

Zebrafish as a new animal model to study lymphangiogenesis

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Abstract The lymphatic system is essential for fluid homeostasis, fat absorption and immune responses, and also plays key roles under pathological conditions, such as tumor metastasis, lymphoedema and inflammation. The main function of the lymphatic vascular system is to return excess interstitial fluid back to the blood vascular system. Lymph, including fluid, macromolecules, leukocytes and activated antigen-presenting cells, is transported from the blind-ended lymphatic capillaries toward the collecting lymphatic vessels; for there, it is returned to the blood circulation through lymphatico-venous junctions (Alitalo et al. in *Nature* 438:946–954, 2005). Despite its importance, lymphangiogenesis remains poorly understood. The lack of specific markers has complicated the identification of lymph vessels, and a small animal model that could be genetically manipulated to discover the function of novel lymphangiogenic candidates has only recently become available (Ny et al. in *Nat Med* 11(9):998–1004, 2005). Since 2004, we have worked to make the zebrafish a new genetic model for unraveling the function of candidate genes involved in lymphangiogenesis. We have demonstrated that zebrafish possess a lymphatic vascular system that shares the morphological, molecular and functional characteristics of the lymphatic vessels found in other vertebrates (Yaniv et al. in *Nat Med* 12(6):711–716, 2006).

In this process, we realized that it was necessary to seek a common definition for the lymph system which would be applicable from fish to man. The aim of this article is to review classical, mainly morphological, studies in order to elucidate the nature of the lymphatic system.

Keywords Animal model · Lymphangiogenesis · Zebrafish

A short history of lymphology

Hippocrates spoke of “white blood”, and Aristotle described vascular structures containing a colorless fluid. These “white vessels” were also observed by the physicians in the Alexandrian medical school, but their function was not understood. Galenus did not distinguish these “lacteals” from arteries and veins, and they were eventually forgotten because the contents of the smaller lymphatic vessels are colorless and their walls thin. Consequently, ancient scientists were less interested in them.

Asellius observed the lacteals (mesenteric lymphatic vessels) during the vivisection of a well-fed dog and believed that they went to the liver where their contents were “concocted” into blood. He demonstrated thereafter that they are present in various mammals. Chyle vessels were first demonstrated in man by Fabricius. In 1651, Pecquet described the thoracic duct and the cisterna chili. Vesalius had discovered the thoracic duct before Pecquet, but he failed to recognize its significance and termed it “Vena alba thoracis”.

It was a “physiological postulate” that the chyle vessels are the only pathway for food absorption from the intestines to the liver, the organ of blood production. In 1653, Rudbeck discovered the thoracic duct, its inflow to the

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large veins and also its connection with the cisterna chyli, first in the calf and then in the cat. He later discovered the lymphatics of the esophagus, colon, rectum and spermatic duct in dogs, sheep and other animals and described the valves of lymphatics. Rudbeck expressed practically no opinion on the function of the lymph vascular system, but he did perceive the practical importance of this system based on the fact that ascites and edema resulted from the occlusion of the lymphatic vessels. In 1653, a few months earlier than Rudbeck, Bartholinus published his description of the “serous vessels” in many parts of the body, calling them “lymphatics”.

William Harvey first described the gross anatomy of the blood circulation in 1628. Much later, in 1661, Malpighi described the capillaries in the lung of a frog and discovered the connection between the arteries and the veins. Van Leeuwenhoek suggested that the thinness of the walls of the smallest vessels might allow the “finest part of the arterial blood passage through those coats, to the end that every part of the body may be continually supplied with necessarily and suitable nourishment”. Although the concept of a microcirculation had been established, the function of the lymphatic vessels was not yet understood at the end of seventeenth century. Hunter (1718–1783) and his pupils Cruickshank and Hewson proposed that the “lymphatic vessels are the absorbing vessels all over the body”. Hewson (1774) made extensive dissections of the lymphatic vessels in fish, birds and mammals and described in all of these organisms a system of lacteals and lymphatics with a common trunk, the thoracic duct. He noted that lymph glands (nodes) were absent in fish and in the turtle, were few in number in birds and well developed only in mammals. He also noted the presence of globules (lymphocytes) in lymph and thought that they originated from the lymph glands, entering the blood via the lymph channels. Cruickshank (1789) and Mascagni (1789) described the topography of lymph vessels in all parts of the human body using a mercury injection method developed by Nuck (1692). By the end of eighteenth century, the anatomical description of the lymph vessels and lymph trunks of major importance had been completed, but the origin of the lymph vessels, lymph capillaries and small lymphatics inside of the organs was still unknown. The structure of the smallest lymphatic vessels and their relation to the tissues they innervated was open to debate. It was assumed that the lymphatics communicated directly with the blood vessels through a very fine and tubular system, the *Vasa serosa*. Virchow (1858) put forward the view that hollow connective tissue cells bridged the gaps between the blood and lymphatic vessels, but in 1862 Von Recklinghausen was able to demonstrate, by means of silver nitrate staining, that the lymphatic vessels were lined throughout by a distinct cell layer of endothelium, while His (1863) claimed that the

lymph vascular apparatus formed a closed system of tubes. Toward the latter part of the nineteenth century, Ludwig developed techniques for collecting lymph from lymphatic vessels and proposed that lymph was a filtrate derived from the blood. Heidenhain, one of his contemporaries, held the opposing view that lymph was produced by a process of secretion. Starling provided a firm foundation to Ludwig’s concept by pointing out the relationship between the hydrostatic pressure of the blood in the capillaries and the colloid osmotic pressure of the plasma proteins, which lead to the realization that capillaries in fact leaked proteins to a small extent. Drinker and his colleagues elaborated the concept of the lymphatics as a system of absorbing vessels, the main function of which was to return to the blood stream those protein molecules that escape from the circulation. It has since become clear that the lymphatic vessels are also essential for migration throughout the body of lymphocytes.

