Are we about to settle
this century old controversy?

Heterogeneity of the origin of the lymphatic system

K. Mattonet, M. Jeltsch

Wihuri Research Institute and Translational Cancer Biology Research Program, Biomedicum Helsinki, University of Helsinki, Finland

Summary

The question "How does the lymphatic system develop?" may be a simple one, but it is fundamental to the understanding of lymphatic malformations in children and the regeneration of lymphatics in adults. The questuion is by no means new and was already explored in the early 20th century. This resulted in a long-lasting controversy, which until recently had been far from being settled. The interest in the lymphatic system has greatly increased in recent years due to its implications in a variety of diseases. Several studies published this year address the heterogeneity of lymphatic endothelial cell development and unite previous controversially discussed data in a coherent model. These remarkable results, as well as the studies that paved their way, are discussed in this review.

Keywords: embryonic development, lymphatic system, lymphatic endothelial cells, lymphvasculogenesis, lymphangiogenesis, centrifugal, centripetal

Über die heterogene Herkunft des Lymphgefäßsystems

Zusammenfassung

Die Beantwortung der Frage nach dem embryonalen Ursprung des Lymphgefäßsystems ist fundamental für unser Verständnis von Fehlentwicklungen des Systems bei Kindern und seiner Regeneration bei Erwachsenen. Doch neu ist die Frage keineswegs und sorgte bereits im frühen 20. Jahrhundert für vehemente Diskussionen. Bis heute ist diese Debatte nicht beigelegt, doch das Interesse am Lymphgefäßsystem hat aufgrund seiner Bedeutung für eine große Zahl verschiedenster Erkrankungen in den letzten Jahren stark zugenommen. Mehrere diesjährige Publikationen zur Heterogenität der Entwicklung von Lymphendothelzellen scheinen nun die Vielzahl bislang kontrovers diskutierter Studien zusammenzuführen und zu einem schlüssigen Modell zu vereinen. Diese bemerkenswerten Ergebnisse, wie auch die Studien, die ihnen den Weg bereitet haben, werden in dieser Übersichtsarbeit dargestellt.

Schlüsselwörter: Embryonalentwicklung, Lymphgefäßsystem, Lymphendothelzelle, Lymphvaskulogenese, Lymphangiogenese, zentrifugal, zentripetal

How does the lymphatic system develop? This question is as simple as it is fundamental to the understanding of the lymphatic system (LS) under physiological and pathological conditions. Moreover, it is also critical for the development of therapeutic interventions. In 1902, the anatomist *Florence Sabin* published her studies on the development of the earliest lymphatic vessels in pigs [1] and thereby laid the foundation for a controversy that would last for more than a century into the present. Sabin visualized lymphatic vessels by dye injections into living embryos, and her results implied that already the earliest lymphatic vessels are connected to the venous system and that the development of the lymphatic system starts by radial sprouting from lymph sacs, which arise from four outgrowths of the venous system. *Sabin* thus provided experimental support for the centrifugal development hypothesis that had been proposed earlier by Ranvier, who, however, had not been able to support his claims experimentally [2].

In 1905, *Frederic T. Lewis*, a proponent of the centrifugal hypothesis, modified Sabin's original idea based on serial sections of rabbit embryos. He observed that the original lymphatic outgrowths from the veins would disappear giving rise to closed lymph sacs in the mesenchyme, and that only later, the permanent openings of the lymphatic system into the cardiovascular system would form [3]. He also described the existence of more than only four venous locations from which the LS would originate. About 70 years later, his view of the lymphatic development received solid support by a detailed histological analysis of mouse and human embryos by van der Putte [4, 5].

Nevertheless, the centrifugal hypothesis of *Sabin* and *Lewis* was already early on challenged by others, notably by *Huntington* and *McClure* in 1908 [6]. They proposed the so-called centripetal model, in which lymphatic spaces could form de-novo by differentiation within the mesoderm. Those spaces would merge in the course of development to ultimately form a continuous vascular system, that would finally connect to the venous system. *Huntington* and *McClure* based their model on serial paraffin sections of early cat embryos, in which they could demonstrate that some lymphatic structures (e.g. the Ductus thoracicus) are never connected to the venous system during the early development of the lymphatic system.

Two different anlagen

A synthesis between these two contrasting models of lymphatic development was proposed by *van der Jagt* in 1932 [7], who found a composite origin of the anterior lymph sacs of turtle embryos and thus proposed, that both mesenchyme-derived and venous-de-

Figure 1.

The arrival of molecular biology inflamed the century old controversy between the centrifugal and centripetal hypotheses of lymphatic development. After initial over-enthusiasm about experimental data in favor of the centrifugal hypothesis, a synthesis of the two hyoptheses seems within reach. This figure shows a comparison of the visualization of lymph vessels by Florence Sabin (dye injection into porcine embryos) with the X-gal staining of heterozygous VEGFR-3+/lacZ mouse embryos.
Lotta Jussila is acknowledged for providing the photograph of the X-gal staining.

rived structures would merge in order to form the LS. Eight decades later, further experimental evidence would show that his proposal came closest to reality.

