A Tale of Two Models: Mouse and Zebrafish as Complementary Models for Lymphatic Studies

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Lymphatic vessels provide essential roles in maintaining fluid homeostasis and lipid absorption. Dysfunctions of the lymphatic vessels lead to debilitating pathological conditions, collectively known as lymphedema. In addition, lymphatic vessels are a critical moderator for the onset and progression of diverse human diseases including metastatic cancer and obesity. Despite their clinical importance, there is no currently effective pharmacological therapy to regulate functions of lymphatic vessels. Recent efforts to manipulate the Vascular Endothelial Growth Factor-C (VEGFC) pathway, which is arguably the most important signaling pathway regulating lymphatic endothelial cells, to alleviate lymphedema yielded largely mixed results, necessitating identification of new targetable signaling pathways for therapeutic intervention for lymphedema. Zebrafish, a relatively new model system to investigate lymphatic biology, appears to be an ideal model to identify novel therapeutic targets for lymphatic research. Zebrafish, which are amenable for time-lapse analysis and genetic manipulation (Gore et al., 2012; Wilkinson and van Eeden, 2014), have recently emerged as an important in vivo model system to investigate development and maintenance of lymphatic vessels (Kołtowska et al., 2013; Yaniv et al., 2006), complementing mammalian and cell culture models. In this review, we will summarize the use of zebrafish as a model system for lymphatic biology, compare the commonality and unique features of zebrafish lymphatic vessels, and provide perspectives on zebrafish as a unique model for lymphatic biology.

LYMPHATIC SYSTEM: A BRIEF OVERVIEW

Anatomical structures associated with the lymphatic system, particularly the lymph nodes, were first described as early as the era of Hippocrates (Chikly, 1997). However, it was not until the 18th century that the 'lymphatic system' was discovered (Chikly, 1997; Kerjaschki, 2014). Like blood vessels, lymphatic vessels display a highly stereotypic hierarchy. Lymphatic capillaries absorb excessive interstitial fluid within tissue and transport it to the larger caliber lymphatic vessels such as lymphatic collecting vessels and lymphatic trunks. While lymphatic capillaries usually lack muscular layers, therefore relying on peristaltic movement to transport lymph, larger lymphatic vessels do possess contractile muscular layers to facilitate forward movement of lymph (Dejana et al., 2009; Pfeiffer et al., 2008). However, compared to blood vessels, the muscular layers encompassing lymphatic vessels are generally thinner, which generate weaker contractile force. Therefore, lymph fluid within the large caliber lymphatic vessels can move in a retrograde manner. To prevent backflow of lymph, large caliber lymphatic vessels have lymphatic valves alongside the lymphatic wall, similar to the valves found in the large veins (Bazigou and Makinen, 2013). Excessive interstitial fluid is returned to blood circulation via subclavian veins and thoracic ducts which complete the lymphatic circulation. In addition to modulating the level of interstitial fluid, the lymphatic system plays an essential role in immune responses: Lymph nodes, where immature immune cells reside, function as a filter for foreign immunogens (Altalo, 2011).
EMERGENCE OF LYMPHATIC VESSELS DURING DEVELOPMENT IN MOUSE AND ZEBRAFISH

The basic building blocks of lymphatic vessels, lymphatic endothelial cells, are known to emerge from blood endothelial cells (BECs) during development (Hägerling et al., 2013; Yang et al., 2012). While lymphatic endothelial cells (LEC) share certain molecular characteristics with BECs, cellular morphology of LECs appears to be distinct from BECs: For instance, both LECs and BECs strongly express PECAM and VE-Cadherin (Oliver and Srinivasan, 2010). However, LECs form button-like discontinuous junctions, which make lymphatic vessels highly permeable to excessive interstitial fluid, macromolecules, as well as migrating leukocytes, while BECs tend to form more organized and compact cell junctions (Neufeld et al., 2014).