Phylogeny and ontogeny of the lymph system in vertebrates

In the following sections, we discuss the specifics of the phylogeny and ontogeny of the lymphatic vascular system in different animals, comparing and contrasting the common and distinct features of each.

Cyclostomata and cartilaginous fishes

Phylogeny and ontogeny

In invertebrates, whose circulatory system is scarcely differentiated, the venous system also performs the function of the lymphatic and the chyle vascular apparatus. The cyclostomata still possesses an undifferentiated haemolymphatic system in which lymphatic sinuses contain blood and are in direct connection with the blood-vessel system (Treakoff 1926–1930; Weidenreich 1931, 1933). Cartilaginous fishes begin to establish a less than complete separation of the two parts of the lymph vascular apparatus, i.e., the visceral system and the parietal system. The visceral lymph vessels form a profuse network on the surface of the organs and unite in the *Vas lymphatic intestino-mesentericum*. The parietal system consists of the longitudinal superficial trunks (dorsallis, ventrallis, laterale) and deeper trunks (spinal and subvertebral). Hoyer and Michaloski (1922) regarded the subvertebral trunk as being identical to the thoracic duct of higher vertebrates. The lymphatic system exists in cartilaginous fish, but it contains blood; as such, it still belongs to the venous system. Certain types of torpedoes form an exception; in these fish, the visceral system has differentiated itself from the venous system.

Teleosts

Phylogeny

In an evolutionary sense, the differentiation of a distinct lymphatic system is first observed in teleosts. In amphibians and reptiles, the lymph sinuses are cavities in direct connection with the lymphatics, and they are lined with endothelium. In fish, sinuses are present between paired fins, between pericardium and bronchial musculature and also between inner organs and parietal musculature.

Ontogeny

Hoyer suggested that cell proliferation, leading to formation of lymphatics, sets in at the confluence of the anterior and posterior cardinal veins in the embryos of trout (1922) and shark (1928). He claimed that it was possible to make injections into the main lymphatics and sinuses of the head and lateral lymphatics, even in freshly emerged trout

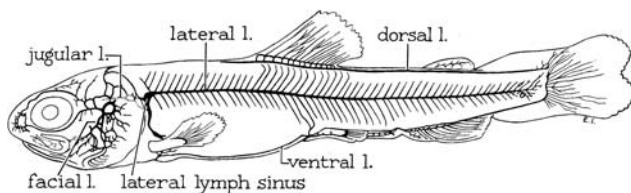
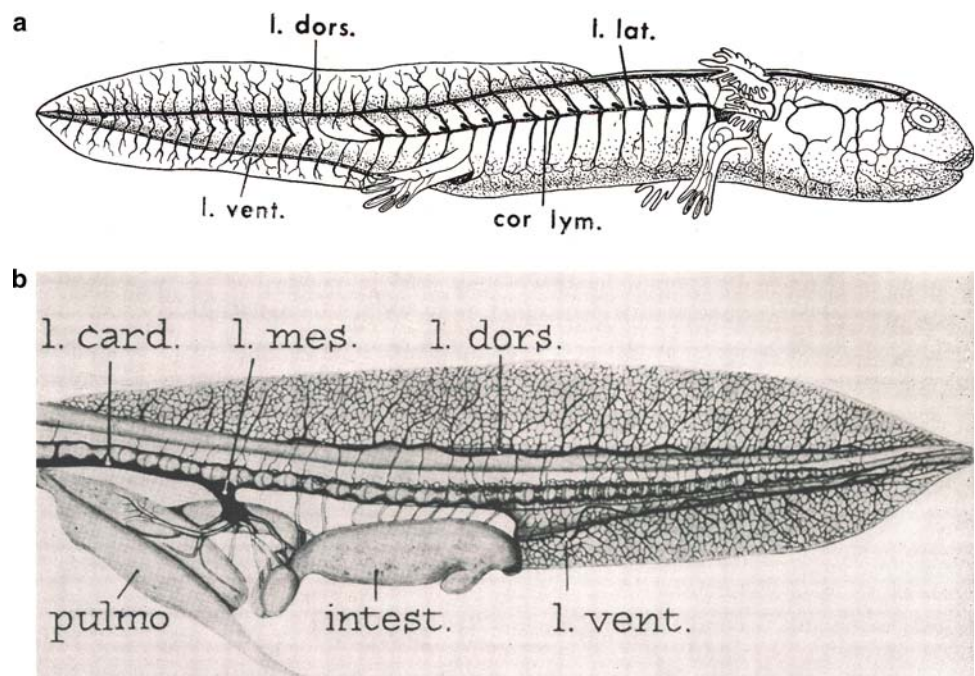


Fig. 1 Lateral view of a 26-mm trout embryo, *Salmo forio*, injected to show the superficial lymph trunks (black lines). Diagram is after Hoyer (1934) and obtained from Kampmeier (1969). Courtesy of Charles C Thomas Publisher, Springfield, IL

