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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Heart failure is the leading cause of death in the US and globally. There is a pressing need for novel therapeutic interventions to prevent heart failure in patients with coronary heart diseases. Tissue regeneration holds great promise of treating organ injures and chronic diseases, including coronary heart disease. We proposed to investigate the roles of cardiac lymphatic vessels in revascularization and immune modulation, two processes important for heart regeneration. Our preliminary data suggest that cardiac lymphatic vessels can carry blood and perfuse myocardium of zebrafish, an animal with remarkable capacity of heart regeneration. These cardiac lymphatic vessels form in close association with coronary arteries, conserved with human hearts. We also found that hearts with impaired cardiac lymphatic vessels fail to regenerate. We will continue to investigate how cardiac lymphatic vessels modulate revascularization and immune cell clearance. Molecular mechanisms underlying cardiac lymphatic vessel formation after heart injury might lead to development of novel therapeutic design of myocardial revascularization and regeneration.

15. SUBJECT TERMS

Heart Regeneration, Cardiac Lymphatic Vessels, revascularization,

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1. INTRODUCTION

The lymphatic vasculature is a specialized network of vessels that drains fluid from tissues and enables immune-cell trafficking and surveillance throughout the body. How lymphatic vessels affect tissue regeneration is not well understood. After ischemic heart injuries, myocardial edema decreases cardiac output and can cause interstitial fibrosis. In addition to fluid homeostasis and immune cell surveillance, our unexpected findings suggest that zebrafish cardiac lymphatic vessels can carry blood and perfuse the myocardium in zebrafish. Zebrafish provide a unique opportunity to study the roles of lymphatic vessels after tissue injuries. They have remarkable regenerative capacity after tramatic injuries. Available forward and reverse genetic mutants and transgenic lines make detailed molecular imaging and mechanistic studies possible. Furthermore, well established injuries models for different zebrafish organs allow us to test different injury types and severities even for the same organ. Our data suggest that new cardiac lymphatic vessel form in response to cryoinjury, a model mimicking the pathogenesis of myocardial infarction. Furthermore, zebrafish with impaired cardiac lymphatic vessels in fail to regenerate their hearts after cryoinjury. We will continue to determine the **roles of lymphatic vessels in revascularization and immune cell modulation, two processes that are important for cardiac regeneration.**

2. KEYWORDS

Heart regeneration

Cardiac lymphatic vessel

revascularization

Immune cell clearance

zebrafish

3. ACCOMPLISHMENTS

What were the major goals of this project?

--To determine how cardiac lymphatic vessels affect myocardial revascularization?

We will continue to investigate whether blood perfusion through cardiac lymphatic vessels increases after heart amputation or cryoinjuries. These experiments will elucidate how lymphatic vessels might affect revascularization after different types of heart injuries.

--To determine how cardiac lymphangiogenesis affects myocardial regeneration and scar resolution

We will ablate the lymphatic vessels to observe how myocardial regeneration is impacted after heart amputation or cryoinjuries. We will also utilize a *cxcr7a/ackr3a* fish mutant that displays increased lymphatic vessels to evaluate whether they have enhanced myocardial regeneration. These experiments will shed light on how lymphatic vessels might regulate fibrotic scar resolution and myocardial regeneration after different type of injuries.

What was accomplished under these goals?

Major Activities 1 (Major Task 1 in SOW): Determine blood prefusion and revascularization via cardiac lymphatic vessels occur in response to physiological stress or heart injury.
Subtask 1. Blood perfusion by cardiac lymphatics during physiological stress.

Subtask 2: Revascularization by cardiac lymphatic vessels during zebrafish heart regeneration

Results: Passage of erythrocytes through the myocardium is usually confined to the blood system, and lymphatic vessels do not carry red blood cells except in pathological conditions that lead to thrombosis (Lippi et al., 2012). Given the extent of the injury and the damage to the coronary vasculature and potentially the lymphatic vessels themselves, we sought to understand if this is a function of the tissue damage or more general myocardial stress. A more rapid and magnified effect was observed by increasing heart rate with adrenaline homolog, isoproterenol (Figure 1), suggesting that this is a regulated flow of blood into the lymphatic vessels. Such flow, however, cannot account of the magnitude of immune cells found within the lymphatic vessels after injury, but rather may represent a separate hemodynamic response to the demand for increased cardiac output.

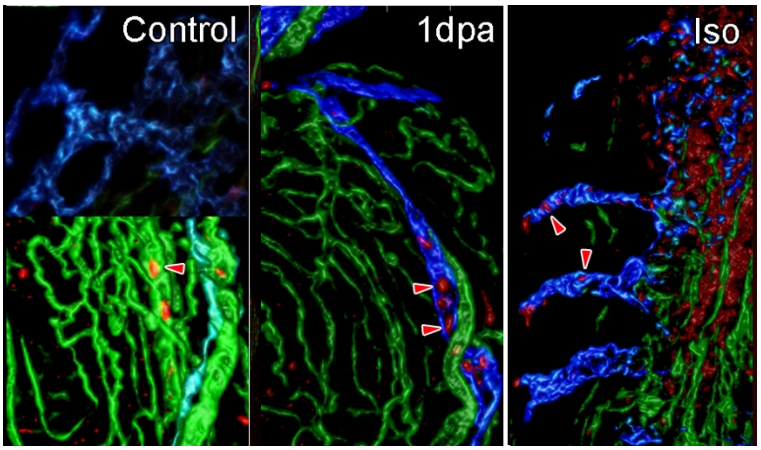

Figure. 1. Zebrafish cardiac lymphatic vessels carry erythrocytes in response to heart injuries and physiological stress. In freshly isolated control hearts, erythrocytes (red) are visible within the coronary vasculature (green), but not in the lymphatic vessels (blue). In 1-day post amputation (1dpa) heart showing erythrocytes within the lymphatic vessels (red arrowheads). Heart from zebrafish treated for 5 minutes with isoproterenol (Iso) with erythrocytes with the lymphatic vessels (red arrowheads).

Tg(gata1:RFP), Tg(flt4:mCitrine), Tg(fli1a:GFP), and control siblings, double and triple transgenic fish generated by crosses. 200 fish have been used for breeding and 54 fish were used for experiments so far.

Major Task 2 Milestones to be achieved: Observe whether fibrotic scar is resolved and heart regeneration is enhanced with revascularization via new lymphatic vessel formation
Results:
1. *cxcr7a*/*ackr3a* is required for heart regeneration after amputation.