During development, LECs, similar to other cell types found in cardiovascular systems of vertebrates, are thought to arise within the mesoderm (Marcelo et al., 2013). In particular, lateral plate mesoderm (LPM), a subset of the mesodermal layer in embryos, has been implicated as the developmental source of LECs. LECs first emerge around embryonic week 6 to 7 in humans, E10 in mice, and about 2 days post-fertilization (dpf) in zebrafish, only after the formation of blood vessels (Bruyère and Noël, 2006; Neufeld et al., 2014; van der Putte, 1975). The majority of LECs appear to originate from veins during development (Fig. 1). At the turn of the 20th century, Florence Sabin elegantly demonstrated that LECs originate from veins, which was validated by recent lineage tracing and in vivo time lapse imaging analyses in mouse, Xenopus, and zebrafish (Francois et al., 2011; Ny et al., 2005; Sabin, 1902; Srinivasan et al., 2007; Yaniv et al., 2006). The earliest event during lymphatic development in mouse is the polarized expression of Lymphatic Vessel Endothelial Hyaluronan Receptor 1 (Lyve1) around E9 within a subset of ECs within the cardinal vein, which is the first sign of lymphatic competency of ECs (Banerji et al., 1999; Prevo et al., 2001). While the cardinal vein appears to be the major source of LECs, recent reports suggest that a significant number of LECs may be derived from ECs within the intersegmental vessels (ISVs) at approximately E10.5 and E11 (Yang et al., 2012). At E11.5, mature jugular lymph sacs are formed. During the specification and differentiation of LECs, the expression of SOX18, a member of SRY-related HMG-box (SOX) family of transcription factors, and venous enriched transcriptional factor Coup-TFII appear to be instrumental (François et al., 2008; Srinivasan et al., 2010): These two transcription factors cooperate to induce the expression of a lymphatic specific transcription factor, PROX1 (Wigle and Oliver, 1999; Wigle et al., 2002), which persists in LECs throughout their lifetime.

Although it is apparent that the PROX1 positive LECs within veins are the source of lymphatic vessels during development (Wigle et al., 2002), it is not clear how the separation between blood and lymphatic vessels occurs. Platelets have been implicated as the major driving force for initiating this process (Abthian et al., 2003; Kim and Koh, 2010). By interacting with Podoplanin on the surface of LECs using CLEC-2 on their surface, platelets promote Syk/Spl76-mediated activation of Integrin signaling, which appears to be an essential step for the segregation of lymphatic vessels from the adjacent blood vessels. However, more recently, an alternative model has been proposed: Using high resolution in vivo imaging, Hägerling and colleagues suggest that individual PROX1 positive cells may delaminate from the cardinal vein, and subsequently aggregate to form the thoracic duct (Hägerling et al., 2013). Further analyses are warranted to resolve the potential discrepancy between these two models and to formulate the unified model. However, considering the high level of developmental, functional, and molecular heterogeneity of LECs (Oliver, 2004; Tammela and Alitalo, 2010), it is conceivable that these two mechanisms are not mutually exclusive, and collectively explain the complex nature of lymphatic development in vertebrates.

Similar to mouse, the progenitors for LECs appear to originate...
Diverse signaling pathways modulate lymphatic development. To date, a number of signaling pathways have been implicated in lymphatic development. VEGF-C/VEGFR3 appears to be the most critical regulator of lymphatic development. In addition, the roles of other signaling pathways such as G Protein Coupled Receptor and BMP/TGFβ signaling, as well as Notch signaling pathways are known to modulate distinct aspects of lymphatic development.

MOLECULAR MECHANISMS MODULATING DEVELOPMENT OF THE LYMPHATIC VESSELS

So far, a number of signaling pathways have been implicated in modulating lymphatic development (Fig. 2). Arguably, the most important signaling pathway for lymphatic development is Vascula Endothelial Growth Factor-C (VEGF-C) signaling. VEGF-C, which serves as a ligand for Vascular Endothelial Growth Factor Receptor 2 (VEGFR2/Flik1) and Vascular Endothelial Growth Factor Receptor 3 (VEGFR3/Flik4), has been identified as a protein with significant homology to VEGF-A and VEGF-B (Eichmann et al., 1998; Enholm et al., 2001; Jeltsch et al., 1997; Kukk et al., 1996). VEGF-C appears to be the main chemoattractant cue for migrating LECs (Karkkainen et al., 2004). In development, VEGF-C is highly expressed in the mesenchymal cells adjacent to developing lymphatic vessels, consistent with its proposed role. Targeted deletion of Vegfc in mouse led to embryonic lethality around E15 due to defects in the cardiovascular system (Karkkainen et al., 2004). In Vegfc null mice, lymphatic vessels did not form, indicating the essential function of VEGF-C in lymphatic development. Similarly, attenuation of VEGFR3, the main receptor for VEGF-C, led to similar lymphatic defects (Irrthum et al., 2000; Karkkainen et al., 2000). Taken together, these observations indicate that the function of VEGF-C/VEGFR3 signaling pathway appears to be critical for the early development of the lymphatic vessels. At later stages, the function of VEGF-C/VEGFR3 for LECs appears to be comparable to that of VEGF-A/VEGFR2 for BECs (Lohela et al., 2003).