Fig. 2 a Lateral view of subcutaneous lymphatics in a larva of the salamander, *Salamandra maculosa*, showing the arrangement of the intersegmental lymph hearts (cor lym.) and the axillary and inguinal lymph sinuses (black lines) (after Hoyer and Udziela 1912, obtained from Kampmeier 1969). **b** Lateral view of the lymphatics (black lines) of the body and tail of a 54-mm larva of the axolotl, *Amblystoma mexicanum*, after removal of skin, musculature (of the left side) and mesonephros (after Grodzinski 1927, from Kampmeier 1969). Courtesy of Charles C Thomas Publisher, Springfield, IL



embryos (Fig. 1). A network of small lymph vessels next to the aorta fuses to form two main trunks, the thoracic ducts. A similar developmental pathway was also observed for the lateral lymphatics. McClure (1913), based on experiments with salmonid and ganoid embryos, reached conclusions contradictory to those of Hoyer. This latter researcher claimed that the lymphatic system initially has no direct communication with the venous system and considered it to have arisen by a fusion of independent clefts, with its communication with the veins being a secondary phenomenon.

Amphibians

Phylogeny

The lymphatic system of amphibians and reptiles is basically similar to that of fish (Fig. 2a, b), but the presence of lymph hearts is a specific characteristic of the former. The presence of these organs are the exception in fishes, and they are also generally absent in adult birds, with the exception of certain species where they are present only in the embryo. In fish, lymph is propelled primarily by the movement caused by contraction of the trunk muscles, so lymph hearts play only a minor role in the transportation of lymph and the maintenance of circulation. Respiratory movements and gill movements in fishes are equally important for the maintenance of lymph flow (Jossifov 1903). The lymphatic system of frogs and anurans represents a special differentiation process. Gaupp (1896) described that, in contrast to other amphibians, anurans

have practically no tubular lymphatics (except lymph capillaries) and that all their lymph accumulates in the lymph cavities (*Spatia lymphatica*). Frogs have no definite system of lymphatics in the strict sense of the term: they are devoid of a system of connected tubes, but are provided with wide-spread lymph sacs. Knowler (1908) suggested that these sacs are not primary formations but the results of the transformations of lymph channels.

Ontogeny

In amphibia (Hoyer 1908; Hoyer and Udziela 1912; Knowler 1908, 1913–1914, 1939; Kampmeier 1915, 1919, 1920, 1922, 1925, 1958) and in reptiles (Huntington 1911; Stromsten 1910, 1911), lymph sacs or plexuses arise in association with several veins. In the frog, the first lymph vessels form a small plexus which drains into the third intersegmental vein. The anterior lymph heart is produced from this plexus by the dilatation and confluence of the channels. A little later, posterior lymph hearts develop from caudal plexuses (Kampmeier 1925; Knowler 1939). Since the lymph hearts arise in an intersegment just next to two adjacent myotomes, Knowler (1908) put forward the view that the source of the muscle of the lymph hearts was these myotomes; lymph sacs not adjacent to myotomes were not transformed to lymph hearts. Kampmeier (1922), however, provided evidence suggesting that the muscle is derived from adjacent mesenchymal cells.

Reptiles

Phylogeny and ontogeny

The lymphatic system of reptiles consists of tubular lymph vessels with plexuses and sinuses similar to those of Urodela. The thoracic duct, paired in its cranial part, coalesces to form a reservoir (*Cisterna lymphatica*) that drains the lymphatics of the intestine and pelvis from the caudal lymph hearts (not more than two).

Birds

Phylogeny

The lymph node is not connected with the lymphatic system in lower vertebrates. Islets of lymphoid tissue embedded in the lymph paths first occur in certain aquatic and wading birds (Jolly 1908, 1919, 1919), but there are only two pairs in the cervical and lumbar regions. The lymphatics of the head and neck empty into the cervical lymph nodes situated before their junction with the vein, but the lymphatics of the heart, lung, trachea and wings still empty directly into the thoracic duct or independently into

veins. Lymphatics empty into two thoracic ducts that run parallel to the aorta and are drained by the Vv. Cavae craniales. Valves are indeed present, but at a considerably lower number than in mammals. The lymphatic network, consisting of a narrow and thin-walled tubular system, is encountered only in birds and mammals, and it is here that a characteristic valve system securing unidirectional lymph flow is first seen.

Ontogeny

Afanasiew (1868) observed “vesicles” which he took for lymph cavities. Budge (1880, 1881, 1887) made the most thorough study of the lymphatic system and its development in chick embryos. The “second lymphatic system” (Budge) is characterized by the development of the paired thoracic ducts on the 8th to 10th embryonic day. By this time, all larger caliber blood vessels are accompanied by two small lymphatic trunks interconnected by numerous small branches, forming a coat of lymphatics around the blood vessels. These lymphatics empty into the veins partly through the thoracic duct and partly through the posterior lymph hearts. Thoracic ducts originate from solid mesenchymal cords that subsequently grow round a cavity; approximately 10 days are needed for the thoracic duct to be formed. It is only after this process is completed that, secondarily, a connection with the venous system is established. In avian embryos, the jugular lymph sac remains a plexus rather than a sac (Miller 1912; Clark 1912; Kampmeier 1960). This plexus ultimately becomes, in part, a lymph node in the chick, but not in all birds (Jolly 1910). The earliest lymphatics in the posterior region of the chick embryo emerge in association with the first five coccygeal veins either as buds (Sala 1900; Clark and Clark 1912) or as mesenchymal spaces (West 1915). These then grow to form a lymphatic plexus in communication with the vein. This plexus is in turn transformed into a pulsating lymph sac—the posterior lymph heart. In the chick, the posterior lymph heart later disappears, although it maintains its function in adult life in some birds.

