for hematoxylin-eosin and immunohistochemical staining. Tissue sections were immersed in 10% formalin or ice-cold acetone for 10 min before staining. Some residual specimens of frozen fishes after collecting tissue sections were further immersed in Karnovsky's fixative for scanning electron microscopy (SEM).

Immunohistochemistry

Several tissue sections from the zebrafishes were immersed in 0.3% H_2O_2 in phosphate buffered saline (PBS; 1/15 M, pH 7.4) containing 0.1% sodium azide at room temperature to block the endogenous peroxidase activity. After rinsing in PBS, the sections were incubated in 10% normal goat serum and then in rabbit antibody against Prox1 (ab38692; Abcam, Tokyo, Japan) at 4°C overnight. Following a rinse in PBS, they were treated with peroxidase (PO)-conjugated goat anti-rabbit IgG (Histofine Simple Stain MAX-PO, Nichirei Bioscience, Tokyo, Japan) for 1 hr at room temperature. The site of the immunoreaction was visualized by the diaminobenzidine (DAB; Dojindo, Kumamoto, Japan) reaction. All stained sections were examined with a BX-60 light microscope (Olympus, Tokyo, Japan).

Some tissue sections were processed for fluorescence-labeled immunostaining. The sections from fishes and murine ovaries were incubated in 10% normal donkey serum, and then in anti-prox1 antibody (Abcam), or in a mixture of an antibody to Prox1 or LYVE-1 (Angiobio, Del Mar, CA, USA), another lymphatic endothelial molecule [3, 4, 16], together with an antibody to plakoglobin (PROGEN Biotechnik, Heidelberg, Germany) or CD31 (BD Biosciences Pharmingen, San Jose, CA, USA) at 4°C overnight. They were then treated with indocarbocyanine (Cy3)-conjugated donkey anti-rabbit IgG (Jackson Immno-Research, West Grove, PA, USA), or with a mixture of Cy3-conjugated donkey anti-rabbit IgG and fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse or rat IgG (Jackson ImmunoResearch) for 1 hr at room temperature. The immunostained sections were examined by confocal laser scanning microscope (LSM 5; Carl Zeiss, Oberkochen, Germany).

For electron microscopic observation, some tissue sections of fish ovaries were fixed with 0.05% glutaraldehyde and 4% paraformaldehyde solution for 10 min and processed for immunostaining for Prox1. Following pre-incubation in 1% bovine serum albumin solution, they were incubated in anti-Prox1 antibody (Abcam) overnight, followed by treatment with ultrasmall (1 nm) gold-conjugated anti-rabbit goat IgG (BBI International, Cardiff, UK) for 1 hr. The immunoreaction sites were then developed by silver enhancing kit (BBI International) for 5 min at room temperature.

Control immunostaining was carried out using the same procedures, except for the utilization of an antibody absorbed with an excess amount of antigen (Prox1: 50–100 μg/ml, Abcam) or a non-immunized serum, instead of the corresponding antibodies; completely negative results were observed (Figs. 1g, 2e).

Scanning electron microscopy

Several residual fish specimens after collecting tissue sections were processed for SEM observation. Following a rinse in PBS, the fixed tissues were immersed in aqueous solution of tannic acid for 1 hr, rinsed in distilled water, and stained with 1% osmium tetroxide for 1 hr at room temperature. The tissues were then dehydrated in a graded ethanol series, freeze-dried by t-butylalcohol, and observed under an S-800 scanning electron microscope (Hitachi, Tokyo, Japan).

Transmission electron microscopy

Small tissue pieces of zebrafish ovaries fixed in Karnovsky's fixative were postfixed in 2% osmium tetroxide solution, dehydrated in a graded ethanol series, and then were embedded in Epon 812 (Oken, Tokyo, Japan). Ultrathin sections were prepared, stained with uranyl acetate and lead citrate, and observed under a JEM-1200 EX TEM (JEOL, Tokyo, Japan).

The immunostained tissues for TEM were also dehydrated in ethanol series, embedded in Epon 812, and cut into ultrathin sections for examination in TEM (JEOL).