VEGF-C/VEGFR3 signaling serves as the main pro-lymphangiogenic cue by promoting proliferation, migration, and survival of LECs (Enholm et al., 2001; Jeltsch et al., 1997). Similar to many secreted ligands, VEGF-C is also produced in a preproform, and secreted as a pro-VEGF-C, which needs to be processed to be fully active (Lee et al., 2005; McColl et al., 2003). A disintegrin and metalloprotease with thrombospondin motifs-3 (ADAMTS3) has been recently shown to mediate proteolytic cleavage of pro-VEGF-C (Jeltsch et al., 2014), and the process is facilitated by Collagen and calcium-binding EGF domain-containing protein 1 (CCBE1) (Bos et al., 2011; Jeltsch et al., 2014). In humans, mutations in VEGF-C, VEGF-R3, and CCBE1 have been shown to cause hereditary lymphedemas (Brouillard et al., 2014); For instance, mutations in VEGF-R3 cause Milroy’s disease (Irrthum et al., 2000; Karkkainen et al., 2000), and mutations in CCBE1 lead to Hennekam lymphan-
Fig. 3. Application of transgenic zebrafish for lymphatic research. Confocal images of 3dpf Tg(kdr:mCherry) (A) and Tg(flil:EGFP) (B). Since kdr promoter is only active within BECs and flil promoter is active in both BECs and LECs, in the double transgenic line which carries both transgenes, BECs and LECs will be shown as yellow and green respectively. Area within the rectangle is shown in detail as (C). (D) Confocal image of 3dpf Tg(proxta:KalTA4-4xUAS-E1b:uncTgRFP)(flil); Tg(kdr:mCherry). Area within the rectangle is shown in (E). (F) In combination with Tg(flil:EGFP) transgenic line, the nucleus of individual LECs can be visualized and easily quantitated.

Zebrafish as a Model for Lymphatic Studies
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stance, it has been shown that a number of G-protein Coupled Receptors (GPCRs) are critical for lymphatic development (Karpinich and Caron, 2014). To date, at least four GPCR ligand/receptor pairs have been implicated; Adrenomedul-lin/RAMP/CALCRL (Fritz-Six et al., 2008; Karpinich et al., 2013), Sphingosine-1-phosphate/EDG1 (Aoyagi et al., 2012; Yoon et al., 2008), CXCL12/CXCR4 (Cha et al., 2012), and Apelin/APJ (Kim et al., 2013). While diverse ligand and extracellular stimuli such as light and shear stress can activate GPCRs (Venkatarkrishnan et al., 2013), virtually all GPCRs require trimeric G-protein complexes, which consist of Gα, Gβ, and Gγ subunits, to mediate their signaling activity and elicit cellular responses (Möhle and Drost, 2012; Smrcka, 2013). Within G-protein complexes, the Gα subunit generally determines the signaling outcomes of GPCRs (Gilman, 1987; Kimple et al., 2011). We have recently identified Gα13 as the main Gα subunit which functions as the essential downstream effector for diverse GPCRs to modulate lymphatic development (unpublished). GPCR signaling appears to be delayed by ERK1/2, AKT1/2 and ERK5 (Goldsmith and Dhanasekaran, 2007; Kidoya and Takakura, 2012; Nithianandarajah-Jones et al., 2012; Simon et al., 1991). Considering many targets of currently available pharmacologi- cal therapies are the components of GPCR signaling (DeWire and Violin, 2011; Dorsam and Gutkind, 2007; Smrcka, 2013), identification and understanding of the molecular and cellular basis of additional GPCRs and their contribution in lymphatic development and maintenance may help to develop effective translational applications for lymphatic disorders and associated diseases in the future.