Mammals

Phylogeny

In mammals, lymph hearts have disappeared, and the lymphatic and the venous system connect generally at only a single point in the jugular region where the thoracic duct and the large lymph trunks join the great vein. The number and situation of the lymph nodes varies among species. The lymph nodes or groups of lymph nodes that are situated in species-specific positions and collect the lymph of an organ or region are termed tributary lymphatics. Another

characteristic of the lymphatic system of mammals is the large number of valves. As described above, valves are not observed in animals whose lymphatics still largely comprise broad sinuses and large hollow spaces. The valvular system constitutes an important factor in the maintenance and regulation of lymph flow in mammals.

Ontogeny

Ranvier (1895) was the first to perform systematic investigations into the ontogenesis of lymphatics in mammals. The lymphatics of the skin and the chyle vessels terminate in a saccular formation in 6- and 10-cm pig embryos. Further growth is by sprouting: buds are formed at the end or on the side of the closed sacs and, with further growth, the buds form channels. Valves arise simultaneously with the development of channels. Ranvier likened the developing lymphatics to huge glands in terms of their secretion as lymph. Based on observations using a dye injection method, Sabin (1902) claimed that, in most mammals, there are two sets of paired lymph sacs lying close to the veins, the jugular and iliac sacs, and two unpaired sacs, the retroperitoneal and cisterna chili. These are preceded by blood-filled capillary plexuses. The jugular sacs arise in association with the anterior cardinal vein in the neck, and all other lymphatic sacs arise from the mesonephric vein and veins in the dorsomedial edge of the Wolffian bodies. Sabin also claimed that, starting from these points, lymph vessels grow and ramify gradually until reaching the periphery. Sabin's work began a long debate among American anatomists over the evolution of lymphatics, which still continues to some extent to this day. The development of the jugular lymph sac has been shown in embryos of man (Sabin 1909), pig (Sabin 1902, 1904; Heuer 1909; Beatjer 1908; Clark and Reichert 1921), cat (Huntington and McClure 1910; Huntington 1911, 1911), rabbit (Lewis 1906), mouse (Higgins 1926), guinea-pig (Schwarz-Karsten 1928), and opossum (Zimmerman 1940).

Recklinghausen's work (1862) led to the concept that peripheral lymphatics originate from unlined canaliculi or "Saftkanälchen" ("juice channels") in the interstitial spaces of tissues. He proposed that these canaliculi turn into lymphatic vessels, develop an endothelial lining, thus giving rise to the subsequent formation of the uniform system of tubes and channels. Lewis (1906) found that part of the V. cardinalis inferior was apparently changed into a lymphatic vessel. Observing additional veins which seemed to be converted into lymphatics in a similar manner, he began to a systematic study of the histogenesis of the developing lymphatics. He found that the main lymphatic trunk next to the aorta (thoracic duct) originates from the Vena azygos, while the cranial portion arises from

the jugular sacs, and the caudal part from the V. subcardinalis. Huntington and McClure (1906, 1907) claimed that lymphatic trunks are formed by the fusion of perivenous mesodermal clefts and have no communication with the veins, and that the lymphatico-venous junctions were secondarily established at certain points. Huntington and McClure (1908, 1908) and Huntington (1914) modified their hypothesis to suggest that the jugular lymphatic sac arises from the capillary network of the embryonic anterior and posterior cardinal veins and that only the rest of the lymphatic system is formed through a fusion of extra-intimal mesenchymal clefts. Huntington's principal object was to prove the primary extra-intimal origin of the main lymphatic trunks.

Early researchers used the living tail of the frog larva (Koelliker 1846; Remak 1850; Langer 1868; Rouget 1873; Clark 1909), and the ear chamber technique in the rabbit (Clark and Clark 1932, 1933, 1937, 1938) to observe the growth of lymphatic vessels. In both mammals and amphibians the growth of the lymphatics is always secondary to that of the blood capillaries. Growing lymphatic capillaries sometimes lie adjacent to venules or blood capillaries, but they never join together with blood capillaries (Clark and Clark 1937).

The course of the thoracic duct is very variable in mammals. In 1908, Pensa claimed that the different types of thoracic duct may be traced back to a basic form with two thoracic ducts, one on either side of the aorta, communicating via transversal anastomoses. Jossifov (1904, 1906) devoted much attention to the variable origin of the thoracic duct in man and wrote that Pensa's theory seemed to be probable but that there was no evidence to show this basic type at a certain stage of mammalian ontogenesis (Baum and Trautmann 1933). Zhdanov (1945, 1952) concerned himself with the variations of the confluence of the thoracic duct and the main lymphatic trunks with the cervical veins.