III. Results

Immunohistochemistry

Immunohistochemistry for Prox1 demonstrated several types of cells with significant immunoreactivity in their cellular nuclei in adult zebrafish (Fig. 1). Like in mammals [16], although Prox1 was immunolocalized in all hepatic and some nervous, intestinal and renal cells (Fig. 1b–f), the expression was restricted in a subpopulation of vascular endothelial cells among the vascular system also in zebrafish (Figs. 1–5). The blood vessels showed no Prox1-immunoreactivity (Figs. 1–5), whereas the immunoreaction products were exclusively detected in endothelial cell nuclei of another vessels (Figs. 1–5), being in proximity to the

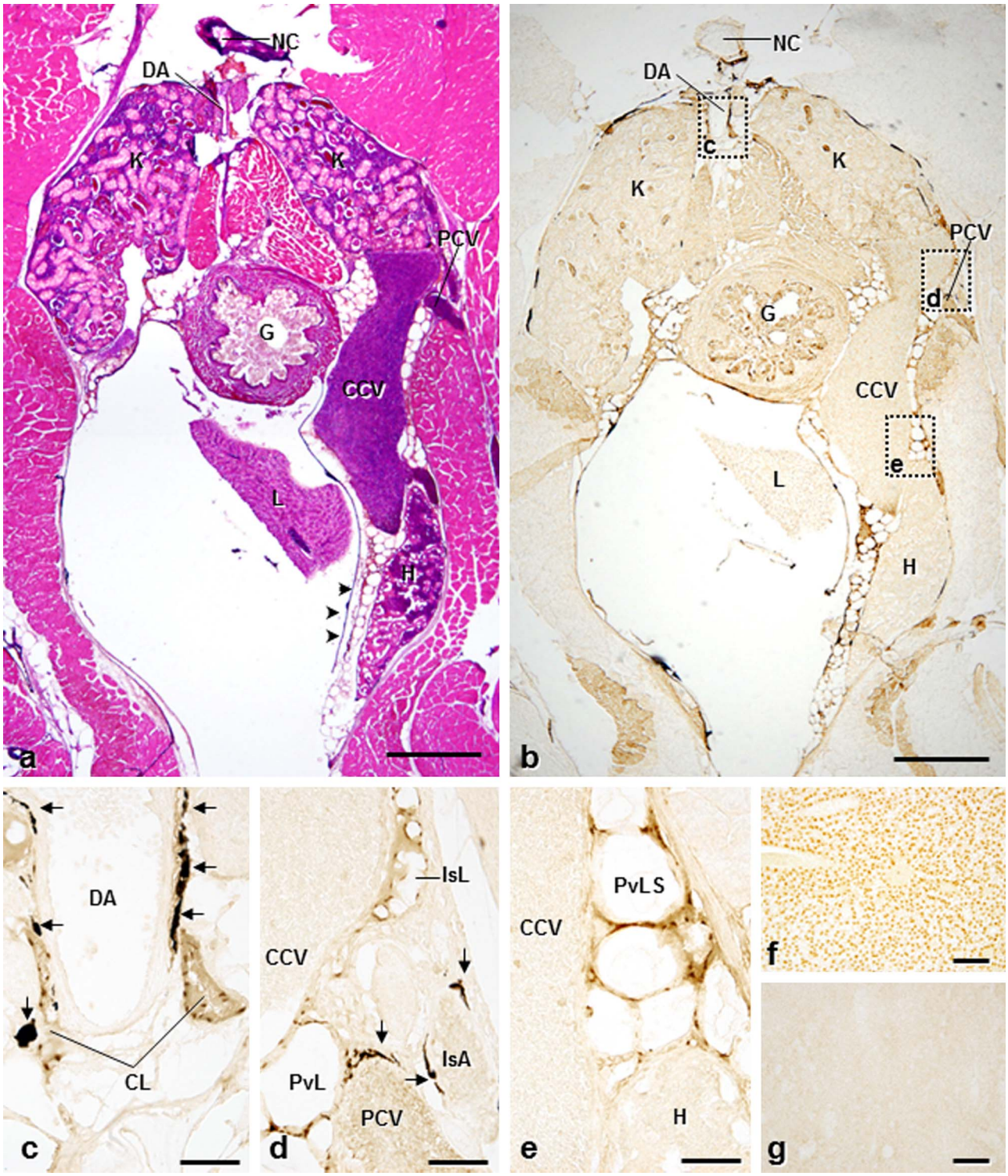

Fig. 1. Hematoxylin and eosin staining (**a**) and immunostaining for Prox1 (**b–g**) on tissue sections of adult zebrafish. **a**: Overview of a transverse section of trunk. Arrowheads indicate peritoneum. **b**: Adjacent section of **a**. The immunoreaction products for Prox1 are seen in cellular nuclei of lymphatic endothelial cells (**c–e**), hepatic cells (**f**) and some mucosal cells of gut. **c–e**: Higher magnification of each boxed area in **b**. Bilateral cardinal (CL), perivenous lymphatic (PvL) and intersegmental (IsL) lymphatics, consisting of Prox1-positive endothelial cells, are shown beneath dorsal aorta (DA) and near posterior cardinal vein (PCV) and intersegmental artery (IsA). The Prox1-positive endothelium also forms perivenous lymphatic sinus (PvLS) around common cardinal vein (CCV). Natural melanin pigments (arrows) are also seen around blood vessels. **f**: The hepatic cells show immunoreaction for Prox1 in their nuclei. **g**: Histochemical control of immunostaining for Prox1 using antigen-absorbed antibody on a tissue section of zebrafish liver. G, gut; H, heart; K, kidney; L, liver; NC, notochord. Bars=200 μm (**a, b**), 50 μm (**c–g**).