Despite their impressive accuracy and meticulous descriptions, the early lymphatic researchers were restricted by the technical limitations of the time. While *Sabin's* work was characterized by a relatively specific detection method, she could observe only functional lymphatics. *Huntington* and *McClure,* on the other hand, suffered from the absence of specific staining methods for the lymphatics and thus were confined to the study of vessels with known localizations [6]. *Huntington* and *McClure* argued that the proponents of the centrifugal hypothesis would only observe functional derivatives of the earliest lymphoid structures [8]. At the same time, the mesodermal lymphatic spaces described by *Huntington* and *McClure* were disregarded as artifacts by proponents of the centrifugal hypothesis [4]. Collectively, all these early studies were restricted to observing lumenized structures and all suffered from the inability to detect their precursors.

Only during the last decade of the millennium novel techniques and lymphatic-specific markers had become available that allowed new insights and thus reopened the debate.

Transplantation experiments in chicken embryos indicated that at least some lymphatic endothelial cells in birds were not of venous origin [9], and this argued for a heterogeneous development of the LS as suggested by *van der Jagt.* Follow-up studies showed that lymphangioblasts contribute to the formation of the lymphatic system of birds [10]. These and similar observations in Xenopus tadpoles [11] and mice [12] suggested that several complex mechanisms might co-operate in the development of the LS.

The Centrifugal Hypothesis

However, around the same time, data from other studies argued strongly for the far more popular hypothesis of centrifugal development of the LS. *Wigle* and *Oliver* showed in 1999 that the homeobox transcription factor Prox1 is indispensable for lymphatic development [13], which begins in mice at embryonic day E9.5 with the appearance of Prox1-positive cells in the jugular region of the anterior cardinal veins. These Prox1-positive, lymphatic precursor cells sprout from the cardinal veins into the surrounding mesenchyme where they form early lymphatic structures [14]. This migration and separation depends on various signaling molecules such as VEGF-C [15], CCBE1 [16] and other molecular markers such as Podoplanin [17]. In

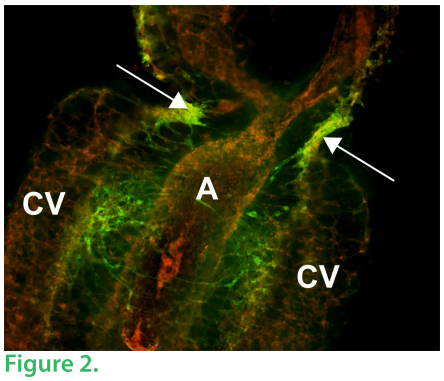

Figure 2.

Centrifugal development of lymph sacs and adjacent lymphoid structures in the mouse (day E11.5). The mode of emigration of LEC progenitor cells from the primitive veins (whether as single cells, as cells strands or as lumenized vessels) appears heterogeneous and seems to depend among other factors on the visualization methodology and investigated species. This immunofluorescence image shows the blood endothelial cells (PECAM-1 staining, red) and the lymphatic endothelial cells and their precursors (LYVE-1 staining, green). The white arrows point to the evolving jugulo-axillary lymph sacs. A, aorta; CV, cardinal veïn. Marika Kärkkäinen is acknowledged for providing this figure.

2007, the lymphatic development was followed meticulously in a mouse model by fluorescent labeling of Prox1-expressing cells, showing that certain venous endothelial cells begin to express Prox1 and that these are the cells, that start to migrate into the surrounding mesoderm to form the early paravenous lymphatic structures [18]. Together with further support from studies in zebrafish [19], *Sabin's* centrifugal hypothesis seemed to emerge from the controversy as winner proving the embryonic veins as the principal and probably only source of lymphatic endothelial cell (LEC) precursors . Indeed, this view dominated the following years [20], and the participation of lymphangioblasts and other sources of lymphatic endothelial cells in the development of the LS was generally regarded as a phenomenon that might play a role in birds and frogs, but not in mammals.

Nevertheless, several studies in the following years revealed phenomena that could not be easily explained while maintaining the view of a purely ve-

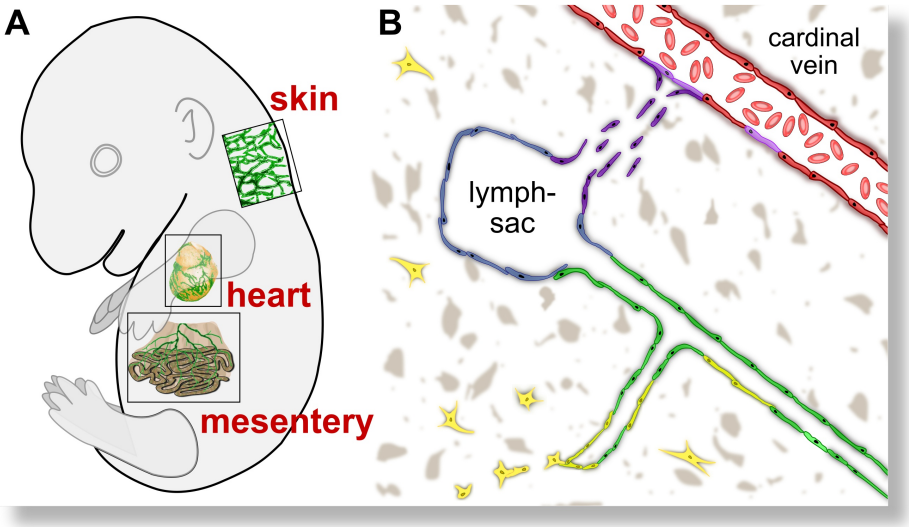

Figure 3.