Early anatomists and embryologists had tried to explain the reason why the course of the thoracic duct shows so many variations in different adult animals. Huntington found that, at a certain stage of a cat's growth, the embryonic structures of the thoracic duct appear as potentially symmetrical lymphatic channels. It is only later, when the left-hand duct has come to drain the lymph from the whole trunk and the posterior extremities, that it alone constitutes the thoracic duct in the proper sense of the term. Kampmeier (1936) investigated the thoracic duct based on the examination of serial sections of human and pig embryos. He divided the duct into three portions—cervical, thoracic, and abdominal (*Cisterna chili*). The cervical part consists of the jugular lymph sac and the arch of the Ductus thoracicus. In general, Kampmeier subscribed to the views of McClure, but he dissented from McClure and

Huntington on one point in claiming that the jugular lymphatic sac is of a non-venous origin. The jugular lymphatic sac connects to the great cervical veins at the jugular angle. The cervical arch of the thoracic duct arises along the dorsomedian collateral branches of the anterior cardinal vein through a fusion of the mesenchymal clefts, independently of the veins. The thoracic part develops along the anterior and posterior cardinal veins and also along an oblique collateral vein in a manner similar to that described by Huntington in cat embryos. The newly formed permanent thoracic duct is drained by the left Saccus jugularis, and the main trunk formed along the V. oblique unites the primitive channels which have arisen on both sides of the azygos veins. The smaller mediastinal lymphatic trunk, which corresponds to the right thoracic duct of birds and lower vertebrates empties, together with the Truncus lymphaticus dexter, into the right jugular lymph sac or its vestigial part. The cysterna chili arises at the height of the kidneys or rather of the Arteria coeliaca on the site of the plexus made up from the supracardinal (azygos) veins, through a fusion of the mesenchymal slits which are originally independent of the venous system; growing, it gradually compresses this venous plexus.

Ontogenesis of the lymphatic vascular system

The ontogenesis of the lymphatic system remains controversial even today. Do the lymph vessels grow from the great veins toward the periphery by means of budding and ramification (the “centrifugal” model), or do they travel toward the great veins by means of fusion of the mesenchymal clefts (the “centripetal” model), or does the process consist of some mixture of both of these? In 1911, Huntington summarized various possible models as follows:

1. The lymphatic system develops independently of the blood vascular system through a fusion of the mesenchymal clefts, or rather through a canalization of differentiated mesenchymal bundles, and its connection with the veins is but a secondary phenomenon.
2. Lymph vessels arise from veins: certain embryonic vessels are directly transformed into lymphatics.
3. All lymph vessels are derived from the fusion of channels abstracted from embryonic veins or, else, it is only the thoracic duct which arises in this manner, while all other lymphatics arise independently.
4. The entire lymphatic system originates from blind buds and ducts growing out from veins. Dilating, they form sacs: these issue lymph vessels toward the periphery which, through continuous growth, reach all organs and spread over the skin and serous membranes.

5. Lymphatics are formed through a fusion of perivenous mesodermal clefts which, in point of fact, develop extra-intimally, quite independently of the vein with which they have no communication except at the point of certain strictly defined lymphaticovenous anastomoses which, however, are the products of a secondary development.
6. There is finally a theory which, combining the above possibilities, holds that part of the lymphatic system (the jugular lymphatic sac) arises from a metamorphosis of the perivenous capillary network, while all other parts owe their existence to a fusion of the perivenous extra-intimal clefts.

Anatomists and embryologists using the injection technique favored the view that the primary lymph sacs bud off from the vein and that the lymphatic vessels grow from these sacs in a centrifugal direction by endothelial budding. Sabin and her students were the chief protagonists of this view (Sabin 1902, 1904, 1908, 1911, 1916; Baetjer 1908; Heuer 1909; Reichert 1912; Knowler 1939). This concept elicited the justified objections from other investigators that injections can only be made into an uninterrupted system of tubes so that failure to demonstrate lymph vessels by way of injections does not necessarily mean that the injected area is void of lymphatics existing in the form of mesodermal clefts.

In contrast, researchers using serial sectioning methods developed the alternative view that lymphatic vessels arise from mesenchymal spaces. The primary sacs arose, and the general systemic lymphatic vessels initially developed along the course of the embryonic veins; subsequently, centripetal extensions made connections with the lymph sacs, which made secondary connections with the venous system (Huntington and McClure 1910; Huntington 1908, 1910, 1910, 1911, 1911, 1911, 1914; Kampmeier 1912, 1922, 1925; McClure 1915, 1921; Miller 1912; Zimmerman 1940). The methods of investigation leading to this concept have also been criticized on the grounds that any study of growing lymphatics in serial sections is unreliable because not all of the lymphatic endothelium present at any period of development can be seen in stained cross-sections and the identification of lymphatics is problematic.

The secondary vascular system theory

Kampmeier (1969) performed a detailed classification of the lymphatics of adult teleosts (see Fig. 3). In the course of our work to establish the zebrafish as a useful model organism for studying lymphangiogenesis, we were able to confirm the existence of every lymphatic vessel classified

Deep Lymphatics of the Trunk and Tail
cardinal lymphatics (thoracic ducts) (l. card)

lymphatica cardinalis collateralis (l. card. Collat):

Depending upon the species, either the cardinal lymphatic or its collateral serve in the capacity of collecting the lymph and chyle from the viscera (gonads, intestine, stomach, esophagus, swim bladder). Principally, according to Glaser, the cardinal lymphatics collect the submuscular drainage from the dorsal part of the body, begin in communication by cross-channels with the spinal and subcutaneous dorsal and lateral lymphatics; in other words, the “thoracic duct” drain chiefly the somatic areas, and the collateral cardinal lymph trunks, the visceral areas.

spinal lymphatic (spin l.):

Superficial Lymphatics of the Trunk and Tail

lateral lymphatic (lateral l.)

dorsal lymphatic (dorsal l.)

ventral lymphatic (ventral l.)

intersegmental lymphatics

Lymphatics of the appendages

Lymphatica pectoralis

Lymphatica pinnalis caudalis

Lymphatics of the Head

MacClure’s description and figures of the lymphatic plan in the head of a 22-day rainbow-trout embryo provides a relatively uncomplicated and basic schema for guidance in their comparisons.

jugular lymphatic

precardinal and lateral pharyngeal lymphatic by MacClure.