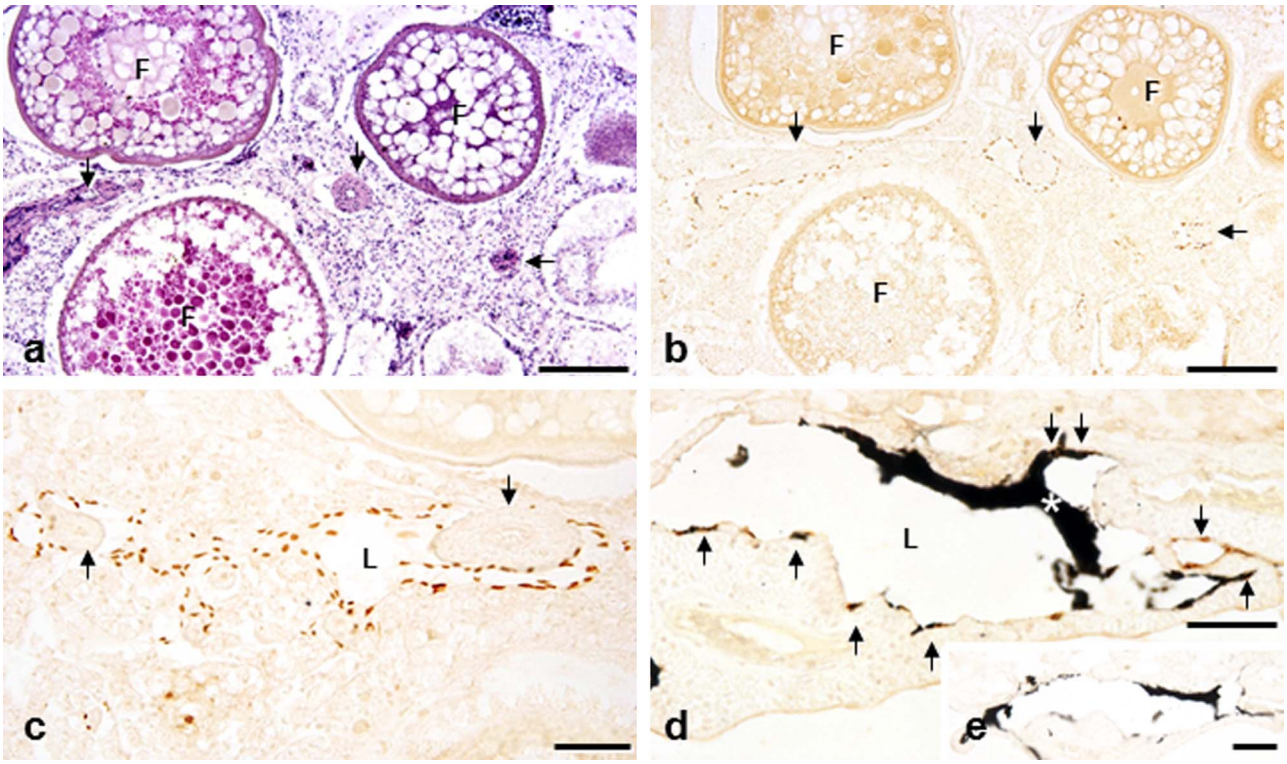

Fig. 2. Light micrographs of tissue sections of adult zebrafish ovary. **a, b**: Hematoxylin-eosin staining (**a**) and immunostaining for Prox1 (**b**) on adjacent sections. Some vascular structures lined with Prox-1-immunopositive endothelium are seen around arterioles (arrows) in interstitium between ovarian follicles (F). **c**: Higher magnification of an irregularly-shaped lymphatic vessel (L) lined with endothelial cells showing Prox1-immunopositive nuclei. Arterioles (arrows) are encompassed by the lymphatic. **d**: Immunostaining for Prox1 on a tissue section of ovary after intraparenchymal injection of India ink. India ink is seen in the lumen (asterisk) and Prox1-positive endothelium (arrows) of a lymphatic (L). **e**: Histochemical control of imunostaining for Prox1 using antigen-absorbed antibody on a tissue section of ovary injected with India ink. Bars=200 μm (**a, b**), 50 μm (**c**), 40 μm (**d**), 50 μm (**e**).

blood vessels. The Prox1-immunopositive vessels were recognized as lymphatic vessels in zebrafish, because they displayed similar morphological and histochemical characteristics to those in mammals [16], as described below.

In thoracicoabdominal trunk of zebrafish, the Prox1-immunopositive lymphatic vessels were disposed along dorsal aorta, intersegmental arteries and posterior cardinal veins, and they were regarded as cardinal lymphatics (thoracic ducts), intersegmental lymphatics and perivenous (collateral) lymphatics, respectively (Fig. 1). The lymphatics were mostly composed of single layer of endothelial cells to be irregular in shape (Figs. 1–5). Both the cardinal and perivenous lymphatics were bilateral, and intersegmental lymphatics connected them (Fig. 1b–d). The perivenous lymphatics exhibited large sinus-like aspects, featured by honeycomb endothelial structures, around common cardinal vein and in close proximity to peritoneum (PvLS in Fig. 1b, e).