In the previous progress report, we showed that *ackr3a* mutant hearts have better immune cell clearance compare to WT controls after cryoinjury. This result prompted us to to investigate whether ackr3a mutants have enhanced heart regeneration. Amputation at ventricle apex was performed in 8-month-old *ackr3a* mutant and WT control fish. The heart regeneration process was then examined by AFOG staining 30 days after amputation(dpa). In *ackr3a* mutants, 6 out of 8 hearts failed to regenerate and left a big scar at the injury site. By comparison, 7 out of 7 WT fish completely finish the heart regeneration process at 30dpa. These data suggest that *ackr3a* mutants showed impaired heart regeneration after amputation. Since the functions of cardiac lymphatic vessels might be different in the hearts after cryoinjuries vs amputations, we are currently investing whether *ackr3a* mutants show increased heart regeneration after cryoinjuries.

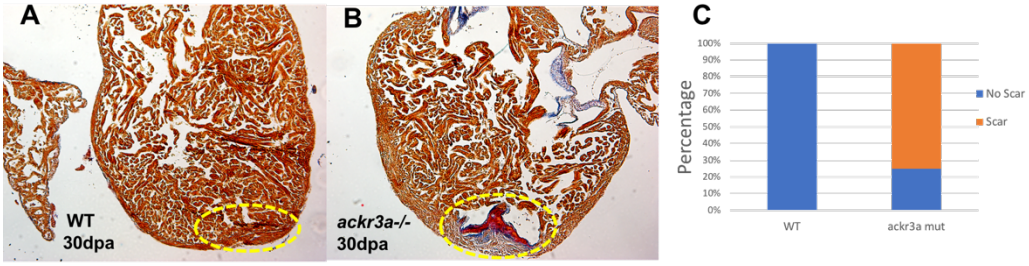

Figure 2. Ackr3a is required for heart regeneration after amputation. A) AFOG staining of WT heart at 30dpa. Sar tissue was resolved and lost cardiomyocytes was replaced in the wound area at 30dpa B) AFOG staining of *ackr3a* mutant heart at 30dpa. Large scar tissues remain at wound area in most *ackr3a* mutants. C) Percentage of hearts with or without scar at 30dpa in WT and ackr3a mutants.

lyve1:NTR-dsRed;fli1:EGFP double transgenic fish and *cxcr7a/ackr3a* mutant and combination of fish generated by crosses. 200 fish have been used for breeding to generate the double transgenic and mutant combination and 67 fish were used for experiments.

What opportunities for training and professional development has the project provided?

This project has provided training and career development opportunity for postdoctoral fellows. Dr. Michael Harrison has completed his training and landed a faculty position at Weill Cornell Medical School.

How are the results disseminated to communities of interest?

The research results were presented as progress report as weekly seminars at the Program of Developmental Biology and Regenerative Medicine, Saban Research Institute, Children's Hospital Los Angeles

The research results were presented at the Gordon Research Conference on Lymphatics

What do you plan to do during the next reporting period to accomplish the goals?

Major Task 1 Milestones to be achieved: Characterize blood perfusion and revascularization by cardiac lymphatic vessels after physiological stress and heart injury

1. Determine and quantify the erythrocytes present in lymphatic vessels at 3 and 7 days post heart injury to assess whether revascularization is persistent throughout heart regeneration.
2. Live imaging of the erythrocytes and blood and lymphatic vessels of injured hearts and isoproterenol treated hearts to identify the point/location where erythrocytes enter lymphatic vessels.
3. Single cell RNAseqs to examine if certain lymphatic (LECs) and blood endothelial cells (BEC) are more permeable than others. These blood vessels might allow erythrocytes to transmigrate from blood vessels into lymphatic vessels.

The potential results from these experiments will reveal more mechanistic insight as to how this phenomenon occurs.

Major Task 2 Milestones to be achieved: Observe whether fibrotic scar is resolved and heart regeneration is enhanced with revascularization via new lymphatic vessel formation

1. Determine whether fibrotic scar is resolved faster after cryoinjury in *ackr3a* mutant hearts that display excess cardiac lymphatic vessels.

4. IMPACT

What was the impact on the development of the principal discipline(s) of the project?

1. This project will reveal a novel mechanism by which cardiac lymphatic vessels regulate revascularization of regenerating zebrafish hearts.

2. The project will identify a novel candidate to enhance cardiac lymphatic vessel formation.

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

5. CHANGES/PROBLEMS

Change in approach and reasons for change
Nothing to report

Actual or anticipated problems or delays and actions or plan to resolve them
The proposed experiments and milestones were delayed due to the second wave of COVID-19. We have resumed more research activities after lab personnel received vaccines at our research institute and will speed up the proposed experiments.

Changes that had significant impact on expenditures
Due to the second wave of COVID-19 related delays, we have experienced delayed purchase requisitions required for the experiments. Therefore, we have less expenditure than proposed for this year.

Significant changes in use or care of human subjects, vertebrate animals, biohazards and/or select agents
Nothing to report

6. PRODUCTS

Publications, Conference papers, and presentation

Feng X, Travisano S, Person CA, Lien CL, Harrison MR. The lymphatic system in zebrafish heart development, regeneration and disease modeling. J. Cardiovas. Dev. Dis. (In press).

Other publications, conference paper and presentations.

None.

Websites or other internet sites

Nothing to report

Technologies or techniques

Nothing to report

Inventions, patent applications, and/or licenses

Nothing to report

Other products

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name	Ching-Ling (Ellen) Lien, Ph.D.
Project Role	PI
Researcher Identifier (e.g. ORCID ID)	0000-0002-5100-9780
Nearest person month worked	1.2
Contribution to Project	Dr. Lien is the PI of this project and oversees the overall direction, data collection, analysis and completion of milestones of the project. She will ensure the project goals are accomplished in a rigorous and timely manner.
Funding support	DoD, NIH, TRDRP,
Name	Michael Harrison, Ph.D.
Project Role	Postdoc Associate
Researcher Identifier (e.g. ORCID ID)	0000-0003-1703-9879
Nearest person month worked	8
Contribution to Project	Dr. Harrison has performed heart injuries, tissue collection, confocal imaging and data analysis of the project
Funding support	DoD, NIH, TRDRP
Name	Stanislao Travisano, Ph.D.
Project Role	Postdoc Fellow
Researcher Identifier (e.g. ORCID ID)	0000-0001-6453-0367
Nearest person month worked	4
Contribution to Project	Dr. Travisano has performed heart injuries, tissue collection, confocal imaging and data analysis of the project
Funding Support	DoD, NIH
Name	Xidi Feng, MS.

Project Role	Ph.D. student
Researcher Identifier (e.g. ORCID ID)	None
Nearest person month worked	2
Contribution to Project	Ms. Feng has performed heart injuries, tissue collection, confocal imaging and data analysis of the project
Funding Support	Saban Research Institute Pre-Doctoral Award

Has there been a change in the active other support of the PD/PIs or senior/key personnel since the last reporting period?