While it is widely accepted that Notch signaling plays an instrumental role during angiogenesis, its function in lymphatic development remains unresolved because of conflicting evidence. For instance, lack of Notch signaling appears to be detrimental for lymphatic development; genetic deletion of Notch1 or inhibition of Notch activity by Dll4-Fc treatment led to lymphatic overgrowth and dilation of lymphatic vessels by promoting excessive sprouting of lymphatic vessels and increasing proliferation and survival of LECs (Murtonaki et al., 2013; Zheng et al., 2011), suggesting that Notch may negatively modulate lymphangiogenesis, similar to its role in angiogenesis. In contrast, Notch signaling appears to positively regulate lymphatic vessels in post-natal development (Niessen et al., 2011). Therefore, the function of Notch signaling during lymphatic development appears to be highly context-dependent. In addition to cell autonomous effects, Notch signaling is also critical for migration of lymphatic vessels. In zebrafish, attenuation of Dll4, Notch1, or Notch6 compromise the extension of lymphatic vessels alongside the arterial ISVs, suggesting that Notch signaling may mediate the interaction between arterial and lymphatic vessels to guide migrating lymphatic vessels (Geudens et al., 2010).

Recently, bone morphogenetic protein (BMP) signaling has been implicated in lymphatic development. While anti-angiogenic BMP9/Alk1 signaling promotes lymphatic development (Larrivee et al., 2012; Levet et al., 2013), pro-angiogenic BMP2/Alk2/3 signaling inhibits lymphatic development (Dunworth et al., 2013; Kim et al., 2012; Wiley et al., 2011), suggesting distinct cellular responses toward BMP signaling in BECs and LECs. In addition, transforming growth factor β (TGFβ), a signaling ligand related to BMPs, appears to provide context-dependent regulation of lymphatic development. While TGFβ signaling can substantially decrease proliferation and migration of human dermal lymphatic microvascular endothelial cells and negatively modulate lymphangiogenesis in vivo during regeneration (Oka et al., 2008), recent evidence suggest that TGFβ is indispensable for lymphangiogenesis in dermal lymphatic ves-}

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lymphedema [reviewed in (Brouillard et al., 2014)]. Connexin 37 (CX37/GJA4), 43 (CX43/GJA1) and 47 (CX47/GJC2) are required for the formation of lymphatic valve leaflets in conjunction with Calcinurin signaling (Meens et al., 2014). Similarly, Integrin A9 (ITGA9) has been also implicated in the formation of lymphatic valve leaflets (Bazigou et al., 2009).

AN OLD MODEL WITH A NEW TRICK?: ZEBRAFISH AS AN EMERGING MODEL FOR LYMPHATIC RESEARCH

Since the first description of the lymphatic vessels in zebrafish less than a decade ago (Yaniv et al., 2006), zebrafish have become an emerging model system for lymphatic research. Many key attributes make zebrafish an attractive model system for studying the lymphatic system. First, zebrafish embryos are externally fertilized and transparent which allows for easy access and manipulations. One of the key challenges in investigating the early phases of lymphatic development in the traditional mouse model is in vivo real time imaging to track the movement of individual LECs over development since mouse embryos develop in utero. Although recent advances allow visualization of developing LECs in mouse embryos, this technique requires specific microscopes and complex preparations (Pollmann et al., 2014). Therefore, zebrafish can complement the mouse model and offers a unique advantage to unravel cellular behaviors during early lymphatic development. Second, like mice, zebrafish are a genetically tractable model with various transgenic lines which help to visualize and track individual cells during development (Ellertsdrött et al., 2010; Kamei et al., 2010). For instance, there are a number of transgenic lines which selectively label developing LECs, each of which offers distinct advantages over others (Koltowska et al., 2013; Okuda et al., 2012). Third, traditional techniques for investigating lymphatic biology, such as lymphangiography, are applicable to zebrafish embryos (Yaniv et al., 2006).

AVAILABLE TOOLS TO INVESTIGATE LYMPHATIC BIOLOGY IN ZEBRAFISH

Diverse tools to manipulate and visualize the lymphatic vessels in zebrafish contribute to its value as the new model system in lymphatic research.