Composite scheme of systemic lymphatics (yellow) in teleosts; veins, black; arteries, cross-lined. Vents I, II and III indicate the three levels of anterior lymphatic communications with the veins favored by different fishes. The caudal lymph propulsor is shown at its best development, as seen, for example, in the eels. (after Kampmeier, 1969).

From Kampmeier O.F., Evolution and comparative morphology of the lymphatic system, 1969, Courtesy of Charles C Thomas Publisher, Springfield, Illinois

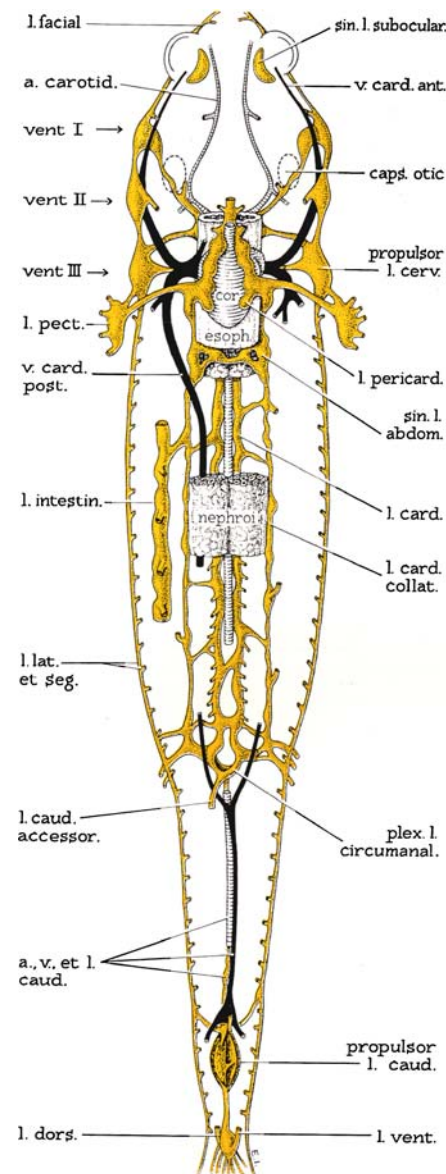
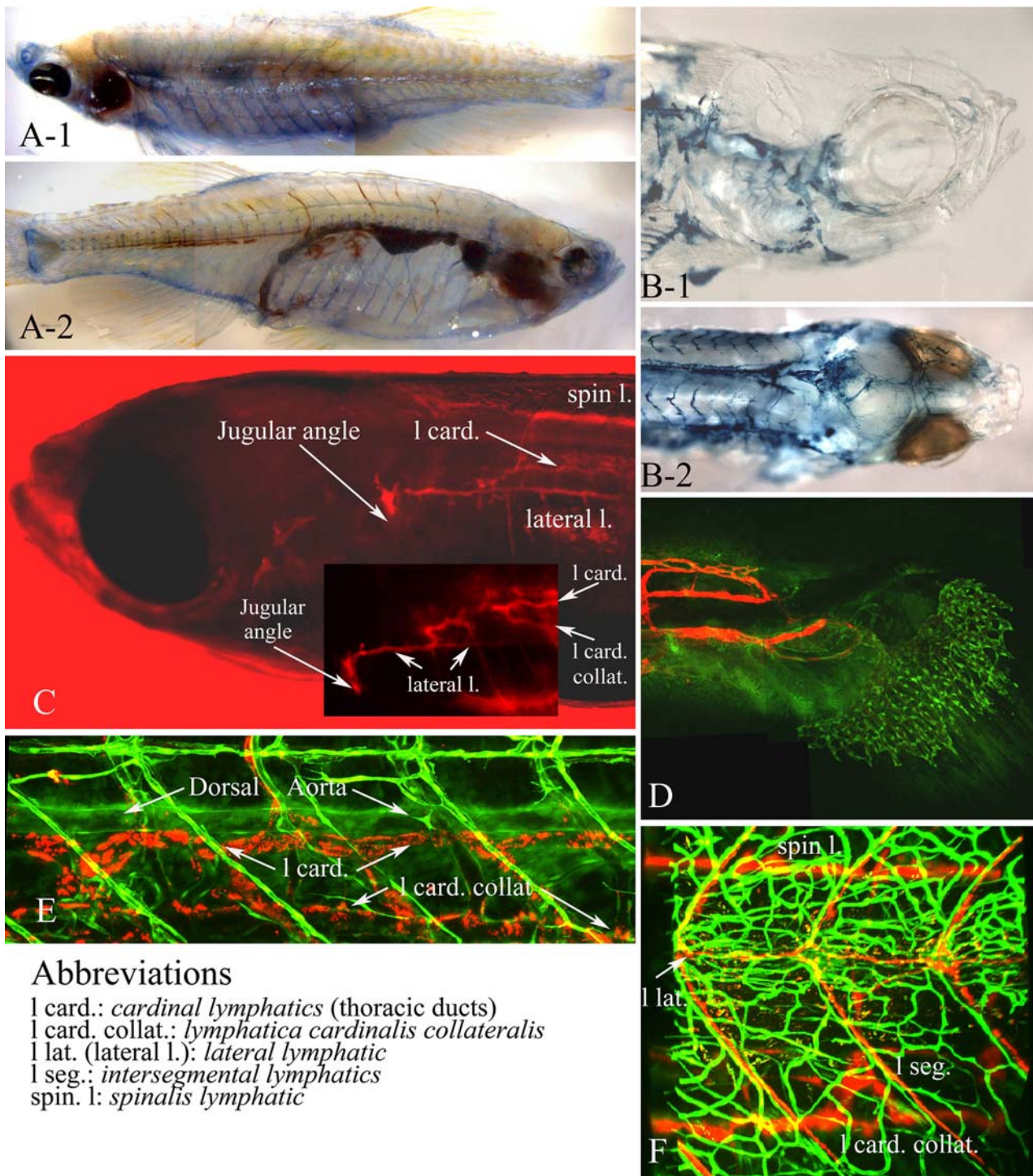