Dr. Ching-Ling Lien has received one new grant support:

1R01HL148706-01A1 (Lien, PI) NIH(NHLBI)	08/28/20—05/31/2024 Annual DC	3.6 calendar Mo.

Title “Cardiac lymphatic vessels in heart development and regeneration”.
The aims of this grant are to determine the molecular mechanisms of coronary artery dependent new lymphatic vessel formation during heart development and myocardium regeneration. We propose to identify the secreted factors from the coronary arteries that provide a scaffold for cardiac lymphatic vessels to form during heart development and regeneration. New candidate genes and factors will be screened using CRISPR F0 mutants. There is no scientific overlap between this project and the current DoD project.
Role: PI

Dr. Michael Harrison, a postdoc associate in the lab was listed as key personnel in this project. Dr. Harrison has completed his postdoctoral training in our institute and is leaving to establish his own independent laboratory at Weill Cornell Medical College on Aug. 15, 2020.

Dr. Stanislao Travisano has been recruited to join the Lien laboratory and has continued to perform proposed experiments after Dr. Michael Harrison’s departure. 66.7% of Dr. Travisano’s effort will be devoted to this project.

Ms. Xidi Feng (Ph.D. student) also helped and contribute to this project (33.3% effort). Ms. Feng is currently supported by Saban Research Institute Pre-Doctoral Award until June 30, 2021.

What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

Collaborative Awards: Not Applicable

Quad Charts: Not application

9.APPENDICES

See attached.

Journal of
Cardiovascular
Development and Disease

MDPI

Review

The Lymphatic System in Zebrafish Heart Development, Regeneration and Disease Modeling

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† These authors contributed equally to this work.

Abstract: Heart disease remains the single largest cause of death in developed countries, and novel therapeutic interventions are desperately needed to alleviate this growing burden. The cardiac lymphatic system is the long-overlooked counterpart of the coronary blood vasculature, but it is important roles in homeostasis and disease are becoming increasingly apparent. Recently, the cardiac lymphatic vasculature in zebrafish has been described and its role in supporting the potent regenerative response of zebrafish heart tissue investigated. In this review, we discuss these findings in the wider context of lymphatic development, evolution and the promise of this system to open new therapeutic avenues to treat myocardial infarction and other cardiopathologies.

Keywords: cardiac lymphatic vessels; zebrafish; heart; development; regeneration

Citation: Feng, X.; Travisano, S.; Pearson, C.A.; Lien, C.-L.; Michael R. M. Harriso The lymphatic system in zebrafish heart development, regeneration and disease modeling. *J. Cardiovasc. Dev. Dis.* **2021**, *8*, x. https://doi.org/10.3390/xxxxx

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1. Introduction

1.1. The Lymphatic System: Discovery and Functions

In the 5th-century BC, Hippocrates described the presence of nodes containing a milky fluid (chyle) in specific subcutaneous and deep organ regions of the body [1]. Through the gallant efforts of a collection of anatomists, including Thomas Bartholin, Olaus Rudbeck and George Joyliffe, the "lymphatic vessels (*vasa lymphatica*)" were defined [1]. However, it was not until the 18th century, with the work of Mascagni and others, that it was appreciated that these lymph-containing vessels and nodes are an integral part of a network, which extends from blind-ended lymphatic capillaries (or initial lymphatics) to collecting lymphatic vessels that eventually connect to the blood circulatory system. In comparison to the blood circulatory system, understanding the role and development of the lymphatic system has been slow. This is in part due to the network being delicate and largely invisible in comparison to the obvious sphygmic blood circulatory system. The term lymph was coined to reflect this property coming from the Greek Nymph, a creature associated with clear streams and the Roman deity Lympha, meaning spring of clear water [1]. Even with the evolution of microscopy investigation, studies on the lymphatic system remained stubbornly hindered by a paucity of good molecular markers and labels. Nonetheless, just as blood vasculature is essential for the supply of oxygen and nutrients in addition to the removal of waste, the lymphatic vasculature also

provides vital support of healthy tissue. As the range of techniques and technologies to study the lymphatic vasculature continues to expand, so does our understanding of the unique and critical roles this system plays in tissue homeostasis and disease [2].

The lymphatic system provides a unidirectional conduit for the essential flow of fluid from the tissue interstitium back to the circulatory system. This fluid regulation is absolutely critical, and malformation (primary lymphedema) or disruption (secondary lymphedema) of lymphatic vessels results in disabling swelling of the tissue [3,4]. This lymph fluid is rich in plasma proteins and also contains immune cells and antigens. Through the various afferent lymphatics, lymph nodes are exposed to intact or degraded microorganisms and toxic stimuli [5]. These must be removed from the fluid before being returned to the blood flow, and recent studies suggest a population of neutrophils and macrophages in the lymphatic system prevent the systemic spread of tissue pathogens [6,7]. In addition to these innate immune cells, the lymphatic vasculature plays a critical role in supporting adaptive immune responses. Immune cells that ingest foreign antigens are brought into contact with lymphocytes in the node where they present antigens to activate the adaptive responses [5]. The lymphatic vessels are not just a passive conduit that is homogenous throughout the body, but they also have tissue-specific roles, including the active absorption of lipids and vitamins. Gut villi lymphatic vessels, called lacteals, take up dietary-fats as triglyceride particles known as chylomicrons packaged by the gut enterocytes [8]. These are then transported to the systemic blood system via collecting vessels and the thoracic duct [8]. As a result, lacteal control of lipid absorption has been implicated in obesity and its sequelae, including the impact on heart disease and function [9,10]. However, as we will discuss in this review, the lymphatic system also has an immerging direct role in supporting cardiovascular health and disease.

1.2. The Evolution of the Lymphatic System

Most invertebrates have an open circulatory system and no distinction between a lymphatic and blood system or their respective functions [11]. Vertebrates have a range of lymphatic system features, including lymphatic vessels, lymph nodes, lymphoid organs and tissues that appear to become increasingly distinct and specialized [12]. Jawless and cartilaginous fish lack lymphatic vessels; however, some thin-walled sinuses provide a conduit for extravascular fluid back into veins in these lower vertebrates [11]. In other vertebrates, lymphatic vasculature has contractile regions, which actively aid the flow of lymph into the venous circulation [11,13]. So-called lymph hearts have been identified in lungfish, amphibians, reptiles and some flightless birds. They are typically found at the junction between lymphatic and venous systems and have been lost in higher vertebrates [11,13]. A lymphatic system that often lacks a lymph heart similar to that of mammals is found in other species of bony fish (teleosts) [11]. However, the connection of this lymphatic system to the blood circulation appears to vary across teleosts species and organ systems. In trout and glassfish, an arterial connection to the blood system has been described, and the fluid of these secondary vasculature systems can become perfused with blood under hypoxic conditions [14]. Zebrafish have been shown to have an extensive lymphatic system throughout the body, and analyses of the zebrafish vasculature system suggest it shares many conserved anatomical features with the mammalian system [15,16]. The zebrafish system has bicuspid valves and a venous connection but lacks nodes [15,17]. The possibility that this lymphatic system also retains the ability to be perfused under extreme conditions has been contested [18–21]. Reflecting the systems increasing specialization, it is likely that a spectrum of blood and lymphatic vasculature interconnectedness exists across teleosts species. Nonetheless, the zebrafish has provided invaluable insight into the molecular regulation of lymphatic development and provides a fascinating evolutionary nexus to gain a deep understanding of lymphatic function in disease.