In zebrafish ovary, the interstitium between ovarian follicles displayed several irregularly-shaped lymphatic vessels lined with the Prox1-immunopositive endothelial cells (Fig. 2a, b). The Prox1-positive lymphatic vessels frequently enveloped arteriolar walls as if the arterioles ran within the lymphatics (Fig. 2b, c). Following an injection of India ink into the ovarian parenchyma, the ink was immediately absorbed into the lymphatic vessels through the Prox1-immunopositive endothelial cells (Fig. 2d). Double immunostaining for Prox1 and plakoglobin further demonstrated the lymphatic vessels surrounding arterioles in zebrafish ovarian stroma precisely, because the immunoreaction products for plakoglobin exaggerated contours of blood vascular and lymphatic endothelium (Fig. 3a). Meanwhile, by means of double immunostaining for LYVE-1 and CD31 on tissue sections of murine ovaries, the LYVE-1-immunopositive lymphatic vessels were clearly shown to embrace arterioles with the CD31-immunopositive endothelium also in murine ovarian interstitium (Fig. 3b).

Electron microscopy

Electron microscopy provided information regarding the fine structure of lymphatic vessels in zebrafish ovary. The lymphatic vessels were recognized under SEM by contrasting with immunohistochemistry for Prox1 on mirror tissue sections (Fig. 4), whereas the lymphatics were readily identified under TEM by their morphology and contrasting with immunoelectron microscopic findings for Prox1 (Fig. 5).

