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Novel zebrafish model reveals a critical role for MAPK in lymphangiogenesis

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Abstract

Purpose: Lymphatic disorders are poorly understood with few animal models. We designed a novel assay to measure lymphatic development using transgenic zebrafish with fluorescently labeled endothelial cells. Two major branches of the vascular endothelial growth factor receptor (VEGFR) signaling pathway were examined: the MAPK and PI3K pathways.

Methods: Direct visualization of lymphatic development was performed in control embryos or under chemical inhibition. Treatment involved a 6-hour pulse of inhibitor at 3 days postfertilization. Fish were analyzed for the presence of the thoracic duct (TD) at 4 days postfertilization ($n > 100$ specimens).

Results: Thoracic duct formation was prevented using selective inhibitors against kinases (MAPK, PI3K/TOR, or VEGFR). These kinases were important for TD formation because the lymphatic vessel failed to form in most of treated animals. Remarkably, MAPK pathway inhibition most robustly reduced lymphangiogenesis, demonstrated by a lack of lymphatic endothelial cells.

Conclusion: We conclude that MAPK pathway function downstream of the VEGFRs is crucial at the early stages of TD development. This study provides a novel animal model and a potential target pathway for further investigation. We suggest further examination of MAPK pathway deregulation as a potential mechanism underlying lymphatic disease in humans.

The lymphatic system normally functions in the transport of fluids and facilitating the return of extravasated cells and macromolecules back into the blood circulation. Disorders of the lymphatic vasculature can lead to a variety of debilitating conditions, including disfigurement, bone overgrowth, bleeding, infection, pleural effusions, and ascites. Lymphatic disease and its complications frequently hinder a child's normal development and induce added emotional distress. Despite centuries of clinical experience, little is known regarding the underlying mechanisms responsible for lymphatic disease. By using a novel zebrafish model, we sought a basic understanding of how this unique unidirectional vascular system develops.

The vascular endothelial growth factors (VEGFs; VEGF-A, VEGF-C, and VEGF-D) and their receptor tyrosine kinases (vascular endothelial growth factor receptor [VEGFR] 1, 2, and 3) are master regulators for the development of blood and lymphatic vessels in vertebrates [1]. In developmental lymphangiogenesis, VEGF-C stimulation

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of VEGFR3 is essential for the proliferation, growth, and survival of lymphatic endothelial cells (LECs) [2], whereas a role for VEGF-D appears to be to linked to pathologic lymphangiogenesis [2,3]. Evidence for overlapping roles for VEGFR2 and VEGFR3 function in angiogenesis and lymphangiogenesis has also been demonstrated, suggesting the expression of heterodimeric receptors consisting of VEGFR2 and VEGFR3 during development [4]. Despite understanding of the endothelial functions of these receptors, the mechanism by which ligand stimulation is converted into an intracellular signal in vivo is largely unknown. Most of the data on VEGFR downstream signaling have been derived from in vitro blood or LEC studies [5,6]. From the first report of isolated LECs, VEGF-C stimulation of VEGFR3 was shown to lead to the downstream phosphorylation of both MAPK and AKT (Fig. 1) [6]. The MAPK and PI3K-AKT-TOR pathways appear to act in parallel to each other. However, the stage-dependent need for these intracellular signaling pathways during lymphangiogenesis has not been clearly defined in vivo.

Currently, the zebrafish is the simplest vertebrate organism system for investigation of lymphatic development. Zebrafish offer the advantages of rapid external development, transparency, high fecundity, homologous genes, and conserved cellular processes with humans and mice [7,8]. Like the mouse model, zebrafish lymphangioblasts initially sprout from venous endothelial cells by approximately 2 days postfertilization (dpf) [9,10]. A distinct subpopulation of endothelial cells (ECs) in the posterior cardinal vein commits to a lymphatic linage through expression of a lymphatic cell fate regulator Prox-1 [11]. In zebrafish, as in mammals, VEGF-C and VEGFR3 signaling are essential in the early formation and migration of lymphangioblasts derived from venous endothelial cells

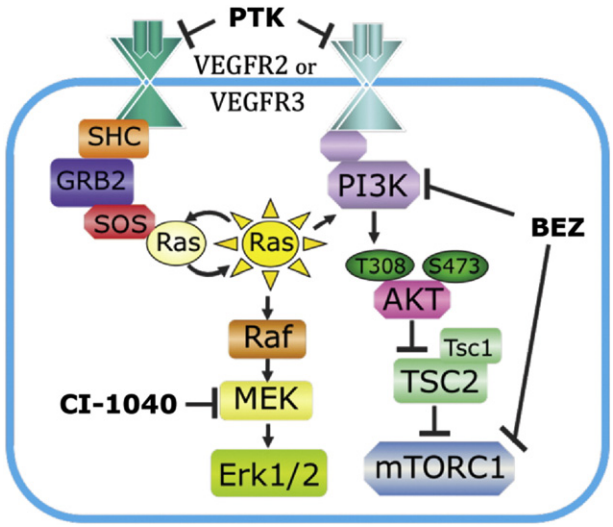

Fig. 1 MAPK and PI3K signaling pathways. VEGFR2 and R3 can stimulate MAPK and PI3K signaling pathways in vitro. Inhibitors CI-1040, BEZ235 (BEZ), and PTK787/ZK2222584 (PTK) were used to target the MAPK pathway, the PI3K pathway, or both pathways, respectively.

[12,13]. In fish, these lymphangioblasts travel a defined path along the intersegmental and parachordal vessels to eventually reside just ventral to the aorta, in isolated patches [9,10]. Over the next couple of days, these LECs coalesce to form the thoracic duct (TD). This central lymphatic duct is complete and functional by the first week of development [12]. Using the presence or absence of a continuous TD over 6 somites as a measurement for lymphatic development, we used selective chemical kinase inhibitors to define signaling cascades activated during lymphangiogenesis in vivo.

1. Materials and methods

1.1. Animals

The Institutional Animal Care and Use Committee of Children's Hospital Boston approved all animal protocols. Zebrafish (*Danio rerio*) were maintained at 28.5°C on a 14-hour-light/10-hour-dark cycle. Embryos were collected by natural spawning and raised in 10% Hanks buffered saline solution at 32°C.

1.2. Chemical treatment with inhibitors

Small-molecule inhibitors were added to the embryo medium [14] at 3 dpf for a period of 6 hours and kept at 32°C. Inhibitors used consisted of CI-1040 (1.5 μmol/L), BEZ235 (500 nmol/L), and PTK787/ZK222584 (5 μmol/L). Inhibitor was then washed out with 3 washes of embryo medium. All inhibitors were purchased commercially from Axon Medchem (Groningen, The Netherlands).

1.3. Quantitation of TD formation

Embryos were anesthetized with tricaine (Sigma (St. Louis, MO)) and mounted in 4% methylcellulose. Thoracic duct formation in a transgenic endothelially driven green fluorescent protein (GFP) line [14] was evaluated at 4 dpf via fluorescence microscopy. The presence of the dorsal aorta and posterior cardinal vein was identified anatomically. In addition, blood flow was ensured by microscopic evaluation in embryos at this time. The TD was identified as a separate tubular structure just ventral to the aorta over a length of the first 6 intersegmental vessels, that is, over 6 somites. This was the only lymphatic vessel directly and clearly observed in the embryo. The duct was counted as present only if it spanned this entire length. Partial formation was rarely observed and scored as absence of TD.

1.4. Statistics analysis

Statistical analysis was performed using Chi squared analysis. A total of 8 separate experiments were combined