1.3. The Zebrafish Lymphatic System

The majority of research has focused on zebrafish lymphatic development during the embryonic stage, taking advantage of various transgenic and tracing tools and body transparency. Lymphangiogenesis of the trunk lymphatic, facial lymphatic and intestinal lymphatic network has been well characterized in zebrafish embryos. The lymphangioblasts of trunk lymphatic vessels are derived from the posterior cardinal vein and migrate to the dorsal myoseptum to become parachordal lymphangioblasts by two days post-fertilization (DPF) [16,22–24]. Those lymphangioblasts migrate along intersomitic arteries dorsally and ventrally, forming intersomitic lymphatic vessels. The fish trunk lymphatic vasculature continues to develop to form the thoracic duct under the dorsal aorta and the dorsal longitudinal lymphatic vessel along the dorsal longitudinal anastomotic vessel by five DPF [15,16].

The development of facial lymphatic vessels starts from the budding of the lymphangioblasts from the common cardinal vein forming the facial lymphatic sprout (FLS) at 36hpf [25,26]. The FLS migrate along the primary head sinus (PHS) towards the head area. The formation of facial lymphatic vessels is not from a single source of lymphangioblasts. As the FLS migrates, lymphanigoblasts originating from the PHS and the ventral aorta join the FLS, making up a complex facial lymphatic network together.

The origin of intestinal lymphatics has not been identified. There is a large lymphatic vessel associated with the entire zebrafish intestine [26,27], indicating the intestinal lymphatics may also play a role in lipid transporting as in mammals. Unlike trunk lymphatic vessels that migrate along arteries, intestinal lymphatic vessels have been found to form along both arteries and veins. This suggests that there may be tissue-specific guidance cues that guide lymphatic endothelial cell (LEC) migration.

Although the development of the lymphatic system is well-studied in zebrafish embryos, the functional studies of lymphatic vessels in regeneration and disease models in different organs are in their infancy. In this review, we will discuss the recent work on zebrafish cardiac lymphatic vessels in heart regeneration and the implications of this for our understanding of lymphatic vessels' role in heart disease.

2. The Development of the Lymphatic System

2.1. Venous and Non-Venous Origins

The lymphatic vasculature includes a network of LECs found in close proximity to, but separate from, the blood vasculature [28]. After carrying out ink-injection experiments in pig embryos, Florence Sabin hypothesized that the majority of lymphatic vessels bud off from the endothelium of the veins and that these primitive lymphatics then spread throughout the entire embryo body to create the lymphatic network. However, after injecting along the aorta, she also concluded that, despite budding from veins, the deep lymphatics follow arteries [29]. Cell lineage studies and grafting experiments in birds validated different sources of the lymphatic vascular system. The deeper parts of the jugular lymph sacs originate from the jugular segments of the cardinal veins and the superficial, dermal lymphatics from local lymphangioblasts in the dermatomes, while the LECs of the lymph heart is of somitic origin [30].

The first cardiac lymphatic described in the human embryo grows from two different plexuses. The first one near the left jugular lymph sac, elongating between the pulmonary trunk and the aorta and following the right coronary artery. The second plexus, described as the main one, terminates in the right jugular sac and follows the left coronary artery around embryonic week eight [31]. This is in contradiction to the mouse, in which the cardiac lymphatic vessels follow the course of the cardiac veins rather than the coronary arteries [32]. In zebrafish, the development of the cardiac lymphatics occurs during late juvenile to early adult stages after two months post-fertilization (MPF) when coronary arteries, not veins, provide a scaffold for the elongation of the lymphatic vessels and the expansion of the network [33,34]. This similarity between zebrafish and human cardiac lymphatic development could represent an ancestral mechanism of essential guidance

cues for the cardiac lymphatic endothelium, which has been altered across mammalian species [35].

The lymphatic vasculature is thought to form exclusively by sprouting from embryonic veins (lymphangiogenesis). Lineage tracing experiments in mice embryos demonstrated that the lymphatic system has largely venous origins [36]. Time-lapse imaging in developing zebrafish embryos demonstrated that this process is well-conserved and that at least the main thoracic duct-like vessel arises embryonically from primitive veins [16]. However, the discovery of an alternative non-venous origin(s) of LECs in mammals that contribute to the lymphatic vasculature of the skin [37], mesentery [38], and heart [32,39,40] has changed the understanding of the mechanisms of embryonic lymphatic vessel development. Furthermore, evidence of a non-venous lymphatic progenitor, named "ventral aorta lymphangioblast" (VA-L), was found to give rise to facial lymphatic in zebrafish, suggesting that the origin and development of lymphatic vessels is tissue context-dependent [41].

2.2. Molecular Mechanism of LEC Identity

The equilibrium between endothelial cell fate regulators, Notch, COUP-TFII, and Prox1 may play a critical role in the specification of endothelial cell (EC) fate during vascular development and arteriovenous-lymphatic cell fate specification [42]. Notch signaling promotes arterial EC differentiation, while in venous ECs, Notch activity is repressed by the COUP-TFII orphan nuclear receptor to maintain the vein identity [43]. The specification of the LECs in mammals is dependent on Prox1, a key transcriptional factor also crucial for maintaining the lymphatic endothelial identity [44,45]. Transcription factors *Sox18* [46], *CoupTFII* [47], *Gata2* [48,49], and *Hhex* [50] have been found to regulate *Prox1* expression in mouse LECs.

LEC progenitors relocate from the cardinal vein through paracrine action of VEGF-C expressed by the neighboring mesenchyme to form the primitive lymph sacs [51,52]. LECs express VEGFR2 and VEGFR3, as well as the co-receptor neuropilin 2 (Nrp2) [52,53]. It was also demonstrated that VEGF-C and VEGF-D act through VEGF receptor 3 (VEGFR-3) to induce lymphangiogenesis [54,55]. LYVE-1, one of the genes expressed in LECs, in a subset of ECs from the large central veins, provides the first signal of lymphatic endothelial competence [56,57].