A) Studies published this year show that the lymphatics of the mesentery, the skin and the heart are formed not only by centrifugal lymphangiogenic growth originating from the embryonic lymph sacs, but also from progenitor cells, which are found within the mesenchyme.
B) The lymphatics of the skin and the mesentery (green) are established from contributions by lymphangiogenic processes from the embryonic lymph sacs (blue) and from precursor cells in the mesenchyme (yellow). The embryonic lymph sacs in turn are established from endothelial cell outgrowths (purple) from the large embryonic veins, most notably the cardinal veins (red). These emigrating cells take up a lymphatic identity. Additionally, it was proposed, that the hemogenic endothelium of the yolk sac represents another alternative, non-venous source of lymphatic endothelial cells specifically for the heart.

nous origin of lymphatic endothelium. For example, *Hägerling et al.* observed between mouse embryonic day E10.0 and E10.5 a massive increase in Prox1-positive LEC precursors outside the veins, although the rate of division of this cell population did not change appreciably at the same time [21]. The origin of these suddenly appearing Prox1-positive cells could not be identified, although *Hägerling* identified the skin veins as an additional venous region from which LEC precursors can emigrate. Such LEC progenitor cells, however, were found in this vein bed only in mutants, in which the major LEC precursor emigration from the cardinal veins was disturbed. This implied both a massive, synchronous migration of LEC precursors between day E10.0 and E10.5, and the existence of other, according to the authors probably venous sources of LEC-precursors, which were not identified. Perhaps, this sudden increase of Prox1-positive cells could be partly explained by a differentiation of cells in the mesenchyme.

Heterogeneous Origin

This year, experimental evidence for such a heterogeneous origin of the lymphatic system has been described in several published studies. By lineage tracing, *Martinez-Corral et al.* showed, that significant numbers of dermal lymphatic vessels have no venous origin, but develop from mesenchymal cells which are negative for the endothelial cell marker Tie2 [22]. These cells form aggregates which further develop into vessels (lymphvasculogenesis), and the resulting, isolated vessel fragments merge with lymphatic vessels of venous origin to form a functional unit. Notably, this constitutes the first direct evidence that lymphatic vessels can for by vasculogenesis, a process that had been observed so far only in the development of the cardiovascular system. In parallel, a second publication from the same group by *Stańczuk et al.* showed, that the lymph vessels of the mesentery are of dual origin [23]. The study demonstrated that hemogenic, c-kit positive progenitor cells in the mesentery can give rise via a vasculogenic mechanism to lymphatic structures, which then are integrated into the venous-system-derived parts of the lymphatic system. Alternative sources for LEC precursors are also discussed for the heart [24, 25], where, in birds, even lymphatico-venous anastomoses were found [25].

New Light Shed on Lymphatic Vascular Development

These experimental findings shed new light on the development of lymphatic vessels (see Figure 3). They confirm central ideas proposed by *van der Jagt*, according to which both *Sabin's* centrifugal hypothesis and the centripetal hypothesis of *Huntington* and *McClure* are partially correct and contribute to the development of the LS. At the same time they show that the development of the LS can be very heterogeneous between different organs and body regions. This could provide an explanation for the peculiar region-specific manifestation of lymphatic diseases like Milroy's disease [26]. The development of the LS appears today more complex than previously thought, and is similar in this respect to the cardiovascular system.

Our knowledge about the development of the LS is still rudimentary, although it plays a significant role in many important human diseases [27]. Many researchers and experimental approaches have contributed in the past 20 years to our growing body of knowledge about the lymphatic system, and solid basic concepts of lymphatic development emerge. We realize in hindsight, that apparent contradictions were largely confined to the interpretation of experimental data. This demonstrates our dependence on and the limitations of our various model systems, which capture only parts of the complex reality of lymphatic development. The recent new insights into the develop-

ment of the LS will inspire the researcher community, and together with the rapid technological development in the life sciences, this will accelerate the expansion of our knowledge and ultimately lead to better treatment modalities for lymphatic diseases.

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Corresponding author
Dr. Michael Jeltsch, adjunct professor
Wihuri Research Institute and Translational Cancer Biology Program
Biomedicum Helsinki
P.O.Box 63 (Haartmaninaktu 8)
FIN-00014 University of Helsinki, Finland
http://lab.jeltsch.org, michael@jeltsch.org