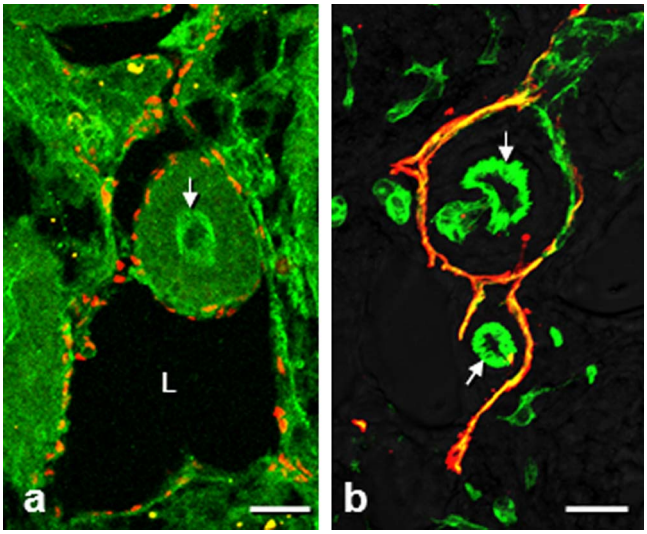

Fig. 3. Fluorescence micrographs of tissue sections of zebrafish ovary (**a**) immunostained for Prox1 (red in **a**) and plakoglobin (green in **a**) and of murine ovary (**b**) immunostained for LYVE-1 (red in **b**) and CD31 (green in **b**). **a**: A lymphatic vessel (L) lined with endothelial cells, showing Prox1-immunopositive cellular nuclei and plakoglobin-immunopositive endothelial contours, embraces arterioles showing plakoglobin-immunopositive endothelium (arrow). **b**: The arterioles with CD31-immunopositive endothelium are seen to be surrounded by the LYVE-1-immunopositive lymphatic. The lymphatic shows a closed configuration presumably due to the high stromal tissue pressure. Bars=20 μm.

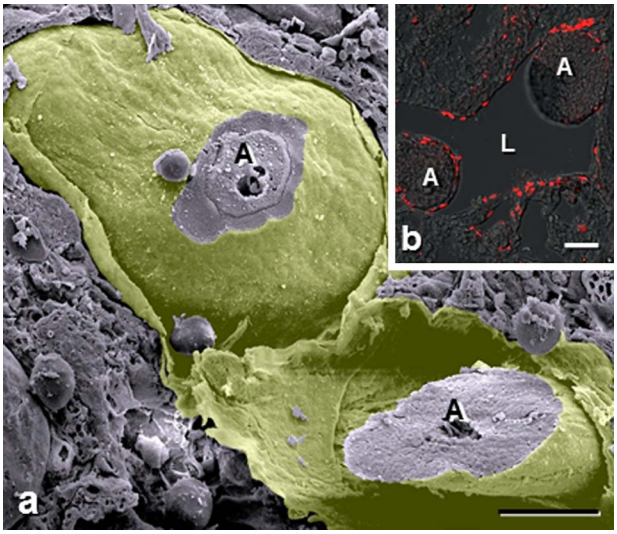

Fig. 4. SEM (**a**) and fluorescence (**b**) images of a lymphatic vessel in interstitium of zebrafish ovary. **a**: Thin lymphatic endothelium (yellow) entirely embraces thick arteriolar wall with the cytoplasmic lamellae within lymphatic lumen. **b**: Immunostaining for Prox1 on an adjacent tissue section of mirror image of **a**. The nuclei of lymphatic (L) endothelium show Prox1-immunoreactivity. A, arteriole. Bars=50 μm (**a**), 40 μm (**b**).