Transient and permanent genetic manipulation in zebrafish embryos

To delineate the function of a specific locus during development in zebrafish, antisense Morpholino oligonucleotides (MOs) are widely used (Ekker, 2000). MOs are 20 to 25 base pair long nucleotides which bind to the target sequence on pre-mature or mature mRNA to block translation or splicing (Bill et al., 2009). The translation blocking-MOs target the area encompassing the initiation codon of the mature mRNA and inhibit translation, while splice blocking-MOs bind to the splicing donor within the exon in pre-mature mRNAs to block proper mRNA splicing, which in turn introduce a premature stop codon to generate non-functional proteins. By targeting existing mRNAs, translation blocking-MOs can interfere with both maternal and zygotic transcripts. In contrast, splice blocking-MOs can only inhibit zygotic transcripts since the maternal mRNA are fully processed transcripts, therefore lacking exon/intron boundaries. MOs can be directly injected into zebrafish embryos at 1-2 cell stages. Similar to an allelic series in classical mutagenesis, a range of phenotypes can be created by titrating the amount of injected MO, which helps to define the function of a specific locus (Bill et al., 2009; Corey and Abrams, 2001; Heasman, 2002). Moreover, MOs can be combined with chemical antagonists/agonists, which allows for a small molecule screen in a sensitized background to identify modulators of lymphatic biology (Kim et al., 2013).

In addition, recent advances in targeted mutagenesis techniques allow fast and relatively easy generation of mutations affecting specific loci (Blackburn et al., 2013; Campbell et al., 2013). For instance, Zinc Finger Nucleases (ZFN) (Doyon et al., 2008; Meng et al., 2008), Transcription activator-like effector nucleases (TALEN) (Bedell et al., 2012), and more recently CRISPR/Cas9-based gene knockout (Hwang et al., 2013) strategies have been shown to be successful in generating mutations.

Visualizing lymphatic vessels in zebrafish embryos

Lymphangiography (lymphography) is a traditional method to visualize the lymphatic vessels and diagnose abnormalities of the lymphatic system in the human body (Guermazi et al., 2003). In zebrafish, a modified version of lymphangiography can be performed to trace lymphatic structures. While injection of a radioactive contrasting agent followed by X-ray imaging is the standard procedure in humans (Guermazi et al., 2003), injection of fluorescently-labeled macromolecules such as Rhodamine dextran (e.g. 2 x 10^6 MW), is shown to be sufficient to visualize lymphatic vessels in zebrafish (Kim et al., 2013; Yaniv et al., 2006). In general, fluorescent spheres which are conjugated with high molecular weight particles, are injected subcutaneously into the tail region or directly into the thoracic duct (TD) in the trunk region of 3-5 dpf embryos or adults (Yaniv et al., 2006). In conjunction with chemical screening or MO-mediated knock-down, lymphangiography allows rapid detection of structural deviations in lymphatic vessels.

In addition, transgenic lines can be used to visualize various vascular structures in zebrafish (Kamei et al., 2010; Wilkinson and van Eeden, 2014) (Fig. 3). Transgenic lines which express fluorescent reporters under vascular specific promoters such as vegfl2kdri (Jin et al., 2005), fil1a (Lawson and Weinstein, 2002) and etv2/etsrp (Kohli et al., 2013), have been generated. More recently, two lymphatic specific transgenic lines have been generated which express fluorescent reporters under prox1a or lyve1 promoters (Durworth et al., 2013; Okuda et al., 2012).

SIMILARITIES AND DIFFERENCES BETWEEN MAMMALIAN AND ZEBRAFISH MODELS

While zebrafish is an attractive model to investigate lymphatic development, there are a number of pending issues to be addressed. For instance, it is possible that the molecular and cellular basis of lymphatic development and physiology in zebrafish may not be readily applicable to humans. Consistent with this idea, it has recently been suggested that zebrafish Prox1 is dispensable for lymphatic development (van Impel et al., 2014). By generating targeted mutagenesis, van Impel and colleague generated zebrafish prox1 and prox1b double mutants, which have no discernible defects in lymphatic development. While intriguing, a number of issues need to be resolved to draw such a conclusion: First, both prox1 and prox1b are maternally deposited transcripts. Even homozygous mutant embryos from the heterozygous mother will display an attenuated phenotype as shown in other mutations affecting loci whose transcripts are maternally provided (Abrams and Mullins, 2009; Lindeman and Pelegrin, 2010; Wagner et al., 2004).
Second, the zebrafish genome has yet another prox1-related gene, *prox1l*/*prox2*, which is ubiquitously expressed. It is conceivable that *Prox1l/Prox2* may compensate for the lack of *Prox1*/*Prox1b* activity given their high sequence similarities (Pistocchi et al., 2008). Therefore, it is still premature to conclude that *Prox1* in zebrafish has a divergent role in lymphatic development compared to their homoeothermic relatives. However, considering that zebrafish is a poikilotherm, physiology of lymphatic anomalies. J. Clin. Invest. 127, 1371-1380.

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