Fig. 3 Composite scheme of systemic lymphatics (yellow) in teleosts. Veins are given in *black* and arteries are *cross-lined*. Vents I, II, III indicate the three levels of anterior lymphatic

communications with the veins favored by different fishes. The caudal lymph propulsor is shown at its best development, as seen, for example, in the eels (from Kampmeier 1969 with permission)

by Kampmeier in the adult zebrafish (Fig. 4; Yaniv et al. 2006). However, Vogel (1985) and Steffensen et al. (1986) proposed the terms inter-arterial anastomoses and secondary vessel system to describe the vessels Kampmeier observed, claiming that the vessels originated from the artery in the fish and did not represent true lymphatics (Fig. 5). In support of this view, Steffensen and Lomholt (1992) wrote that, in contrast to the lymphatics of other vertebrates, the “secondary vessels” of fish were in open communication with systemic arteries via a large number

of anastomoses of capillary dimensions. As evidence, they showed live images, dye-injected specimens and plastic casts. The work of these authors has led to the recent view that zebrafish, an important model organism for blood vascular studies, lacks a well-established lymphatic vasculature (Ny et al. 2005). However, our recent studies show very clearly that the secondary vascular system theory put forward by Steffensen et al. (1986) is incorrect and that zebrafish possess a lymphatic vascular system that has many features in common with those of other vertebrates.



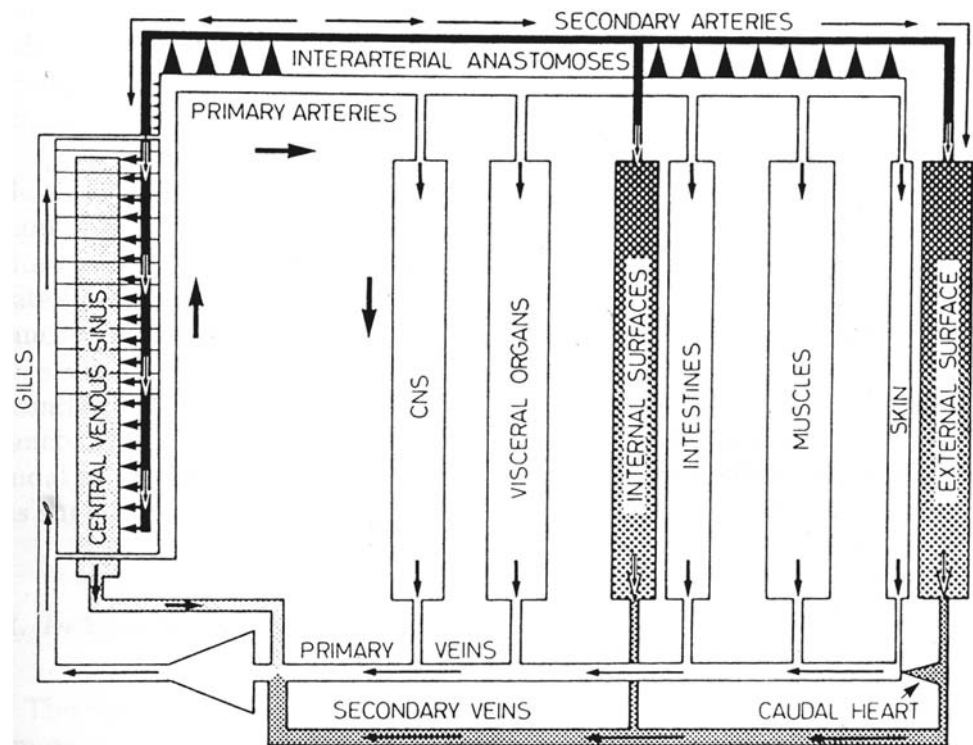
Abbreviations

l card.: *cardinal lymphatics* (thoracic ducts)
 l card. collat.: *lymphatica cardinalis collateralis*
 l lat. (lateral l.): *lateral lymphatics*
 l seg.: *intersegmental lymphatics*
 spin. l.: *spinalis lymphatic*

Fig. 4 **a** Lymphatic systems of adult zebrafish (trypan blue is taken up by the endothelium cells of lymphatic): **a-1** dorso-lateral view, **a-2** median plane after removal of skin and musculature of the right side. **b** Cranial lymphatic systems of adult zebrafish (injected with Berlin blue): **b-1** lateral view, **b-2** dorsal view. **c** Drainage point (jugular angle) of lymphatic vessels into the common cardinal vein (CCV) at 3 wpf (weeks post-fertilized) zebrafish. Thoracic duct merges into the lateral lymphatics and then drains into CCV just caudal to the optic capsule. **d** Lymphatic system of tail in zebrafish at 3 wpf (red fluorescence beads are injected into the lymphatic system of

fli-enhanced green fluorescent protein (EGFP) transgenic zebrafish, in which the *fli-1* promoter drives the expression of EGFP in vascular and lymphatic endothelial cells. **e** Left lateral trunk view of the *fli* EGFP transgenic zebrafish at 3 wpf. Fully developed thoracic duct extends along the dorsal aorta. **f** Left lateral trunk view of the *fli* EGFP transgenic zebrafish at 5 wpf. Superficial lateral lymphatic appears at this stage. Red fluorescence beads are injected into the lymphatic system, and so the blood vascular system remains green in **e** and **f**