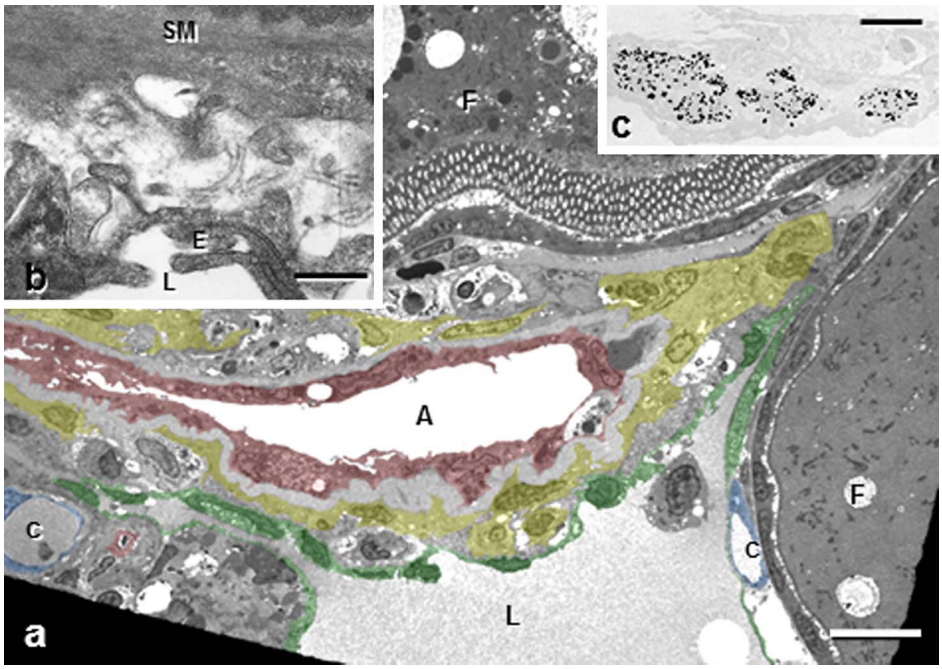

Fig. 5. TEM images of a lymphatic vessel in zebrafish ovary. **a**: Large and irregularly-shaped lymphatic vessel (L), which is composed of attenuated endothelial cells (green) arranging in a single layer, is seen in interstitium between ovarian follicles (F). The lymphatic endothelium (green) closely surrounds an arteriole (A) consisting of endothelium (red) and tunica media containing smooth muscles (yellow). Small blood capillaries (C) lined with flat endothelium (blue) are shown between lymphatic (L) and ovarian follicles (F). **b**: Higher magnification of lymphatic endothelium (E) and arteriolar smooth muscle (SM). A lymphatic (L) is endowed with neither continuous basal lamina nor periendothelial cells, but smooth muscle cells (SM) of an arteriole are in parts closely located to lymphatic endothelium (E). The flattened lymphatic endothelial cells (E) are connected with each other by overlapping and interdigitation. **c**: Immunostaining for Prox1. The silver-enhanced reaction products are immunolocalized in nuclei of lymphatic endothelial cells. Bars=10 μm (**a**), 500 nm (**b**), 5 μm (**c**).

SEM examination demonstrated three-dimensional luminal aspects of lymphatic vessels in the fish ovary. The lymphatic vessels consisted only of smooth and thin endothelial cells extending cytoplasmic folds and ensheathed thick arteriolar walls entirely with the lymphatic endothelial lamellae (Fig. 4). No specific structural devices including periendothelial cells were seen around the lymphatics (Fig. 4).

By TEM, the lymphatic vessels were shown to be composed of attenuated endothelial cells, which connected with each other by direct apposition, overlapping and interdigitation, and free from periendothelial cells and continuous basal lamina (Fig. 5), like mammalian lymphatic capillaries [16]. Immunoelectron microscopy for Prox1 further confirmed the lymphatic endothelial cells by exhibiting silver immunoreaction precipitates in their nuclei (Fig. 5c). The arterioles were embraced by a part of the lymphatic endothelium, as demonstrated by light and scanning electron microscopy (Figs. 2–4). Some smooth muscle cells of the arteriolar walls were often in close location to the lymphatic endothelium covering the arterioles (Fig. 5a, b). Blood capillaries were distributed around ovarian follicles to interpose between each follicle and the lymphatics (Fig. 5a).