Similar to mammals, venous-derived lymphatic progenitors in zebrafish can be detected with *prox1a* expression [22,24], and lymphatic sprouting is reliant on *vegfr3* (known as *flt4* in zebrafish) [15,58]. However, the functionally related transcription factors Coup-TFII (Nr2f2) and Sox18 were found to be dispensable for lymphatic specification in zebrafish, suggesting that transcriptional regulation of lymphatic commitment may have diverged somewhat between zebrafish and mice [59]. However, it is not known if other Nr2f factors can compensate for the loss of Nr2f2.

3. The Development of Cardiac Lymphatic System in Zebrafish

A cardiac lymphatic vessel system in adult zebrafish has been identified [33,34,60] (Table 1). The zebrafish cardiac lymphatic vessels express common LEC markers discussed above, including *prox1a*, *lyve1b*, *flt4* and also *mrc1a* and *stab1* [33,34,60]. When cardiac LECs migrate, the very first 1–5 tip cells are primarily labeled by *flt4* [34]. Unlike mammals that develop their cardiac lymphatic vessels at embryonic stages, lymphatic vessels are found to develop in the zebrafish post-embryonically [33,34]. The zebrafish cardiac lymphatic vessels arise from ventral facial lymphatics, which migrate along the ventral aorta [34]. The cardiac lymphatic vessel sprouts are visible at the tip of bulbus arteriosus (BA) at 21–28 DPF before any coronary vasculature development on the zebrafish heart ventricle has occurred [33,34].

The emergence of cardiac lymphatic vessels on the heart has been shown to be correlated with the heart rate increase during the larval to the juvenile transition [34]. Reducing

heart rate with the β-blocker Atenolol attenuates cardiac lymphatic sprouts on the BA and impacts the BA lymphatic branch. The BA is the fish cardiac outflow tract with a special thick-wall chamber to adjust the blood flow pressure from the fish ventricle [61]. The sprouts on the BA continue to develop and expand to form an extensive lymphatic network by eight weeks post-fertilization (WPF) [33,34]. The cardiac lymphatic vessels on the BA remain stable and do not bud from this until young adult stages when the LECs emerge onto the heart ventricle around 12–16 WPF (Figure 1a,b). These cells migrate and form vessels along the main coronary arteries, verified by *dll4*, *kdrl*, *flt1*, and *cxcr4a* expression, and also within subepicardial fat tissue [33,34]. The functional significance of this expansion into adipocytes is not known, but interestingly an upregulation of lipid metabolism genes occurs in cardiac lymphatic defective zebrafish, indicating that lymphatic vessels in zebrafish may also have a role in lipid flux in cardiac tissue [60]. Zebrafish cardiac lymphatic vessels do not appear to have open connections with the blood vasculature in resting states, as confirmed by intravascular injection [34]. However, if this does occur under stress remains to be determined.

Table 1. Summary of the genetic tools and findings of papers describing the zebrafish cardiac lymphatic system.

	Vivien et al. [60]	**Harrison et al. [33]**	**Gancz et al. [34]**
Cardiac lymphatic vessels (LVs)	*prox1a*$^{+}$ *lyve1b*$^{+}$	*prox1a*$^{+}$ *flt4*$^{+}$ *lyve1b*$^{+}$ *(high BA; low ventricle) mrc1a*$^{+}$ *stab1*$^{+}$	*prox1a*$^{+}$ *flt4*$^{+}$ *lyve1b*$^{+}$ *mrc1a*$^{+}$
LVs in regeneration (cryoinjury)	*prox1a*$^{+}$	*prox1a*$^{+}$ *flt4*$^{+}$ *lyve1b*$^{+}$*(low) mrc1a*$^{+}$	*prox1a*$^{+}$ *flt4*$^{+}$ *lyve1b*$^{+}$ *mrc1a*$^{+}$
Roles of LVs	Cardiac hypertrophy, metabolic homeostasis, and inflammation resolution	Cardiac regeneration, cell debris clearance and inflammation resolution	Cardiac regeneration
Mutants lacking BA LECs	*vegfc*$^{hy-/-}$; *vegfd*$^{-/-}$	-	*flt4*$^{-/-}$
Mutants/Tg lacking ventricular LECs	*vegfc*$^{hy+/-}$; *vegfd*$^{-/-}$ *vegfc*$^{hy-/-}$; *vegfd*$^{+/-}$	*sFlt4* *cxcr4a*$^{-/-}$ *(majority)*	*vegfc*$^{+/-}$ *cxcr4a*$^{-/-}$ *(isolated LECs unaffected)*
Mutants with hypertrophy	-	-	*vegfc*$^{hy-/-}$; *vegfd*$^{-/-}$
Mutants/Tg defective scar resolution	*vegfc*$^{+/-}$ *flt4*$^{-/-}$	*cxcr4a*$^{-/-}$	*vegfc*$^{hy-/-}$; *vegfd*$^{-/-}$ *cxcr4a*$^{-/-}$

The development of zebrafish cardiac lymphatic vessels is dependent on Vegfc-Flt4 signaling [33,34,60] (Table 1). The deletion of the *flt4* receptor completely blocks the emergence of cardiac LECs on both BA and heart ventricles [34]. Since *vegfc* mutation is embryonic lethal in zebrafish, cardiac lymphatic vessel dependence on Vegfc was characterized in *vegfc* heterozygotes. The reduction of Vegfc ligand dramatically affected the lymphatic coverage and branching on BA. The sprouts and growth were reduced in the *vegfc* heterozygotes [34] (Table 1). Similar results were observed in hypomorphic *vegfc* mutants on a *vegfd* mutant background [60]. Cardiac lymphatic vessels were still detectable on the BA in zebrafish with either one functional *vegfc* or *vegfd* allele but lacking on heart ventricles of these zebrafish. In hypomorphic *vegfc* and amorphic *vegfd* double mutants, the cardiac lymphatic vessels were absent on both BA and heart ventricles [60]. In order to investigate the role of Vegfc signaling in cardiac lymphatic vessel extension in isolation of the more systemic effects on lymphatic development at earlier stages, Harrison et al. blocked the Vegfc signaling by a heat-inducible expression of soluble Flt4 (sFlt4) receptor [33]. The induction of *sflt4* after the establishment of cardiac lymphatic vessels on BA resulted in no lymphatic vessel formation on the zebrafish ventricle. This indicates that the ventricular extension of the lymphatic vessels specifically requires Vegfc signaling and addition to

any prior requirement in the specification. The coronary vessels are also required for normal cardiac lymphatic vessel growth providing a scaffold that can promote the extension of the lymphatic vessels onto the ventricle [33,34]. Phenylhydrazine hydrochloride (PHZ)-induced coronary vasculature overgrowth also promoted cardiac lymphatic development in zebrafish [34]. In contrast, in *cxcr4a* mutants without normal coronary vasculature, the growth of cardiac lymphatic vessels was also blocked on the ventricles [33,34]. Notably, the VFL and cardiac lymphatic vessels on the BA did not show obvious defects in mutants, indicating that the lack of cardiac lymphatic vessels extension onto the heart ventricle is mainly due to the loss of coronary vasculature [33,34].