Fig. 5 Vogel's concept of the primary and secondary circulation systems in a typical teleost (after Vogel 1985). From Steffensen (1992). Copyright Elsevier



The key evidence for the secondary vessel theory was that the authors proposing this theory described open connections between the arteries and secondary vessels (lymphatics) in all parts of the animal. Steffensen et al. (1986) showed plastic casts of *Oncorhynchus mykiss* with apparently lymphatic-like vessels originating from arteries as evidence for their theory.

In contrast, Ura (1950 and unpublished data), and Isogai and Horiguchi (1997 and unpublished data) injected Berlin blue dye into the vascular system of the rainbow trout and prepared a complete series of developmental anatomical atlases of the trunk vascular system, without finding any connection between the lymphatics and arteries. To further check this, we injected dye (Berlin blue 0.5% solution) into the vascular system of zebrafish at stages varying from the embryo through to the adult, without observing any lymphatic–arterial connections. We also performed microangiographic injection (Weinstein et al. 1955) of fluorescent dye into both the blood and lymphatic vasculature and, by means of high-resolution confocal imaging, confirmed that there is no such connection in the zebrafish (Fig. 4; Yaniv et al. 2006). However, when we injected plastic resin into the vascular system of the adult zebrafish, we observed the connections which Steffensen et al. (1986) showed with their plastic casts.

In order to obtain well-remodeled blood vascular casts, researchers often inject resin media into the heart or major arteries at relatively high pressures, resulting in leakage

from small blood vessels (artery, capillary and vein) into the interstitial tissue. It is well known that fine lymph vessels can be cast after the injection of methacrylate or other resins into the interstitial tissue (Castenholz 1989). Consequently, both blood vessels and lymphatic vessels are often remodeled via direct connections. As mentioned in a short history of lymphology, His (1863) claimed that the lymph vascular apparatus formed a closed system of tubes, although it had been assumed that the lymphatics were directly communicating with the blood vessels through a very fine and tubular system. However, few modern researchers who used resin-casting method still assumed that the fine lymphatics were directly communicating with peripheral blood vessels through a very fine and tubular system, even in mammals. Using corrosion cases, Castenholz (1989) examined the fine structure of the vessels of the terminal blood circulation and the initial lymphatic system of rats, guinea pigs and tree shrews and showed that resin fills the initial lymphatics after interstitial injection. There are different explanations for the mode of filling: (1) the resin may invade the vessel's lumen through the numerous small open junctions of the lymphatic endothelium (Wenzl-Hora et al. 1987); (2) the casting medium fills the vessel via a direct prelymphatic-lymphatic connection which passively exists in different places of the connective tissue; (3) there is a direct communication between prelymphatic spaces or channels and true lymphatics in the mesentery (Hauck 1973; Hauck et al. 1987) and also in living and

fixed tongue tissue (Castenholz 1989). Castenholz concluded that the initial lymphatics are filled from the interstice via such performed or artificial tissue-vessel communications. We have performed resin casting of the vascular system for over 20 years, using the mammals, birds, reptile, amphibians and teleosts. We have occasionally collected the same sorts of images that Steffensen et al. (1986) reported as the anastomoses. Based on our experience, we know that the lymphatics were often cast well accidentally when trying to perform blood vascular casts. Together, our conclusions and those of other researchers mentioned above lead us to the conclusions that Steffensen et al. (1986) failed to recognize the resin casting artifacts that occur in vascular casts of all vertebrates, which appear as arterial-lymphatic anastomoses in the fish.

Zebrafish as a new animal model to study lymphangiogenesis

As mentioned above, we reviewed classical papers using the great books written by Rusznyák et al. (1960) (*Lymphatics and lymph circulation*), Yoffey and Courtice (1970) (*Lymphatics lymph and the lymphomyeloid complex*) and Kampmeier (1969) (*Evolution and comparative morphology of the lymphatic system*) in order to draw conclusions about the common and distinct aspects of the lymphatic system of different vertebrates and to compare zebrafish lymphatics with those of other teleosts and vertebrates from the morphological point of view. In order for the zebrafish to serve as a new genetic model for studying lymphangiogenesis, it is important that it possesses a lymphatic vascular system that shares the morphological, molecular and functional characteristics of the lymphatic vessels found in other vertebrates. Our recent work has shown clearly that this is the case (Yaniv et al. 2006). The purpose of this article is to provide a firm morphological foundation for the molecular genetic research of lymphangiogenesis using zebrafish.

Classical studies using cyclostomes and cartilaginous fishes suggest that the lymphatic systems of bony fishes, amphibians, reptiles, birds and mammals are phylogenetically evolved from the venous system. However, as reviewed above, the ontogenesis of the lymphatic system is

still controversial because of the difficulties in observing lymphatic cells in vivo and in performing defined genetic and experimental manipulation of the lymphatic system in currently available animals. We believe that use of the zebrafish will lead to valuable new insights into the origins of the vertebrate lymphatic system.

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