IV. Discussion

The present study has demonstrated lymphatic vessels in adult zebrafish by immunohistochemistry and electron microscopy. Zebrafish has currently developed into a powerful vertebrate model for developmental biology [7, 19], pharmacological testing [1, 13] and for investigating genetic [1, 13], metabolic [9] and malignant [17] disorders. Hence, a precise anatomy of every organ in zebrafish has to be defined, but little information was available as regards the lymphatic system. Several studies have described lymphatic vasculature in zebrafish using dye injection and transgenic techniques [5, 6, 8, 12, 15, 18, 21], but no report, to our knowledge, has immunohistochemically delineated lymphatic vessels in the fish trunk and visceral organ.

Our immunohistochemical data have shown immunoreaction products for Prox1 in cellular nuclei of a subpopulation of vascular endothelial cells, but not in blood vessels. Since Prox1 is a master transcription factor for lymphatic lineage [20], it is demanded that the molecule localizes in cellular nuclei of the lymphatic endothelial cells. Meanwhile, the present ultrastructural examination has exhibited lymphatic vessels, which were distinguishable from blood vessels by the morphological characteristics similar to those in mammals [16], and immunoreaction for Prox1 in the lymphatic endothelial cell nuclei. Furthermore, the lymphatic vessels demonstrated by electron microscopy corresponded exactly to those shown by light microscopy combined with immunostaining for Prox1. On these accounts, the Prox1-immunopositive vascular structures are certainly regarded as lymphatic vessels. This paper is therefore the first to report morphological profiles of lymphatic vessels in zebrafish by immunohistochemistry. The present results indicate the usefulness of immunohistochemical methods for Prox1 for visualization of lymphatic vessels, but also for evaluation of functional roles and dynamics of lymphatics in various pathophysiological conditions in zebrafish, as well as in mammals.

The present immunohistochemical investigation has demonstrated two organized longitudinal lymphatic vessels in zebrafish thoracicoabdominal trunk, viz., cardinal and perivenous lymphatics, running along dorsal aorta and large veins, respectively. The intersegmental lymphatics connected both vessels. This finding coincides to our previous view obtained by dye injection method [10]. Both types of the longitudinal lymphatics were bilateral, and this is considered to be a model of main lymphatic trunk in somatic trunk of vertebrate including humans. The lymph in the lymphatic trunks presumably transported to junction of cardinal veins [11] by movements of blood vessels and skeletal muscles. Each lymphatic trunk was mainly composed of thin and flat endothelium and lacked distinct external wall, although further investigation throughout the whole body is required. This may represent certain retention potential and slow transportation of lymph, as in the case of mammalian lymphatics [16].

Noteworthy is the occurrence of lymphatic sinus with honeycomb structure being in close contact with peritoneum and around common cardinal veins and heart. This lymphatic sinus is perhaps equivalent to subvertebral or pericardial sinus appeared in frog or snake [11]. This peculiar structure is likely to sufficiently absorb and accumulate tissue fluid excreted from peritoneal cavity and/or large veins and to contribute to tissue homeostasis by draining lymph into circulation under support of heart beat.

In the present study, the lymphatic vessels were investigated in ovary of zebrafish. The zebrafish ovary displayed large vessel-like structures lined with the Prox1-immunopositive cells, which ultrastructurally extended thin lamellar cytoplasm to form vascular lumen, in the interstitium among ovarian follicles, and India ink injected into the parenchyma was immediately absorbed into those vascular spaces. The Prox1-immunopositive vessels are therefore regarded as lymphatic vessels in both views of morphological and functional aspects. The lymphatic vessels frequently embraced arterioles in zebrafish ovary as if the arterioles passed through the lymphatics, and this was confirmed by our SEM examination. Kotani [11] has described that arteries are encompassed by lymphatics in amphibian and reptile, and our investigation in murine ovary also disclosed the lymphatics surrounding the arterioles. This unique form of lymphatics detected in zebrafish ovary is presumably conserved in vertebrates beyond species, and this might play a role in regulation of temperature of blood stream and of tissue microenvironment, as reported by Kotani [11]. The ovarian lymphatics were composed only of endothelial cells, but smooth muscles in the arteriolar wall were often in close proximity to the opposite portion of lymphatic endothelium. This structural device

may contribute to transport of lymph in zebrafish ovary.

The lymphatic system in zebrafish is starting to be elucidated; structural organization of lymphatic vessels and their development in every organ of zebrafish will be explored in future studies.

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