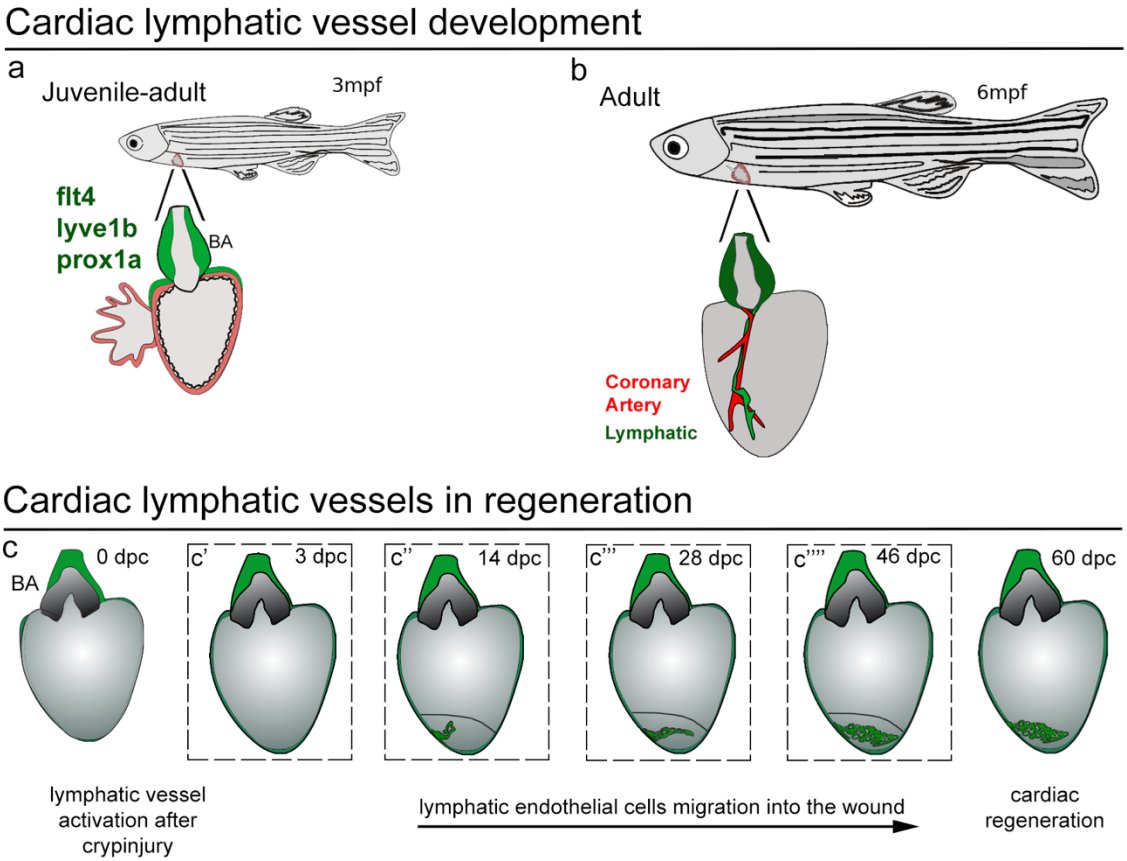

Figure 1. Cardiac lymphatic vessel development and regeneration. (**a**),(**b**) Cardiac lymphatic vessel development. (**a**) Cardiac lymphatic vessels (*flt4+*, *lyve1b+*, *prox1a+*) that reside on bulbus arteriosus (BA) start to migrate down to the ventricle after 3 months post-fertilization (MPF) when juvenile fish mature to adults. (**b**) Cardiac lymphatic vessels follow the course of the coronary artery to populate the ventricle. (**c**) Lymphatic activation during heart regeneration. After cryoinjury, the lymphatic vasculature starts to migrate into the wound at 14 days post-cryoinjury (DPC), over the wound site and is crucial for supporting the regenerative response.

4. The Role of the Cardiac Lymphatic System in Heart Homeostasis, Disease, and Regeneration

4.1. Roles of Lymphatic Vessels in Cardiovascular Diseases

The lymphatic vessels play a prominent role in lipid metabolism. Intestinal lymphatics take up dietary lipids in the form of lipoprotein particles known as chylomicrons to transport them to the bloodstream [62]. Furthermore, lymphatic endothelium is a passive exchange perimeter indispensable for the transport of cholesterol [63]. Although vascular smooth muscle cells are the major cell type responsible for plaque formation in murine models of atherosclerosis, contributing to almost 70% of all plaque cells [64], the hypothesis that atherosclerosis is a chronic inflammatory disease of the arterial wall has gained

widespread acceptance [65]. Elevated serum cholesterol levels and hypertension are very well-known risk factors for cardiovascular disease [66]. Despite the fact that blood vessels are more frequent than lymphatics in the collagenous outside (*adventitia*) surrounding a coronary plaque, the lymphatic vessels are highly present in the inner layers (*intima* and *media*) of progressive atherosclerotic lesions of coronary arteries and their growth is associated with areas characterized by scattered calcium deposits and cholesterol crystals [67]. In addition, it has been shown that the specific blockage of the VEGFR-3 decreases lymphatic vessel activation and local cardiac inflammation after transplantation and could be used as a novel lymphatic vessel–targeted immunomodulatory therapy [68]. A better understanding of the cardiac lymphatic system may offer new possibilities for therapeutic interventions in the future.

The blockage of coronary arteries by an atherosclerotic plaque results in the death of surrounding cardiac muscle in events known as myocardial infarction (MI). The necrotic tissue will further cause acute inflammation response, edema and tissue remodeling at the infarcted site, leading to fibrotic scar, arrhythmia and eventually heart failure [69]. Recently, an increasing number of studies have demonstrated the importance of cardiac lymphatic vessels in MI. The lymphangiogenesis at the infarcted area has been observed in artery ligation induced MI mice [32] and rats [70] and in post-MI human patient samples [71]. It has been shown that cardiac lymphatic vasculature has a protective role in post-MI recovery in mice [72–74]. The blockage of VEGF-C signaling by soluble decoy VEGFR3 (sVEGFR3) results in impaired morphology of cardiac lymphatic vessels [72]. The survival rate in sVEGFR3 mice after MI was dramatically reduced compared to WT controls. Further analysis revealed an increase in scar size and intramyocardial hemorrhages in sVEGFR3 mice. Furthermore, the scar composition measured by non-invasive MRI in sVEGFR3 mice was found different from that of WT controls. Apelin (also known as APLN), the ligand for the G-protein-coupled APJ receptor, is important for lymphatic vasculature maturation [75]. The knockout of apelin in mice affected the cell–cell junction integrity in LECs and resulted in dilated lymphatic vessels [73]. Without healthy cardiac lymphatic vasculature, apelin knockout mice suffered a more serious inflammation response after MI.

One of the important functions of lymphatic vessels is immune cell clearance at the inflammation site, which has been shown to be essential for cardiac function after MI [74]. LYVE-1 deletion in mice did not affect the overall development of lymphatic vessels [76] but was deleterious to leukocyte docking [77]. The LYVE-1 mutant mice with defective immune cell clearance exhibited more fibrotic tissue and reduced percentage LV ejection fraction and stroke volume in the hearts after MI [74].

Besides its physiologic function in MI, the cardiac lymphatic vessels also secrete signal molecules in regulating heart repair. Lui et al. have shown that reelin (Reln), an extracellular matrix protein mainly expressed by cardiac LECs, regulates heart growth and promotes cardiomyocyte (CM) proliferation during development in mice hearts [78]. During heart repair in neonatal mice, Reln expression was highly induced at the injury site [78]. The deletion of Reln diminished the heart repair with increased scar size and reduced heart function [78]. Consistent with its role in heart development, CM proliferation was reduced and CM apoptosis elevated in *Reln* mutants after MI. Together these studies suggest cardiac lymphatic vessels have a supportive role in post-MI recovery.

Therapeutic induction of cardiac lymphangiogenesis by VEGF-C appears to have a beneficial role in MI. The application of VEGF-C promoted lymphangiogenesis and improved cardiac function in both rats [70] and mice [32] after MI. In VEGF-C-treated rats, immune cell clearance was increased, and cardiac edema and collagen deposition were decreased compared to controls [70]. The potential roles of cardiac lymphatic vessels in heart disease and regeneration are summarized in Figure 2.

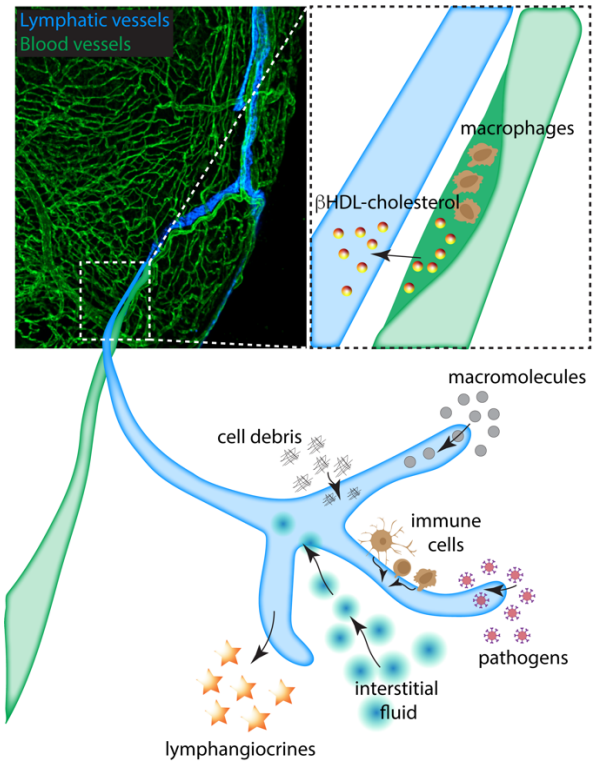

Figure 2. The roles of the cardiac lymphatic system in heart disease and regeneration. Coronary artery occlusion by atherosclerotic plaque causes myocardial infarction. The plaque is composed of infiltrated macrophage and cholesterol deposits; cardiac lymphatics running along the artery provide a conduit for cholesterol as a βHDL complex to be removed from the heart and returned to the liver. During development, homeostats and disease, the cardiac lymphatics uptake cell debris, macromolecules, immune cells, pathogens and fluid. Insufficiency of such removal can result in inflammation and edema induced fibrosis, which is detrimental for clinical outcomes. The lymphatic vasculature responds to such insufficiency by the expansion of the lymphatic capillaries after damage to the myocardium. The lymphatic endothelial cells are also a source of lymphangiocrines, excreted proteins that promote regeneration and growth of the myocardial tissue.

4.2. The Function of Cardiac Lymphatics in Zebrafish Heart Regeneration

Compared to mammals, zebrafish have the amazing capacity to fully regenerate heart tissue after injury, making it an ideal model to study the function of cardiac lymphatic vessels in heart regeneration [79]. Cardiac lymphatics have been shown to have distinct responses in different injury models [33,34]. After amputation, few hearts had limited cardiac lymphatic vessel growth into the wound area during heart regeneration [33,60]. The amputation has less inflammation and only minor collagen/fibrin deposition due to clean removal of the cardiac tissue. In contrast, a dramatic lymphangiogenesis response was induced in zebrafish hearts after cryoinjury, with a large number of lymphatic vessels migrating into the wound area and forming a network with increased branches and enlarged vessel diameter [33,34]. Compared to amputation, cryoinjury is a more complex heart regeneration model, which incorporates components of necrosis and inflammation, with injured tissue and ECM persisting in the wound area. A similar response occurs with injury to the fin suggesting necrotic tissue is important for neo-lymphatic growth after injury [60]. The lymphangiogenesis response during heart regeneration is also regulated by Vegfc-Flt4 signaling. *Vegfc* expression in zebrafish heart became undetectable after 14 days post-amputation (DPA) while still remaining in the heart wound area after 42 days post-cryoinjury (DPC) [33]. In addition, cardiac lymphatic vessel growth was completely absent in *flt4* mutants and highly reduced in *vegfc* hets after cryoinjury [34]. Consistent with this lymphangiogenic response, cardiac lymphatic vessels also show important roles in heart regeneration after cryoinjury. In the hearts with defective cardiac

lymphatic vessel development, heart regeneration after cryoinjury was also impacted compared to WT controls; this was not seen in the heart without cardiac lymphatic vessels after amputation [33,34,60]. The difference in zebrafish heart regeneration after amputation and cryoinjury suggests that it is important to use appropriate injury models in disease studies. Cryoinjury may be a more suitable injury model to study the functions of cardiac lymphatic vessels since there are server inflammation and necrotic tissue at infarctional sites in post-MI human hearts [69].

The functions of zebrafish cardiac lymphatic vessels in cryoinjury appear to include homeostasis maintenance and immune cell clearance. The cardiac lymphatic vessels were able to absorb intramyocardial injected Qdots (<10 nm diameter) and transport *mpx*+ neutrophils recruited after cryoinjury [33]. However, *mpx*+ neutrophil clearance was attenuated in zebrafish heart without cardiac lymphatic vessels after cryoinjury [33]. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, which detects DNA breaks in apoptosis, revealed an accumulation of TUNEL-positive signals at the infarcted area in cardiac lymphatic vessel impacted hearts in zebrafish [60]. These results indicate that the functions of cardiac lymphatic vessels in immune cell clearance and necrotic cell removal are essential for efficient heart regeneration after cryoinjury in zebrafish. This suggests a therapeutic benefit in targeting a patient's cardiac lymphatic vessels after MI. According to zebrafish heart regeneration results, the induction of cardiac lymphangiogenesis after MI may prevent long-term inflammation and fibrotic scar deposition. It will be interesting to investigate further the dysregulation of myocardial metabolism in the zebrafish lacking cardiac lymphatics and potential effects on myocardial proliferation and regeneration.

5. Future Directions

The zebrafish is an emerging model to study development, regeneration, and model human disease due to their amenability for imaging and available forward and reverse genetic tools. The studies of trunk lymphatic vessels in zebrafish embryos have provided valuable insights into lymphatic development. Different organs, including the heart, may utilize organ/tissue-specific mechanisms to regulate fluid homeostasis and immune cell modulation to accommodate their physiological demands, and this is currently under intense study. For the roles of cardiac lymphatic vessels, the following aspects can be further clarified and studied.

5.1. Cardiac Lymphatic Formation and Populations

We and others have performed a detailed characterization of cardiac lymphatic vessel development and neo-lymphangiogenesis during zebrafish heart regeneration as a basis for future studies. One unexpected aspect of the cardiac lymphatic vessels in zebrafish is their discontinuous nature over the ventricle. A conduit is formed as observed with Qdot uptake following intramyocardial injection [33], but also individual or small groups of LECs were often observed in connection with the main cardiac lymphatic vessel or isolated from it [33,34,60]. Interestingly, Gancz et al. found that this population has a different sensitivity to signaling changes suggesting that isolated cells may not require the scaffold of the coronary arteries. Furthermore, additional signaling pathways and sources may be directing cardiac lymphatic development. Understanding the development of cardiac lymphatic vessels at the cellular level and the signaling that shapes them will be critical to therapeutically encourage (or discourage) their formation.

It remains unclear whether these isolated lymphatic cells and clusters are truly a distinct population or if they are an artifact of the formation of this delicate lymphatic vessel. They may reciprocally dissociate and associate from the main vessel as it expands during development and regeneration. This is consistent with the observed reduced sensitivity of isolated LECs to loss of *cxcr4a,* which manifests as a range of phenotypic severity [33,80]. In *cxcr4a* mutant zebrafish that develop some coronary vasculature, this may be

sufficient to support limited LEC outgrowth and expansion but still insufficient for complete vessel formation. Significantly, these isolated LEC clusters were transiently observed in mouse embryonic hearts, but the origins of the clusters were found to be indistinguishable from the main vessel [34]. This suggests that the LEC clusters could be derived from the main lymphatic vessel in a process that may be similar to that observed during lung development [81]. The clusters are transient in the mouse, not being identifiable at later stages. As the development of the cardiac vessel progresses, these clusters may progressively fuse with the main vessel.

Regardless of origin, it is also possible that isolated LEC populations can contribute to heart regeneration. They appear in zebrafish heart during regeneration after cryoinjury [34]. Furthermore, the identification of the first lymphangiocrine, Reln, suggests that the positive benefits post-MI are not limited to the lymphatics acting as a conduit in the classical sense [78]. Individual cells could excrete pro-regenerative factors or provide scavenger functions much like those described of brain LECs/fluorescent granular perithelial cells [82,83]. It will be fascinating to further uncover the unexpected support functions and morphogenic events of LECs in developmental and regenerative contexts.

5.2. Signaling Pathways Regulating Cardiac Lymphatic Vessel Expansion

Many different signaling pathways emanating from coronary vasculature or otherwise might be further explored. One candidate signaling pathway is Notch, which is known to regulate EC proliferation, motility, filopodia formation, adhesion, and vessel stabilization [84]. Notch receptors and ligands such as Notch1 and Dll4 are predominantly expressed in arterial endothelial cells during embryonic development and arterial cell specification [85,86]. Activation of Notch 1 by Dll4 venous ECs has been shown to induce a lymphatic transcription profile, so transcriptional activation of Notch signaling may be required to reprogram VEC into LEC [87]. Moreover, genetic targeting of Notch impaired LEC migration during embryonic zebrafish development [87] and blocking its activation by Dll4-expression leads to downregulation of LYVE1 and EphrinB2 both in vitro [42] and in vivo [88]. Conversely, the lack of Notch activity resulted also in enhanced lymphatic sprouting leading to an increased LEC proliferation/survival in mice [89,90]. The role of Notch signaling in cardiac LECs is less well understood and will require further study in the future.

5.3. Role of Cardiac Lymphatics in MI

A key role of lymphatic vasculature is the clearance of interstitial fluid. Loss of cardiac lymphatic vessels on the ventricle did not appear to give rise to overt interstitial edema [33,34,60]. Only with loss of *vegfd* together with compromised Vegfc function was hypertrophy observed, but it is not clear if this is caused by interstitial edema. In most conditions of compromised Vegfc-Flt4 and/or coronary vessel signaling, the BA lymphatic vessels remain largely unaffected, and this may be sufficient to provide a conduit for fluid removal. It remains to be determined if the hypertrophy observed in the *vegfc* hypermorph; *vegfd* double mutant ($vegfc^{hy-/-}$; $vegfd^{-/-}$) is due to loss of the BA populations, misregulation of Flt4/Vegfr3-independent signaling or a compensatory effect of earlier reductions in cardiomyocyte proliferation due to loss of mitogens as observed in the mouse [78]. The phenotypic variability observed with the $vegfc^{hy-/-}$; $vegfd^{-/-}$ combination, indeed all the variability in reported phenotypes across the three studies using various mutant alleles and reporters, needs to be considered in light of varying modifiers in the genetic background [91].

The damage to heart tissue that occurs in response to MI is complex, involving hypoxia, necrosis, inflammation and fibrosis. The cryoinjury model of zebrafish heart incorporates these features more robustly than the amputation injury. Complexity in a model can occlude analysis of specific processes; however, the cost of this simplicity is that not

all features of the regenerative response are captured with the amputation model. In amputation, there is a lack of lymphangiogenesis, and the regenerative response is not perturbed with loss of lymphatics on the ventricle, both, however, are observed after cryoinjury [33,34,60]. Comparison of the models provides a useful insight into what processes are driving the expansion of lymphatic vessels, their roles at the wound site and how these can be utilized to resolve the complex post-MI environment observed in patients.

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